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EVIDENCE REVIEW

Pancreatic adenocarcinoma: Beyond first line, where are we?

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Abstract

Pancreatic cancer is considered one of the most aggressive cancers, with an increasing incidence in recent years. To date, chemotherapy is still the standard of care for advanced metastatic disease, unfortunately providing only a slight advantage in terms of survival. The molecular and cellular characteristics of pancreatic cancer cells, as well as the cells that characterize the pancreatic tumour microenvironment, are the basis of the mechanisms of resistance to treatment. After progression during first-line treatment, few patients are eligible for second-line treatment due to the loss of performance status. To date, a clear survival advantage has not yet been demonstrated for second-line chemotherapy. Precision medicine could be the key to increasing responses to cancer treatment and finally impacting survival in this difficult-to-treat disease. In this review, we analyze current recommendations in the second-line setting and potential future prospects.

Key Words: Pancreatic adenocarcinoma; Second-line; Chemotherapy; Targeted therapy; Immunotherapy

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Core Tip: The incidence of pancreatic ductal adenocarcinoma is increasing, with anticipation of a large impact on the population. Despite achieving a survival gain in first-line treatment in the last decade, to date, little has been achieved in second-line treatment. The molecular and genetic characteristics of this tumour represent a fundamental challenge for preclinical and clinical research. In this review, we illustrate current clinical practice in second-line treatment for advanced pancreatic adenocarcinoma and the research landscape of potential future prospects.

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INTRODUCTION

Pancreatic cancer remains one of the deadliest malignancies, recording 432242 new deaths in 2018, with 458918 new pancreatic cancer cases reported globally[1]. Adenocarcinoma is the most common type of exocrine (non-endocrine) pancreatic cancer, accounting for over 90 percent of pancreatic cancer diagnoses. In most cases, it originates from the pancreatic ducts (ductal adenocarcinoma), in a smaller percentage of cases it can originate from the acini (acinar cell carcinoma). Rarer forms of pancreatic cancer are squamous cell carcinoma, adenosquamous carcinoma and colloid carcinoma. Despite advances in pancreatic cancer detection and management, the 5-year survival rate is still very low, only approximately 9%[2]. It is expected to become the second most common cause of cancer-related death by 2030[3]. Unfortunately, most cases are diagnosed in locally advanced or metastatic stages, for which chemotherapy remains the standard of care[4]. Progress in the treatment of pancreatic ductal adenocarcinoma (PDAC) has been very limited; in particular, gemcitabine (GEM) has been used as a monotherapy agent for first-line treatment for approximately 20 years. Subsequently, in 2011, there was a breakthrough in the treatment of metastatic PDAC (mPDAC) with the introduction of the FOLFIRINOX regimen [5-fluorouracil (5FU), folinic acid, irinotecan (IRI) and oxaliplatin (OX)] as a first-line standard of treatment[5]. However, this regimen is not suitable for all patients. Eventually, the combination of nab-paclitaxel and GEM (NabGem) also demonstrated an overall survival (OS) gain in mPDAC compared to GEM monotherapy[6]. However, no prospective randomized studies have demonstrated a benefit in terms of OS for a second-line treatment; moreover, there is currently no standard regarding the sequencing of treatments.

CURRENT CLINICAL PRACTICE IN SECOND LINE MPDAC

Chemotherapy

mPDAC is a biologically aggressive cancer that is often characterized by clinically evident disease progression during first-line treatment (pain, fatigue, anorexia, weight loss, constipation, fever, diabetic decompensation, *etc.*) with a deterioration of the patient performance status (PS) that limits subsequent treatments. Several complications can also arise, such as duodenal stenosis, obstruction of biliary stents and cholangitis, gastrointestinal bleeding and intestinal obstructions, which further limit the possibility of accessing second-line chemotherapy. In this context, it is not surprising that few data from large randomized trials are available. To date, there are no clear data on the superiority of a specific chemotherapy regimen due to the lack of adequate comparisons.

In advanced PDAC, the choice of which chemotherapy to use in the second-line setting basically depends on the treatments used in the first-line setting, residual toxicities (*e.g.*, peripheral neuropathy), patient PS, age and comorbidities. The ability of patients in different countries to access a specific treatment should also be considered due to the limitations of regulatory agencies.

Currently, in first-line treatment for patients with a good PS, Eastern Cooperative Oncology Group (ECOG) 0-1, two main regimens are indicated based on evidence of an OS benefit highlighted by randomized phase III trials: FOLFIRINOX and NabGem. In fact, the PRODIGE4/ACCORD11 trial showed the superiority of FOLFIRINOX over GEM in terms of OS (11.1 mo *vs* 6.8 mo), progression-free survival (PFS, 6.4 mo *vs* 3.3 mo) and the objective response rate (ORR, 31.6% *vs* 9.4%)[5], while in the Metastatic Pancreatic Adenocarcinoma Clinical Trial (MPACT), NabGem showed superiority over GEM (OS 8.5 mo *vs* 6.7 mo, PFS 5.5 mo *vs* 3.7 mo, ORR 23% *vs* 7%, respectively)[6]. It is significant to consider how many patients received a subsequent therapy after progression on first-line therapy in these trials. In the PRODIGE4/ACCORD11 trial, second-line therapy was administered in approximately 50% of patients in both arms, while in the MPACT trial, second-line therapy was administered in 38% of patients in the NabGem group and in 42% of patients in the GEM group. Real-life data are very different; in fact, even in the best cases, they do not seem to include half of patients receiving second-line therapy. It is remarkable that patients

treated in high-volume centres, especially those aged < 65 years and with an ECOG PS of 0-1, receive more lines of therapy. According to Abrams *et al*[7], in the United States in 2015, approximately 56% of patients received second-line therapy and 22% received third-line therapy. The percentages reported in other recent databases are even lower: 38.2% in the United States[8], 33% in Sweden[9], and 44%-48% in British Columbia and Canada[10]. Higher percentages are reported in Austria, where the proportions of patients who had access to second- and third-line therapy were 62%-65% and 29%-37%, respectively[11].

The choice of subsequent treatment has to consider the chemotherapy drugs received in the first line. Therefore, there are two main scenarios: after a first-line treatment with GEM-based chemotherapy, the advice of the main guidelines is to choose a 5FU-based chemotherapy; in the case of a front line therapy with a 5FU-based scheme, the indication is a GEM-based therapy[12,13]. The choice between a multidrug combination regimen and monotherapy depends on the patient's PS (ECOG 0-1 or 2, respectively). A summary of the current possible options is reported in Figure 1. The study by Taieb *et al*[14] evaluated first-line and second-line treatment regimens and their geographic variation across European countries between 2014 and 2016, highlighting that the most common first-line treatments were FOLFIRINOX (35.6%), the first choice in France and in the United Kingdom; NabGem (25.7%); and GEM monotherapy (20.5%). Overall, GEM was the most frequently used second-line therapy (27.1%), followed by NabGem (17.8%), FOLFOX [5FU+ leucovorin (LV) + OX, 17.6%] and 5FU monotherapy (16.7%)[14]. It should be noted that nab-paclitaxel beyond the first line is not approved in all countries, and at that time, pegylated liposomal IRI (Nal-IRI) was not yet available.

Pancreatic cancers with specific molecular characteristics, such as microsatellite instability, fusion of the *NTRK* gene, and *BRCA 1-2* mutations, require a separate discussion. In fact, currently, it is recommended by National Comprehensive Cancer Network guidelines to evaluate at least these three genetic features, but unfortunately, this is not accessible yet for everyone in several countries.

For a better understanding of the data available in the literature, we considered the following possible scenarios: (1) Second-line chemotherapy after treatment with FOLFIRINOX; and (2) Second-line chemotherapy after GEM-based regimens (Figure 1).

Second-line chemotherapy after treatment with FOLFIRINOX

There is no clear consensus on the second-line treatment after progression to FOLFIRINOX since no prospective randomized trials have been conducted in this setting. The choice is generally a GEM-based treatment, which could be GEM monotherapy or a GEM-based therapy. Table 1 summarizes the main second-line trials and their results, divided according to the type of study.

GEM in monotherapy: Only a series of retrospective studies have evaluated the efficacy of GEM as a second-line monotherapy after FOLFIRINOX failure[15,16]. The analysis conducted by Viaud *et al*[17] showed a median OS with GEM of 3.7 mo [95% confidence interval (CI): 2.5-5.2], a median PFS of 2.1 mo (95%CI: 2.0-2.6) and a disease control rate (DCR) of 40%, highlighting that age at diagnosis and PS were independently associated with OS in a multivariate analysis [hazard ratio (HR) of 1.86; $P = 0.0055$ and 2.42; $P < 0.0001$, respectively] and suggesting that GEM is beneficial for patients with a good PS. A multicentre retrospective study in the same setting showed an ORR of 11% and a clinical benefit of 44% for patients, regardless of their previous response to the first-line treatment, concluding that some patients benefit from a second-line treatment[18].

GEM based treatment: No randomized trials have evaluated the efficacy of the NabGem combination as second-line therapy. Zhang *et al*[19] published retrospective data collected from a total of 146 patients treated with FOLFIRINOX as the first-line treatment. Of those, 30 received the NabGem combination, 8 received GEM as monotherapy, and 22 received best supportive care (BSC). The median PFS and OS were 3.61 mo and 5.69 mo in the NabGem group and 2.51 mo and 3.82 mo in the GEM monotherapy group, respectively. In a second retrospective study[10], the percentage of patients receiving NabGem compared to GEM alone was different depending on the region considered and the respective possibility for reimbursement[20]. In this study, the OS outcomes favour the NabGem combination regardless of funded access to the second-line combination. The efficacy of the combination in the second-line setting was confirmed in a third multicentre retrospective analysis, although without a comparison with GEM alone[21]. A prospective study showed that the DCR with

Table 1 Studies of second-line treatment in metastatic pancreatic ductal adenocarcinoma

Ref.	Type of study	Patients (*n*)	1st-line regimen	2nd-line regimen	Median 2nd-line OS (mo)	Median 2nd-line PFS (mo)	2nd-line ORR (%)	2nd-line DCR (%)
Pelzer *et al*[35], 2011	Phase III	46[1]	GEM monotherapy	OFF; BSC	4.8; 2.3	-	-	-
Oettle *et al*[36], 2014, CONKO-003	Phase III	160	GEM monotherapy	OFF; FF	5.9; 3.3	2.9; 2.0	-	-
Gill *et al*[37], 2016, PANCREOX	Phase III	108	GEM-based (approximately 75% monotherapy)	mFOLFOX6; FU/LV	6.1; 9.9	3.1; 2.9	13.2; 8.5	60; 63.8
Wang-Gillam *et al*[37], 2016, NAPOLI-1	Phase III	417	GEM-based[2]	Nal-IRI; FU/LV; Nal-IRI + FU/LV	4.9; 4.2; 6.1	2.7; 1.5; 3.1	6; 1; 16	44; 24; 52
Chung *et al*[29], 2018	Phase II	48	GEM-based	mFOLFIRINOX	9.0	5.8	18.8	62.5
Tsavaris *et al*[33], 2005	Phase II	30	GEM	OX 50 mg/mq + FU/LV (1-h iv infusion), weekly	6.25	-	23.3	53.3
Pelzer *et al*[32], 2009	Phase II	37	GEM	OFF	5.5	3.0	6	49
Yoo *et al*[34], 2009	Phase II	61	GEM-based	mFOLFIRI.3; mFOLFOX	3.9; 3.5	1.9; 1.4	0; 7	23; 17
Zaniboni *et al*[49], 2012	Phase II	50	GEM ± platinoid	FOLFIRI	5	3.2	8	36
Chung *et al*[29], 2018, SWOG S1115	Phase II	137	GEM-based	Selumetinib+ MK-2206; mFOLFOX	3.9; 6.7	1.9; 2.0	1.7; 8	22.4; 30.6
Portal *et al*[22], 2015	Prospective cohort	57	FOLFIRINOX	NabGem	8.8	5.1	17.5	58
Zaanan *et al*[47], 2014	Prospective cohort	46	GEM/FOLFIRI.3 in FIRGEM trial	FOLFOX	4.3	1.7	0	36
Wainberg *et al*[45], 2020	Meta-analysis	454	GEM-based	FOLFOX; Nal-IRI	6.3; 6.1	-	-	-
Sonbol *et al*[51], 2017	Meta-analysis	895	GEM-based	FPOX; FPIRIFP	FPIRI *vs* FP: HR OS 0.7, PFS 0.64; FPOX *vs* FP: HR OS 1.0, PFS 0.81			
Citterio *et al*[52], 2018	Meta-analysis	1587	GEM-based	FP, OX or IRI-based	Most effective IRI-based regimens (results cannot be translated into the table)			
Rahma *et al*[43], 2013	Systematic analysis	1503	GEM-based	GEM + platinum; FPOXBSC	6.0; 5.7; 2.8	4; 2.9; -	-	-
Petrelli *et al*[53], 2017	Systematic analysis	-	GEM-based	OX-based; IRI-based	5.3; 5.5	2.9; 2.7	11.9; 8.7	41.1; 29.4
Berk *et al*[48], 2012	Comparative	85	GEM-based	FOLFOX4; XELOX	5.8; 4.9	3.7; 3.7	17; 18	43; 59
Zhang *et al*[19], 2018	Retrospective	146	FOLFIRINOX	NabGem; Gem alone	5.69; 3.82	3.61; 2.51	-	-
Chae *et al*[21], 2020	Retrospective	102	FOLFIRINOX	NabGem	9.8	4.6	8.5	73.6
Viaud *et al*[17], 2017	Retrospective	96	FOLFIRINOX	GEM monotherapy	3.7	2.1	-	40
Gilabert *et al*[18], 2017	Retrospective	72	FOLFIRINOX	GEM monotherapy	-	2.5	11	-

Pointet *et al*[38], 2020	Retrospective	137	NabGem	FOLFOX; FOLFIRI; FOLFIRINOX	3.5; 9.7; 6.1	2; 6.6; 3.4	0; 9.5; 6.3	29.2; 61.9; 50
Lee *et al*[41], 2020	Retrospective	120	GEM-based	FPOX; FP	7.04; 7.43	2.89; 3.81	6.4; 5.4	52.6; 59.5
Neuzillet *et al*[50], 2012	Retrospective	63	GEM ± platinoid	FOLFIRI	6.6	3.0	7.9	39.7
Kieler *et al*[24], 2019	Retrospective	52	GEM-based	Nal-IRI + FU/LV	6.79	3.84	19.2	46.2

[1]The trial was prematurely stopped due to insufficient accrual.
[2]Approximately 45% of gemcitabine alone and 55% in combination. About 30% of patients had received ≥ 2 previous lines for metastatic disease, with 45% of patients pretreated with fluorouracil/leucovorin-based regimens. GEM: Gemcitabine; DCR: Disease control rate; BSC: Best supportive care; OFF: Oxaliplatin, folinic acid and 5-fluorouracil; 5FU: 5-Fluorouracil; OX: Oxaliplatin; LV: Leucovorin; FF: Folinic acid and 5-fluorouracil; Nal-IRI: Liposomal irinotecan; FOLFIRINOX: 5-Fluorouracil, folinic acid, irinotecan and oxaliplatin; FOLFOX: 5-fluorouracil + leucovorin + oxaliplatin; FPOX: Fluoropyrimidine and oxaliplatin-based regimens; HR: Hazard ratio; OS: Overall survival; NabGem: Nb-paclitaxel and gemcitabine; FP: Fluoropyrimidine; IRI: Irinotecan.

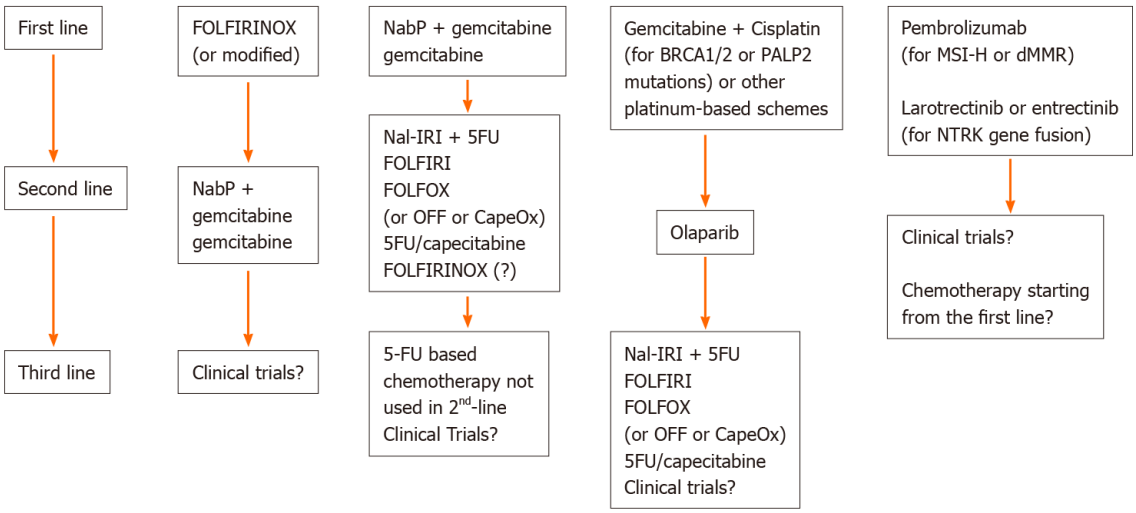


Figure 1 Current therapeutic possibilities for metastatic pancreatic ductal adenocarcinoma. NabP: Nab-paclitaxel; Nal-IRI: Nanoliposomal irinotecan; 5FU: 5-Fluorouracil; FOLFIRI: 5-Fluorouracil + irinotecan; FOLFOX: 5-fluorouracil + leucovorin + oxaliplatin; OFF: Oxaliplatin + 5-fluorouracil + folinic acid; CapeOx: Capecitabine + oxaliplatin; MSI-H: Microsatellite instability high; dMMR: DNA mismatch repair deficiency; Clinical trials?: Evaluate the availability of clinical trials suitable for the patient.

NabGem was 58% (ORR 17.5%), OS was 8.8 mo (95%CI: 6.2-9.7) and the PFS was 5.1 mo (95%CI: 3.2-6.2)[22].

To date, there are no second-line treatment recommendations after progression on the FOLFIRINOX scheme, and the use of GEM alone or in combination with nabpaclitaxel is generally dictated by patient characteristics and by the possibility of reimbursement in individual countries.

Second-line chemotherapy after treatment with GEM based combination therapy

For patients previously treated with GEM-based regimens, the main international guidelines recommend 5FU-based therapies, which include FOLFIRI, Nal-IRI+5FU, OX, folinic acid and 5FU (OFF), FOLFOX or CapeOX and monotherapy with 5FU or capecitabine.

Nal-IRI ± 5FU/LV: Nal-IRI + 5FU/LV is the regimen with the most evidence and therefore a higher degree of recommendation[13]. This indication comes from the results of the NAPOLI-1 study, which compared 5FU/LV alone *vs* monotherapy with Nal-IRI or the combination of 5FU/LV + Nal-IRI in 417 patients with mPDAC and a Karnofsky PS ≥ 70 who were previously treated with GEM-based therapy[23]. In particular, 12% of patients received GEM-based therapy in the adjuvant, neoadjuvant, or locally advanced setting; 56% had received one previous line of metastatic treatment; and 32% had received two or more lines of metastatic treatment. It should be emphasized that few patients received NabGem in the first-line setting since the

GEM combination is preferred in current clinical practice, and 43% of patients had already received previous 5FU-based therapy (10% IRI and 32% platinum). Patients in the 5FU/LV + Nal-IRI group achieved a longer OS than patients in the 5FU/LV group (6.1 mo *vs* 4.2 mo; P = 0.012, HR: 0.67); however, no significant difference in OS was observed between the 5FU/LV and Nal-IRI monotherapy groups (4.2 mo *vs* 4.9 mo; P = 0.94, HR: 0.99).

These data were confirmed by a retrospective study that included 52 patients[24] and a similar Korean study[25]. However, in some countries, including Italy, this combination is not approved due to the methodological limitations of the study, such as the heterogeneity of the patient population, the study design without a comparison with the classic FOLFIRI regimen, and the inclusion of patients pretreated with 5FU or IRI[26].

Fluoropyrimidine and OX-based regimens: The efficacy and safety data of second-line treatment with FOLFIRINOX are based on retrospective analyses[27,28] and on some phase II studies[29,30]. In the single-arm multicentre phase 2 study performed by Chung *et al*[29] (48 patients) of modified FOLFIRINOX (IRI 120 mg/m^2 and OX 60 mg/m^2), the ORR, DCR, median PFS and OS were 18.8%, 62.5%, 5.8 mo and 9.0 mo, respectively, with significant toxicity (neutropenia grade 3 or 4 rates of 64.6%, febrile neutropenia 16.7%). A highly toxic triplet therapy is not very suitable for second-line palliative treatment in patients with a non-optimal PS and is reserved for only a few cases. Furthermore, in a recent real-world analysis, triplet therapy with FOLFIRINOX did not seem to have an advantage over sequential chemotherapy with FOLFIRI-FOLFOX regimens[31].

For the other OX-based regimens, the data are controversial. Based on the promising results of some phase II studies[32-34], three main phase III trials have been conducted[35-37]. In the CONKO-003 trial, 160 patients were randomized to receive FF (folinic acid 200 mg/m^2 followed by a continuous IV infusion of fluorouracil 2000 mg/m^2 over 24 h on days 1, 8, 15, and 22 every 42 d) or OFF (FF and OX 85 mg/m^2 IV administered before FF on days 8 and 22). Compared to FF, OFF achieved a significant increase in both OS (5.9 *vs* 3.3 mo) and PFS (2.9 *vs* 2 mo)[36]. In phase III by Pelzer *et al*[35], OFF compared to BSC significantly prolonged OS (4.82 mo *vs* 2.30 mo, respectively) despite the premature closure for insufficient accrual (only 46 patients) due to the difficulty of clinicians and patients accepting BSC[35]. However, these results of the superiority of OFF over FF and BSC were not confirmed by the phase III PANCREOX trial[37]. In particular, in this study, the addition of OX to FF (in the mFOLFOX6 scheme) did not translate into an increase in OS; in contrast, it seemed detrimental (6.1 mo *vs* 9.9 mo) at the expense of increased toxicity.

In the literature, different retrospective trials and reviews have dealt with the same topic, with discordant results[38-47].

A comparative study evaluated the XELOX and FOLFOX schemes, highlighting their comparable results in terms of efficacy and toxicity profile[48].

IRI and 5FU-based regimens (fluoropyrimidine IRI): The use of second-line FOLFIRI in patients who progressed on first-line therapy of GEM and platinum (cisplatin or OX) was evaluated in a prospective multicentre study[49]. Among the 50 patients enrolled, four partial responses (8%) were observed with disease stability in 28% of patients, while PFS and OS were 3.2 mo and 5.0 mo, respectively. Similar results were obtained from another study that evaluated FOLFIRI after progression on GEM and platinoids[50]. Unlike the previous study, patients (n = 63) could receive more than one treatment line in the metastatic setting. In particular, most patients had received two previous lines. DCR was achieved in 25 patients (39.7%; partial response: n = 5, stable disease: n = 20) with FOLFIRI. The median time to progression (TTP) was 3.0 mo, and the median OS was 6.6 mo. An ECOG PS of 2 was significantly associated with a poor TTP and OS, limiting the efficacy of FOLFIRI to patients with a good PS (PS 0-1).

Some meta-analyses have concluded that fluoropyrimidine (FP) IRI (FPIRI) is superior to FP and OX-based regimens (FPOX) after first-line treatment with gem-based chemotherapy. In particular, Sonbol *et al*[51] collected randomized controlled trials comparing FP monotherapy *vs* FPOX or FPIRI and showed that FPOX or FPIRI improved PFS compared with single-agent FP, but only FPIRI reported an OS advantage. Similarly, in the network meta-analysis by Citterio *et al*[52] and the meta-analysis by Catalano *et al*[40], FPIRI seemed superior to FPOX in terms of OS. Conversely, in the systematic review of 24 studies by Petrelli *et al*[53], FPOX and FPIRI were associated with a similar efficacy, with a pooled ORR, DCR, PFS and OS of 11%, 37.9%, 2.87 mo and 5.48 mo, respectively.

In conclusion, in patients with preserved PS (ECOG PS 0-1), without relevant comorbidities, it is reasonable to propose a second-line treatment with a 5FU-based or GEM-based treatment, depending on the first-line treatment used. Within the 5FU-based regimens, any residual toxicities of the first-line treatment can lead to choose a scheme rather than another. For example, if the patient has residual neurotoxicity (*e.g.*, from Nab-paclitaxel) the choice could be FOLFIRI or Nal-IRI-5FU; if he has diarrhea or bone marrow toxicity, FOLFOX. However, some treatments, such as NabGem or Nal-IRI, are not reimbursed for the second-line in all countries, thus inevitably influencing the choice of treatment.

Targeted therapy

The introduction of increasingly accurate techniques for molecular sequencing and a better understanding of the pathogenetic role of genes related to PDAC have led to the drafting of numerous clinical trials to study potential targeted treatments in chemorefractory disease. Studies in the literature suggest that the use of precision medicine can have a substantial effect on survival in patients affected with PDAC and that molecular-guided treatments targeting oncogenic drivers promise potential developments in clinical practice[54]. Despite countless studies, to date, few biologic treatments have been approved for advanced PDAC. In particular, the FDA (Food and Drug Administration)- and EMA (European Medicines Agency)-approved targeted drugs for second-line treatment are erlotinib, larotrectinib and entrectinib. Olaparib is approved for maintenance after response to a first-line platinum-containing agent[55].

Erlotinib: The approval of erlotinib, an EGFR (epidermal growth factor receptor) TK inhibitor, in combination with GEM comes from a phase III study that demonstrated a statistically significant, albeit modest, improvement in survival in PDAC compared to GEM alone[56]. These data have been confirmed in other prospective[57] and retrospective studies[58].

Larotrectinib and entrectinib: Fusions involving *NTRK1*, *NTRK2* and *NTRK3* lead to the expression of chimeric rearrangements in tropomyosin receptor kinases (TRKs) A, B, and C, respectively, with constitutively active kinase function. TRK fusions are oncogenic drivers in numerous cancer histotypes, including pancreatic cancer, albeit in a very low percentage of cases, approximately 0.34%[59]. A peculiarity of the studies that evaluated TRK inhibitor drugs is that the efficacy of the specific treatment on a genomic alteration is evaluated independent of the tumour histology. No randomized trials have been conducted, but the high ORR that exceeded the predetermined minimum of the investigators (30%), which was 75% for larotrectinib and 57% for entrectinib, led to the approval of these drugs. However, data from studies of these two drugs are not comparable given the heterogeneity of the study populations involved.

The approval of entrectinib for solid tumours with *NTRK* gene fusions is based on the results of three clinical trials: ALKA-372-001, STARTRK-1[60] and STARTRK-2 (NCT02568267). An integrated analysis of the following phase I and II studies included a total of 54 patients with *NTRK* fusion-positive advanced solid tumours for a total of 19 different histotypes[61]. The median follow-up was 12.9 mo and showed 50% partial responses and 7% complete responses. This response to treatment has been maintained over time with a median duration of response of 10 mo and a good toxicity profile.

The approval of larotrectinib is based on data from three multicentre, open-label, single-arm clinical studies: LOXO-TRK-14001 (NCT02122913), SCOUT (NCT02637687) and NAVIGATE (NCT02576431). A pooled analysis of these studies by Hong *et al*[62] included 55 patients treated with larotrectinib. The ORR was 79% (95%CI: 72-85) in 153 evaluable patients, with a 16% complete response rate and a good safety profile.

Given the clinical benefit, even considering the low prevalence of *NTRK* fusions in patients with pancreatic cancer and the lack of easy access to Next Generation Sequencing services, patients should be tested at diagnosis for such gene alterations to guide treatment decisions as well as for gaining access to potential clinical trials.

Unfortunately, in real life clinical practice it is not possible to require such molecular insights in all patients with mPDAC due to the cost sustainability. The lack of access to these drugs in different countries represents the current gap between what precision medicine for mPDAC could be in the future and current clinical practice in different oncology contexts. The use of resources and the high costs of oncological treatments will be an increasingly important topic in the near future and it is inevitable to take this into account.

Immunotherapy

Immunotherapy has changed the natural history of various cancer pathologies, especially melanoma and lung cancer, providing results in terms of increased OS in other neoplastic pathologies, such as cancer of the head and neck, bladder cancer, renal carcinoma, Merkel cell carcinoma and triple-negative breast tumours. However, to date, immune checkpoint inhibitors (ICI) have not shown any efficacy in controlling advanced PDAC, either in monotherapy[63,64] or in combination with chemotherapy[65,66]. Several actors are known to be responsible for the response/resistance mechanisms to ICI. Among these actors, the ability of the tumour to express antigens recognizable by the cells of the immune system and the characteristics of the tumour microenvironment in which the balance of immuno-sensitizing/immuno-suppressive factors is in favour of the latter[67]. In particular, PDAC are characterized by the presence of an abundant desmoplastic stroma composed of fibroblasts, extracellular matrix, immune cells and stellate cells. Immune cells infiltrating this stroma are mostly represented by tumour-associated macrophages, myeloid-derived suppressor cells and Treg cells, with very few effector T cells. Numerous trials are underway aimed at converting the pancreatic tumour microenvironment from immunosuppressive to immunosensitive[68,69]; however, to date, there is no indication for second-line immunotherapy treatments in chemorefractory disease. A PD-1 (programmed cell death protein 1) ICI, Pembrolizumab, has been approved by the FDA for diseases with microsatellite instability, regardless of tumour site[70]. However, this indication has not yet been approved by EMA, and in Europe, it is therefore not possible to prescribe immunotherapy in this setting outside of clinical trials.

FUTURE DIRECTIONS

As seen from current clinical practice in second-line treatment of mPDAC, there are fundamental open questions. These questions include therapeutic possibilities for treatment after progression on TRK inhibitors in TRK fusion-positive cancers, an increase in targeted therapies, and overcoming the immuno-resistance of metastatic pancreatic disease.

Therapeutic possibilities for treatment after progression on TRK inhibitors in TRK fusion-positive cancers

Patients may acquire resistance to first-generation *TRK* inhibitors; however, to date, the mechanisms of resistance to *TRK* inhibitor drugs are not known, and the only secondary resistance mechanism identified is the acquisition of targeted mutations in the *NTRK* kinase domain of the oncogenic fusion. Currently, several trials of newer molecules, such as LOXO-195[71] and TPX-00005[72], have been performed to evaluate the efficacy of targeted therapies after progression on *TRK* inhibitors with very promising results. Such molecules could become the second-line standard in the future after the failure of first-generation *TRK* inhibitors in *TRK* fusion-positive pancreatic adenocarcinoma.

Increase of targeted therapies

Despite the high ORR of *TRK* inhibitor drugs, unfortunately, the percentage of PDAC patients susceptible to this targeted treatment is extremely low. Furthermore, identification of the *BRCA* mutation allows the prescription of olaparib in maintenance after a response to a first-line platinum-based treatment, so there is currently no possibility of second-line targeted treatment in mutated *BRCA* patients[73].

To date, there are few data on the efficacy of targeted treatments in advanced PDAC; however, this deficiency should not discourage clinicians from requiring a genomic study in these patients. Indeed, knowledge of the prospective genomic profile can predict the response to chemotherapy treatment[74]. Furthermore, these data could be useful for the enrolment of second-line patients in a clinical trial. Research is currently progressing by targeting the recombination deficits of DNA as well as considering the driver genes in pancreatic carcinogenesis. Alongside drugs that target pancreatic tumour cells, there is a large amount of research addressing the peculiar tumour microenvironment that is partly responsible for the poor response to cancer treatments of advanced PDAC (Figure 2).

Regarding tumours with recombination deficiency of DNA repair, research is also underway to determine the extent of the cancer risk in patients with the so-called

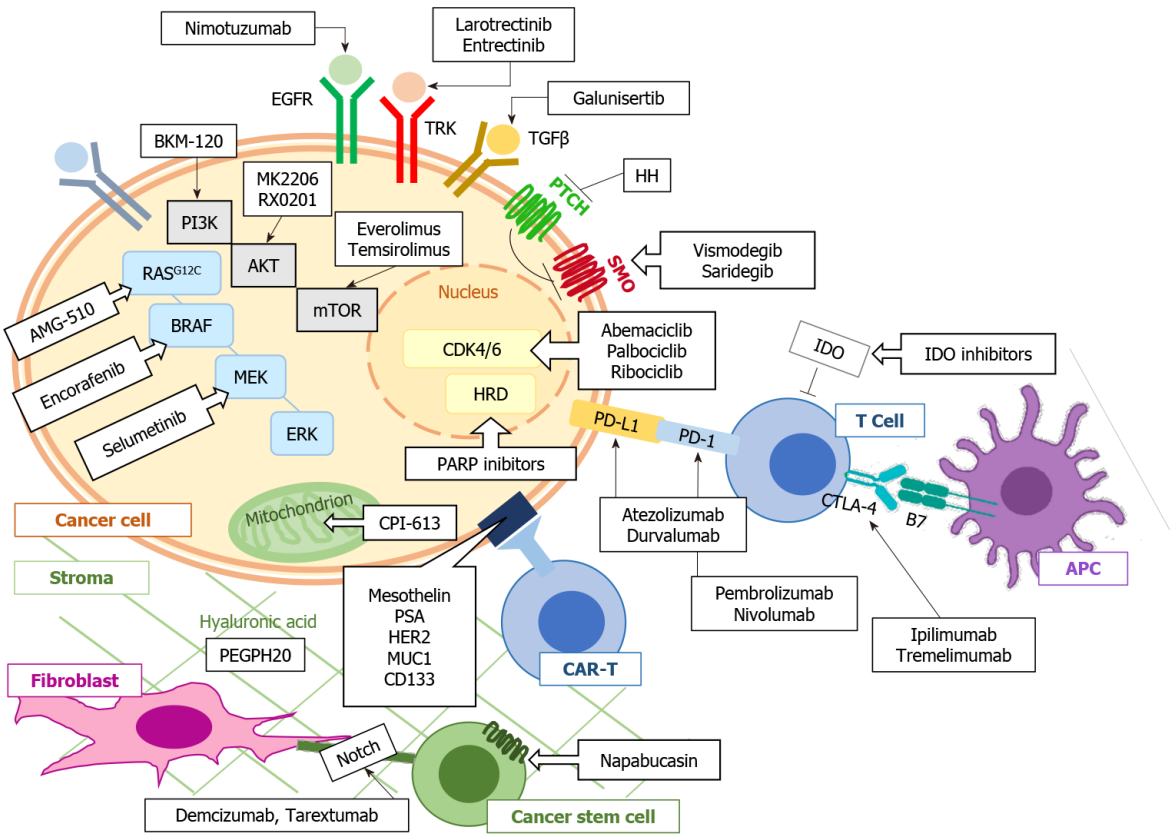


Figure 2 Main targeted drugs under study for patients with advanced pancreatic ductal adenocarcinoma. EGFR: Epidermal growth factor receptor; TGFβ: Transforming growth factor β; APC: Antigen presenting cells; PD-1: Programmed cell death protein 1; PD-L1: Programmed cell death protein ligand 1; CAR-T: Chimeric antigen receptor T cells.

'bracness' phenotype, or rather the genetic alterations that result in a defect in homologous recombination repair, mimicking the loss of *BRCA1* or *BRCA2*. Among these mutations is the *PALB2* mutation, which occurs in 3%-4% of familial pancreatic cancer cases[75,76]. Most studies of *PARP* inhibitors are conducted as maintenance after a response or stability after treatment with a platinum-based first line, *i.e.*, the current indication for olaparib. However, studies have also been conducted on the second line in patients pretreated and in progression after a first-line chemotherapy treatment[77-79]. However, in this setting, the data are currently conflicting, promising for olaparib and rucaparib and not significant for veliparib. Nevertheless, phase 3 studies are lacking.

Considering the major driver genes in pancreatic carcinogenesis, pancreatic tumours are characterized in most cases by activating mutations in *KRAS* (> 90%) and loss-of-function mutations in *TP53* (50%) and *CDKN2A* (80%). Several studies are underway with the aim of targeting such drivers; however, to date, the potential therapeutic target genes are limited to *KRASG12C* and *CDKN2A*, which are found in only a small percentage of patients. AMG 510 is a novel small molecule that specifically and irreversibly inhibits *KRASG12C* and shows antitumour activity when administered as monotherapy in pretreated patients[80,81]. Since the loss of p16INK4a is a standard feature in *KRAS*-driven PDAC, pharmacological specific inhibition of CDK4/6 represents a possible targeted treatment. However, monotherapy treatment with CDK4/6 inhibitors does not appear to be remarkably effective for pancreatic cancer[82]. It has therefore been hypothesized that the activity of CDK4/6 inhibitors can be exploited by combination therapies, such as mTOR inhibitors or chemotherapy[83]. Numerous clinical trials are currently underway (Table 2).

PI3K/Akt signalling is one of the most deregulated signalling pathways in cancer, including PDAC, and has a mediating role of the cellular signalling not only for tumour cells but also for stromal cells. Indeed, *KRAS* activates various signalling pathways of downstream effectors, including the PI3K pathway, which can, in turn, be activated by different signal transduction pathways linked to various growth factor receptors. In the last decade, there has been considerable interest in molecules inhibiting the PI3K/Akt-mediated transduction pathway, including PDAC[84,85]. One

Table 2 Main ongoing targeted therapy studies (clinicaltrial.gov) for advanced pancreatic adenocarcinoma

Treatment	Target	Phase of study	Setting
Ribociclib plus trametinib; NCT02703571	CDK4/6	Phase I/II trial, open label single arm	Advanced or metastatic pancreatic cancer and *KRAS*-mutant colorectal cancer
Palbociclib + the PI3K/mTOR inhibitor, gedatolisib; NCT03065062	CDK4/6	Phase I, open label single arm	Advanced squamous cell lung, pancreatic, head and neck and other solid tumours
Abemaciclib in combination with the TGF-β inhibitor galunisertib or other agents; NCT02981342	CDK4/6	Phase II, open label, randomized	Previously treated metastatic pancreatic ductal adenocarcinoma
BKM120 + mFOLFOX6; NCT01571024	PI3K	Phase I, open label, single arm	Advanced solid tumours including metastatic pancreatic cancer
Metformin + Gemcitabine + Erlotinib; NCT01210911	PI3K	Phase II, randomized, placebo controlled	Locally advanced or metastatic pancreatic cancer
Capecitabine + Cetuximab + Everolimus; NCT01077986	mTOR	Phase I/II, open label, single arm	Metastatic pancreatic cancer
Temsirolimus; NCT00075647	mTOR	Phase II, open label, single arm	Locally advanced or metastatic pancreatic cancer
MK2206 + Fluorouracil + Oxaliplatin + Selumetinib; NCT01658943	Akt	Phase II, open label, randomized	Metastatic pancreatic cancer
RX-0201 + Gemcitabine; NCT01028495	Akt	Phase II, open label, single arm	Metastatic pancreatic cancer
Gemcitabine ± nimotuzumab; NCT02395016	EGFR	Phase III, prospective, randomized, controlled, double-blind	Locally advanced or metastatic pancreatic cancer
MRTX849 (inhibitor of KRAS G12C) + TNO155 (inhibitor of SHP2); NCT04330664	KRASG12C	Phase I/II, open label, non-randomized	Advanced or metastatic cancer with a KRAS *G12C* mutation
AMG 510 Monotherapy; NCT03600883	KRASG12C	Phase I/II, open label, non-randomized	*KRAS* p.G12C mutant advanced solid tumours
Gemcitabine + M7824 (TGF-β ligand trap); NCT03451773	TGF-β	Phase I/II, open label, single arm	Locally advanced or metastatic pancreatic cancer
FFX *vs* CPI-613 + mFFX; NCT03504423	CPI-613	Pase III, open-label randomized	Mtastatic adenocarcinoma of the pancreas

TGF-β: Transforming growth factor β.

of the major challenges contributing to the suboptimal response to PI3K inhibitor drug monotherapies is the development of resistance mechanisms. Therefore, in this case, the current standard is the identification of new targeted combination therapies[86]. Table 2 reports the current ongoing clinical trials targeting the phosphoinositide signalling cascade for the treatment of pancreatic cancer.

The transformation of growth factor beta (TGF-β) signalling regulates cell proliferation and plays a fundamental role in the process of metastasis, angiogenesis and escape from immune surveillance. Several TGF-β inhibitory molecules are being studied, including oral inhibitors of the TGF-β receptor kinase, such as galunisertib (LY2157299), which specifically downregulates SMAD2 phosphorylation, blocking the activation of the canonical pathway[87], and trabedersen (AP 12009), a TGF-β2-specific antisense phosphorothioate oligodeoxynucleotide[88]. For example, data from a phase Ib study using galunisetinib in combination with second-line durvalumab suggests possible second-line activity of the combination[89].

CPI-613 is a new anticancer drug that selectively targets the altered form of mitochondrial energy metabolism in cancer cells, compromising the activity leading to apoptosis of cancer cells. Following the promising results of Phase I and II studies[90], a Phase III study is underway to compare this combination of FOLFIRINOX and CPI-613 with FOLFIRINOX alone.

Numerous other potential targets have been studied, such as c-KIT, VEGFR, and RET. Unfortunately, both masatinib, an anti-cKIT tyrosine kinase inhibitor, and vandetanib, an anti-VEGFR2, -RET, and -EGFR tyrosine kinase inhibitor, have failed to demonstrate a benefit over standard therapy[91,92].

A possible explanation for the failure of targeted therapies is the adaptive response to drug inhibition, for example, through the blockage of downstream signalling and the activation of other signalling transduction pathways. The future is trending towards the identification of combinations of treatments, with the aim of overcoming resistance mechanisms with an acceptable toxicity profile. Alongside this trend, there

is the need to identify predictive molecular markers of response to treatment.

Overcome immuno-resistance of metastatic pancreatic disease

As previously reported, the initial enthusiasm for immunotherapy in advanced pancreatic cancer waned due to the not very encouraging data from early clinical trials. However, the improved understanding of resistance mechanisms has led to further clinical studies aiming to overcome the immuno-resistance of the pancreatic tumour microenvironment.

To date, data from clinical trials that evaluated the combination of ICI drugs in second-line treatment are not promising. The study conducted by O'Reilly *et al*[93] that evaluated the efficacy of the combination of durvalumab and tremelimumab in patients who progressed to first-line FP or GEM did not yield the desired results.

For the association of immunotherapy drugs with chemotherapy, the data seem to be encouraging; however, numerous association trials are also underway with cancer vaccines, adoptive T cell transfer, and direct targeted treatments in the tumour microenvironment (JAK/STAT inhibitors, CSF1R blockers, BTK inhibitors)[94].

Again, there is a lack of factors that allow us to predict the response to treatment; greater knowledge of the individual genetic characteristics together with the molecular characteristics of the disease could in the future lead to a broader selection of patients for immunotherapy treatments.

CONCLUSION

Treatment of patients with mPDAC has improved in recent years thanks to the introduction of more effective chemotherapy regimens in the first-line setting. Consequently, the proportion of patients who are candidates for second- and third-line regimens is increasing. However, to date, chemotherapy remains the second-line standard of care, and neither personalized medicine nor immunotherapy has in fact provided important positive results in the treatment of pancreatic cancer. There are many ongoing studies aiming to overcome the multiple resistance mechanisms to treatment; however, the key to overcoming these mechanisms and providing personalized medicine to patients who have progressed to a first line of treatment is far from being identified. The small improvements shown by ongoing clinical trials could be considered a first step in what could be the future of treatment for advanced pancreatic cancer.

REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Rawla P**, Sunkara T, Gaduputi V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J Oncol* 2019; **10**: 10-27 [PMID: 30834048 DOI: 10.14740/wjon1166]

3 **Rahib L**, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; **74**: 2913-2921 [PMID: 24840647 DOI: 10.1158/0008-5472.CAN-14-0155]

4 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020; **70**: 7-30 [PMID: 31912902 DOI: 10.3322/caac.21590]

5 **Conroy T**, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup. FOLFIRINOX *vs* gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817-1825 [PMID: 21561347 DOI: 10.1056/NEJMoa1011923]

6 **Von Hoff DD**, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* 2013; **369**: 1691-1703 [PMID: 24131140 DOI: 10.1056/NEJMoa1304369]

7 **Abrams TA**, Meyer G, Meyerhardt JA, Wolpin BM, Schrag D, Fuchs CS. Patterns of Chemotherapy Use in a U.S.-Based Cohort of Patients with Metastatic Pancreatic Cancer. *Oncologist* 2017; **22**: 925-933 [PMID: 28476943 DOI: 10.1634/theoncologist.2016-0447]

8 **O'Reilly EM**, Cockrum P, Surinach A, Wu Z, Dillon A, Yu KH. Reducing nihilism in metastatic

pancreatic ductal adenocarcinoma: Treatment, sequencing, and effects on survival outcomes. *Cancer Med* 2020; **9**: 8480-8490 [PMID: 32997898 DOI: 10.1002/cam4.3477]

9 **Gränsmark E**, Bågenholm Bylin N, Blomstrand H, Fredrikson M, Åvall-Lundqvist E, Elander NO. Real World Evidence on Second-Line Palliative Chemotherapy in Advanced Pancreatic Cancer. *Front Oncol* 2020; **10**: 1176 [PMID: 32850339 DOI: 10.3389/fonc.2020.01176]

10 **Tsang ES**, Spratlin J, Cheung WY, Kim CA, Kong S, Xu Y, Gill S. Real-world Outcomes Among Patients Treated With Gemcitabine-based Therapy Post-FOLFIRINOX Failure in Advanced Pancreatic Cancer. *Am J Clin Oncol* 2019; **42**: 903-908 [PMID: 31693510 DOI: 10.1097/COC.0000000000000625]

11 **Kieler M**, Unseld M, Bianconi D, Schindl M, Kornek GV, Scheithauer W, Prager GW. Impact of New Chemotherapy Regimens on the Treatment Landscape and Survival of Locally Advanced and Metastatic Pancreatic Cancer Patients. *J Clin Med* 2020; **9** [PMID: 32121198 DOI: 10.3390/jcm9030648]

12 **National Comprehensive Cancer Network**. National comprehensive cancer network (NCCN) guidelines. [cited 17 January 2021]. In: National Comprehensive Cancer Network [Internet]. Available from: https://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf

13 **European Society for Medical Oncology**. Cancer of the pancreas: ESMO clinical practice guidelines. [cited 17 January 2021]. In: European Society for Medical Oncology [Internet]. Available from: https://www.esmo.org/guidelines/gastrointestinal-cancers/pancreatic-cancer

14 **Taieb J**, Prager GW, Melisi D, Westphalen CB, D'Esquermes N, Ferreras A, Carrato A, Macarulla T. First-line and second-line treatment of patients with metastatic pancreatic adenocarcinoma in routine clinical practice across Europe: a retrospective, observational chart review study. *ESMO Open* 2020; **5** [PMID: 31958291 DOI: 10.1136/esmoopen-2019-000587]

15 **Sarabi M**, Mais L, Oussaid N, Desseigne F, Guibert P, De La Fouchardiere C. Use of gemcitabine as a second-line treatment following chemotherapy with folfirinox for metastatic pancreatic adenocarcinoma. *Oncol Lett* 2017; **13**: 4917-4924 [PMID: 28599496 DOI: 10.3892/ol.2017.6061]

16 **da Rocha Lino A**, Abrahão CM, Brandão RM, Gomes JR, Ferrian AM, Machado MC, Buzaid AC, Maluf FC, Peixoto RD. Role of gemcitabine as second-line therapy after progression on FOLFIRINOX in advanced pancreatic cancer: a retrospective analysis. *J Gastrointest Oncol* 2015; **6**: 511-515 [PMID: 26487945 DOI: 10.3978/j.issn.2078-6891.2015.041]

17 **Viaud J**, Brac C, Artru P, Le Pabic E, Leconte B, Bodère A, Pracht M, Le Sourd S, Edeline J, Lièvre A. Gemcitabine as second-line chemotherapy after Folfirinox failure in advanced pancreatic adenocarcinoma: A retrospective study. *Dig Liver Dis* 2017; **49**: 692-696 [PMID: 28256401 DOI: 10.1016/j.dld.2017.02.007]

18 **Gilabert M**, Chanez B, Rho YS, Giovanini M, Turrini O, Batist G, Kavan P, Raoul JL. Evaluation of gemcitabine efficacy after the FOLFIRINOX regimen in patients with advanced pancreatic adenocarcinoma. *Medicine (Baltimore)* 2017; **96**: e6544 [PMID: 28422841 DOI: 10.1097/MD.0000000000006544]

19 **Zhang H**, Kellett C, Lambert P, Kim CA. Efficacy and Tolerability of Second-line Nab-paclitaxel and Gemcitabine After Failure of First-line FOLFIRINOX for Advanced Pancreas Cancer: A Single-institution Experience. *Clin Colorectal Cancer* 2018; **17**: e451-e456 [PMID: 29631907 DOI: 10.1016/j.clcc.2018.03.003]

20 **Tehfe M**, Dowden S, Kennecke H, El-Maraghi R, Lesperance B, Couture F, Letourneau R, Liu H, Romano A. nab-Paclitaxel Plus Gemcitabine Versus Gemcitabine in Patients with Metastatic Pancreatic Adenocarcinoma: Canadian Subgroup Analysis of the Phase 3 MPACT Trial. *Adv Ther* 2016; **33**: 747-759 [PMID: 27085323 DOI: 10.1007/s12325-016-0327-4]

21 **Chae H**, Jeong H, Cheon J, Chon HJ, Ryu H, Kim IH, Kang MJ, Jeong JH, Ryoo BY, Kim KP, Yoo C. Efficacy and safety of second-line nab-paclitaxel plus gemcitabine after progression on FOLFIRINOX for unresectable or metastatic pancreatic ductal adenocarcinoma: multicenter retrospective analysis. *Ther Adv Med Oncol* 2020; **12**: 1758835920923424 [PMID: 32523632 DOI: 10.1177/1758835920923424]

22 **Portal A**, Pernot S, Tougeron D, Arbaud C, Bidault AT, de la Fouchardière C, Hammel P, Lecomte T, Dréanic J, Coriat R, Bachet JB, Dubreuil O, Marthey L, Dahan L, Tchoundjeu B, Locher C, Lepère C, Bonnetain F, Taieb J. Nab-paclitaxel plus gemcitabine for metastatic pancreatic adenocarcinoma after Folfirinox failure: an AGEO prospective multicentre cohort. *Br J Cancer* 2015; **113**: 989-995 [PMID: 26372701 DOI: 10.1038/bjc.2015.328]

23 **Wang-Gillam A**, Li CP, Bodoky G, Dean A, Shan YS, Jameson G, Macarulla T, Lee KH, Cunningham D, Blanc JF, Hubner RA, Chiu CF, Schwartsmann G, Siveke JT, Braiteh F, Moyo V, Belanger B, Dhindsa N, Bayever E, Von Hoff DD, Chen LT; NAPOLI-1 Study Group. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global, randomised, open-label, phase 3 trial. *Lancet* 2016; **387**: 545-557 [PMID: 26615328 DOI: 10.1016/S0140-6736(15)00986-1]

24 **Kieler M**, Unseld M, Bianconi D, Scheithauer W, Prager GW. A real-world analysis of second-line treatment options in pancreatic cancer: liposomal-irinotecan plus 5-fluorouracil and folinic acid. *Ther Adv Med Oncol* 2019; **11**: 1758835919853196 [PMID: 31360237 DOI: 10.1177/1758835919853196]

25 **Yoo C**, Im HS, Kim KP, Oh DY, Lee KH, Chon HJ, Kim JH, Kang M, Kim I, Lee GJ, Oh SY, Choi Y, Choi HJ, Kim ST, Park JO, Ryoo BY. Real-world efficacy and safety of liposomal irinotecan plus fluorouracil/Leucovorin in patients with metastatic pancreatic adenocarcinoma: a study by the Korean Cancer Study Group. *Ther Adv Med Oncol* 2019; **11**: 1758835919871126 [PMID: 31489036 DOI:

10.1177/1758835919871126]
26 **Associazione Italiana di Oncologia Medica**. Carcinoma del pancreas esocrino. [cited 17 January 2021]. In: Associazione Italiana di Oncologia Medica [Internet]. Available from: https://www.aiom.it/wp-content/uploads/2020/10/2020_LG_AIOM_Pancreas.pdf
27 **Assaf E**, Verlinde-Carvalho M, Delbaldo C, Grenier J, Sellam Z, Pouessel D, Bouaita L, Baumgaertner I, Sobhani I, Tayar C, Paul M, Culine S. 5-fluorouracil/Leucovorin combined with irinotecan and oxaliplatin (FOLFIRINOX) as second-line chemotherapy in patients with metastatic pancreatic adenocarcinoma. *Oncology* 2011; **80**: 301-306 [PMID: 21778770 DOI: 10.1159/000329803]
28 **Lee MG**, Lee SH, Lee SJ, Lee YS, Hwang JH, Ryu JK, Kim YT, Kim DU, Woo SM. 5-Fluorouracil/Leucovorin combined with irinotecan and oxaliplatin (FOLFIRINOX) as second-line chemotherapy in patients with advanced pancreatic cancer who have progressed on gemcitabine-based therapy. *Chemotherapy* 2013; **59**: 273-279 [PMID: 24457620 DOI: 10.1159/000356158]
29 **Chung MJ**, Kang H, Kim HG, Hyun JJ, Lee JK, Lee KH, Noh MH, Kang DH, Lee SH, Bang S; Pancreatobiliary Cancer Study Group of Korean Society of Gastrointestinal Cancer. Multicenter phase II trial of modified FOLFIRINOX in gemcitabine-refractory pancreatic cancer. *World J Gastrointest Oncol* 2018; **10**: 505-515 [PMID: 30595804 DOI: 10.4251/wjgo.v10.i12.505]
30 **Kobayashi N**, Shimamura T, Tokuhisa M, Goto A, Endo I, Ichikawa Y. Effect of FOLFIRINOX as second-line chemotherapy for metastatic pancreatic cancer after gemcitabine-based chemotherapy failure. *Medicine (Baltimore)* 2017; **96**: e6769 [PMID: 28489753 DOI: 10.1097/MD.0000000000006769]
31 **Tezuka S**, Ueno M, Kobayashi S, Morimoto M, Nagashima S, Sano Y, Asama H, Kawano K, Tanaka S, Fukushima T. Modified FOLFIRINOX *vs* sequential chemotherapy (FOLFIRI/FOLFOX) as second-line treatment for advanced pancreatic adenocarcinoma: A real-world analysis. *J Clin Oncol* 2020; **38**: 711 [DOI: 10.1200/JCO.2020.38.4_suppl.711]
32 **Pelzer U**, Stieler J, Roll L, Hilbig A, Dörken B, Riess H, Oettle H. Second-line therapy in refractory pancreatic cancer. results of a phase II study. *Onkologie* 2009; **32**: 99-102 [PMID: 19295247 DOI: 10.1159/000197769]
33 **Tsavaris N**, Kosmas C, Skopelitis H, Gouveris P, Kopterides P, Loukeris D, Sigala F, Zorbala-Sypsa A, Felekouras E, Papalambros E. Second-line treatment with oxaliplatin, leucovorin and 5-fluorouracil in gemcitabine-pretreated advanced pancreatic cancer: A phase II study. *Invest New Drugs* 2005; **23**: 369-375 [PMID: 16012797 DOI: 10.1007/s10637-005-1446-y]
34 **Yoo C**, Hwang JY, Kim JE, Kim TW, Lee JS, Park DH, Lee SS, Seo DW, Lee SK, Kim MH, Han DJ, Kim SC, Lee JL. A randomised phase II study of modified FOLFIRI.3 *vs* modified FOLFOX as second-line therapy in patients with gemcitabine-refractory advanced pancreatic cancer. *Br J Cancer* 2009; **101**: 1658-1663 [PMID: 19826418 DOI: 10.1038/sj.bjc.6605374]
35 **Pelzer U**, Schwaner I, Stieler J, Adler M, Seraphin J, Dörken B, Riess H, Oettle H. Best supportive care (BSC) *vs* oxaliplatin, folinic acid and 5-fluorouracil (OFF) plus BSC in patients for second-line advanced pancreatic cancer: a phase III-study from the German CONKO-study group. *Eur J Cancer* 2011; **47**: 1676-1681 [PMID: 21565490 DOI: 10.1016/j.ejca.2011.04.011]
36 **Oettle H**, Riess H, Stieler JM, Heil G, Schwaner I, Seraphin J, Görner M, Mölle M, Greten TF, Lakner V, Bischoff S, Sinn M, Dörken B, Pelzer U. Second-line oxaliplatin, folinic acid, and fluorouracil *vs* folinic acid and fluorouracil alone for gemcitabine-refractory pancreatic cancer: outcomes from the CONKO-003 trial. *J Clin Oncol* 2014; **32**: 2423-2429 [PMID: 24982456 DOI: 10.1200/JCO.2013.53.6995]
37 **Gill S**, Ko YJ, Cripps C, Beaudoin A, Dhesy-Thind S, Zulfiqar M, Zalewski P, Do T, Cano P, Lam WYH, Dowden S, Grassin H, Stewart J, Moore M. PANCREOX: A Randomized Phase III Study of Fluorouracil/Leucovorin With or Without Oxaliplatin for Second-Line Advanced Pancreatic Cancer in Patients Who Have Received Gemcitabine-Based Chemotherapy. *J Clin Oncol* 2016; **34**: 3914-3920 [PMID: 27621395 DOI: 10.1200/JCO.2016.68.5776]
38 **Pointet AL**, Tougeron D, Pernot S, Pozet A, Béchade D, Trouilloud I, Lourenco N, Hautefeuille V, Locher C, Williet N, Desrame J, Artru P, Soularue E, Le Roy B, Taieb J. Three fluoropyrimidine-based regimens in routine clinical practice after nab-paclitaxel plus gemcitabine for metastatic pancreatic cancer: An AGEO multicenter study. *Clin Res Hepatol Gastroenterol* 2020; **44**: 295-301 [PMID: 31607641 DOI: 10.1016/j.clinre.2019.08.009]
39 **Foschini F**, Napolitano F, Servetto A, Marciano R, Mozzillo E, Carratù AC, Santaniello A, De Placido P, Cascetta P, Butturini G, Frigerio I, Regi P, Silvestris N, Delcuratolo S, Vasile E, Vivaldi C, Bianco C, De Placido S, Formisano L, Bianco R. FOLFIRINOX after first-line gemcitabine-based chemotherapy in advanced pancreatic cancer: a retrospective comparison with FOLFOX and FOLFIRI schedules. *Ther Adv Med Oncol* 2020; **12**: 1758835920947970 [PMID: 33062062 DOI: 10.1177/1758835920947970]
40 **Catalano M**, Conca R, Petrioli R, Ramello M, Roviello G. FOLFOX *vs* FOLFIRI as Second-line of Therapy After Progression to Gemcitabine/Nab-paclitaxel in Patients with Metastatic Pancreatic Cancer. *Cancer Manag Res* 2020; **12**: 10271-10278 [PMID: 33116881 DOI: 10.2147/CMAR.S267393]
41 **Lee K**, Bang K, Yoo C, Hwang I, Jeong JH, Chang HM, Oh D, Song TJ, Park DH, Lee SS, Lee SK, Kim MH, Park JH, Kim KP, Ryoo BY. Clinical Outcomes of Second-Line Chemotherapy after Progression on Nab-Paclitaxel Plus Gemcitabine in Patients with Metastatic Pancreatic Adenocarcinoma. *Cancer Res Treat* 2020; **52**: 254-262 [PMID: 31291709 DOI:

10.4143/crt.2019.190]
42 **Gebbia V**, Maiello E, Giuliani F, Borsellino N, Caruso M, Di Maggio G, Ferraù F, Bordonaro R, Verderame F, Tralongo P, Di Cristina L, Agueli R, Russo P, Colucci G. Second-line chemotherapy in advanced pancreatic carcinoma: a multicenter survey of the Gruppo Oncologico Italia Meridionale on the activity and safety of the FOLFOX4 regimen in clinical practice. *Ann Oncol* 2007; **18** Suppl 6: vi124-vi127 [PMID: 17591805 DOI: 10.1093/annonc/mdm240]
43 **Rahma OE**, Duffy A, Liewehr DJ, Steinberg SM, Greten TF. Second-line treatment in advanced pancreatic cancer: a comprehensive analysis of published clinical trials. *Ann Oncol* 2013; **24**: 1972-1979 [PMID: 23670093 DOI: 10.1093/annonc/mdt166]
44 **Nagrial AM**, Chin VT, Sjoquist KM, Pajic M, Horvath LG, Biankin AV, Yip D. Second-line treatment in inoperable pancreatic adenocarcinoma: A systematic review and synthesis of all clinical trials. *Crit Rev Oncol Hematol* 2015; **96**: 483-497 [PMID: 26481952 DOI: 10.1016/j.critrevonc.2015.07.007]
45 **Wainberg ZA**, Feeney K, Lee MA, Muñoz A, Gracián AC, Lonardi S, Ryoo BY, Hung A, Lin Y, Bendell J, Hecht JR. Meta-analysis examining overall survival in patients with pancreatic cancer treated with second-line 5-fluorouracil and oxaliplatin-based therapy after failing first-line gemcitabine-containing therapy: effect of performance status and comparison with other regimens. *BMC Cancer* 2020; **20**: 633 [PMID: 32641104 DOI: 10.1186/s12885-020-07110-x]
46 **Chiorean EG**, Von Hoff DD, Tabernero J, El-Maraghi R, Wee Ma W, Reni M, Harris M, Whorf R, Liu H, Shiansong Li J, Manax V, Romano A, Lu B, Goldstein D. Second-line therapy after nab-paclitaxel plus gemcitabine or after gemcitabine for patients with metastatic pancreatic cancer. *Br J Cancer* 2016; **115**: e13 [PMID: 27657342 DOI: 10.1038/bjc.2016.306]
47 **Zaanan A**, Trouilloud I, Markoutsaki T, Gauthier M, Dupont-Gossart AC, Lecomte T, Aparicio T, Artru P, Thirot-Bidault A, Joubert F, Fanica D, Taieb J. FOLFOX as second-line chemotherapy in patients with pretreated metastatic pancreatic cancer from the FIRGEM study. *BMC Cancer* 2014; **14**: 441 [PMID: 24929865 DOI: 10.1186/1471-2407-14-441]
48 **Berk V**, Ozdemir N, Ozkan M, Aksoy S, Turan N, Inal A, Balakan O, Yasar N, Unal OU, Benekli M, Durnali A, Colak D, Sonmez OU. XELOX vs. FOLFOX4 as second line chemotherapy in advanced pancreatic cancer. *Hepatogastroenterology* 2012; **59**: 2635-2639 [PMID: 22534542 DOI: 10.5754/hge12181]
49 **Zaniboni A**, Aitini E, Barni S, Ferrari D, Cascinu S, Catalano V, Valmadre G, Ferrara D, Veltri E, Codignola C, Labianca R. FOLFIRI as second-line chemotherapy for advanced pancreatic cancer: a GISCAD multicenter phase II study. *Cancer Chemother Pharmacol* 2012; **69**: 1641-1645 [PMID: 22576338 DOI: 10.1007/s00280-012-1875-1]
50 **Neuzillet C**, Hentic O, Rousseau B, Rebours V, Bengrine-Lefèvre L, Bonnetain F, Lévy P, Raymond E, Ruszniewski P, Louvet C, Hammel P. FOLFIRI regimen in metastatic pancreatic adenocarcinoma resistant to gemcitabine and platinum-salts. *World J Gastroenterol* 2012; **18**: 4533-4541 [PMID: 22969226 DOI: 10.3748/wjg.v18.i33.4533]
51 **Sonbol MB**, Firwana B, Wang Z, Almader-Douglas D, Borad MJ, Makhoul I, Ramanathan RK, Ahn DH, Bekaii-Saab T. Second-line treatment in patients with pancreatic ductal adenocarcinoma: A meta-analysis. *Cancer* 2017; **123**: 4680-4686 [PMID: 28817187 DOI: 10.1002/cncr.30927]
52 **Citterio C**, Baccini M, Orlandi E, Di Nunzio C, Cavanna L. Second-line chemotherapy for the treatment of metastatic pancreatic cancer after first-line gemcitabine-based chemotherapy: a network meta-analysis. *Oncotarget* 2018; **9**: 29801-29809 [PMID: 30038721 DOI: 10.18632/oncotarget.25639]
53 **Petrelli F**, Inno A, Ghidini A, Rimassa L, Tomasello G, Labianca R, Barni S; GISCAD (Gruppo Italiano per lo Studio dei Carcinomi dell'Apparato Digerente) and Cremona Hospital. Second line with oxaliplatin- or irinotecan-based chemotherapy for gemcitabine-pretreated pancreatic cancer: A systematic review. *Eur J Cancer* 2017; **81**: 174-182 [PMID: 28633088 DOI: 10.1016/j.ejca.2017.05.025]
54 **Pishvaian MJ**, Blais EM, Brody JR, Lyons E, DeArbeloa P, Hendifar A, Mikhail S, Chung V, Sahai V, Sohal DPS, Bellakbira S, Thach D, Rahib L, Madhavan S, Matrisian LM, Petricoin EF 3rd. Overall survival in patients with pancreatic cancer receiving matched therapies following molecular profiling: a retrospective analysis of the Know Your Tumor registry trial. *Lancet Oncol* 2020; **21**: 508-518 [PMID: 32135080 DOI: 10.1016/S1470-2045(20)30074-7]
55 **Sohal DPS**, Kennedy EB, Cinar P, Conroy T, Copur MS, Crane CH, Garrido-Laguna I, Lau MW, Johnson T, Krishnamurthi S, Moravek C, O'Reilly EM, Philip PA, Pant S, Shah MA, Sahai V, Uronis HE, Zaidi N, Laheru D. Metastatic Pancreatic Cancer: ASCO Guideline Update. *J Clin Oncol* 2020; JCO2001364 [PMID: 32755482 DOI: 10.1200/JCO.20.01364]
56 **Moore MJ**, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W; National Cancer Institute of Canada Clinical Trials Group. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960-1966 [PMID: 17452677 DOI: 10.1200/JCO.2006.07.9525]
57 **Wang JP**, Wu CY, Yeh YC, Shyr YM, Wu YY, Kuo CY, Hung YP, Chen MH, Lee WP, Luo JC, Chao Y, Li CP. Erlotinib is effective in pancreatic cancer with epidermal growth factor receptor mutations: a randomized, open-label, prospective trial. *Oncotarget* 2015; **6**: 18162-18173 [PMID: 26046796 DOI: 10.18632/oncotarget.4216]

58 **Wang Y**, Hu GF, Zhang QQ, Tang N, Guo J, Liu LY, Han X, Wang X, Wang ZH. Efficacy and safety of gemcitabine plus erlotinib for locally advanced or metastatic pancreatic cancer: a systematic review and meta-analysis. *Drug Des Devel Ther* 2016; **10**: 1961-1972 [PMID: 27358556 DOI: 10.2147/DDDT.S105442]

59 **Vaishnavi A**, Le AT, Doebele RC. TRKing down an old oncogene in a new era of targeted therapy. *Cancer Discov* 2015; **5**: 25-34 [PMID: 25527197 DOI: 10.1158/2159-8290.CD-14-0765]

60 **Drilon A**. TRK inhibitors in TRK fusion-positive cancers. *Ann Oncol* 2019; **30**: viii23-viii30 [DOI: 10.1093/annonc/mdz282]

61 **Doebele RC**, Drilon A, Paz-Ares L, Siena S, Shaw AT, Farago AF, Blakely CM, Seto T, Cho BC, Tosi D, Besse B, Chawla SP, Bazhenova L, Krauss JC, Chae YK, Barve M, Garrido-Laguna I, Liu SV, Conkling P, John T, Fakih M, Sigal D, Loong HH, Buchschacher GL Jr, Garrido P, Nieva J, Steuer C, Overbeck TR, Bowles DW, Fox E, Riehl T, Chow-Maneval E, Simmons B, Cui N, Johnson A, Eng S, Wilson TR, Demetri GD; trial investigators. Entrectinib in patients with advanced or metastatic NTRK fusion-positive solid tumours: integrated analysis of three phase 1-2 trials. *Lancet Oncol* 2020; **21**: 271-282 [PMID: 31838007 DOI: 10.1016/S1470-2045(19)30691-6]

62 **Hong DS**, DuBois SG, Kummar S, Farago AF, Albert CM, Rohrberg KS, van Tilburg CM, Nagasubramanian R, Berlin JD, Federman N, Mascarenhas L, Geoerger B, Dowlati A, Pappo AS, Bielack S, Doz F, McDermott R, Patel JD, Schilder RJ, Tahara M, Pfister SM, Witt O, Ladanyi M, Rudzinski ER, Nanda S, Childs BH, Laetsch TW, Hyman DM, Drilon A. Larotrectinib in patients with TRK fusion-positive solid tumours: a pooled analysis of three phase 1/2 clinical trials. *Lancet Oncol* 2020; **21**: 531-540 [PMID: 32105622 DOI: 10.1016/S1470-2045(19)30856-3]

63 **Royal RE**, Levy C, Turner K, Mathur A, Hughes M, Kammula US, Sherry RM, Topalian SL, Yang JC, Lowy I, Rosenberg SA. Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. *J Immunother* 2010; **33**: 828-833 [PMID: 20842054 DOI: 10.1097/CJI.0b013e3181eec14c]

64 **Brahmer JR**, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012; **366**: 2455-2465 [PMID: 22658128 DOI: 10.1056/NEJMoa1200694]

65 **Aglietta M**, Barone C, Sawyer MB, Moore MJ, Miller WH Jr, Bagalà C, Colombi F, Cagnazzo C, Gioeni L, Wang E, Huang B, Fly KD, Leone F. A phase I dose escalation trial of tremelimumab (CP-675,206) in combination with gemcitabine in chemotherapy-naive patients with metastatic pancreatic cancer. *Ann Oncol* 2014; **25**: 1750-1755 [PMID: 24907635 DOI: 10.1093/annonc/mdu205]

66 **Kamath SD**, Kalyan A, Kircher S, Nimeiri H, Fought AJ, Benson A 3rd, Mulcahy M. Ipilimumab and Gemcitabine for Advanced Pancreatic Cancer: A Phase Ib Study. *Oncologist* 2020; **25**: e808-e815 [PMID: 31740568 DOI: 10.1634/theoncologist.2019-0473]

67 **Sunami Y**, Häußler J, Kleeff J. Cellular Heterogeneity of Pancreatic Stellate Cells, Mesenchymal Stem Cells, and Cancer-Associated Fibroblasts in Pancreatic Cancer. *Cancers (Basel)* 2020; **12** [PMID: 33333727 DOI: 10.3390/cancers12123770]

68 **Torphy RJ**, Zhu Y, Schulick RD. Immunotherapy for pancreatic cancer: Barriers and breakthroughs. *Ann Gastroenterol Surg* 2018; **2**: 274-281 [PMID: 30003190 DOI: 10.1002/ags3.12176]

69 **Rosenberg A**, Mahalingam D. Immunotherapy in pancreatic adenocarcinoma-overcoming barriers to response. *J Gastrointest Oncol* 2018; **9**: 143-159 [PMID: 29564181 DOI: 10.21037/jgo.2018.01.13]

70 **U**. S. Food and Drug Administration. FDA grants accelerated approval to pembrolizumab for first tissue/site agnostic indication. [cited 10 January 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-grants-accelerated-approval-pembrolizumab-first-tissuesite-agnostic-indication

71 **Drilon A**, Nagasubramanian R, Blake JF, Ku N, Tuch BB, Ebata K, Smith S, Lauriault V, Kolakowski GR, Brandhuber BJ, Larsen PD, Bouhana KS, Winski SL, Hamor R, Wu WI, Parker A, Morales TH, Sullivan FX, DeWolf WE, Wollenberg LA, Gordon PR, Douglas-Lindsay DN, Scaltriti M, Benayed R, Raj S, Hanusch B, Schram AM, Jonsson P, Berger MF, Hechtman JF, Taylor BS, Andrews S, Rothenberg SM, Hyman DM. A Next-Generation TRK Kinase Inhibitor Overcomes Acquired Resistance to Prior TRK Kinase Inhibition in Patients with TRK Fusion-Positive Solid Tumors. *Cancer Discov* 2017; **7**: 963-972 [PMID: 28578312 DOI: 10.1158/2159-8290.CD-17-0507]

72 **Drilon A**, Ou SI, Cho BC, Kim DW, Lee J, Lin JJ, Zhu VW, Ahn MJ, Camidge DR, Nguyen J, Zhai D, Deng W, Huang Z, Rogers E, Liu J, Whitten J, Lim JK, Stopatschinskaja S, Hyman DM, Doebele RC, Cui JJ, Shaw AT. Repotrectinib (TPX-0005) Is a Next-Generation ROS1/TRK/ALK Inhibitor That Potently Inhibits ROS1/TRK/ALK Solvent- Front Mutations. *Cancer Discov* 2018; **8**: 1227-1236 [PMID: 30093503 DOI: 10.1158/2159-8290.CD-18-0484]

73 **Rainone M**, Singh I, Salo-Mullen EE, Stadler ZK, O'Reilly EM. An Emerging Paradigm for Germline Testing in Pancreatic Ductal Adenocarcinoma and Immediate Implications for Clinical Practice: A Review. *JAMA Oncol* 2020; **6**: 764-771 [PMID: 32053139 DOI: 10.1001/jamaoncol.2019.5963]

74 **Aung KL**, Fischer SE, Denroche RE, Jang GH, Dodd A, Creighton S, Southwood B, Liang SB, Chadwick D, Zhang A, O'Kane GM, Albaba H, Moura S, Grant RC, Miller JK, Mbabaali F, Pasternack D, Lungu IM, Bartlett JMS, Ghai S, Lemire M, Holter S, Connor AA, Moffitt RA, Yeh JJ, Timms L, Krzyzanowski PM, Dhani N, Hedley D, Notta F, Wilson JM, Moore MJ, Gallinger S, Knox JJ. Genomics-Driven Precision Medicine for Advanced Pancreatic Cancer: Early Results from the

COMPASS Trial. *Clin Cancer Res* 2018; **24**: 1344-1354 [PMID: 29288237 DOI: 10.1158/1078-0432.CCR-17-2994]

75 **Janssen B**, Bellis S, Koller T, Tischkowitz M, Liau SS. A systematic review of predicted pathogenic PALB2 variants: an analysis of mutational overlap between epithelial cancers. *J Hum Genet* 2020; **65**: 199-205 [PMID: 31619740 DOI: 10.1038/s10038-019-0680-7]

76 **Villarroel MC**, Rajeshkumar NV, Garrido-Laguna I, De Jesus-Acosta A, Jones S, Maitra A, Hruban RH, Eshleman JR, Klein A, Laheru D, Donehower R, Hidalgo M. Personalizing cancer treatment in the age of global genomic analyses: PALB2 gene mutations and the response to DNA damaging agents in pancreatic cancer. *Mol Cancer Ther* 2011; **10**: 3-8 [PMID: 21135251 DOI: 10.1158/1535-7163.MCT-10-0893]

77 **Lowery MA**, Kelsen DP, Capanu M, Smith SC, Lee JW, Stadler ZK, Moore MJ, Kindler HL, Golan T, Segal A, Maynard H, Hollywood E, Moynahan M, Salo-Mullen EE, Do RKG, Chen AP, Yu KH, Tang LH, O'Reilly EM. Phase II trial of veliparib in patients with previously treated BRCA-mutated pancreas ductal adenocarcinoma. *Eur J Cancer* 2018; **89**: 19-26 [PMID: 29223478 DOI: 10.1016/j.ejca.2017.11.004]

78 **Kaufman B**, Shapira-Frommer R, Schmutzler RK, Audeh MW, Friedlander M, Balmaña J, Mitchell G, Fried G, Stemmer SM, Hubert A, Rosengarten O, Steiner M, Loman N, Bowen K, Fielding A, Domchek SM. Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. *J Clin Oncol* 2015; **33**: 244-250 [PMID: 25366685 DOI: 10.1200/JCO.2014.56.2728]

79 **Domchek SM**, Hendifar AE, McWilliams RR, Geva R, Epelbaum R, Biankin A, Vonderheide RH, Wolff RA, Alberts SR, Giordano H, Goble S, Lin KK, Shroff RT. RUCAPANC: An open-label, phase 2 trial of the PARP inhibitor rucaparib in patients (pts) with pancreatic cancer (PC) and a known deleterious germline or somatic BRCA mutation. *J Clin Oncol* 2016; **34** [DOI: 10.1200/JCO.2016.34.15_suppl.4110]

80 **Fakih M**, O'Neil B, Price TJ, Falchook GS, Desai J, Kuo, J Govindan R, Rasmussen E, Morrow PKH, Ngang J, Henary HA, Hong DS. Phase 1 study evaluating the safety, tolerability, pharmacokinetics (PK), and efficacy of AMG 510, a novel small molecule KRASG12C inhibitor, in advanced solid tumors. *J Clin Oncol* 2019; **37** [DOI: 10.1200/JCO.2019.37.15_suppl.3003]

81 **Gillson J**, Ramaswamy Y, Singh G, Gorfe AA, Pavlakis N, Samra J, Mittal A, Sahni S. Small Molecule KRAS Inhibitors: The Future for Targeted Pancreatic Cancer Therapy? *Cancers (Basel)* 2020; **12** [PMID: 32456277 DOI: 10.3390/cancers12051341]

82 **Baghdadi TA**, Halabi S, Garrett-Mayer E, Mangat PK, Ahn ER, Sahai V, Alvarez RH, Kim ES, Yost KJ, Rygiel AL, Antonelli KR, Butler NL, Bruinooge SS, Schilsky RL. Palbociclib in Patients With Pancreatic and Biliary Cancer With CDKN2A Alterations: Results From the Targeted Agent and Profiling Utilization Registry Study. *JCO Precis Oncol* 2019; **3**: 1-8

83 **García-Reyes B**, Kretz AL, Ruff JP, von Karstedt S, Hillenbrand A, Knippschild U, Henne-Bruns D, Lemke J. The Emerging Role of Cyclin-Dependent Kinases (CDKs) in Pancreatic Ductal Adenocarcinoma. *Int J Mol Sci* 2018; **19** [PMID: 30340359 DOI: 10.3390/ijms19103219]

84 **Ebrahimi S**, Hosseini M, Shahidsales S, Maftouh M, Ferns GA, Ghayour-Mobarhan M, Hassanian SM, Avan A. Targeting the Akt/PI3K Signaling Pathway as a Potential Therapeutic Strategy for the Treatment of Pancreatic Cancer. *Curr Med Chem* 2017; **24**: 1321-1331 [PMID: 28176634 DOI: 10.2174/0929867324666170206142658]

85 **Baer R**, Cintas C, Therville N, Guillermet-Guibert J. Implication of PI3K/Akt pathway in pancreatic cancer: When PI3K isoforms matter? *Adv Biol Regul* 2015; **59**: 19-35 [PMID: 26166735 DOI: 10.1016/j.jbior.2015.05.001]

86 **Murthy D**, Attri KS, Singh PK. Phosphoinositide 3-Kinase Signaling Pathway in Pancreatic Ductal Adenocarcinoma Progression, Pathogenesis, and Therapeutics. *Front Physiol* 2018; **9**: 335 [PMID: 29670543 DOI: 10.3389/fphys.2018.00335]

87 **Herbertz S**, Sawyer JS, Stauber AJ, Gueorguieva I, Driscoll KE, Estrem ST, Cleverly AL, Desaiah D, Guba SC, Benhadji KA, Slapak CA, Lahn MM. Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-beta signaling pathway. *Drug Des Devel Ther* 2015; **9**: 4479-4499 [PMID: 26309397 DOI: 10.2147/DDDT.S86621]

88 **Schlingensiepen KH**, Jaschinski F, Lang SA, Moser C, Geissler EK, Schlitt HJ, Kielmanowicz M, Schneider A. Transforming growth factor-beta 2 gene silencing with trabedersen (AP 12009) in pancreatic cancer. *Cancer Sci* 2011; **102**: 1193-1200 [PMID: 21366804 DOI: 10.1111/j.1349-7006.2011.01917.x]

89 **Melisi D**, Hollebecque A, Oh DY, Calvo E, Varghese AM, Borazanci EH, Mercade TM, Simionato F, Park JO, Bendell JC, Faivre SJ, Zhao Y, Gueorguieva I, Man M, Estrem S, Benhadji KA, Lanasa M, Guba SC, Garcia-Carbonero R. A phase Ib dose-escalation and cohort-expansion study of safety and activity of the transforming growth factor (TGF) β receptor I kinase inhibitor galunisertib plus the anti-PD-L1 antibody durvalumab in metastatic pancreatic cancer. *J Clin Oncol* 2019; **37**: 4124

90 **Alistar A**, Morris BB, Desnoyer R, Klepin HD, Hosseinzadeh K, Clark C, Cameron A, Leyendecker J, D'Agostino Jr R, Topaloglu U, Boteju LW, Boteju AR, Shorr R, Zachar Z, Bingham PM, Ahmed T, Crane S, Shah R, Migliano JJ, Pardee TS, Miller L, Hawkins G, Jin G, Zhang W, Pasche B. Safety and tolerability of the first-in-class agent CPI-613 in combination with modified FOLFIRINOX in patients with metastatic pancreatic cancer: a single-centre, open-label, dose-escalation, phase 1 trial. *Lancet Oncol* 2017; **18**: 770-778 [DOI: 10.1016/S1470-2045(17)30314-5]

91 **Deplanque G**, Demarchi M, Hebbar M, Flynn P, Melichar B, Atkins J, Nowara E, Moyé L, Piquemal D, Ritter D, Dubreuil P, Mansfield CD, Acin Y, Moussy A, Hermine O, Hammel P. A randomized,

placebo-controlled phase III trial of masitinib plus gemcitabine in the treatment of advanced pancreatic cancer. *Ann Oncol* 2015; **26**: 1194-1200 [PMID: 25858497 DOI: 10.1093/annonc/mdv133]

92 **Middleton G**, Palmer DH, Greenhalf W, Ghaneh P, Jackson R, Cox T, Evans A, Shaw VE, Wadsley J, Valle JW, Propper D, Wasan H, Falk S, Cunningham D, Coxon F, Ross P, Madhusudan S, Wadd N, Corrie P, Hickish T, Costello E, Campbell F, Rawcliffe C, Neoptolemos JP. Vandetanib plus gemcitabine *vs* placebo plus gemcitabine in locally advanced or metastatic pancreatic carcinoma (ViP): a prospective, randomised, double-blind, multicentre phase 2 trial. *Lancet Oncol* 2017; **18**: 486-499 [PMID: 28259610 DOI: 10.1016/S1470-2045(17)30084-0]

93 **O'Reilly EM**, Oh DY, Dhani N, Renouf DJ, Lee MA, Sun W, Fisher G, Hezel A, Chang SC, Vlahovic G, Takahashi O, Yang Y, Fitts D, Philip PA. Durvalumab With or Without Tremelimumab for Patients With Metastatic Pancreatic Ductal Adenocarcinoma: A Phase 2 Randomized Clinical Trial. *JAMA Oncol* 2019 [PMID: 31318392 DOI: 10.1001/jamaoncol.2019.1588]

94 **Looi CK**, Chung FF, Leong CO, Wong SF, Rosli R, Mai CW. Therapeutic challenges and current immunomodulatory strategies in targeting the immunosuppressive pancreatic tumor microenvironment. *J Exp Clin Cancer Res* 2019; **38**: 162 [PMID: 30987642 DOI: 10.1186/s13046-019-1153-8]

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REVIEW

Non-alcoholic fatty liver and chronic kidney disease: Retrospect, introspect, and prospect

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Abstract

With the growing prevalence of obesity and diabetes in the United States and across the world, a rise in the overall incidence and prevalence of non-alcoholic fatty liver disease (NAFLD) is expected. The risk factors for NAFLD are also associated with the development of chronic kidney disease (CKD). We review the epidemiology, risk factors, genetics, implications of gut dysbiosis, and specific pathogenic mechanisms linking NAFLD to CKD. Mechanisms such as ectopic lipid accumulation, cellular signaling abnormalities, and the interplay between fructose consumption and uric acid accumulation have led to the emergence of potential therapeutic implications for this patient population. Transplant evaluation in the setting of both NAFLD and CKD is also reviewed. Potential strategies for surveillance and management include the monitoring of comorbidities, the use of non-invasive fibrosis scoring systems, and the measurement of laboratory markers. Lastly, we discuss the management of patients with NAFLD and CKD, from preventative measures to experimental interventions.

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Core Tip: Patients with non-alcoholic fatty liver disease (NAFLD) are at higher risk for the development of chronic kidney disease (CKD) than the general population. The prevalence of mutual comorbidities in addition to direct pathogenic mechanisms linking NAFLD to the development of CKD can explain this finding. With the breadth of data linking NAFLD to CKD, there are minimal options for treating this patient population. Regardless, we have presented strategies that can be implemented at various levels including surveillance, preventative, and management level.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of chronic liver disease ranging from steatosis on one end to fibrosis and cirrhosis on the other end[1]. NAFLD and non-alcoholic steatohepatitis (NASH) are the hepatic manifestations of metabolic syndrome (MetS), which is a driving force for a multitude of comorbidities, such as insulin resistance, cardiovascular disease (CVD), chronic kidney disease (CKD), obstructive sleep apnea (OSA), as well as increased malignancy risk[2]. While NASH is the second leading indication for liver transplantation (LT), it is expected that NASH will overtake hepatitis C virus (HCV) as the leading cause, given the efficacy of direct-acting antiviral therapy[3]. A recent epidemiological study has already confirmed a downward trend for HCV-related LT in the United States[4].

NAFLD is tightly linked to underlying insulin resistance and is associated with other comorbidities related to MetS[5]. Growing evidence suggests that NAFLD is a risk factor for CKD[6] due to shared metabolic risk factors[7]. Of note, several studies have shown an association between the severity of NASH and CKD[8-11]. Interestingly, a meta-analysis of 33 studies showed that diabetes status and metabolic risk factors had no impact on the positive correlation between the severity of NASH and CKD[12], suggesting a possible unique pathogenic link between NAFLD and CKD irrespective of their shared metabolic risk factors. We review the genetic, epidemiologic, and pathogenic links between NAFLD and CKD in addition to potential preventative and management strategies.

PREVALENCE OF CKD IN NAFLD AND POTENTIAL PREDISPOSING RISK FACTORS

Two meta-analyses and a retrospective cohort analysis suggest that the incidence and prevalence of CKD increase in patients with NAFLD compared to patients without NAFLD (Table 1). In all analyses, the magnitude and direction of effects remained unaffected by diabetes status, even after adjustment for other risk factors[12-14]. Moreover, the association was stronger in patients with advanced fibrosis or decompensated cirrhosis as compared to compensated cirrhosis. The studies that were included in these major meta-analyses defined advanced fibrosis was defined by histological parameters, imaging findings, and/or elevations in the NAFLD fibrosis score (NFS). Of note, among 42 studies included in these two meta-analyses, only 13 (n = 2205) utilized liver histology, which is the gold standard in diagnosing NAFLD[15]. The majority of the studies established diagnosis of NAFLD *via* abdominal ultrasound, liver enzyme elevation [including serum gamma-glutamyl transferase (GGT)

Table 1 Incidence and prevalence of chronic kidney disease in patients with varying degrees of non-alcoholic fatty liver disease severity

Ref.	Year	*n*	NAFLD diagnostic modalities	Conclusion(s)
Musso *et al*[12]. A meta-analysis of 33 studies	2014	63902	Liver biopsy, abdominal ultrasound, elevated liver enzymes	(1) 20 cross-sectional studies: Nearly two-fold increased risk of CKD in patients with NAFLD (OR 2.12, 95%CI 1.69-2.66); (2) 11 longitudinal studies: 1.8-fold increased risk of CKD in patients with NAFLD (HR 1.79, 95%CI 1.65–1.95); and (3) advanced fibrosis associated with increased prevalence (OR 5.20, 95%CI 3.14-8.61) and incidence (HR 3.29, 95%CI 2.30-4.71) of CKD in patients with NAFLD
Mantovani *et al*[13]. A meta-analysis of 9 studies	2018	96595	Abdominal ultrasound; FLI; serum GGT	Incidence of CKD: (1) 1.4-fold increased long-term risk (HR 1.37, 95%CI 1.20–1.53) in patients with NAFLD with a median follow-up period of 5.2 years; and (2) 1.5-fold increased risk (HR 1.50, 95%CI 1.25-1.74) in patients with severe NAFLD (defined as NFS ≥ -1.455 or serum GGT ≥ 109 U/L)
Park *et al*[14]. Retrospective Cohort with Propensity Score Matching (1:3)	2019	262619	ICD-9	Incidence of CKD: 1.4-fold increased risk (aHR 1.41; 95%CI, 1.36-1.46) in patients with NAFLD after adjusting for demographics, baseline covariates, and ACEi/ARB use; Risk of incident CKD increases as the severity of NAFLD increases: (1) compensated cirrhosis (aHR, 1.47; 95%CI 1.36-1.59); and (2) decompensated cirrhosis (aHR, 2.28; 95%CI 2.12-2.46)

NAFLD: Non-alcoholic fatty liver disease; CKD: Chronic kidney disease; HR: Hazard ratio; FLI: Fatty liver index; GGT: Gamma glutamyl transferase; NFS: NAFLD fibrosis score; CI: Confidence interval; OR: Odds ratio; ACEi: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin receptor blocker; ICD-9: International classification of disease-9.

elevation], or using international classification of disease-9 code.

Previous review articles estimated that the prevalence of CKD was 20% to 55% in patients with NAFLD, whereas the prevalence of CKD in patients without NAFLD was 5% to 30%[16,17]. However, most of these reviews evaluated the same pool of data[8,9,11,18-22], which were also included in the two meta-analyses mentioned above. Our conclusions were based on studies that were published before 2015 as several more recent studies did not use histology or imaging for NAFLD diagnosis[23-26].

Many non-hepatic and hepatic risk factors are associated with CKD in those with NAFLD.

Non-hepatic risk factors

There is minimal data on non-hepatic risk factors to predict which patients will go on to develop CKD. However, there are a few studies outlined below to identify which patients may be at higher risk (Table 2).

Smoking

Current cigarette smoking is associated with CKD or death from end-stage renal disease. Mainstream cigarette smoke includes over 4000 compounds, and nicotine is one of many biologically stable and active compounds present in tobacco. Nicotine causes kidney damage by modulating α7nAChR, NLRP6 inflammasome, ER stress, and autophagy[27,28]. Studies examining the relationship between smoking and NAFLD are lacking; however, in a cohort study of 1525 CKD patients who underwent repeated health check-up examinations over 10 years, the decline in estimated glomerular filtration rate (eGFR) associated with NAFLD was greater in current smokers, hypertensive patients, or those with lower eGFR at baseline had greater age- and sex-adjusted decline in eGFR[29].

Diabetes

Around one-third of patients with NAFLD have impaired renal function and its prevalence in patients with NAFLD is dependent on the severity of liver disease and presence of diabetes mellitus[30]. The development of NAFLD in patients with diabetes appears to be an important event in its natural history predisposing these patients to a higher risk for developing CKD. Type 2 diabetes mellitus (T2DM) increases the risk of serious NASH and advanced fibrosis in patients with NAFLD[31,32]. Patients with T2DM or type 1 diabetes mellitus and NAFLD are at an increased risk of developing CKD compared to diabetics without NAFLD[20,33,34]. Despite accumulating evidence for NAFLD as a driver for CKD, the shared common risk factors make it difficult to isolate diabetes as an independent risk factor for CKD in NAFLD patients.

Table 2 Summary of studies assessing non-hepatic risk factors for chronic kidney disease in patients with non-alcoholic fatty liver disease

Ref.	Risk factor(s)	Year	*n*	Comparison	Findings
Önnerhag *et al*[147]	Older age	2019	120	Biopsy-proven NAFLD *vs* non-NAFLD	Higher prevalence of CKD in patients ≥ 55 years old
Targher *et al*[20]	Diabetes mellitus	2008	2103	NAFLD and T2DM *vs* T2DM only	Patients with NAFLD and T2DM independently associated with increased risk of CKD (OR 1.87; 95%CI 1.3-4.1, P = 0.020)
Targher *et al*[33]	Diabetes mellitus	2010	301	NAFLD and T1DM *vs* T1DM only	Patients with NAFLD and T1DM independently associated with increased risk of CKD
Jang *et al*[29]	Elevated baseline eGFR, HTN, and current smoking	2018	1525	NAFLD *vs* Non-NAFLD	The decline in eGFR associated with NAFLD appeared to be stronger among patients who were current smokers, hypertensive, and lower eGFR at baseline

NAFLD: Non-alcoholic fatty liver disease; CKD: Chronic kidney disease; T2DM: Type 2 diabetes mellitus; OR: Odds ratio; CI: Confidence interval; T1DM: Type 1 Diabetes Mellitus; eGFR: Estimated glomerular filtration rate; HTN: Hypertension.

Hypothyroid

Proper thyroid function is implicated in renal blood flow, glomerular and tubular function, electrolyte homeostasis, hepatic lipid metabolism, and fatty acid beta-oxidation[35]. Hypothyroidism can cause NAFLD through fat accumulation, while hyperthyroid can cause NAFLD through reactive oxygen species formation[36]. Additionally, the prevalence of hypothyroidism increases for each 10 mL/min/1.73 m^2 decrement in eGFR[37], and patients with hypothyroidism were more than 2 times likely to have NAFLD and 4 times more likely to have NASH[38].

Hepatic risk factors

NAFLD-related advanced fibrosis: Patients with NAFLD-related advanced fibrosis are more likely to have CKD compared to patients with NAFLD but without advanced fibrosis[39]. The risk of albuminuria increases with the severity of NAFLD-related advanced fibrosis, according to a 2017 study of 1763 Chinese diabetic patients[40]. After adjusting for common CKD risk factors such as diabetes and other metabolic comorbidities, advanced fibrosis but not steatosis was associated with a higher risk of albuminuria (OR: 1.52; 95%CI: 1.02-2.28; P = 0.039). In a 2019 study of 594 patients with T2DM, significant liver fibrosis as detected by elastography (LSM ≥ 7.0/6.2 kPa) was independently associated with a higher risk of CKD (adjusted OR: 3.6, 95%CI: 1.3-10.1; P = 0.01) in addition to CVD and other microvascular complications[41]. Increased liver stiffness as detected by transient elastography is a predictor of CKD in patients with ultrasound-diagnosed NAFLD[42]

In a 12-year prospective cohort, patients with non-obese NAFLD had a higher risk of developing CKD than patients with obese NAFLD[43]. A recent study has noted that the risk of developing CKD is higher in metabolically unhealthy non-obese NAFLD patients than their counterparts with metabolically healthy status defined by the lack of metabolic risk factors (*i.e.* diabetes mellitus, low High-density lipoprotein, hypertriglyceridemia, arterial hypertension)[44].

Pathophysiology: CKD secondary to fatty liver is thought to be due to systemic low-grade inflammation[45], which may involve upregulation of the nuclear factor-κB (NF-κB) pathway[45,46]. As discussed earlier, there is circumstantial evidence to suggest that patients with NASH-related advanced fibrosis have an increased prevalence of CKD. Progression of NASH may be partly mediated by the altered renin-angiotensin-aldosterone system due to CKD has also been proposed as a mechanism for NAFLD progression[47]. Although direct pathogenic links between NAFLD and CKD seem to be confounded by common metabolic comorbidities, novel mechanisms have been described.

Insulin resistance: Increased adiposity leads to increased free fatty acids and pro-inflammatory cytokine release that causes systemic insulin resistance (IR), which is an established mediator of NAFLD. IR is further exacerbated by the progression of NAFLD, leading to atherogenic dyslipidemia and further release of inflammatory cytokines resulting in CKD as shown in animal models[48]. Proinflammation occurs through the NF-κB and Jun-N-terminal kinase (JNK) pathways; activation of adipose-specific JNK pathways has been shown to cause insulin resistance[48,49]. As NAFLD

progresses to NASH, the inflammatory component is neutrophil-predominant and can cause systemic endothelial dysfunction (Figure 1)[50,51]. Notably, IR leads to increased production of very-low-density lipoprotein and endoplasmic reticulum stress, both of which can cause podocyte damage in glomeruli[52]. These latter two mechanisms have been linked to proteinuria and subsequent hastening of CKD[53,54].

Ectopic lipid accumulation: In animal models, a high fat/fructose diet resulted in increased urinary albumin excretion, elevated transaminases, and increased incidence of liver tumors when compared to a standard diet. Microscopically, lipid deposition leads to accelerated hepatorenal pathologies, suggesting that intracellular lipid accumulation may link NAFLD to CKD[55]. When treated with fenofibrate, slower intracellular lipid accumulation was noted in co-incidence with slower progression of renal and hepatic pathologies[55].

Wnt signaling abnormalities: Alterations in cellular pathways critical for homeostasis play an important role in the development of CKD in patients with NAFLD. Specifically, abnormalities in the Wnt (named as a fusion of the Drosophila gene wingless and its vertebrate homolog, integrated) signaling pathway have been linked to lipid accumulation, chronic inflammation, and fibrosis in the development of both NAFLD and CKD[56].

Sterol regulatory element-binding proteins: Sterol regulatory-element binding proteins are activated in a nutrient-rich (*i.e.*, anabolic) state that leads to insulin-signaling and increased endoplasmic reticulum stress, which can cause increased lipogenesis and hepatosteatosis. These changes cause the progression of other metabolic phenomena such as CKD and MetS[57].

Fructose consumption and uric acid accumulation: Fructose intake has been linked to hepatorenal injury *via* uric acid accumulation by altering the gut microbiome (Figure 2)[45,58]. Patients with a normal body mass index (BMI) and elevated serum uric acid levels (> 10 mg/dL) have an increased prevalence of MetS when compared to patients with a serum uric acid < 6 mg/dL[59], which is corroborated by other studies[60-62]. An increase in serum uric acid levels is also associated with an increase in the incidence of NAFLD[63]. In patients with NAFLD, elevated uric acid levels are known to be pathogenic in CKD progression[42,64]. These studies suggest that MetS, NAFLD, and CKD are interconnected through elevated serum uric acid levels[65].

Uric acid stimulates fructokinase, which sensitizes hepatocytes to fructose metabolism, subsequently leading to fat deposition in the liver, thereby explaining the link between elevated uric acid and NAFLD[66]. Elevated uric acid levels in animal models lead to glomerular hypertension and tubulointerstitial fibrosis, two processes that preclude the development of CKD[64]. Decreased urate clearance in CKD patients may further exacerbate this pathology. Interestingly, xanthine oxidase inhibitors are currently being tested in patients with CKD to monitor for disease progression in the CKD-FIX[67].

Gut dysbiosis: Changes in the gut microbiome play a role in the pathogenesis of NAFLD and CKD[45]. Dietary conditions such as increased fructose intake and vitamin D deficiency are shown to cause dysbiosis, which may directly lead to low-grade inflammation responsible for the development of NAFLD and CKD[45]. Dysbiosis and subsequent microbial fermentation lead to increased production of uremic toxins indoxyl sulfate and p-cresyl sulfate, which correlate directly with the progression of CKD[68]. The liver cytochrome P450 enzymes are directly regulated by these uremic toxins derived from alterations in gut microbial metabolism, hence the gut-liver-kidney axis[69]. Animal models have also shown the gut microbiota's ability to metabolize choline into trimethylamine N-oxide (TMAO), which is considered both nephrotoxic and hepatotoxic. In a 2015 study comparing TMAO levels in patients with CKD (n = 521) to healthy patients (n = 3166), median TMAO levels among CKD patients were significantly higher (P < 0.001)[70]. Similarly, a 2019 case-control study comparing patients with NAFLD (n = 34) to those without (n = 14) showed that TMAO has a role in aggravating liver steatosis[71]. Lastly, certain species in the gut microbiota produce short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate and diffuse through gut mucosa, which can disrupt the integrity of the intestinal barrier. In the bloodstream, SCFAs can cause systemic inflammation, the common pathogenic link between NAFLD and CKD[45].

Genetic links between NAFLD and CKD: Two gene variants associated with both CKD and NAFLD are the G allele of the *patatin-like phospholipase domain-containing* (

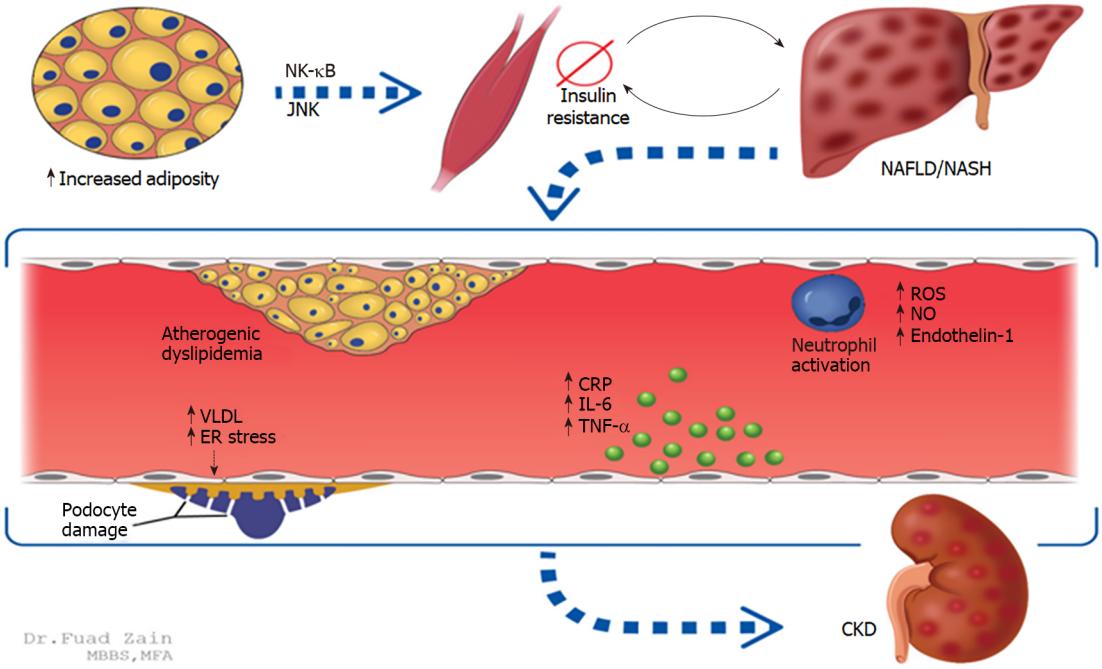


Figure 1 Two established mechanisms between non-alcoholic fatty liver disease and the development of chronic kidney disease are increased adiposity and insulin resistance. NF-κB: Nuclear factor-κB; JNK: Jun N-terminal kinases; NAFLD/NASH: Non-alcoholic fatty liver disease/Non-alcoholic steatohepatitis; ROS: Reactive oxygen species; NO: Nitric oxide; CRP: C-reactive protein; IL-6: Interleukin-6; VLDL: Very low-density lipoprotein; TNF-α: Tumor necrosis factor alpha; CKD: Chronic kidney disease

PNPLA3) gene and the T allele of the *transmembrane 6 superfamily member 2* (*TM6SF2*) gene. The G allele in the rs738409 polymorphism of the *PNPLA3* gene has been shown to play a major role in the progression of NASH[72,73]. Patients with the G allele also have been shown to have lower eGFR, increased incidence of microalbuminuria, and increased prevalence of CKD, regardless of NAFLD/NASH status[74,75]. The patient population that was found to have the highest risk of CKD and NAFLD in a 2015 study were patients who carried the G allele of the *PNPLA3*; furthermore, these patients were not obese, which is an important risk factor for CKD[75]. Another study showed that Chinese patients with normal alanine aminotransferase levels who carried the rs738409 polymorphism in the *PNPLA3* gene were at risk for early glomerular and tubular damage, which could explain why these patients develop CKD even in the absence of well-known risk factors, such as obesity or diabetes[76]. In postmenopausal women with T2DM, having the G/G allele leads to a higher prevalence of CKD, regardless of NAFLD status, further supporting the argument that this polymorphism may be an independent predictor for CKD[77]. Patients who are found to have the G/G allele in the polymorphism rs738409 should have close monitoring for the development of NAFLD as well as renal dysfunction, even in normal-weight patients[75]. On the other hand, the rs58542926 polymorphism on the *TM6SF2* gene, also known as the T allele of the *TMS6F2* gene, has been associated with the development of NAFLD[78] but has also been associated with a higher eGFR and lower prevalence of microalbuminuria[74]. Thus, this specific polymorphism in *TM6SF2* may be nephroprotective in patients with NAFLD.

Identifying NAFLD patients at risk for progression of CKD

Role of non-invasive fibrosis scoring systems: Non-invasive scoring systems are utilized in assessing the severity of various chronic liver diseases. However, they have also been shown in several studies to be useful in predicting CKD in patients with NAFLD (Table 3). Incremental increases in the fatty liver index are an independent risk factor for developing CKD in a 10-year prospective analysis of 6238 adults (age 40-69 years) without CKD at baseline[23]. In another study of 11376 Taiwanese subjects, the NFS was negatively correlated with eGFR[6]. Multiple studies show that patients who have an intermediate and high-risk category of fibrosis-4 index (FIB-4)-index and NFS are at an increased risk of CKD[79,80], while a 2019 cross-sectional study of 11836

Table 3 Summary of studies assessing non-invasive scoring systems for advanced fibrosis to assess risk for chronic kidney disease in patients with nonalcoholic fatty liver disease

Ref.	Year	*n*	Scoring system(s) assessed	Results
Ciardullo *et al*[82]	2020	2770	APRI, FIB-4, FLI, NFS	NAFLD-related fibrosis as measured with FIB-4 associated with CKD ($P < 0.01$)
Hsieh *et al*[6]	2020	11376	NFS	Higher NFS associated with impaired eGFR ($P < 0.0001$)
Choi *et al*[81]	2019	11836	APRI, BARD, FIB-4, FLI	FIB-4 ($P = 0.0258$) most precise in predicting kidney dysfunction
Önnerhag *et al*[79]	2019	144	APRI, BARD, NFS, FIB-4	High-risk NFS ($P < 0.001$), FIB-4 ($P < 0.001$), APRI ($P = 0.008$) predict CKD
Wijarnpreecha *et al*[80]	2018	4142	APRI, BARD, NFS, FIB-4	High/intermediate probability of liver fibrosis on NFS (AUC = 0.75) and FIB-4 (AUC = 0.77) independently predict CKD
Huh *et al*[23]	2017	6238	FLI	NAFLD cut-off for NAFLD is an independent RF for CKD ($P < 0.0001$)

NFS: Nonalcoholic fatty liver disease fibrosis score; FIB-4: Fibrosis-4 index; APRI: Aspartate aminotransferase to platelet ratio index; FLI: Fatty liver index; CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; AUC: Area under the curve; RF: Risk factor; BARP: Biologically-oriented Alveolar Ridge Preservation; NAFLD: Nonalcoholic fatty liver disease.

patients showed that FIB-4 is the most precise tool when estimating renal dysfunction attributable to NAFLD (area under the curve = 0.6227, 95%CI: 0.5929-0.6526, $P = 0.0258$) after adjusting for various demographic and clinical variables[81]. FIB-4 is the most superior predictor in other studies as well[80,82]. In summary, patients with NAFLD-related fibrosis are at increased risk for CKD, and these patients should undergo proper surveillance *via* non-invasive fibrosis scoring systems and/or advanced imaging techniques (*i.e.* Fibroscan, TE) (Figure 3).

Cystatin C: Serum creatinine, a widely used biomarker in assessing renal function, is inaccurate in determining GFR in patients with cirrhosis[83]. This is due to muscle wasting that occurs in cirrhosis, thus leading to diminished creatinine formation, increased tubular secretion of creatinine, and impaired assay interpretation caused by elevated bilirubin[83]. Alternatively, the measurement of cystatin C does not have the same limitations as serum creatinine due to its low molecular weight and because it does not require adjustment for gender, mass, or bilirubin level[84]. A combination of serum creatinine and cystatin C is more accurate in determining GFR than serum creatinine alone[85]. However, serum creatinine alone is superior for patients without cirrhosis[85]. Measurement of cystatin C in addition to serum creatinine may have utility for accurately assessing renal function in transplant candidates and for monitoring the development of CKD in patients with NASH cirrhosis. Although the cost of measuring eGFR using Cystatin C in addition to serum creatinine is higher, the burden of over-diagnosing CKD in patients with cirrhosis is lessened, which may lead to an overall reduction in unnecessary medical expenses for patients with cirrhosis who truly have CKD[86].

Alkaline phosphatase and GGT: In diabetic patients with NAFLD, serum alkaline phosphatase (ALP), a NAFLD-associated marker when elevate, was also significantly associated with impaired renal function[87,88]. Interestingly, ALP is associated with the release of proinflammatory cytokines from the liver that are known to disrupt the glomerular endothelial glycocalyx, leading to albuminuria, which may explain why ALP is a potential surveillance marker in patients with NAFLD who are at risk for developing CKD[89]. Furthermore, elevated serum GGT is associated with an increased risk of CKD[24,90,91]. GGT is associated with increased inflammatory markers and insulin resistance, both of which play central roles in patients with NAFLD who develop CKD[24,92]. However, elevated GGT may not be an accurate CKD parameter in Caucasian men, as GGT is confounded by BMI, lifestyle factors, and lipids, as noted in a 2017 study[25]. Therefore, elevated GGT in Caucasian men with NAFLD should be interpreted with caution when monitoring for CKD. Of importance, NAFLD was diagnosed by elevated GGT levels (in addition to ultrasound in only one study[91]; therefore, these findings may not apply to patients diagnosed by more

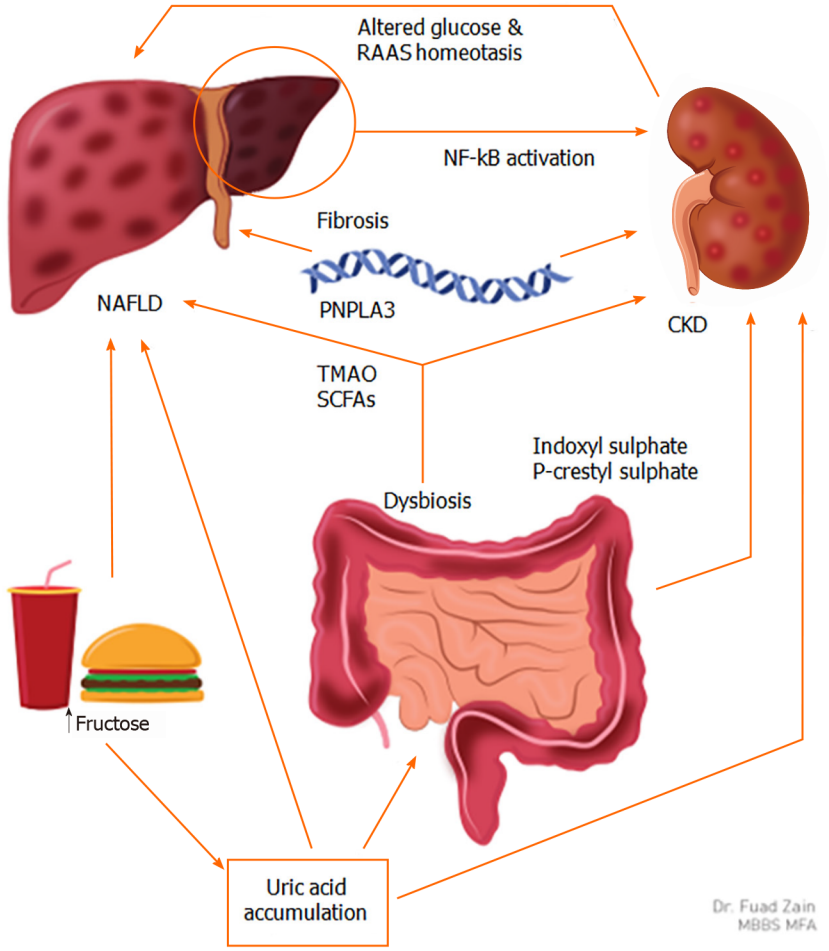


Figure 2 Fructose consumption and uric acid accumulation play a key role in patients with non-alcoholic fatty liver disease who develop chronic kidney disease. TMAO: Trimethylamine N-oxide; SCFAs: Short-chain fatty acids; RAAS: Renin-angiotensin-aldosterone system; PNPLA3: Patatin-like phospholipase domain-containing protein 3; NAFLD: Non-alcoholic fatty liver disease; CKD: Chronic kidney disease; NF-κB: Nuclear factor-κB.

invasive parameters (*i.e.* liver biopsy).

Managing the progression of CKD

Surveillance of comorbidities: In general, we recommend patients with diabetes and NAFLD undergo frequent surveillance for underlying kidney dysfunction, more so than patients with diabetes only. Monitoring thyrotropin and thyroid hormone levels may have clinical utility when evaluating the risk of developing CKD in patients with NAFLD; however, future studies are needed to specifically address the risk of CKD in patients with NAFLD and hypothyroidism (Table 4).

Body weight

Waist-to-hip ratio: Few studies have evaluated the impact of weight loss on the progression of CKD in patients with NAFLD. Recent studies have shown that a decrease in the waist-to-hip ratio (WHR) in patients with NAFLD decreases the risk of CKD development[43]. Serial monitoring of WHR may be beneficial in identifying patients with NAFLD at risk for CKD. A drawback to this finding is that a reduction in WHR does not differentiate between a reduction in visceral fat *vs* subcutaneous fat. Studies have shown that visceral fat, but not subcutaneous fat, is the key driver in NAFLD pathogenesis *via* increased insulin resistance[93]. However, with regards to reducing the risk of CKD, the significance of reducing visceral *vs* subcutaneous fat is not well-studied.

Weight loss: Data from a post-hoc analysis of a clinical trial involving 261 patients with NAFLD showed a statistically significant relationship between reduction in body weight and changes in eGFR (when calculated by CKD-Epidemiology collaboration and modification of diet in renal disease equations), even after adjusting for

Table 4 Summary of Interventions for patients with nonalcoholic fatty liver disease and chronic kidney disease

Intervention	Ref.	Year	*n*	Findings	Recommendation
Decreasing WHR	Chon *et al*[43]. 12-yr prospective cohort	2020	6137	A decrease in the WHR of more than 5% in patients with NAFLD leads to a significantly reduced risk of CKD development, even in non-obese patients	Serial Monitoring WHR may be beneficial in identifying patients with NAFLD at risk of developing CKD and reduction can ameliorate the progression
Weight loss	Vilar-Gomez *et al*[94]. Post-hoc analysis	2017	261	Improvement in liver histology due to weight loss linked to improved renal outcomes, even after adjusting for medication profile, diabetes, and hypertension	Advocate for weight loss
SGLT2 Inhibitors	Shimizu *et al*[96]. RCT	2019	57	SGLT inhibitor (Dapagliflozin) improved liver steatosis in patients with T2DM and NAFLD and attenuates liver fibrosis in patients with NAFLD-related advanced fibrosis	Although data is not sufficient, consider using SGLT2 inhibitors in T2DM patients with NAFLD and CKD
	Perkovic *et al*[95]. CREDENCE trial	2019	4401	SGLT2 inhibitor (Canagliflozin) decreased the risk of renal failure in patients with T2DM and CKD	
GLP-1	Armstrong *et al*[100]. LEAN trial	2016	52	Liraglutide led to weight loss, glycemic control, and histological resolution of NASH	GLP-1's in NASH is considered effective in improving components of MetS, however, long-term studies are needed to determine NASH-related outcomes
	Tuttle *et al*[101]. AWARD-7 trial	2018	577	Once-weekly dulaglutide is associated with reduced decline in eGFR, while being as effective as insulin in achieving glycemic control	GLP-1 is a safe option for patients with CKD and is associated with slower progression of CKD
Coenzyme Q10	Farhangi *et al*[109] and Farsi *et al*[110]. RCT	2014[109] and 2016[110]	44[109] and 41[110]	100 mg of oral CoQ10/d improve biochemical variables of NAFLD after 4 wk[109] and 12 wk[110] of treatment	Due to lack of data in patients with both NAFLD and CKD, the benefit of CoQ10 supplementation is unknown; however, in separate trials with regards to both NAFLD and CKD, CoQ10 supplementation is beneficial
	Yeung *et al*[111]. RCT	2015	15	Oral CoQ10 supplementation in patients with CKD showed significant improvement in serum creatinine when compared to placebo	

WHR: Waist-to-hip ratio; NAFLD: Nonalcoholic fatty liver disease; CKD: Chronic kidney disease; SGLT2: Sodium-glucose co-transporter-2; RCT: Randomized controlled trial; T2DM: Type 2 Diabetes Mellitus; GLP-1: Glucagon-like peptide receptor agonist; NASH: Non-alcoholic steatohepatitis.

medication profile, diabetes, and hypertension[94]. Additionally, patients with improvement in liver histology due to lifestyle modifications such as weight loss were linked with significantly improved renal outcomes[94]**.** Overall, patients with NAFLD who had more than 5% weight loss and/or more than a 5% reduction in WHR had improved renal outcomes.

Sodium-glucose cotransporter type-2 inhibitors: In patients with T2DM, sodium-glucose cotransporter type-2 (SGLT2) inhibitors have an established role in improving glycemic control, weight loss, cardiovascular outcomes, and lowering serum uric acid levels. In patients with type 2 diabetes, the landmark CREDENCE trial showed that patients treated with an SGLT2 inhibitor (*i.e.*, canagliflozin) were shown to have improved outcomes related to CKD[95]. Furthermore, recent evidence has shown that SGLT2 inhibitors can also improve NAFLD progression as determined by TE[96] and biomarkers in NAFLD (*i.e.*, liver enzymes)[96,97]. As SGLT2 inhibitors decrease serum uric acid levels, this may also contribute to this class's positive effects on both diseases. In addition to facilitating glucosuria, SGLT2 inhibitors are thought to decrease inflammation and reactive oxygen species formation[98], which is key in the pathogenesis of NAFLD and NASH[99].

Glucagon-like peptide 1 receptor agonists: Among its multiple mechanisms of action, glucagon-like peptide 1 **(**GLP-1)'s aid in increasing insulin secretion, delaying gastric

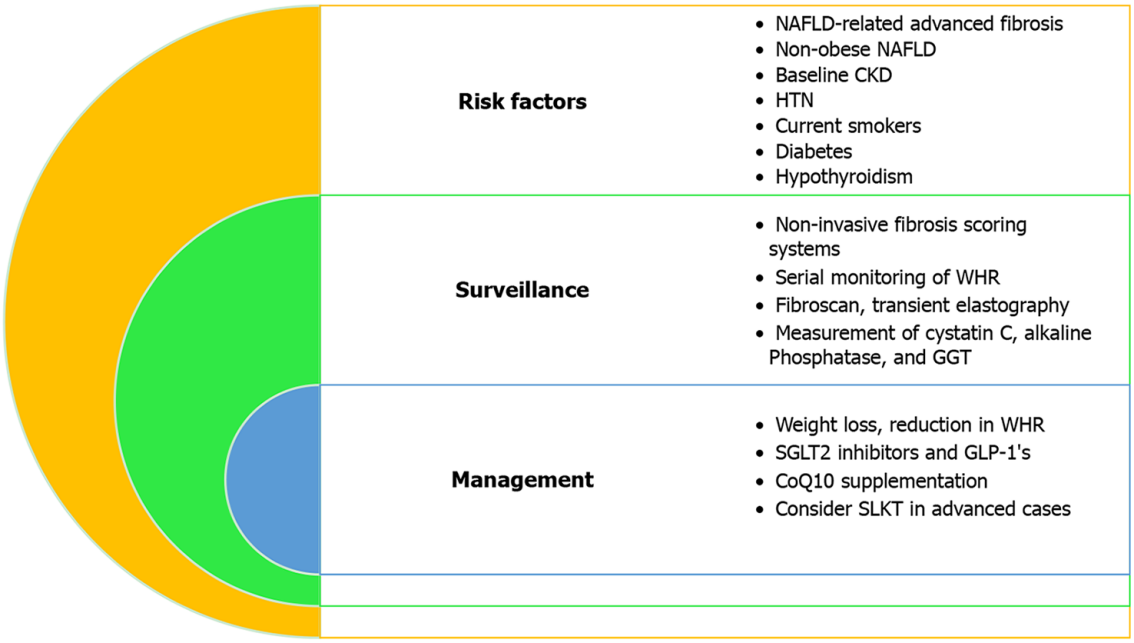


Figure 3 Identifying and managing non-alcoholic fatty liver disease patients who are at risk for developing chronic kidney disease.
NAFLD: Non-alcoholic fatty liver disease; CKD: Chronic kidney disease; HTN: Hypertension; WHR: Waist-to-Hip ratio; GGT: Gamma-glutamyl transferase; T2DM: Type 2 diabetes mellitus; SLKT: Simultaneous liver-kidney transplantation; SGLT2: Sodium-glucose cotransporter type-2; GLP-1: Glucagon-like peptide 1.

emptying, and decreasing appetite, all of which can lead to improved glycemic control and weight loss. Additionally, a possible anti-inflammatory mechanism makes GLP-1's an attractive agent in NAFLD and NASH. For instance, GLP-1 Liraglutide, when compared to placebo, led to histological resolution of NASH; however, larger studies are still needed[100]. In CKD, GLP-1's are shown to be nephroprotective, which could be due to GLP-1's ability to lower blood pressure in addition to the aforementioned mechanisms[101,102]. GLP-1's and SGLT2 inhibitors exhibit cardioprotective effects, and as discussed previously, patients with NAFLD and CKD are at high risk for CV events. Therefore, the use of these agents is recommended in patients with NAFLD and CKD. However, while there is landmark data to support the use of GLP-1's and SGLT2 inhibitors to prevent CV events in patients with established CVD, data on primary prevention in patients with NAFLD and CKD is lacking[103,104]. Regardless, in patients with T2DM, CKD, and NAFLD, SGLT2 inhibitors or GLP-1's are highly recommended not only for glycemic control but for the cardio-, hepato-, and nephro-protective effects as well.

Coenzyme Q10: Coenzyme Q10 (CoQ10) is produced endogenously and has antioxidant and anti-inflammatory effects[105]. CoQ10 also serves as an electron carrier in cellular respiration and a cofactor in pyrimidine synthesis for DNA repair and replication, among other important roles. Patients with NAFLD, CKD, and/or CVD have been reported to have CoQ10 deficiency[106]. A majority of endogenous CoQ10 is produced in the liver, and patients with NAFLD had diminished CoQ10 production[106,107]. CoQ10 deficiency will lead to oxidative stress, which plays a key pathogenic factor in NAFLD[108]. Results from separate trials assessing oral CoQ10 supplementation in patients with NAFLD and CKD are summarized in Table 4. Briefly, CoQ10 has been shown to improve NAFLD parameters and CKD parameters in separate trials[109-111]. Specific findings are summarized in Table 4. CoQ10 has positive effects on the progression of CVD as well, which is notable because patients with CKD and NAFLD are at risk for cardiovascular events[112,113]. Supplementation may be beneficial in patients with NAFLD who have CKD, but clinical data in this population is lacking.

Experimental interventions

Thiazolidinediones: Thiazolidinediones are agonists of peroxisome proliferator-activated receptors (PPARs), and they play a physiologic role in metabolism and cellular differentiation. PPARs have proven clinical utility in diseases such as hyperlipidemia (PPARα) and T2DM (PPARγ)[114]. Because CKD is a manifestation of a

metabolic and/or inflammatory process, the use of PPAR agonists has been studied in patients with CKD. Specifically, pioglitazone, a PPARγ agonist, has been shown to improve cardiovascular outcomes in patients with CKD and diabetes[115]. Several RCTs have shown the beneficial effects that pioglitazone has on histopathology and metabolic function in patients with NASH[116-120]. Pioglitazone has been endorsed as a pharmacological agent in biopsy-proven NASH by the American Association for the Study of Liver Diseases[121]. Rosiglitazone has been shown to improve histological components of NASH through increasing insulin sensitivity[122] while also improving liver function[123] in a separate study, although both studies did not show improvements in liver fibrosis[122,123]. Interestingly, an extension trial showed that rosiglitazone was only beneficial in the first year of treatment, without substantial benefit noted with longer use[124]. However, Rosiglitazone is not available in most countries and its use is limited in the United States due to data concerning for increased coronary events. The most widely studied PPAR agonist, Pioglitazone has shown favorable outcomes in patients with CKD and patients with NAFLD, but data assessing the efficacy in patients with both CKD and NAFLD is lacking[114].

Vitamin D: Vitamin D deficiency is associated with increased severity of NAFLD[125] and is also associated with CKD[126]. These findings may be explained by the physiology of vitamin D activation, which requires hydroxylation by both the kidney and liver, and therefore the presence of CKD and NAFLD inevitably leads to vitamin D resistance[58]. Furthermore, experimental models have demonstrated the role hypovitaminosis D plays in the pathogenesis of both CKD and NAFLD[58]. In patients with CKD, therapeutic implications of higher vitamin D supplementation showed an ability to correct hypovitaminosis D[127], but a meta-analysis yielded a higher incidence of hypercalcemia[128]. In patients with NAFLD, vitamin D supplementation did not correct hypovitaminosis D[129], however, trials are underway for assessing the use of Vitamin D supplementation in CKD and NAFLD/NASH[130-132] (NCT00893451, NCT01623024, and NCT02098317, www.clinicaltrials.gov).

Probiotics: In rodent models, fecal microbiota transplantation[133], antibiotics in fructose-fed models[134] reduced NAFLD severity, whereas specific probiotics (*Lactobacillaceae* or *Bifidobacteriales*) alleviated proteinuria and reduced systemic inflammation in rodents with CKD. While much of this data is based on studies from animal models, human trials are needed to further evaluate the therapeutic implications of the gut-liver-kidney axis.

LT for patients with NAFLD/NASH and CKD

In recent decades, NASH has become more prevalent and will become the most common indication for LT[135]. Patients with NASH have a higher incidence of CKD compared with other etiologies, and therefore, NASH is rapidly growing as a cause for not only LT[136,137] but also simultaneous liver-kidney transplantation (SLKT) in the United States given serum creatinine and dialysis status are important components of the model for end-stage liver disease (MELD) score[138]. Considering the increased incidence of renal dysfunction at LT due to prioritization based on the MELD allocation system in the United States, SLKT rates climbed from 2.7% of all LT in 2000 to 9.3% in 2016[138-140]. NASH is currently the leading and most rapidly growing indication for SLKT in the United States[138,140] with a 200% increase for SLKT from 2002 to 2010[138]. Patients with NASH have a high probability to undergo SLKT rather than LT alone since they are highly incident for CKD for a prolonged duration, which can fulfill criteria for SLKT (patients with CKD: GFR ≤ 60 mL/min for ≥ 3 mo with recent GFR ≤ 30 mL/min or on hemodialysis, patients with AKI: Dialysis for > 6 wk GFR ≤ 25 for > 6 wk)[141].

Patients with NASH were independently associated with a higher risk of CKD or advanced kidney damage after LT compared with those without NASH[142-144]. In general, renal dysfunction after LT is affected not only by immunosuppressant medications, especially calcineurin inhibitors, pre-LT kidney dysfunction, but also persistent or de novo metabolic co-morbidities such as hypertension, diabetes, and obesity - all of which are highly likely in NASH patients undergoing LT[142,145]. Special attention to the recognition of CKD is needed for patients with NASH patients when deciding LT *vs* SLKT. Controlling for metabolic complications and avoiding or keeping a low dose of calcineurin inhibitors as much as possible seems to be crucially important to reduce the risk of incident CKD, and risk of progression of CKD after liver transplant in NASH patients.

CONCLUSION

Despite the breadth of research, minimal guideline-based management of patients with both NAFLD and CKD is available. However, important pathogenic links and shared risk factors between NAFLD and CKD underscore the importance of earlier surveillance and strict control of shared metabolic risk factors. Although preventative strategies for CKD in NAFLD are limited, treatment directed specifically for NASH in the future will hopefully ameliorate the progression of renal dysfunction in affected patients. There is a plethora of clinical trials underway, and if these drugs show safety and efficacy in improving NASH, they may translate into improving renal function[146]. Specific interventions for preventing CKD progression using SGLT2 inhibitors, PPAR agonists, SAM, XO inhibitors, and Vitamin D have been tried but need further confirmation. Progression from NAFLD to NAFLD-related advanced fibrosis is linked to an increased risk of CKD, and earlier intervention in those with renal dysfunction is warranted. Genetic links between NAFLD and CKD have also been proposed, specifically in the G allele of *PNPLA3* and the T allele of *TM6SF2*, and future studies targeting patients with such genetic profiles to prevent progression to CKD is needed.

REFERENCES

1 **Satapathy SK**, Sanyal AJ. Epidemiology and Natural History of Nonalcoholic Fatty Liver Disease. *Semin Liver Dis* 2015; **35**: 221-235 [PMID: 26378640 DOI: 10.1055/s-0035-1562943]

2 **Kovalic AJ**, Cholankeril G, Satapathy SK. Nonalcoholic fatty liver disease and alcoholic liver disease: metabolic diseases with systemic manifestations. *Transl Gastroenterol Hepatol* 2019; **4**: 65 [PMID: 31620647 DOI: 10.21037/tgh.2019.08.09]

3 **Noureddin M**, Vipani A, Bresee C, Todo T, Kim IK, Alkhouri N, Setiawan VW, Tran T, Ayoub WS, Lu SC, Klein AS, Sundaram V, Nissen NN. NASH Leading Cause of Liver Transplant in Women: Updated Analysis of Indications For Liver Transplant and Ethnic and Gender Variances. *Am J Gastroenterol* 2018; **113**: 1649-1659 [PMID: 29880964 DOI: 10.1038/s41395-018-0088-6]

4 **Gadiparthi C**, Spatz M, Greenberg S, Iqbal U, Kanna S, Satapathy SK, Broder A, Ahmed A. NAFLD Epidemiology, Emerging Pharmacotherapy, Liver Transplantation Implications and the Trends in the United States. *J Clin Transl Hepatol* 2020; **8**: 215-221 [PMID: 32832402 DOI: 10.14218/JCTH.2020.00014]

5 **Byrne CD**, Targher G. NAFLD: a multisystem disease. *J Hepatol* 2015; **62**: S47-S64 [PMID: 25920090 DOI: 10.1016/j.jhep.2014.12.012]

6 **Hsieh MH**, Wu KT, Chen YY, Yang JF, Lin WY, Chang NC, Lin CY, Huang CK, Wang CL, Chuang HY, Lin SC, Hsu YK, Tsai YS, Chuang WL, Yu ML, Dai CY. Higher NAFLD fibrosis score is associated with impaired eGFR. *J Formos Med Assoc* 2020; **119**: 496-503 [PMID: 31353118 DOI: 10.1016/j.jfma.2019.07.007]

7 **Locatelli F**, Pozzoni P, Del Vecchio L. Renal manifestations in the metabolic syndrome. *J Am Soc Nephrol* 2006; **17**: S81-S85 [PMID: 16565254 DOI: 10.1681/ASN.2005121332]

8 **Yilmaz Y**, Alahdab YO, Yonal O, Kurt R, Kedrah AE, Celikel CA, Ozdogan O, Duman D, Imeryuz N, Avsar E, Kalayci C. Microalbuminuria in nondiabetic patients with nonalcoholic fatty liver disease: association with liver fibrosis. *Metabolism* 2010; **59**: 1327-1330 [PMID: 20096896 DOI: 10.1016/j.metabol.2009.12.012]

9 **Yasui K**, Sumida Y, Mori Y, Mitsuyoshi H, Minami M, Itoh Y, Kanemasa K, Matsubara H, Okanoue T, Yoshikawa T. Nonalcoholic steatohepatitis and increased risk of chronic kidney disease. *Metabolism* 2011; **60**: 735-739 [PMID: 20817213 DOI: 10.1016/j.metabol.2010.07.022]

10 **Park CW**, Tsai NT, Wong LL. Implications of worse renal dysfunction and medical comorbidities in patients with NASH undergoing liver transplant evaluation: impact on MELD and more. *Clin Transplant* 2011; **25**: E606-E611 [PMID: 21958082 DOI: 10.1111/j.1399-0012.2011.01497.x]

11 **Machado MV**, Gonçalves S, Carepa F, Coutinho J, Costa A, Cortez-Pinto H. Impaired renal function in morbid obese patients with nonalcoholic fatty liver disease. *Liver Int* 2012; **32**: 241-248 [PMID: 22098270 DOI: 10.1111/j.1478-3231.2011.02623.x]

12 **Musso G**, Gambino R, Tabibian JH, Ekstedt M, Kechagias S, Hamaguchi M, Hultcrantz R, Hagström H, Yoon SK, Charatcharoenwitthaya P, George J, Barrera F, Hafliðadóttir S, Björnsson ES, Armstrong MJ, Hopkins LJ, Gao X, Francque S, Verrijken A, Yilmaz Y, Lindor KD, Charlton M, Haring R, Lerch MM, Rettig R, Völzke H, Ryu S, Li G, Wong LL, Machado M, Cortez-Pinto H, Yasui K, Cassader M. Association of non-alcoholic fatty liver disease with chronic kidney disease: a systematic review and meta-analysis. *PLoS Med* 2014; **11**: e1001680 [PMID: 25050550 DOI: 10.1371/journal.pmed.1001680]

13 **Mantovani A**, Zaza G, Byrne CD, Lonardo A, Zoppini G, Bonora E, Targher G. Nonalcoholic fatty liver disease increases risk of incident chronic kidney disease: A systematic review and meta-analysis. *Metabolism* 2018; **79**: 64-76 [PMID: 29137912 DOI: 10.1016/j.metabol.2017.11.003]

14 **Park H**, Dawwas GK, Liu X, Nguyen MH. Nonalcoholic fatty liver disease increases risk of

incident advanced chronic kidney disease: a propensity-matched cohort study. *J Intern Med* 2019; **286**: 711-722 [PMID: 31359543 DOI: 10.1111/joim.12964]

15 **Jennison E**, Patel J, Scorletti E, Byrne CD. Diagnosis and management of non-alcoholic fatty liver disease. *Postgrad Med J* 2019; **95**: 314-322 [PMID: 31085617 DOI: 10.1136/postgradmedj-2018-136316]

16 **Corey KE**, Kartoun U, Zheng H, Shaw SY. Development and Validation of an Algorithm to Identify Nonalcoholic Fatty Liver Disease in the Electronic Medical Record. *Dig Dis Sci* 2016; **61**: 913-919 [PMID: 26537487 DOI: 10.1007/s10620-015-3952-x]

17 **Targher G**, Byrne CD. Non-alcoholic fatty liver disease: an emerging driving force in chronic kidney disease. *Nat Rev Nephrol* 2017; **13**: 297-310 [PMID: 28218263 DOI: 10.1038/nrneph.2017.16]

18 **Campos GM**, Bambha K, Vittinghoff E, Rabl C, Posselt AM, Ciovica R, Tiwari U, Ferrel L, Pabst M, Bass NM, Merriman RB. A clinical scoring system for predicting nonalcoholic steatohepatitis in morbidly obese patients. *Hepatology* 2008; **47**: 1916-1923 [PMID: 18433022 DOI: 10.1002/hep.22241]

19 **Targher G**, Bertolini L, Rodella S, Lippi G, Zoppini G, Chonchol M. Relationship between kidney function and liver histology in subjects with nonalcoholic steatohepatitis. *Clin J Am Soc Nephrol* 2010; **5**: 2166-2171 [PMID: 20724519 DOI: 10.2215/CJN.05050610]

20 **Targher G**, Bertolini L, Rodella S, Zoppini G, Lippi G, Day C, Muggeo M. Non-alcoholic fatty liver disease is independently associated with an increased prevalence of chronic kidney disease and proliferative/laser-treated retinopathy in type 2 diabetic patients. *Diabetologia* 2008; **51**: 444-450 [PMID: 18058083 DOI: 10.1007/s00125-007-0897-4]

21 **Targher G**, Pichiri I, Zoppini G, Trombetta M, Bonora E. Increased prevalence of chronic kidney disease in patients with Type 1 diabetes and non-alcoholic fatty liver. *Diabet Med* 2012; **29**: 220-226 [PMID: 21883436 DOI: 10.1111/j.1464-5491.2011.03427.x]

22 **Li G**, Shi W, Hug H, Chen Y, Liu L, Yin D. Nonalcoholic fatty liver disease associated with impairment of kidney function in nondiabetes population. *Biochem Med (Zagreb)* 2012; **22**: 92-99 [PMID: 22384523 DOI: 10.11613/bm.2012.010]

23 **Huh JH**, Kim JY, Choi E, Kim JS, Chang Y, Sung KC. The fatty liver index as a predictor of incident chronic kidney disease in a 10-year prospective cohort study. *PLoS One* 2017; **12**: e0180951 [PMID: 28738057 DOI: 10.1371/journal.pone.0180951]

24 **Shen ZW**, Xing J, Wang QL, Faheem A, Ji X, Li J, Bian WW, Jiang Z, Li XJ, Xue FZ, Liu J. Association between serum γ-glutamyltransferase and chronic kidney disease in urban Han Chinese: a prospective cohort study. *Int Urol Nephrol* 2017; **49**: 303-312 [PMID: 27704320 DOI: 10.1007/s11255-016-1429-2]

25 **Kunutsor SK**, Laukkanen JA. Gamma-glutamyltransferase and risk of chronic kidney disease: A prospective cohort study. *Clin Chim Acta* 2017; **473**: 39-44 [PMID: 28811239 DOI: 10.1016/j.cca.2017.08.014]

26 **Sinn DH**, Kang D, Jang HR, Gu S, Cho SJ, Paik SW, Ryu S, Chang Y, Lazo M, Guallar E, Cho J, Gwak GY. Development of chronic kidney disease in patients with non-alcoholic fatty liver disease: A cohort study. *J Hepatol* 2017; **67**: 1274-1280 [PMID: 28870674 DOI: 10.1016/j.jhep.2017.08.024]

27 **Zheng CM**, Lee YH, Chiu IJ, Chiu YJ, Sung LC, Hsu YH, Chiu HW. Nicotine Causes Nephrotoxicity through the Induction of NLRP6 Inflammasome and Alpha7 Nicotinic Acetylcholine Receptor. *Toxics* 2020; **8** [PMID: 33114531 DOI: 10.3390/toxics8040092]

28 **Rezonzew G**, Chumley P, Feng W, Hua P, Siegal GP, Jaimes EA. Nicotine exposure and the progression of chronic kidney disease: role of the α7-nicotinic acetylcholine receptor. *Am J Physiol Renal Physiol* 2012; **303**: F304-F312 [PMID: 22552933 DOI: 10.1152/ajprenal.00661.2011]

29 **Jang HR**, Kang D, Sinn DH, Gu S, Cho SJ, Lee JE, Huh W, Paik SW, Ryu S, Chang Y, Shafi T, Lazo M, Guallar E, Cho J, Gwak GY. Nonalcoholic fatty liver disease accelerates kidney function decline in patients with chronic kidney disease: a cohort study. *Sci Rep* 2018; **8**: 4718 [PMID: 29549269 DOI: 10.1038/s41598-018-23014-0]

30 **Nampoothiri RV**, Duseja A, Rathi M, Agrawal S, Sachdeva N, Mehta M, Dhaliwal HS, Dhiman RK, Chawla Y. Renal Dysfunction in Patients With Nonalcoholic Fatty Liver Disease is Related to the Presence of Diabetes Mellitus and Severity of Liver Disease. *J Clin Exp Hepatol* 2019; **9**: 22-28 [PMID: 30765935 DOI: 10.1016/j.jceh.2017.12.005]

31 **Bian H**, Zhu X, Xia M, Yan H, Chang X, Hu X, Pan B, Guo W, Li X, Gao X. Impact of type 2 diabetes on nonalcoholic steatohepatitis and advanced fibrosis in patients with nonalcoholic fatty liver disease. *Endocr Pract* 2020; **26**: 444-453 [PMID: 31968197 DOI: 10.4158/EP-2019-0342]

32 **Reddy YK**, Marella HK, Jiang Y, Ganguli S, Snell P, Podila PSB, Maliakkal B, Satapathy SK. Natural History of Non-Alcoholic Fatty Liver Disease: A Study With Paired Liver Biopsies. *J Clin Exp Hepatol* 2020; **10**: 245-254 [PMID: 32405181 DOI: 10.1016/j.jceh.2019.07.002]

33 **Targher G**, Bertolini L, Chonchol M, Rodella S, Zoppini G, Lippi G, Zenari L, Bonora E. Non-alcoholic fatty liver disease is independently associated with an increased prevalence of chronic kidney disease and retinopathy in type 1 diabetic patients. *Diabetologia* 2010; **53**: 1341-1348 [PMID: 20369224 DOI: 10.1007/s00125-010-1720-1]

34 **Manco M**, Ciampalini P, DeVito R, Vania A, Cappa M, Nobili V. Albuminuria and insulin resistance in children with biopsy proven non-alcoholic fatty liver disease. *Pediatr Nephrol* 2009; **24**: 1211-1217 [PMID: 19242728 DOI: 10.1007/s00467-009-1134-9]

35 **Iglesias P**, Díez JJ. Thyroid dysfunction and kidney disease. *Eur J Endocrinol* 2009; **160**: 503-515 [PMID: 19095779 DOI: 10.1530/EJE-08-0837]

36 **van Tienhoven-Wind LJ**, Dullaart RP. Low-normal thyroid function and the pathogenesis of common cardio-metabolic disorders. *Eur J Clin Invest* 2015; **45**: 494-503 [PMID: 25690560 DOI: 10.1111/eci.12423]

37 **Rhee CM**, Kalantar-Zadeh K, Streja E, Carrero JJ, Ma JZ, Lu JL, Kovesdy CP. The relationship between thyroid function and estimated glomerular filtration rate in patients with chronic kidney disease. *Nephrol Dial Transplant* 2015; **30**: 282-287 [PMID: 25246335 DOI: 10.1093/ndt/gfu303]

38 **Pagadala MR**, Zein CO, Dasarathy S, Yerian LM, Lopez R, McCullough AJ. Prevalence of hypothyroidism in nonalcoholic fatty liver disease. *Dig Dis Sci* 2012; **57**: 528-534 [PMID: 22183820 DOI: 10.1007/s10620-011-2006-2]

39 **Chen PC**, Kao WY, Cheng YL, Wang YJ, Hou MC, Wu JC, Su CW. The correlation between fatty liver disease and chronic kidney disease. *J Formos Med Assoc* 2020; **119**: 42-50 [PMID: 30876789 DOI: 10.1016/j.jfma.2019.02.010]

40 **Yeung MW**, Wong GL, Choi KC, Luk AO, Kwok R, Shu SS, Chan AW, Lau ESH, Ma RCW, Chan HL, Chan JC, Wong VW, Kong AP. Advanced liver fibrosis but not steatosis is independently associated with albuminuria in Chinese patients with type 2 diabetes. *J Hepatol* 2017 [PMID: 28989092 DOI: 10.1016/j.jhep.2017.09.020]

41 **Lombardi R**, Airaghi L, Targher G, Serviddio G, Maffi G, Mantovani A, Maffeis C, Colecchia A, Villani R, Rinaldi L, Orsi E, Pisano G, Adinolfi LE, Fargion S, Fracanzani AL. Liver fibrosis by FibroScan® independently of established cardiovascular risk parameters associates with macrovascular and microvascular complications in patients with type 2 diabetes. *Liver Int* 2020; **40**: 347-354 [PMID: 31612634 DOI: 10.1111/liv.14274]

42 **Qin S**, Wang S, Wang X, Wang J. Liver stiffness assessed by transient elastography as a potential indicator of chronic kidney disease in patients with nonalcoholic fatty liver disease. *J Clin Lab Anal* 2019; **33**: e22657 [PMID: 30239032 DOI: 10.1002/jcla.22657]

43 **Chon YE**, Kim HJ, Choi YB, Hwang SG, Rim KS, Kim MN, Lee JH, Ha Y, Lee MJ. Decrease in waist-to-hip ratio reduced the development of chronic kidney disease in non-obese non-alcoholic fatty liver disease. *Sci Rep* 2020; **10**: 8996 [PMID: 32488147 DOI: 10.1038/s41598-020-65940-y]

44 **Ampuero J**, Aller R, Gallego-Durán R, Banales JM, Crespo J, García-Monzón C, Pareja MJ, Vilar-Gómez E, Caballería J, Escudero-García D, Gomez-Camarero J, Calleja JL, Latorre M, Albillos A, Salmeron J, Aspichueta P, Lo Iacono O, Francés R, Benlloch S, Fernández-Rodríguez C, García-Samaniego J, Estévez P, Andrade RJ, Turnes J, Romero-Gómez M; HEPAmet Registry. The effects of metabolic status on non-alcoholic fatty liver disease-related outcomes, beyond the presence of obesity. *Aliment Pharmacol Ther* 2018; **48**: 1260-1270 [PMID: 30353552 DOI: 10.1111/apt.15015]

45 **Raj D**, Tomar B, Lahiri A, Mulay SR. The gut-liver-kidney axis: Novel regulator of fatty liver associated chronic kidney disease. *Pharmacol Res* 2020; **152**: 104617 [PMID: 31881272 DOI: 10.1016/j.phrs.2019.104617]

46 **Willy JA**, Young SK, Stevens JL, Masuoka HC, Wek RC. CHOP links endoplasmic reticulum stress to NF-κB activation in the pathogenesis of nonalcoholic steatohepatitis. *Mol Biol Cell* 2015; **26**: 2190-2204 [PMID: 25904325 DOI: 10.1091/mbc.E15-01-0036]

47 **Marcuccilli M**, Chonchol M. NAFLD and Chronic Kidney Disease. *Int J Mol Sci* 2016; **17**: 562 [PMID: 27089331 DOI: 10.3390/ijms17040562]

48 **Targher G**, Chonchol MB, Byrne CD. CKD and nonalcoholic fatty liver disease. *Am J Kidney Dis* 2014; **64**: 638-652 [PMID: 25085644 DOI: 10.1053/j.ajkd.2014.05.019]

49 **Sabio G**, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, Barrett T, Kim JK, Davis RJ. A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. *Science* 2008; **322**: 1539-1543 [PMID: 19056984 DOI: 10.1126/science.1160794]

50 **Khukhlina OS**, Antoniv AA, Mandryk OY, Smandych VS, Matushchak MR. The role of endothelial dysfunction in the progression mechanisms of non-alcoholic steatohepatitis in patients with obesity and chronic kidney disease. *Wiad Lek* 2019; **72**: 523-526 [PMID: 31055525]

51 **Hübscher SG**. Histological assessment of non-alcoholic fatty liver disease. *Histopathology* 2006; **49**: 450-465 [PMID: 17064291 DOI: 10.1111/j.1365-2559.2006.02416.x]

52 **Sieber J**, Lindenmeyer MT, Kampe K, Campbell KN, Cohen CD, Hopfer H, Mundel P, Jehle AW. Regulation of podocyte survival and endoplasmic reticulum stress by fatty acids. *Am J Physiol Renal Physiol* 2010; **299**: F821-F829 [PMID: 20668104 DOI: 10.1152/ajprenal.00196.2010]

53 **Joles JA**, van Goor H, van der Horst ML, van Tol A, Elema JD, Koomans HA. High lipid levels in very low density lipoprotein and intermediate density lipoprotein may cause proteinuria and glomerulosclerosis in aging female analbuminemic rats. *Lab Invest* 1995; **73**: 912-921 [PMID: 8558854]

54 **Lindenmeyer MT**, Rastaldi MP, Ikehata M, Neusser MA, Kretzler M, Cohen CD, Schlöndorff D. Proteinuria and hyperglycemia induce endoplasmic reticulum stress. *J Am Soc Nephrol* 2008; **19**: 2225-2236 [PMID: 18776125 DOI: 10.1681/ASN.2007121313]

55 **Monteillet L**, Gjorgjieva M, Silva M, Verzieux V, Imikirene L, Duchampt A, Guillou H, Mithieux G, Rajas F. Intracellular lipids are an independent cause of liver injury and chronic kidney disease in non alcoholic fatty liver disease-like context. *Mol Metab* 2018; **16**: 100-115 [PMID: 30100243 DOI: 10.1016/j.molmet.2018.07.006]

56 **Ackers I**, Malgor R. Interrelationship of canonical and non-canonical Wnt signalling pathways in chronic metabolic diseases. *Diab Vasc Dis Res* 2018; **15**: 3-13 [PMID: 29113510 DOI:

10.1177/1479164117738442]
57 **Shimano H**, Sato R. SREBP-regulated lipid metabolism: convergent physiology - divergent pathophysiology. *Nat Rev Endocrinol* 2017; **13**: 710-730 [PMID: 28849786 DOI: 10.1038/nrendo.2017.91]
58 **Musso G**, Cassader M, Cohney S, De Michieli F, Pinach S, Saba F, Gambino R. Fatty Liver and Chronic Kidney Disease: Novel Mechanistic Insights and Therapeutic Opportunities. *Diabetes Care* 2016; **39**: 1830-1845 [PMID: 27660122 DOI: 10.2337/dc15-1182]
59 **Choi HK**, Ford ES. Prevalence of the metabolic syndrome in individuals with hyperuricemia. *Am J Med* 2007; **120**: 442-447 [PMID: 17466656 DOI: 10.1016/j.amjmed.2006.06.040]
60 **Babio N**, Martínez-González MA, Estruch R, Wärnberg J, Recondo J, Ortega-Calvo M, Serra-Majem L, Corella D, Fitó M, Ros E, Becerra-Tomás N, Basora J, Salas-Salvadó J. Associations between serum uric acid concentrations and metabolic syndrome and its components in the PREDIMED study. *Nutr Metab Cardiovasc Dis* 2015; **25**: 173-180 [PMID: 25511785 DOI: 10.1016/j.numecd.2014.10.006]
61 **Zurlo A**, Veronese N, Giantin V, Maselli M, Zambon S, Maggi S, Musacchio E, Toffanello ED, Sartori L, Perissinotto E, Crepaldi G, Manzato E, Sergi G. High serum uric acid levels increase the risk of metabolic syndrome in elderly women: The PRO.V.A study. *Nutr Metab Cardiovasc Dis* 2016; **26**: 27-35 [PMID: 26643208 DOI: 10.1016/j.numecd.2015.10.007]
62 **Sun HL**, Pei D, Lue KH, Chen YL. Uric Acid Levels Can Predict Metabolic Syndrome and Hypertension in Adolescents: A 10-Year Longitudinal Study. *PLoS One* 2015; **10**: e0143786 [PMID: 26618358 DOI: 10.1371/journal.pone.0143786]
63 **Yuan H**, Yu C, Li X, Sun L, Zhu X, Zhao C, Zhang Z, Yang Z. Serum Uric Acid Levels and Risk of Metabolic Syndrome: A Dose-Response Meta-Analysis of Prospective Studies. *J Clin Endocrinol Metab* 2015; **100**: 4198-4207 [PMID: 26308292 DOI: 10.1210/jc.2015-2527]
64 **Johnson RJ**, Nakagawa T, Jalal D, Sánchez-Lozada LG, Kang DH, Ritz E. Uric acid and chronic kidney disease: which is chasing which? *Nephrol Dial Transplant* 2013; **28**: 2221-2228 [PMID: 23543594 DOI: 10.1093/ndt/gft029]
65 **Sharaf El Din UAA**, Salem MM, Abdulazim DO. Uric acid in the pathogenesis of metabolic, renal, and cardiovascular diseases: A review. *J Adv Res* 2017; **8**: 537-548 [PMID: 28748119 DOI: 10.1016/j.jare.2016.11.004]
66 **Lanaspa MA**, Sanchez-Lozada LG, Cicerchi C, Li N, Roncal-Jimenez CA, Ishimoto T, Le M, Garcia GE, Thomas JB, Rivard CJ, Andres-Hernando A, Hunter B, Schreiner G, Rodriguez-Iturbe B, Sautin YY, Johnson RJ. Uric acid stimulates fructokinase and accelerates fructose metabolism in the development of fatty liver. *PLoS One* 2012; **7**: e47948 [PMID: 23112875 DOI: 10.1371/journal.pone.0047948]
67 **Kimura K**, Hosoya T, Uchida S, Inaba M, Makino H, Maruyama S, Ito S, Yamamoto T, Tomino Y, Ohno I, Shibagaki Y, Iimuro S, Imai N, Kuwabara M, Hayakawa H, Ohtsu H, Ohashi Y; FEATHER Study Investigators. Febuxostat Therapy for Patients With Stage 3 CKD and Asymptomatic Hyperuricemia: A Randomized Trial. *Am J Kidney Dis* 2018; **72**: 798-810 [PMID: 30177485 DOI: 10.1053/j.ajkd.2018.06.028]
68 **Wu IW**, Hsu KH, Lee CC, Sun CY, Hsu HJ, Tsai CJ, Tzen CY, Wang YC, Lin CY, Wu MS. p-Cresyl sulphate and indoxyl sulphate predict progression of chronic kidney disease. *Nephrol Dial Transplant* 2011; **26**: 938-947 [PMID: 20884620 DOI: 10.1093/ndt/gfq580]
69 **Liu H**, Narayanan R, Hoffmann M, Surapaneni S. The Uremic Toxin Indoxyl-3-Sulfate Induces CYP1A2 In Primary Human Hepatocytes. *Drug Metab Lett* 2016; **10**: 195-199 [PMID: 27449409 DOI: 10.2174/1872312810666160719143703]
70 **Tang WH**, Wang Z, Kennedy DJ, Wu Y, Buffa JA, Agatisa-Boyle B, Li XS, Levison BS, Hazen SL. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. *Circ Res* 2015; **116**: 448-455 [PMID: 25599331 DOI: 10.1161/CIRCRESAHA.116.305360]
71 **Tan X**, Liu Y, Long J, Chen S, Liao G, Wu S, Li C, Wang L, Ling W, Zhu H. Trimethylamine N-Oxide Aggravates Liver Steatosis through Modulation of Bile Acid Metabolism and Inhibition of Farnesoid X Receptor Signaling in Nonalcoholic Fatty Liver Disease. *Mol Nutr Food Res* 2019; **63**: e1900257 [PMID: 31095863 DOI: 10.1002/mnfr.201900257]
72 **Anstee QM**, Seth D, Day CP. Genetic Factors That Affect Risk of Alcoholic and Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2016; **150**: 1728-1744. e7 [PMID: 26873399 DOI: 10.1053/j.gastro.2016.01.037]
73 **Macaluso FS**, Maida M, Petta S. Genetic background in nonalcoholic fatty liver disease: A comprehensive review. *World J Gastroenterol* 2015; **21**: 11088-11111 [PMID: 26494964 DOI: 10.3748/wjg.v21.i39.11088]
74 **Musso G**, Cassader M, Gambino R. PNPLA3 rs738409 and TM6SF2 rs58542926 gene variants affect renal disease and function in nonalcoholic fatty liver disease. *Hepatology* 2015; **62**: 658-659 [PMID: 25482317 DOI: 10.1002/hep.27643]
75 **Oniki K**, Saruwatari J, Izuka T, Kajiwara A, Morita K, Sakata M, Otake K, Ogata Y, Nakagawa K. Influence of the PNPLA3 rs738409 Polymorphism on Non-Alcoholic Fatty Liver Disease and Renal Function among Normal Weight Subjects. *PLoS One* 2015; **10**: e0132640 [PMID: 26200108 DOI: 10.1371/journal.pone.0132640]
76 **Sun DQ**, Zheng KI, Xu G, Ma HL, Zhang HY, Pan XY, Zhu PW, Wang XD, Targher G, Byrne CD, Chen YP, Yuan WJ, Zheng MH. PNPLA3 rs738409 is associated with renal glomerular and tubular

injury in NAFLD patients with persistently normal ALT levels. *Liver Int* 2020; **40**: 107-119 [PMID: 31519069 DOI: 10.1111/liv.14251]

77 **Mantovani A**, Zusi C, Sani E, Colecchia A, Lippi G, Zaza GL, Valenti L, Byrne CD, Maffeis C, Bonora E, Targher G. Association between PNPLA3rs738409 polymorphism decreased kidney function in postmenopausal type 2 diabetic women with or without non-alcoholic fatty liver disease. *Diabetes Metab* 2019; **45**: 480-487 [PMID: 30763699 DOI: 10.1016/j.diabet.2019.01.011]

78 **Sookoian S**, Castaño GO, Scian R, Mallardi P, Fernández Gianotti T, Burgueño AL, San Martino J, Pirola CJ. Genetic variation in transmembrane 6 superfamily member 2 and the risk of nonalcoholic fatty liver disease and histological disease severity. *Hepatology* 2015; **61**: 515-525 [PMID: 25302781 DOI: 10.1002/hep.27556]

79 **Önnerhag K**, Hartman H, Nilsson PM, Lindgren S. Non-invasive fibrosis scoring systems can predict future metabolic complications and overall mortality in non-alcoholic fatty liver disease (NAFLD). *Scand J Gastroenterol* 2019; **54**: 328-334 [PMID: 30907181 DOI: 10.1080/00365521.2019.1583366]

80 **Wijarnpreecha K**, Thongprayoon C, Scribani M, Ungprasert P, Cheungpasitporn W. Noninvasive fibrosis markers and chronic kidney disease among adults with nonalcoholic fatty liver in USA. *Eur J Gastroenterol Hepatol* 2018; **30**: 404-410 [PMID: 29215435 DOI: 10.1097/MEG.0000000000001045]

81 **Choi JW**, Lee CH, Park JS. Comparison of laboratory indices of non-alcoholic fatty liver disease for the detection of incipient kidney dysfunction. *PeerJ* 2019; **7**: e6524 [PMID: 30867987 DOI: 10.7717/peerj.6524]

82 **Ciardullo S**, Muraca E, Perra S, Bianconi E, Zerbini F, Oltolini A, Cannistraci R, Parmeggiani P, Manzoni G, Gastaldelli A, Lattuada G, Perseghin G. Screening for non-alcoholic fatty liver disease in type 2 diabetes using non-invasive scores and association with diabetic complications. *BMJ Open Diabetes Res Care* 2020; **8** [PMID: 32049637 DOI: 10.1136/bmjdrc-2019-000904]

83 **Angeli P**, Ginès P, Wong F, Bernardi M, Boyer TD, Gerbes A, Moreau R, Jalan R, Sarin SK, Piano S, Moore K, Lee SS, Durand F, Salerno F, Caraceni P, Kim WR, Arroyo V, Garcia-Tsao G. Diagnosis and management of acute kidney injury in patients with cirrhosis: revised consensus recommendations of the International Club of Ascites. *J Hepatol* 2015; **62**: 968-974 [PMID: 25638527 DOI: 10.1016/j.jhep.2014.12.029]

84 **Piano S**, Romano A, Di Pascoli M, Angeli P. Why and how to measure renal function in patients with liver disease. *Liver Int* 2017; **37** Suppl 1: 116-122 [PMID: 28052631 DOI: 10.1111/liv.13305]

85 **Mindikoglu AL**, Dowling TC, Weir MR, Seliger SL, Christenson RH, Magder LS. Performance of chronic kidney disease epidemiology collaboration creatinine-cystatin C equation for estimating kidney function in cirrhosis. *Hepatology* 2014; **59**: 1532-1542 [PMID: 23744636 DOI: 10.1002/hep.26556]

86 **Shardlow A**, McIntyre NJ, Fraser SDS, Roderick P, Raftery J, Fluck RJ, McIntyre CW, Taal MW. The clinical utility and cost impact of cystatin C measurement in the diagnosis and management of chronic kidney disease: A primary care cohort study. *PLoS Med* 2017; **14**: e1002400 [PMID: 29016597 DOI: 10.1371/journal.pmed.1002400]

87 **Bulum T**, Kolarić B, Duvnjak M, Duvnjak L. Alkaline phosphatase is independently associated with renal function in normoalbuminuric type 1 diabetic patients. *Ren Fail* 2014; **36**: 372-377 [PMID: 24455970 DOI: 10.3109/0886022X.2013.872569]

88 **Pantsari MW**, Harrison SA. Nonalcoholic fatty liver disease presenting with an isolated elevated alkaline phosphatase. *J Clin Gastroenterol* 2006; **40**: 633-635 [PMID: 16917408 DOI: 10.1097/00004836-200608000-00015]

89 **Satchell SC**, Tooke JE. What is the mechanism of microalbuminuria in diabetes: a role for the glomerular endothelium? *Diabetologia* 2008; **51**: 714-725 [PMID: 18347777 DOI: 10.1007/s00125-008-0961-8]

90 **Ryu S**, Chang Y, Kim DI, Kim WS, Suh BS. gamma-Glutamyltransferase as a predictor of chronic kidney disease in nonhypertensive and nondiabetic Korean men. *Clin Chem* 2007; **53**: 71-77 [PMID: 17110470 DOI: 10.1373/clinchem.2006.078980]

91 **Arase Y**, Suzuki F, Kobayashi M, Suzuki Y, Kawamura Y, Matsumoto N, Akuta N, Sezaki H, Saito S, Hosaka T, Ikeda K, Kumada H, Ohmoto Y, Amakawa K, Tsuji H, Hsieh SD, Kato K, Tanabe M, Ogawa K, Hara S, Kobayashi T. The development of chronic kidney disease in Japanese patients with non-alcoholic fatty liver disease. *Intern Med* 2011; **50**: 1081-1087 [PMID: 21576832 DOI: 10.2169/internalmedicine.50.5043]

92 **Thamer C**, Tschritter O, Haap M, Shirkavand F, Machann J, Fritsche A, Schick F, Häring H, Stumvoll M. Elevated serum GGT concentrations predict reduced insulin sensitivity and increased intrahepatic lipids. *Horm Metab Res* 2005; **37**: 246-251 [PMID: 15952086 DOI: 10.1055/s-2005-861411]

93 **Kim D**, Kim WR. Nonobese Fatty Liver Disease. *Clin Gastroenterol Hepatol* 2017; **15**: 474-485 [PMID: 27581063 DOI: 10.1016/j.cgh.2016.08.028]

94 **Vilar-Gomez E**, Calzadilla-Bertot L, Friedman SL, Gra-Oramas B, Gonzalez-Fabian L, Villa-Jimenez O, Lazo-Del Vallin S, Diago M, Adams LA, Romero-Gomez M, Chalasani N. Improvement in liver histology due to lifestyle modification is independently associated with improved kidney function in patients with non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2017; **45**: 332-344 [PMID: 27862096 DOI: 10.1111/apt.13860]

95 **Perkovic V**, Jardine MJ, Neal B, Bompoint S, Heerspink HJL, Charytan DM, Edwards R, Agarwal

R, Bakris G, Bull S, Cannon CP, Capuano G, Chu PL, de Zeeuw D, Greene T, Levin A, Pollock C, Wheeler DC, Yavin Y, Zhang H, Zinman B, Meininger G, Brenner BM, Mahaffey KW; CREDENCE Trial Investigators. Canagliflozin and Renal Outcomes in Type 2 Diabetes and Nephropathy. *N Engl J Med* 2019; **380**: 2295-2306 [PMID: 30990260 DOI: 10.1056/NEJMoa1811744]

96 **Shimizu M**, Suzuki K, Kato K, Jojima T, Iijima T, Murohisa T, Iijima M, Takekawa H, Usui I, Hiraishi H, Aso Y. Evaluation of the effects of dapagliflozin, a sodium-glucose co-transporter-2 inhibitor, on hepatic steatosis and fibrosis using transient elastography in patients with type 2 diabetes and non-alcoholic fatty liver disease. *Diabetes Obes Metab* 2019; **21**: 285-292 [PMID: 30178600 DOI: 10.1111/dom.13520]

97 **Leiter LA**, Forst T, Polidori D, Balis DA, Xie J, Sha S. Effect of canagliflozin on liver function tests in patients with type 2 diabetes. *Diabetes Metab* 2016; **42**: 25-32 [PMID: 26575250 DOI: 10.1016/j.diabet.2015.10.003]

98 **Scheen AJ**. Beneficial effects of SGLT2 inhibitors on fatty liver in type 2 diabetes: A common comorbidity associated with severe complications. *Diabetes Metab* 2019; **45**: 213-223 [PMID: 30708071 DOI: 10.1016/j.diabet.2019.01.008]

99 **Angulo P**, Lindor KD. Non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 2002; **17** Suppl: S186-S190 [PMID: 12000605 DOI: 10.1046/j.1440-1746.17.s1.10.x]

100 **Armstrong MJ**, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, Guo K; LEAN trial team, Abouda G, Aldersley MA, Stocken D, Gough SC, Tomlinson JW, Brown RM, Hübscher SG, Newsome PN. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet* 2016; **387**: 679-690 [PMID: 26608256 DOI: 10.1016/S0140-6736(15)00803-X]

101 **Tuttle KR**, Lakshmanan MC, Rayner B, Busch RS, Zimmermann AG, Woodward DB, Botros FT. Dulaglutide versus insulin glargine in patients with type 2 diabetes and moderate-to-severe chronic kidney disease (AWARD-7): a multicentre, open-label, randomised trial. *Lancet Diabetes Endocrinol* 2018; **6**: 605-617 [PMID: 29910024 DOI: 10.1016/S2213-8587(18)30104-9]

102 **Sumida Y**, Yoneda M, Toyoda H, Yasuda S, Tada T, Hayashi H, Nishigaki Y, Suzuki Y, Naiki T, Morishita A, Tobita H, Sato S, Kawabe N, Fukunishi S, Ikegami T, Kessoku T, Ogawa Y, Honda Y, Nakahara T, Munekage K, Ochi T, Sawada K, Takahashi A, Arai T, Kogiso T, Kimoto S, Tomita K, Notsumata K, Nonaka M, Kawata K, Takami T, Kumada T, Tomita E, Okanoue T, Nakajima A; Japan Study Group Of Nafld Jsg-Nafld. Common Drug Pipelines for the Treatment of Diabetic Nephropathy and Hepatopathy: Can We Kill Two Birds with One Stone? *Int J Mol Sci* 2020; **21** [PMID: 32668632 DOI: 10.3390/ijms21144939]

103 **Kristensen SL**, Rørth R, Jhund PS, Docherty KF, Sattar N, Preiss D, Køber L, Petrie MC, McMurray JJV. Cardiovascular, mortality, and kidney outcomes with GLP-1 receptor agonists in patients with type 2 diabetes: a systematic review and meta-analysis of cardiovascular outcome trials. *Lancet Diabetes Endocrinol* 2019; **7**: 776-785 [PMID: 31422062 DOI: 10.1016/S2213-8587(19)30249-9]

104 **McMurray JJV**, Solomon SD, Inzucchi SE, Køber L, Kosiborod MN, Martinez FA, Ponikowski P, Sabatine MS, Anand IS, Bělohlávek J, Böhm M, Chiang CE, Chopra VK, de Boer RA, Desai AS, Diez M, Drozdz J, Dukát A, Ge J, Howlett JG, Katova T, Kitakaze M, Ljungman CEA, Merkely B, Nicolau JC, O'Meara E, Petrie MC, Vinh PN, Schou M, Tereshchenko S, Verma S, Held C, DeMets DL, Docherty KF, Jhund PS, Bengtsson O, Sjöstrand M, Langkilde AM; DAPA-HF Trial Committees and Investigators. Dapagliflozin in Patients with Heart Failure and Reduced Ejection Fraction. *N Engl J Med* 2019; **381**: 1995-2008 [PMID: 31535829 DOI: 10.1056/NEJMoa1911303]

105 **Hargreaves IP**. Coenzyme Q10 as a therapy for mitochondrial disease. *Int J Biochem Cell Biol* 2014; **49**: 105-111 [PMID: 24495877 DOI: 10.1016/j.biocel.2014.01.020]

106 **Mantle D**, Hargreaves I. Coenzyme Q10 and Degenerative Disorders Affecting Longevity: An Overview. *Antioxidants (Basel)* 2019; **8** [PMID: 30781472 DOI: 10.3390/antiox8020044]

107 **Yesilova Z**, Yaman H, Oktenli C, Ozcan A, Uygun A, Cakir E, Sanisoglu SY, Erdil A, Ates Y, Aslan M, Musabak U, Erbil MK, Karaeren N, Dagalp K. Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic Fatty liver disease. *Am J Gastroenterol* 2005; **100**: 850-855 [PMID: 15784031 DOI: 10.1111/j.1572-0241.2005.41500.x]

108 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]

109 **Farhangi MA**, Alipour B, Jafarvand E, Khoshbaten M. Oral coenzyme Q10 supplementation in patients with nonalcoholic fatty liver disease: effects on serum vaspin, chemerin, pentraxin 3, insulin resistance and oxidative stress. *Arch Med Res* 2014; **45**: 589-595 [PMID: 25450583 DOI: 10.1016/j.arcmed.2014.11.001]

110 **Farsi F**, Mohammadshahi M, Alavinejad P, Rezazadeh A, Zarei M, Engali KA. Functions of Coenzyme Q10 Supplementation on Liver Enzymes, Markers of Systemic Inflammation, and Adipokines in Patients Affected by Nonalcoholic Fatty Liver Disease: A Double-Blind, Placebo-Controlled, Randomized Clinical Trial. *J Am Coll Nutr* 2016; **35**: 346-353 [PMID: 26156412 DOI: 10.1080/07315724.2015.1021057]

111 **Yeung CK**, Billings FT 4th, Claessens AJ, Roshanravan B, Linke L, Sundell MB, Ahmad S, Shao B, Shen DD, Ikizler TA, Himmelfarb J. Coenzyme Q10 dose-escalation study in hemodialysis patients: safety, tolerability, and effect on oxidative stress. *BMC Nephrol* 2015; **16**: 183 [PMID: 26531095 DOI: 10.1186/s12882-015-0178-2]

112 **Mortensen SA**, Rosenfeldt F, Kumar A, Dolliner P, Filipiak KJ, Pella D, Alehagen U, Steurer G, Littarru GP; Q-SYMBIO Study Investigators. The effect of coenzyme Q10 on morbidity and mortality in chronic heart failure: results from Q-SYMBIO: a randomized double-blind trial. *JACC Heart Fail* 2014; **2**: 641-649 [PMID: 25282031 DOI: 10.1016/j.jchf.2014.06.008]

113 **Alehagen U**, Johansson P, Björnstedt M, Rosén A, Dahlström U. Cardiovascular mortality and N-terminal-proBNP reduced after combined selenium and coenzyme Q10 supplementation: a 5-year prospective randomized double-blind placebo-controlled trial among elderly Swedish citizens. *Int J Cardiol* 2013; **167**: 1860-1866 [PMID: 22626835 DOI: 10.1016/j.ijcard.2012.04.156]

114 **Cheng HS**, Tan WR, Low ZS, Marvalim C, Lee JYH, Tan NS. Exploration and Development of PPAR Modulators in Health and Disease: An Update of Clinical Evidence. *Int J Mol Sci* 2019; **20** [PMID: 31614690 DOI: 10.3390/ijms20205055]

115 **Schneider CA**, Ferrannini E, Defronzo R, Schernthaner G, Yates J, Erdmann E. Effect of pioglitazone on cardiovascular outcome in diabetes and chronic kidney disease. *J Am Soc Nephrol* 2008; **19**: 182-187 [PMID: 18057215 DOI: 10.1681/ASN.2007060678]

116 **Promrat K**, Lutchman G, Uwaifo GI, Freedman RJ, Soza A, Heller T, Doo E, Ghany M, Premkumar A, Park Y, Liang TJ, Yanovski JA, Kleiner DE, Hoofnagle JH. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. *Hepatology* 2004; **39**: 188-196 [PMID: 14752837 DOI: 10.1002/hep.20012]

117 **Belfort R**, Harrison SA, Brown K, Darland C, Finch J, Hardies J, Balas B, Gastaldelli A, Tio F, Pulcini J, Berria R, Ma JZ, Dwivedi S, Havranek R, Fincke C, DeFronzo R, Bannayan GA, Schenker S, Cusi K. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med* 2006; **355**: 2297-2307 [PMID: 17135584 DOI: 10.1056/NEJMoa060326]

118 **Aithal GP**, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, Austin AS, Freeman JG, Morgan L, Webber J. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. *Gastroenterology* 2008; **135**: 1176-1184 [PMID: 18718471 DOI: 10.1053/j.gastro.2008.06.047]

119 **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR; NASH CRN. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]

120 **Cusi K**, Orsak B, Bril F, Lomonaco R, Hecht J, Ortiz-Lopez C, Tio F, Hardies J, Darland C, Musi N, Webb A, Portillo-Sanchez P. Long-Term Pioglitazone Treatment for Patients With Nonalcoholic Steatohepatitis and Prediabetes or Type 2 Diabetes Mellitus: A Randomized Trial. *Ann Intern Med* 2016; **165**: 305-315 [PMID: 27322798 DOI: 10.7326/M15-1774]

121 **Chalasani N**, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM, Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; **67**: 328-357 [PMID: 28714183 DOI: 10.1002/hep.29367]

122 **Neuschwander-Tetri BA**, Brunt EM, Wehmeier KR, Oliver D, Bacon BR. Improved nonalcoholic steatohepatitis after 48 weeks of treatment with the PPAR-gamma ligand rosiglitazone. *Hepatology* 2003; **38**: 1008-1017 [PMID: 14512888 DOI: 10.1053/jhep.2003.50420]

123 **Ratziu V**, Giral P, Jacqueminet S, Charlotte F, Hartemann-Heurtier A, Serfaty L, Podevin P, Lacorte JM, Bernhardt C, Bruckert E, Grimaldi A, Poynard T; LIDO Study Group. Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. *Gastroenterology* 2008; **135**: 100-110 [PMID: 18503774 DOI: 10.1053/j.gastro.2008.03.078]

124 **Ratziu V**, Charlotte F, Bernhardt C, Giral P, Halbron M, Lenaour G, Hartmann-Heurtier A, Bruckert E, Poynard T; LIDO Study Group. Long-term efficacy of rosiglitazone in nonalcoholic steatohepatitis: results of the fatty liver improvement by rosiglitazone therapy (FLIRT 2) extension trial. *Hepatology* 2010; **51**: 445-453 [PMID: 19877169 DOI: 10.1002/hep.23270]

125 **Eliades M**, Spyrou E, Agrawal N, Lazo M, Brancati FL, Potter JJ, Koteish AA, Clark JM, Guallar E, Hernaez R. Meta-analysis: vitamin D and non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2013; **38**: 246-254 [PMID: 23786213 DOI: 10.1111/apt.12377]

126 **LaClair RE**, Hellman RN, Karp SL, Kraus M, Ofner S, Li Q, Graves KL, Moe SM. Prevalence of calcidiol deficiency in CKD: a cross-sectional study across latitudes in the United States. *Am J Kidney Dis* 2005; **45**: 1026-1033 [PMID: 15957131 DOI: 10.1053/j.ajkd.2005.02.029]

127 **Parikh A**, Chase HS, Vernocchi L, Stern L. Vitamin D resistance in chronic kidney disease (CKD). *BMC Nephrol* 2014; **15**: 47 [PMID: 24641586 DOI: 10.1186/1471-2369-15-47]

128 **Xu L**, Wan X, Huang Z, Zeng F, Wei G, Fang D, Deng W, Li Y. Impact of vitamin D on chronic kidney diseases in non-dialysis patients: a meta-analysis of randomized controlled trials. *PLoS One* 2013; **8**: e61387 [PMID: 23626678 DOI: 10.1371/journal.pone.0061387]

129 **Dasarathy J**, Varghese R, Feldman A, Khiyami A, McCullough AJ, Dasarathy S. Patients with Nonalcoholic Fatty Liver Disease Have a Low Response Rate to Vitamin D Supplementation. *J Nutr* 2017; **147**: 1938-1946 [PMID: 28814531 DOI: 10.3945/jn.117.254292]

130 **NIH**. Effect on Liver Histology of Vitamin D in Patients With Non-alcoholic Steatohepatitis. [cited 7 November 2020]. Available from: https://clinicaltrials.gov/ct2/results?cond=&term=NCT01623024&cntry=&state=&city=&dist=

131 **NIH**. Study of Vitamin D3 Supplementation in Patients With Chronic Kidney Disease. [cited 7 November 2020]. Available from:

https://clinicaltrials.gov/ct2/results?cond=&term=NCT00893451&cntry=&state=&city=&dist=
132 **NIH**. DHA and Vitamin D in Children With Biopsy-proven NAFLD. [cited 7 November 2020]. Available from: https://clinicaltrials.gov/ct2/results?cond=&term=+NCT02098317&cntry=&state=&city=&dist=
133 **García-Lezana T**, Raurell I, Bravo M, Torres-Arauz M, Salcedo MT, Santiago A, Schoenenberger A, Manichanh C, Genescà J, Martell M, Augustin S. Restoration of a healthy intestinal microbiota normalizes portal hypertension in a rat model of nonalcoholic steatohepatitis. *Hepatology* 2018; **67**: 1485-1498 [PMID: 29113028 DOI: 10.1002/hep.29646]
134 **Bergheim I**, Weber S, Vos M, Krämer S, Volynets V, Kaserouni S, McClain CJ, Bischoff SC. Antibiotics protect against fructose-induced hepatic lipid accumulation in mice: role of endotoxin. *J Hepatol* 2008; **48**: 983-992 [PMID: 18395289 DOI: 10.1016/j.jhep.2008.01.035]
135 **Younossi Z**, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 11-20 [PMID: 28930295 DOI: 10.1038/nrgastro.2017.109]
136 **Wong RJ**, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, Ahmed A. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. *Gastroenterology* 2015; **148**: 547-555 [PMID: 25461851 DOI: 10.1053/j.gastro.2014.11.039]
137 **Calzadilla-Bertot L**, Jeffrey GP, Jacques B, McCaughan G, Crawford M, Angus P, Jones R, Gane E, Munn S, Macdonald G, Fawcett J, Wigg A, Chen J, Fink M, Adams LA. Increasing Incidence of Nonalcoholic Steatohepatitis as an Indication for Liver Transplantation in Australia and New Zealand. *Liver Transpl* 2019; **25**: 25-34 [PMID: 30609187 DOI: 10.1002/lt.25361]
138 **Singal AK**, Hasanin M, Kaif M, Wiesner R, Kuo YF. Nonalcoholic Steatohepatitis is the Most Rapidly Growing Indication for Simultaneous Liver Kidney Transplantation in the United States. *Transplantation* 2016; **100**: 607-612 [PMID: 26479282 DOI: 10.1097/TP.0000000000000945]
139 **Miles CD**, Westphal S, Liapakis A, Formica R. Simultaneous Liver-Kidney Transplantation: Impact on Liver Transplant Patients and the Kidney Transplant Waiting List. *Curr Transplant Rep* 2018; **5**: 1-6 [PMID: 29564203 DOI: 10.1007/s40472-018-0175-z]
140 **Singal AK**, Salameh H, Kuo YF, Wiesner RH. Evolving frequency and outcomes of simultaneous liver kidney transplants based on liver disease etiology. *Transplantation* 2014; **98**: 216-221 [PMID: 24621538 DOI: 10.1097/TP.0000000000000048]
141 **Formica RN**, Aeder M, Boyle G, Kucheryavaya A, Stewart D, Hirose R, Mulligan D. Simultaneous Liver-Kidney Allocation Policy: A Proposal to Optimize Appropriate Utilization of Scarce Resources. *Am J Transplant* 2016; **16**: 758-766 [PMID: 26603142 DOI: 10.1111/ajt.13631]
142 **Houlihan DD**, Armstrong MJ, Davidov Y, Hodson J, Nightingale P, Rowe IA, Paris S, Gunson BK, Bramhall SB, Mutimer DJ, Neuberger JM, Newsome PN. Renal function in patients undergoing transplantation for nonalcoholic steatohepatitis cirrhosis: time to reconsider immunosuppression regimens? *Liver Transpl* 2011; **17**: 1292-1298 [PMID: 21761549 DOI: 10.1002/lt.22382]
143 **Mikolasevic I**, Orlic L, Hrstic I, Milic S. Metabolic syndrome and non-alcoholic fatty liver disease after liver or kidney transplantation. *Hepatol Res* 2016; **46**: 841-852 [PMID: 26713425 DOI: 10.1111/hepr.12642]
144 **Molnar MZ**, Joglekar K, Jiang Y, Cholankeril G, Abdul MKM, Kedia S, Gonzalez HC, Ahmed A, Singal A, Bhamidimarri KR, Aithal GP, Duseja A, Wong VW, Gulnare A, Puri P, Nair S, Eason JD, Satapathy SK; Global NAFLD Consortium. Association of Pretransplant Renal Function With Liver Graft and Patient Survival After Liver Transplantation in Patients With Nonalcoholic Steatohepatitis. *Liver Transpl* 2019; **25**: 399-410 [PMID: 30369023 DOI: 10.1002/lt.25367]
145 **Mikolasevic I**, Filipec-Kanizaj T, Mijic M, Jakopcic I, Milic S, Hrstic I, Sobocan N, Stimac D, Burra P. Nonalcoholic fatty liver disease and liver transplantation - Where do we stand? *World J Gastroenterol* 2018; **24**: 1491-1506 [PMID: 29662288 DOI: 10.3748/wjg.v24.i14.1491]
146 **Ganguli S**, DeLeeuw P, Satapathy SK. A Review Of Current And Upcoming Treatment Modalities In Non-Alcoholic Fatty Liver Disease And Non-Alcoholic Steatohepatitis. *Hepat Med* 2019; **11**: 159-178 [PMID: 31814783 DOI: 10.2147/HMER.S188991]
147 **Önnerhag K**, Dreja K, Nilsson PM, Lindgren S. Increased mortality in non-alcoholic fatty liver disease with chronic kidney disease is explained by metabolic comorbidities. *Clin Res Hepatol Gastroenterol* 2019; **43**: 542-550 [PMID: 30827925 DOI: 10.1016/j.clinre.2019.02.004]

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REVIEW

Repurposing metformin for the treatment of gastrointestinal cancer

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Abstract

Diabetes mellitus type 2 and cancer share many risk factors. The pleiotropic insulin-dependent and insulin-independent effects of metformin might inhibit pathways that are frequently amplified in neoplastic tissue. Particularly, modulation of inflammation, metabolism, and cell cycle arrest are potential therapeutic cancer targets utilized by metformin to boost the anti-cancer effects of chemotherapy. Studies *in vitro* and *in vivo* models have demonstrated the potential of metformin as a chemo- and radiosensitizer, besides its chemopreventive and direct therapeutic activity in digestive system (DS) tumors. Hence, these aspects have been considered in many cancer clinical trials. Case-control and cohort studies and associated meta-analyses have evaluated DS cancer risk and metformin usage, especially in colorectal cancer, pancreatic cancer, and hepatocellular carcinoma. Most clinical studies have demonstrated the protective role of metformin in the risk for DS cancers and survival rates. On the other hand, the ability of metformin to enhance the actions of chemotherapy for gastric and biliary cancers is yet to be investigated. This article reviews the current findings on the anti-cancer mechanisms of metformin and its apparatus from pre-clinical and ongoing studies in DS malignancies.

Key Words: Antidiabetic treatments; Gastrointestinal tumors; Therapeutic target

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Core Tip: Modulation of cell function into the neoplastic and around the microenvironment tissue are possible cancer targets utilized by metformin to raise

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chemotherapy's anti-tumor outcomes. Herein we review the studies that have demonstrated the likelihood of metformin as chemo and radiosensitizer, in addition to its chemopre-ventive and direct therapeutic activity in gastrointestinal tumors.

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INTRODUCTION

Diabetes mellitus type 2 (DM2) and cancer share several risk factors[1]. Notably, obesity and metabolic syndrome, with their inherent biological connections, such as hyperinsulinemia[2] and chronic inflammation[3]. Furthermore, some antihyperglycemic medications (*e.g.*, sulfonylureas and insulin) used for the treatment of DM2 may increase cancer risk[4]. Particularly, central obesity, physical inactivity, and perhaps a low dietetic polyunsaturated fat to saturated fat ratio are major risk factors for insulin resistance and hyperinsulinemia and seem to be related to cancer risk[5]. All of them have been recognized as proposed gears holding those relationships[6-9]. Epidemiologic studies and meta-analyses have suggested that patients with DM2 have a higher incidence and mortality from malignancies[10,11], including digestive system (DS) cancers[12-15].

Metformin is a well-known oral hypoglycemic drug that belongs to the biguanide class and has been used to treat DM2 for almost a century[16]. Importantly, those patients with DM2 with long-term use of metformin have a decreased tumor incidence and lower cancer-associated mortality[17-21]. Furthermore, recent research indicates that metformin can have direct anti-cancer activity against many tumor cells, including tumor stem cells[22,23], therefore, carrying out pleiotropic effects in both the cancer cell and the neoplastic microenvironment[24]. Their potential mechanisms are insulin-dependent [*via* insulin growth factor (IGF) receptor, phosphatidyl inositol 3 kinase (PI3K), and Akt/mammalian target of rapamycin (mTOR)][25,26] and insulin-independent [*via* adenosine kinase monophosphate (AMPK), tuberous sclerosis complex (TSC), and mTOR][27,28]. Moreover, it promotes antitumor immunity-related metabolic checkpoints in T-cells, cancer cells, as well as associated with immunosuppressive cells of the tumor milieu[29]. Furthermore, it might interfere with the gut microbiota and have systemic impacts on body metabolism[30,31]. This article aims to review the rationale of metformin as a drug that might be repurposed for DS cancer treatment.

MECHANISM OF ACTION OF METFORMIN AS AN ANTI-CANCER AGENT

Two potential mechanisms for the antineoplastic action of metformin have been suggested (Figure 1). First, metformin can directly activate AMPK, resulting in inhibition of downstream Akt/mTOR signaling and consequent suppression of cell proliferation[32,33]. Second, metformin-induced reductions in circulating insulin and IGF concentrations may reduce activation of the IGF receptor signaling axis, resulting in decreased growth promotion and mitogenesis[2,34]. Hence, the anti-cancer effects of metformin are mediated through a systemic improvement in the metabolic milieu or directly on tumor cells[35].

The noticeable intracellular metabolic change caused by metformin is the decreased accumulation of glycolytic intermediates and a coordinated decrease in tricarboxylic acid (TCA) cycle intermediates[36,37]. Moreover, the activation of AMPK reduces fatty acid synthase (FAS) gene expression in the synthesis of fatty acids[32]. Furthermore, metformin offers other direct anti-tumor effects by (1) decreasing specific protein (Sp) transcription factors and Sp-related oncogenic proteins[38,39]; (2) decreasing AMPK-dependent c-Myc oncogene; (3) increasing other miRNAs, such as mir33a[40]; (4) increasing other miRNAs, such as miR-26a[41]; (5) reducing endogenous reactive oxygen species and associated DNA damage[42]; (6) reducing Sonic hedgehog

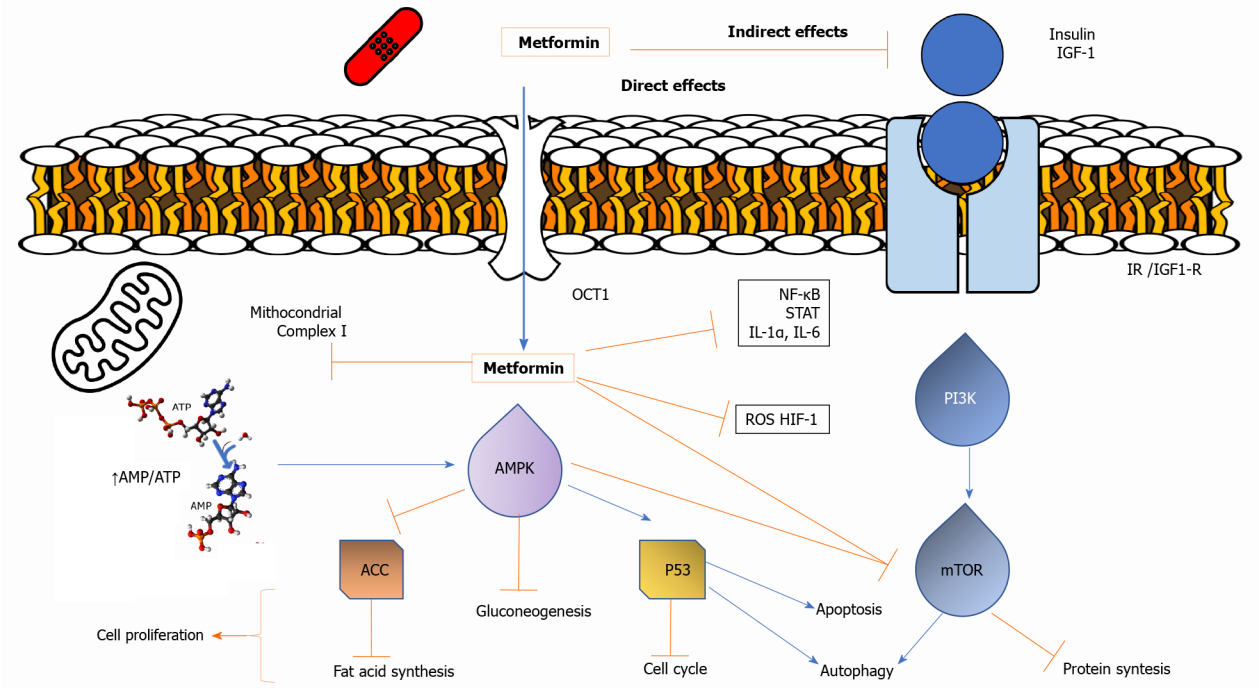


Figure 1 Overview of cellular mechanisms of metformin in cancer. Metformin inhibits mitochondria complex I, stimulates the adenosine monophosphate-activated protein kinase signaling pathway, and/or inhibits the insulin signaling pathway. Blue lines represent activated pathways while red lines represent inhibitory pathways. AMPK: Adenosine monophosphate-activated protein kinase; ACC: Acetyl-CoA carboxylase; HIF-1α: Hypoxia-inducible factor-1 alpha; IGF: Insulin growth factor; IGF-1: Insulin-like growth factor-1; IGF-1R: Insulin-like growth factor-1 receptor; IR: Insulin receptor; IL-1: Interleukin 1; IL-6: Interleukin-6; NF-κB: Nuclear factor kappa; OCT1: Organic cation transporter 1; ROS: Reactive oxygen species; STAT: Signal transducer and activator of transcription; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; PI3K: Phosphoinositide 3-kinase; mTOR: Mechanistic target of rapamycin.

expression[43]; (7) reducing expression of angiogenic factor CCN1, which inhibits invasion induced by the stromal cell-derived factor-1 and reducing levels of type 4 chemokine receptor[44]; and (8) inhibiting Rac1 GTPase activity[45]. Finally, metformin might interfere with the gut microbiota[30,31], as well as interfere with the balance between T-cells and associated immunosuppressive cells in the tumor milieu[29].

Insulin-dependent or indirect effects

A central signal transduction pathway involved in cancer is the PI3K/Akt/mTOR pathway, which, when hyperactivated, leads to deregulation of survival and cell growth[28,46,47]. IGF-1 is a more potent mitogen than insulin and, like insulin, binds to its particular growth factor receptor and stimulates cell growth and anti-apoptotic activity *via* MAPK/ERK or Ras/Raf/MEK/ERK and PI3K/Akt/mTOR signaling [2,34]. In addition, IGF-1 inhibits PTEN, a phosphatase that deactivates PI3K/Akt/mTOR[2]. The indirect mechanisms of metformin action include inhibition of hepatic gluconeogenesis and stimulation of peripheral glucose absorption, which ultimately lead to decreased blood glucose and insulin levels. Thus, the most apparent mechanism of insulin-dependent metformin involves decreasing insulin levels, which reduces insulin binding to the insulin receptor (IR), inhibiting tumor growth[48]. A reduction of insulin/IGF-1 levels is, at least in part, involved in the antiproliferative activity of metformin[49]. Additionally, metformin downregulates IGF-R and IR by decreasing the promoter activity of receptor genes with subsequent Akt/mTOR and MAPK/ERK signaling inhibition[50,51].

Insulin-independent or direct effects

Metformin activates AMPK by inhibiting mitochondrial complex I, which leads to impaired mitochondrial function, decreased adenosine triphosphate synthesis, increased adenosine monophosphate, and subsequent phosphorylation and activation by LKB1[52]. Activated AMPK then phosphorylates TSC2, which negatively regulates mTOR activity[53]. Activation of LKB1 and AMPK, AMPK-induced stabilization of TSC1-TSC2 (inhibitor of Rheb, an mTORC1 activator), and activation of the tumor suppressor p53[54]. Moreover, independent of AMPK, metformin impedes mTORC1 by raising p53-dependent expression of REDD1 and repressing Rags[55]. Metformin

also retards transformation by inhibiting mediators of the inflammatory response, including transcription factors (nuclear factor kappa, Signal transducer and activator of transcription 3, and Forkhead box O signaling), and downregulating Lin28B, most Let-7 miRNA family members, and inflammatory molecules [interleukin (IL)-1α, IL-1β, IL-6, and vascular endothelial growth factor (VEGF)][56].

Metformin has other AMPK-mediated actions that may be implicated in cancer. Through the activation of AMPK, metformin causes the suppression of FAS gene expression, which is involved in the synthesis of fatty acids, resulting in reduced lipogenesis, increased fatty acid oxidation, and decreased cell proliferation[32,57]. The activation of AMPK also modulates cyclin D1 (cell cycle protein), p21 and p27 (cyclin-dependent protein kinase), which further contribute to its anti-cancer effects[55,58]. Interestingly, metformin may act as a chemosensitizer, for example, increasing the 5-fluorouracil (5-FU) and paclitaxel sensitivities of cancer cell lines[59,60]. The ability of metformin to disconnect the electron transport chain by inhibiting complex I (NADH dehydrogenase) strongly induces cell death when glucose is limited. Metformin also reduces the hypoxic activation of hypoxia inducing factor (HIF-1), suggesting that the effects of metformin are increased in hypoglycemic and hypoxic conditions[61].

Other mechanisms

As a drug that controls metabolism, metformin promotes a coordinated decrease of TCA cycle intermediates, including succinate, fumarate, malate, citrate, and α-ketoglutarate[36,37]. The dependency of neoplastic cells on glutamine metabolism has been shown to be reprogrammed by the Kras oncogenic pathway through a single pathway involving serum glutamic-oxaloacetic transaminase, which maintains the cellular redox states essential to mitochondria and offers innovative therapeutic targets in combination with metformin[62].

Metformin can exert antitumor activity by increasing CD8+ T-cells[63,64]. It might inhibit apoptosis of CD8+ tumor infiltrating lymphocytes and prevent immune exhaustion[63,65]. Furthermore, metformin might adjust the expression profile of immune checkpoints[66], such as programmed death ligand 1, in the context of the neoplasm[37], thereby suggesting that a combination of metformin might have the potential to enhance the strength of cancer immunotherapy[63].

There is evidence that epigenomic modifications by metformin may contribute to its anti-cancer properties[67]. Metformin might regulate the activity of numerous epigenetic modifying enzymes, principally by modulating the activation of AMPK. Activated AMPK can phosphorylate several substrates, comprising epigenetic enzymes, such as histone acetyltransferases (HATs), class II histone deacetylases (HDACs) and DNA methyltransferases (DNMTs), usually resulting in their inhibition; however, HAT1 activity may be increased. Metformin has also been related to the diminished expression of various histone methyltransferases[68], enhancing the activity of the class III HDAC SIRT1 and minimizing the influence of DNMT inhibitors[69,70].

METFORMIN STUDIES IN DIGESTIVE SYSTEM MALIGNANCIES

Metformin in colorectal cancer

Cell lines and animal models: Metformin promotes cell cycle arrest in the G0/G1 phase in colorectal cancer (CRC) cell lines. It also decreases the expression of c-Myc and causes down-regulation of IGF-1R[71]. Consequently, up-regulation of the adenosine A1 receptor induces apoptosis[72]. Additionally, it was shown that metformin enhances the activity of the *Sprouty2* gene, which suppresses colon cancer growth[73].

The combination of metformin with 5-FU was investigated on the SW620 CRC cell line and on patients with DM2. The study showed that metformin plus 5-FU treatment significantly inhibited the proliferation of SW620 cells compared with that in monotherapy. Additionally, the examination of 86 CRC tissue samples obtained from patients with DM2 revealed that treatment with metformin decreased the proportion of poorly differentiated tumors[74]. Moreover, a synergistic effect of 5-FU and metformin was observed in a 5-FU resistant cell line[74] and metformin radiosensitizer CRC cells, with reduced survival of ionization-resistant cells[75]. Consistently, the association of oxaliplatin, 5-FU and metformin also demonstrated a superior anti-tumor activity in chemoresistant HT-29, and HCT-116 cells compared to that with the drugs separately[76].

In 1977, it was firstly reported that phenformin inhibits metabolic immunosuppression in rats[77]. Since then, several reports have demonstrated that metformin has both chemopreventive and therapeutic activities in animal models of CRC. For instance, metformin treated ApcMin/+ mice showed significantly smaller polyps[78], and carcinogen-induced animal models that received metformin had a reduction of aberrant crypt foci[79], indicating the chemopreventive effect of metformin. Moreover, the association of metformin with D3 vitamin demonstrated a chemopreventive effect against 1,2-dimethylhydrazine (DMH)-induced CRC in rats and DMH-dextran sodium sulfate-induced colitis-associated CRC in mice[80]. On the other hand, treatment with metformin, 5-FU and oxaliplatin demonstrated superior antiproliferative effects in SCID mice bearing CRC[76]. In avatar models, metformin suppressed the tumor growth in the patient-derived xenografts by 50%[81]. In the same study, when metformin was combined with 5-FU, the tumor growth was inhibited up to 85%[81].

Clinical use of metformin

Metformin and CRC risk: As shown in Table 1 many case-control and cohort studies and associated meta-analyses have evaluated DS cancer risk and metformin use. Specifically, a decreased risk of CRC was found in the majority of studies[82-86], but no association or an increased risk of CRC was found in some of them[87-91]. Although these different results may be related to biases, a large cohort study that used adequate methods to minimize biases also concluded that metformin use decreased the risk of CRC[92].

Saliently, Cardel *et al*[82] demonstrated, in a case-control study, that the risk of CRC was decreased by 17% (OR: 0.83, 95%CI: 0.74-0.92) among patients treated with metformin compared to that among patients not using metformin[82], while Liu *et al*[93] showed a 22% risk reduction for the development of CRC[93]. Importantly, the role of metformin for CRC prophylaxis was addressed in a prospective Japanese phase III trial that demonstrated that low metformin doses for 1-year reduced polyp formation and colorectal adenomas in non-diabetic patients at high risk for new polyps[94]. However, further studies are necessary to draw a definitive conclusion.

Metformin and CRC treatment

Table 2 summarizes the clinical studies of metformin on DS cancers treatment. Specifically related to CRC, a Korean study of 595 patients with diabetes who had CRC with clinical stages I to IV showed that patients using metformin had higher overall survival (OS) and specific cancer survival compared to patients who did not use it[95]. In accordance, metformin use in 424 diabetic patients with CRC was associated with an OS of 76.9 mo *vs* 56.9 mo in patients not using metformin (P = 0.048)[95]. After adjusting for possible confounding factors, the study showed that patients with DM2 treated with metformin had a 30% increase in OS when compared to patients with DM2 treated with other antidiabetic drugs[95]. Recent meta-analyses demonstrated that metformin increases the OS of patients with CRC, as well as a 10% reduction in the incidence of the disease[96,97]. The ASAMET trial, an ongoing randomized, phase II, double-blind, placebo-controlled trial aims to determine the effect of low-dose aspirin and metformin in patients with stage I-III CRC in reducing CRC mortality rates and adenoma recurrence[98]. The 160 patients with CRC were divided in four arms: aspirin, metformin, aspirin plus metformin and placebo for a duration of 1 year.

The radiotherapy-induced tumor response was improved with metformin in a Korean retrospective study that evaluated patients with localized rectal cancer. The diabetic patients receiving metformin had significantly more tumor regression grade 3-4 (P = 0.029) and higher lymph node downstaging (P = 0.006) as compared to patients not receiving the medication. However, the disease-free survival (DFS) and OS was not affected[99]. Consistently, a study with 482 patients examined the effect of metformin use on pathologic complete response (pCR) rates and outcomes in patients submitted to neoadjuvant chemoradiotherapy for rectal cancer. The pCR rates were higher in patients with DM2 taking metformin (35%) compared with those in nondiabetic patients (16.6%) and patients with DM2 not using metformin (7.5%). Additionally, significantly increased DFS and OS was found in patients taking metformin[100].

A phase II clinical trial addressed the combination of metformin with 5-FU in patients with refractory CRC. It demonstrated a disease control rate in 8 wk of 22%, with a median OS of 7.9 mo and progression-free survival (PFS) of 1.8 mo[101]. A trial of our group with a similar design that analyzed the combination of irinotecan with metformin found 41% disease control rate and OS of 8.2 mo[102]. Further randomized prospective studies are needed to establish metformin as a modern drug for the treatment of refractory CRC.

Table 1 Selected clinical studies of metformin on digestive system cancers chemoprevention

Ref.	Study design and population	Inclusion criteria	Combined interventions /drugs	Main findings
			Comparison groups	Risk estimates and 95%CI
Colorectal cancer				
Cardel *et al*[82], 2014	Case-control study. Cases-controls: 2088:9060	Cases: DM2 with CRC. Controls: DM2 without CRC	Metformin user *vs* nonuser	OR: 0.83 (0.68-1.00)
Lee *et al*[83], 2011	Prospective Cohort, Taiwan. *n* = 480984	DM2 and cancer free subjects	Metformin user *vs* nonuser	HR: 0.36 (0.13-0.98)
Sehdev *et al*[84], 2015	Case control study. Cases-controls: 2682:5365	Cases: DM2 with CRC. Controls: DM2 without CRC	Metformin user *vs* nonuser	OR: 0.85 (0.76-0.95)
Tseng *et al*[84]., 2012	Retrospective Cohort. Men: 493704. Women: 502139	Subjects covered by National Health Insurance without CRC	Metformin user *vs* nonuser	RR: 0.64 (0.49-0.84)
Zhang *et al*[86], 2011	Meta-analysis. 108161 DM2 patients	Studies conducted in humans that evaluate metformin and CRC	Metformin user *vs* nonuser	RR: 0.63 (0.47-0.84)
Kowall *et al*[87], 2015	Retrospective Cohort, United Kingdom. 80263 DM2 patients	Patients aged 30-89 years with DM2 diagnosis	Metformin user *vs* sulfonylurea user	HR: 1.04 (0.82-1.31)
Lin *et al*[88], 2015	Prospective Cohort. 36270 DM2 patients. 145080 non DM2	Patients older than 20 years old DM2 and Cancer- free	Metformin user *vs* nonuser	HR: 0.74 (0.53-1.03)
Smiechowski *et al*[89], 2013	Case-control, United Kingdom. Cases-controls: 607:5837	DM patients treated with non-insulin antidiabetic agents	Metformin user *vs* nonuser	RR: 0.93 (0.73-1.18)
Bodmer *et al*[90], 2012	Case control, United Kingdom. Cases-controls: 920:5519	Cases: DM2 with CRC. Controls: DM2 without CRC	Metformin user *vs* nonuser	Men: OR: 1.81 (1.25-2.62). Women: OR: 1.00 (0.63-1.58)
Knapen *et al*[91], 2013	Retrospective Cohort, Denmark. 177281 DM2 with OHA	Oral antidiabetic drug users were matched 1:3 with population-based reference group	Biguanide user *vs* non-diabetic	HR: 1.19 (1.08-1.30)
Bradley *et al*[92], 2018	Retrospective Cohort, Northern California. 47351 DM2 patients	DM2 and no history of cancer or metformin use	Long-term metformin use (5 years) *vs* nonuser	All population: HR: 0.78 (0.60-1.02). Men: HR: 0.65 (0.45-0.94)
Liu *et al*[175], 2017	Meta-analysis. 20 case-control and cohort studies	Studies about metformin therapy and risk of adenoma/CRC in DM2 patients	Metformin user *vs* nonuser	Adenoma: OR: 0.75 (0.59-0.97). Carcinoma: OR: 0.781 (0.7-0.87)
Higurashi *et al*[94], 2016	RCT, phase 3. *n* = 151 patients with resected adenomas or polyps	Non- diabetic adult patients who had previously had single or multiple colorectal adenomas or polyps resected by endoscopy	Metformin 250 daily or placebo (1:1) for 1 yr	Adenoma: RR 0.60 (0.39-0.92)
Gastric cancer				
Tseng *et al*[107], 2016	Retrospective Cohort, Taiwan. 287971 DM2 with metformin. 16217 DM2 without metformin	DM2 patients newly treated with antidiabetic drugs	Metformin user *vs* nonuser	HR: 0.45 (0.36-0.56)
Dulskas *et al*[108], 2020	Retrospective Cohort study. *n* = 99992	DM2 patients with gastric cancer	Metformin user *vs* nonuser	SIR: 0.75 (0.66-0.86)
Ruiter *et al*[109], 2012	Retrospective Cohort study. 85289 DM2 patients	DM2 with more than one prescription of antidiabetic drugs	Metformin user *vs* sulfonylurea user	HR: 0.90 (0.88-0.91)
Kim *et al*[110], 2014	Retrospective cohort study. 39978 DM2 patients	DM2 receiving oral antidiabetic drugs	Metformin user and non-insulin user *vs* nonuser	HR: 0.73 (0.53-1.01)
Cheung *et al*[112], 2019	Prospective Cohort study. 7266 DM2	DM2 with prescription of therapy for H. pylori. Exclusion: history of GC	Metformin user *vs* nonuser	HR: 0.49 (0.24-0.98)
Tsilidis *et al*[113], 2014	Retrospective Cohort study. 51484 metformin. 18264 sulfonylureas	DM2 receiving oral antidiabetic drugs	Metformin user *vs* sulfonylurea user	HR: 0.96 (0.60-1.56)
de Jong *et al*[114], 2017	Retrospective Cohort study, Netherlands. 57621 DM2 with OHA	DM2 receiving oral antidiabetic drugs	Metformin user *vs* nonuser	HR: 0.97 (0.82-1.15)

Zheng *et al*[115], 2019	Prospective Cohort study. 544130 DM2 patients	Diabetes Cohort: DM2 receiving antidiabetic drugs. Matched cohort: common-medication users. Exclusion: History of GC or gastrectomy	Metformin user *vs* nonuser	Non-cardia: HR: 0.93 (0.78-1.12). Cardia: HR: 1.49 (1.09-2.02)
Shuai *et al*[116], 2020	Meta-analysis. 11 cohort studies	Studies conducted in humans that evaluate metformin and GC risk	Metformin user *vs* nonuser	HR: 0.79 (0.62-1.00)
Zhou *et al*[117], 2017	Meta-analysis. 7 Cohort studies. *n* = 591077	Studies conducted in humans that evaluate metformin and GC risk	Metformin user *vs* nonuser	HR: 0.76 (0.64-0.91)
Pancreatic ductal adenocarcinoma				
Currie *et al*[123], 2009	Retrospective cohort study. *n* = 62.809 DM2. Comparison between treatment: Metformin alone; Sulfonylurea alone; metformin plus sulfonylurea; insulin	DM2 developed > 40 years of age; United Kingdom residents	Metformin *vs* Sulfonylurea. Metformin *vs* Insulin	HR: 0.20 (0.11-0.36). HR: 0.22 (0.12-0.38)
Li *et al*[124], 2009	Hospital-based case control. Cases-controls: 973:863. Comparison between treatment: Metformin; insulin secretagogues; Other antidiabetic medications; insulin	DM subjects; cases: Newly PDAC diagnosed. Controls: Nonblood relative controls; United States residents	Metformin user *vs* nonuser	OR: 0.38 (0.22-0.69)
Soranna *et al*[126], 2012	Meta-analysis of 17 case-control and cohort studies. Any cancer: 17 case-control and cohort studies; 37632 cases. PDAC: 4 case-controls and retrospective cohort studies; 1192 cases	DM2 patients exposed to metformin alone or combined to sulfonylurea	Metformin user *vs* nonuser	RR: 0.38 (0.14-0.91)
Zhang *et al*[125], 2013	Meta-analysis of 37 case-control and cohort studies. *n* = 1535636	DM2 patients on treatment	Metformin user *vs* nonuser	SRR: 0.54 (0.35-0.83)
Hepatocellular carcinoma				
Donadon *et al*[159], 2010	Clinic-hospitalbased case control. Cases-controls: 190:359	Cases: HCC patients. Controls: Liver cirrhosis patients and healthy controls	Metformin *vs* sulfonylurea. Metformin *vs* insulin	OR: 0.39 (0.22-0.73). OR: 0.21 (0.11-0.42)
Hassan *et al*[158], 2010	Hospital-based case control. Cases-controls: 122:86	Cases: HCC. Controls: Healthy controls	Metformin user *vs* nonuser	OR: 0.30 (0.20-0.60)
Ma *et al*[160], 2017	Meta-analysis of 19 case-control and cohort studies and post hoc analysis of RCT of DM2 patients. *n* = 550.882	DM2 exposed to metformin or biguanide	Metformin user *vs* nonuser	OR: 0.52 (0.40-0.68)
Intrahepatic cholangiocarcinoma				
Chaiteerakij *et al*[168], 2013	Clinic-hospital based case-control. Cases-controls: 612:594	Cases: ICC patients. Controls: Non-cancer patients	Metformin user *vs* nonuser	OR: 0.40 (0.20-0.90)

PDAC: Pancreatic ductal adenocarcinoma; HR: Hazard ratio; OHA: Oxidized hyaluronate; RCT: Randomized clinical trial; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; CRC: Colorectal cancer.

Interestingly, a randomized trial that included 40 patients with stage III CRC evaluated the use of metformin in preventing oxaliplatin-induced neuropathy. After the 12thcycle of the FOLFOX-4 regimen, in the metformin group, there were fewer patients with grade 2 and 3 neuropathy as compared to the control arm (60% *vs* 95%, P = 0.009). Moreover, significantly higher total scores on the Ntx-12 questionnaire and pain score were found in the metformin arm. The serum levels of neurotensin and malondialdehyde were also significantly lower in the metformin arm after 6 and 12 cycles[103].

Furthermore, there are ongoing trials evaluating the role of metformin in CRC. We highlight, in adjuvant setting, a phase 3 trial (NCT02614339) with high-risk stage II and stage III CRC that aims to evaluate the impact of metformin for 48 mo on disease free survival. In refractory CRC setting, there is an interesting phase 2 trial is recruiting patients to explore the combination of the immune checkpoint inhibitors, such as nivolumab and metformin (NCT03800602).

Metformin and gastric cancer

The effect of metformin alone or in combination with cisplatin or rapamycin was studied in a tumor xenograft model[104]. It demonstrated that metformin alone decreased tumor volume. The combination of metformin with cisplatin, rapamycin or both increase the effect of each drug alone and inhibited the peritoneal dissemination of gastric cancer (GC)[104]. In accordance, Wu performed an *in vitro* study with AGS cell lines that analyzed how the association of metformin with cisplatin or adriamycin or paclitaxel enhanced the effects of each drug alone[105]. In striking contrast, Lesan

Table 2 Selected clinical studies of metformin on digestive system cancers treatment

Ref.	Study design and population	Inclusion criteria	Combined interventions /drugs	Main findings
Colorectal cancer				
Ramjeesingh *et al*[99], 2016	Retrospective cohort. 1394 all stages CRC patients	Patients with CRC	Metformin user *vs* nonuser	HR: 0.81 (0.60-1.08)
Skinner *et al*[100], 2013	Retrospective cohort. 482 locally rectal cancer patients	Locally advanced rectal adenocarcinoma treated with chemoradiation and surgery	Metformin user *vs* nonuser	pCR: OR: 16.8 (1.6-181.1). OS at 5 and 10 years (metformin *vs* non): 81% and 79% *vs* 56% and 39% (P = 0.022)
Miranda *et al*[101], 2016	Phase 2 Clinical trial. 50 refractory CRC patients	Refractory CRC patients	Metformin 850 mg twice a day+ 5-FU 425 mg/m^2 weekly	PFS: 1.8 mo. OS: 7.9 mo. Obese *vs* lean: 12.4 *vs* 5.8 mo
Bragagnoli *et al*[102], 2021	Phase 2 Clinical trial, 41 refractory CRC patients	Refractory CRC patients	Metformin 2500 mg a day+ Irinotecan 125 mg/m^2 D1, D8, every 21 d	PFS: 2.4 mo, CI 95%, 2.0-4.5 mo. OS: 8.4 mo, CI 95%, 5.9-10.8 mo
El-Fatatry *et al*[103], 2018	Clinical Trial, 40 Stage III CRC patients	Stage III CRC patients	FOLFOX 4 12 cycles + metformin 500 mg 3 times a day	Neuropathy grade 2-3 (metformin *vs* non): 60% *vs* 95% (P = 0.009)
Gastric cancer				
Lee *et al*[118], 2016	Retrospective Cohort, single center in Korea. 1974 GC resected patients: – 132 DM2 with metformin; –192 DM2 without metformin; –1648 non-diabetic	GC patients who underwent curative gastrectomy	Metformin user *vs* nonuser	OS-HR: 0.58 (0.37-0.93). RFS-HR: 0.63 (0.41-0.98)
Lacroix *et al*[120], 2018	Retrospective Cohort. 371 Patients	Stage I to III GC patients	Metformin user *vs* nonuser	OS-HR: 0.73 (0.52-1.01); cancer specific mortality-HR: 0.86 (0.56-1.33)
Baglia *et al*[121], 2019	Prospective cohort study in Shangai. 543 GC patients	Breast, CRC, lung and GC patients	Metformin user *vs* nonuser	OS-HR: 1.11 (0.81-1.53). Disease-specific survival-HR: 1.03 (0.73-1.43)
Seo *et al*[119], 2019	Retrospective cohort study. 2187 GC resected patients: – 103 DM2 with metformin; –139 DM2 without metformin; –1945 non-diabetic	GC patients who underwent curative gastrectomy	Metformin user *vs* nonuser	HR: 0.45 (0.30-0.66)
PDAC				
Sadeghi *et al*[128], 2012	Retrospective cohort. n = 302	DM2 patients. All stages. United States single center	Metformin user *vs* nonuser	HR: 0.64 (0.48-0.86)
Chaiteerakij *et al*[129], 2016	Retrospective cohort. n = 980	DM2 patients. All stages. United States single center	Metformin user *vs* nonuser	HR: 0.93 (0.81-1.07)
Lee *et al*[133], 2016	Retrospective cohort. n = 237	DM2 patients. All stages. Korean single center	Use of metformin ≥ 1-mo post-diagnosis *vs* nonuser	HR: 0.61 (0.46-0.81)
Ambe *et al*[130], 2016	Prospective cohort study n = 44	DM2 patients. Resected PDAC, stage I-II. United States single center	Metformin user *vs* nonuser	HR: 0.54 (0.16-1.86)
Cerullo *et al*[131], 2016	Retrospective cohort. n = 3393	Resected PDAC United States population based	Metformin use after surgery *vs* nonuser	HR: 0.79 (0.67-0.93)
Jang *et al*[132], 2017	Prospective cohort. n = 764	DM2, OHA user. Resected Korean population based	Metformin user *vs* nonuser	HR: 0.73 (0.61-0.87)
Hwang *et al*[135], 2013	Retrospective cohort. n = 516	DM2 patients. Locally advanced and metastatic. United Kingdom population based	Use of metformin peridiagnosis *vs* nonuser	HR: 1.11 (0.89-1.38)
Choi *et al*[134], 2016	Retrospective cohort. n = 183	DM2 patients. Locally advanced and metastatic. Korean single center	Metformin user *vs* nonuser	HR: 0.69 (0.49-0.97)
Kordes *et al*[137], 2015	RCT, n = 121	Locally advanced and metastatic. Multicentric. Netherlands	Gemcitabine-everolimus (1000 mg/m^2 D1, 8, 15-every 28 d-1.000 mg/d) +/- metformin (2000 mg/d)	HR: 1.05 (0.72-1.55)
Reni *et al*[138], 2016	RCT. n = 60	Metastatic. Single center. Italian	PEXG (cisplatin-epirubicin-capecitabine-gemcitabine: 30 mg/m^2	HR: 1.56 (0.87-2.80)

			D1,14- 30 mg/m^2 D1,14-2500 mg/m^2 D1–28 – 800 mg/m^2 D1–14) +/- metformin 2000 mg/d	
Zhou *et al*[136], 2017	Meta-analysis12 cohort studies and 2 RCT. *n* = 94778	Studies that investigated metformin exposition. All stages PDAC	Metformin user *vs* nonuser	HR: 0.77 (0.68-0.87)
Li *et al*[139], 2017	Meta-analysis. 9 cohort study and 2 RCT. *n* = 8089	Studies that investigated metformin exposition. All stages PDAC	Metformin user *vs* nonuser	HR: 0.86 (0.76-0.97)
Wan *et al*[140], 2018	Meta-analysis 15 cohort studies and 2 RCT, *n* = 36791	Studies that investigated metformin exposition. All stages PDAC	Metformin user *vs* nonuser	HR: 0.88 (0.80-0.97). Asians only HR: 0.74 (0.58-0.94); Stage I-II HR: 0.76 (0.68-0.86); Stage III-IV HR: 1.08 (0.82-1.43)
Braghiroli *et al*[141], 2015	Single-arm phase II. *n* = 20	Locally advanced or metastatic. 2nd line treatment. Single center. Brazilian	Paclitaxel (80 mg/m^2 D1, 8, 15 every 28 d) + metfomin 1750 mg/d	DCR at 8 wk 31, 6%
Pancreatic neuroendocrine tumor				
Pusceddu *et al*[153], 2018	Retrospective cohort. *n* = 445	Locally advanced or metastatic. Multicentric. Italian	No DM2 *vs* DM2. Metformin user *vs* nonuser	HR: 0.45 (0.32-0.62). HR: 0.49 (0.34-0.69)
Hepatocellular carcinoma				
Chen *et al*[163], 2011	Retrospective cohort. *n* = 53	DM2. Early-stage HCC. RFA treated. Single center. Taiwanese	Metformin user *vs* nonuser	HR: 0.24 (0.07-0.90)
Ma *et al*[164], 2016	Meta-analysis. 11 cohort studies. *n* = 3452	Studies that investigated metformin exposition. HCC patients	Metformin user *vs* nonuser	HR: 0.59 (0.42-0.83)
Intrahepatic cholangiocarcinoma				
Yang *et al*[169], 2016	Retrospective cohort. *n* = 250	DM2. Newly diagnosed ICC. United States single center	Metformin user *vs* nonuser	HR: 0.80 (0.60-1.20)

PDAC: Pancreatic ductal adenocarcinoma; ORC: Origin recognition complex; HR: Hazard ratio; PCR: Polymerase chain reaction; OS: Overall survival; PFS: Progression-free survival; RFS: Regarding refeeding syndrome; OHA: Oxidized hyaluronate; RCT: Randomized clinical trial; PEXG: Pseudoexfoliative glaucoma; DCR: Dacryocystorhinostomy; HCC: Hepatocellular carcinoma; RAF: Rapidly accelerated fibrosarcoma; ICC: Intrahepatic cholangiocarcinoma; CRC: Colorectal cancer; DM2: Diabetes mellitus type 2; GC: Gastric cancer.

et al[106] showed *in vitro* that metformin and cisplatin in combination decreased the effects of cisplatin alone[106].

In recent years, several observational studies have shown that metformin reduces the risk of GC[107-112]. The study of Tseng *et al*[107] demonstrated that GC risk was reduced using metformin, especially when the cumulative duration was more than 2 years[107]. In addition, metformin reduced the risk of GC, while opposite results were observed with sulfonylureas[108].

On the other hand, a study conducted in United Kingdom did not show a difference in GC incidence in patients receiving metformin compared to sulfonylureas[113]. Other reports also could not find any reduction in GC risk associated with metformin us[83,114,115]. Despite that, a meta-analysis showed a 21% reduction in the risk of GC with the use of metformin, in Asians the benefit was more prominent than in Westerners[116]. Another meta-analysis of cohort studies that included 591077 patients found a significantly lower incidence of GC with metformin therapy than other types of therapy (HR: 0.763; 95%CI: 0.642-0.905)[117].

Two retrospective studies conducted by Lee *et al*[118] and Seo *et al*[119] concluded that metformin reduced GC recurrence in patients undergoing gastrectomy[118,119]. Lacroix *et al*[120] showed that metformin improved OS but not cancer specific survival, in contrast, Baglia *et al*[121] observed that metformin use did not impact patient's survival[120,121].

More studies are needed to confirm the effect of metformin in GC treatment and chemoprevention. Unfortunately, there are few clinical trials that are ongoing to analyze this question. An interesting phase 2 randomized trial (NCT04114136) are ongoing to evaluate the synergistic effect of metformin, rosiglitazone and anti-PD-1 on

the treatment of refractory solid tumors including GC. Metformin could reduce tumor oxygen consumption creating a less hypoxic T cell environment leading to restore its anti-tumor cell function. The trial NCT04033107 analyze the combination of metformin and vitamin C in DS tumors including GC.

Metformin and pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer death in the United States and its prognosis remains dismal, encouraging research to discover innovative agents active in its treatment is an urgent unmet need[122]. Pancreatic ductal adenocarcinoma (PDAC) is its most common histologic type. An association between metformin use and decreased PDAC incidence in patients with DM2 was first recognized by two large clinical studies. In a large general practice retrospective cohort, Currie *et al*[123] reported risk reduction in metformin users related to sulfonylurea users (HR: 0.20; 95%CI: 0.11-0.36) and to insulin-based-treatment users (HR: 0.22; 95%CI: 0.12-0.38). Likewise, in a hospital-based case-control study, Li *et al*[124] encountered risk reduction in metformin users compared to those who did not use metformin (OR: 0.38; 95%CI: 0.21-0.67). Several meta-analyses have strongly reinforced PDAC risk reduction with metformin use in patients with DM2[18,125-127]. However, this effect should prospectively be confirmed in large prospective clinical trials.

Regarding survival, in a retrospective study, Sadeghi *et al*[128] reported a 36% lower risk of death (HR: 0.64; 95%CI: 0.48-0.86), OS benefit of 4 mo (15.2 mo *vs* 11.1 mo) and approximately 2-fold increase in 2-year survival rate (30.1% *vs* 15.4%) in patients who took metformin compared to those inpatients who did not take metformin. Interestingly, longer survival was only observed in non-metastatic disease, when stratified by disease stage[128]. Further evidence also encountered survival improvement in the subgroups of resected or locally advanced but not in patients with metastatic disease[129]. Specifically, among resected patients with PDAC, metformin use seemed to improve OS after 18 mo[130-132]. Related to locally advanced or metastatic disease, further evidence was contradictory on survival gains in patients with PDAC exposed to metformin with benefit being reported only in an Asian cohort[133-135]. A large meta-analysis analyzed data from 12 retrospective cohorts demonstrating OS improvement in metformin users at various stages (HR: 0.77; 95%CI: 0.68-0.87)[136].

Stimulated by this retrospective evidence, two European groups explored, in randomized clinical trials (RCTs), the association of metformin with gemcitabine-based chemotherapy as first-line treatment of advanced PDAC with negative results on OS improvement[137,138]. Recently, a meta-analysis, with inclusion of two RCTs, re-analyzed the improvement in OS and confirmed benefit in the whole population of diabetic patients with PDAC (HR: 0.86; 95%CI: 0.76-0.97)[139]. Analysis of subgroups in this study demonstrated improved survival in patients with resected or locally advanced tumors but not in the metastatic group. Similar results were observed in another group with a benefit in OS at various stages, which was more evident in the subgroups of less advanced stages and Asian patients[140]. Considering second-line treatment, a single arm prospective study did not reach survival gain of metformin associated to paclitaxel[141]. Results of ongoing clinical trials recently completed are expected with substantial interest. NCT01666730 explores overall survival improvement of metformin associated with modified FOLFOX6 in metastatic patients, NCT02005419 evaluates DFS at 1 year with the combination of metformin and gemcitabine in resected subjects and NCT02048384 analyses safety of metformin with or without rapamycin after disease stabilization on first line chemotherapy in metastatic individuals.

This clinical evidence is associated with the pre-clinical data that pancreatic cancer cells are sensitive to inhibition of oxidative phosphorylation, decreases in insulin-IGF signaling and inhibition of the mTOR pathway through AMPK activation, which are some of the major antineoplastic effects of metformin[39,142-145]. Identifying predictive or prognostic factors of response to metformin should be of relevance to select patients most likely to benefit from the effects of metformin[39]. Recent advances in molecular characterization might distinguish different biology and response to therapy in patients with morphologically similar PDAC and may be incorporated into clinical trials[146-148]. Moreover, the recently experienced challenge of standard of care in advanced pancreatic cancer treatment with polychemotherapy also brings new perspectives, as patients experience longer survival with the need to combine other active agents[149,150]. Future trials would include disease stage, identification of biomarkers and concentrations of metformin in neoplastic tissue to powerfully evaluate the benefit of metformin in the treatment of PDAC.

Another pancreatic neoplasm with rising incidence is pancreatic neuroendocrine tumors (panNETs)[151]. Few studies have evaluated the clinical benefit of metformin in the treatment of panNETs[152]. Pusceddu *et al*[153], in a multicentric retrospective cohort of patients receiving everolimus with or without somatostatin analogues, reported increased PFS in diabetic patients exposed to metformin compared to diabetic patients not exposed to metformin or non-diabetic patients [44.2 *vs* 20.8 mo (HR: 0.49; 95%CI: 0.34-0.69) or 15.1 mo (HR: 0.45; 95%CI: 0.32-0.62), respectively][153]. This result correlates with *in vitro* evidence that metformin decreases proliferation in human panNET cell lines[154,155]. A recent study demonstrated that the combination of metformin and everolimus strongly inhibited human panNET cell proliferation through mTOR suppression, compared to each agent used alone[156]. Results of the ongoing NCT02294006 prospective trial are expected to better evaluate the effects of this experimental treatment on PFS at 12 mo.

Metformin and hepatocellular carcinoma

The incidence of hepatocellular carcinoma (HCC) has strongly increased in last two decades, as well as the prevalence of its metabolic risk factors[156,157]. Hassan *et al*[158] and Donadon *et al*[159], in hospital-based case-control studies, first observed the strong association of metformin use and reduced risk of HCC in subjects with DM2 (HR: 0.15; 95%CI: 0.04-0.50) (HR: 0.30; 95%CI: 0.20-0.60)[156,158,159]. This protective effect was validated by accumulated evidence of observational studies including more than 0.5 million subjects (OR: 0.52; 95%CI: 0.40-0.68), being more evident in case-control than in cohort studies and without significance in the *post hoc* analysis of RCTs[160-162]. These data suggest an association between metformin use and reduced HCC incidence that needs to be confirmed in prospective clinical trials.

Improvement in HCC survival was first reported by Chen *et al*[163] in an early-stage cohort of patients treated with radiofrequency ablation with longer OS in metformin users compared to non-users (HR: 0.24; 95%CI: 0.07-0.90)[163]. A meta-analysis of 11 cohort studies was in accordance with better prognosis related to metformin use in patients with HCC related to their counterparts (HR: 0.59; 95%CI: 0.42-0.83)[164].

Although the antineoplastic effects of metformin in liver cancer are not completely understood, pre-clinical evidence observed inhibition of proliferation and induction of cell cycle arrest and apoptosis in HCC cells through AMPK activation[165,166]. Future prospective trials should explore the potential benefit of metformin in prevention and treatment of HCC.

Metformin and intrahepatic cholangiocarcinoma and gallbladder cancer

Intrahepatic cholangiocarcinoma (ICC) is the second most common hepatic cancer, and its incidence has markedly increased in the last decades[167]. Chaiteerakij *et al*[168], in a clinic-hospital-based retrospective cohort, reported 60% reduced risk of ICC in patients with DM2 who used metformin related to non-users (OR: 0.4; 95%CI: 0.2-0.9)[168]. The same group, however, did not encounter better prognosis in patients with DM2 with ICC taking metformin (HR: 0.8; 95%CI: 0.6-1.2)[169]. Although gallbladder cancer (GBC) is the most common biliary tract cancer[170], no clinical data and scarce basic evidence have explored the antineoplastic effects of metformin and its potential mechanisms of action in GBC.

Regarding comprehension of the possible mechanistic effects of metformin on ICC and GBC there are some *in vitro* and *in vivo* evidence. Overall, the studies observed the induction of apoptosis and cell cycle arrest mediated by activation of the AMPK-mTOR axis[171-173]. The association of metformin in combination with gemcitabine and cisplatin (the standard of care for advanced ICC) enhanced the antiproliferative effects of treatment in a cell model through their effects on AMPK, cyclin D1 and caspase-3[174]. Furthermore, Liu *et al*[175] first observed the decreased survival of GBC cells *via* inhibition of phosphorylated Akt (p-Akt) and Bcl-2 signaling[175]. Likewise, metformin inhibited GBC cell proliferation *via* downregulation of HIF-1α and VEGF and promoted cell cycle arrest by reduction of cyclin D1 expression in different animal experiments[176,177]. The association of metformin with cisplatin also promoted reduced expression of p-Akt and cyclin D1 downregulation, resulting in a synergistic antiprolife-rative effect in GBC cells[178].

These pre-clinical and preliminary clinical evidence highlights the need for metformin to be more deeply explored in clinical studies of ICC and GBC prevention and treatment. Considering the rationale that metformin may be active in the prevention and treatment of ICC and limited clinical data, exploratory studies should address this issue for a better understanding of its benefit in these clinical settings.

PERSPECTIVES

DS tumors are often associated to high morbidity and mortality and their incidence has increased over recent decades[179]. Recognition of its main risk factors and conditions of worse prognosis as well as development of strategies for prevention and treatment urges. In this context, projection of a worldwide burden of cancer attributable to diabetes and excess weight for the near future is an alarming public health concern[180]. Association of several cancers, including many DS tumors to diabetes and obesity have already been recognized (IARC, WCRF). Further strategies of prevention and treatment urge to be known. The large amount of evidence presented herein supports the idea of an important effect of metformin in decreasing risk and improving prognosis of several DS tumors.

Although most clinical studies presented here are retrospective that are often limited by immortal time and selection bias, recent discoveries of pre-clinical research on antineoplastic effects of metformin establish biological plausibility for the clinical data and reinforce the interest on its effects in carcinogenesis and cancer progression. These preclinical and clinical evidence supports running of adequately powered trials to investigate clinical use of metformin on DS tumors treatment. This should consider diabetic status, predictive biomarkers, disease stage and treatment setting. Concerning chemoprevention, safety, low cost, and widespread access are key to its feasibility. Therefore, repurposing metformin for DS cancer treatment is a scientific field of remarkable interest as it focuses on a global public health problem.

Currently, clinical research is considered a job with its inherent needed professional skills[181]. Taking in consideration that the low metformin cost does not impact the expensive process of drug repurposing, the development of this potential anti-cancer drug has been hampered. Moreover, the current stage of metformin clinical development needs testing in large, randomized, genome-guided, multicenter trials. These aspects explain, at least in part, the shortage of current studies on metformin in cancer prevention and treatment despite the large number of pre-clinical and clinical evidence indicating its potential benefit. We hope that this comprehensive review integrating the potential mechanisms, pre-clinical and clinical studies of metformin as anticancer agent alert the DS cancer community for the need of studying metformin effects in more specific clinical scenarios.

CONCLUSION

The remarkable intracellular pathway change caused by oncogenesis and the potential mechanisms of the antitumoral action of metformin have been supported. They have revealed novel target molecules and newly discovered treatment possibilities. In connection with epidemiological, pre-clinical, and clinical research, data support that metformin benefits some patients with DS tumors, requiring strict clinical trials to identify those who might obtain advantage from metformin combinations. Given that the survival outcomes are affected by a multitude of factors, such as cancer type, differentiation, staging and treatment, for adequately repurposing the use of metformin in DS cancers it is essential to take into consideration patient characteristics that may serve as predictive biomarkers of metformin antitumoral effects, such as insulin resistance, diabetes, body composition, and chronic diseases related to inflammation, as well as the specific tumor driven oncogenic pathway, which may interfere with the direct and indirect antitumoral effects of metformin.

REFERENCES

1 **Giovannucci E**, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, Pollak M, Regensteiner JG, Yee D. Diabetes and cancer: a consensus report. *CA Cancer J Clin* 2010; **60**: 207-221 [PMID: 20554718 DOI: 10.3322/caac.20078]

2 **Pollak M**. Insulin, insulin-like growth factors and neoplasia. *Best Pract Res Clin Endocrinol Metab* 2008; **22**: 625-638 [PMID: 18971123 DOI: 10.1016/j.beem.2008.08.004]

3 **Iyengar NM**, Gucalp A, Dannenberg AJ, Hudis CA. Obesity and Cancer Mechanisms: Tumor Microenvironment and Inflammation. *J Clin Oncol* 2016; **34**: 4270-4276 [PMID: 27903155 DOI: 10.1200/JCO.2016.67.4283]

4 **Wu L**, Zhu J, Prokop LJ, Murad MH. Pharmacologic Therapy of Diabetes and Overall Cancer Risk and Mortality: A Meta-Analysis of 265 Studies. *Sci Rep* 2015; **5**: 10147 [PMID: 26076034 DOI: 10.1038/srep10147]

5 **Giovannucci E**. Insulin and colon cancer. *Cancer Causes Control* 1995; **6**: 164-179 [PMID: 7749056 DOI: 10.1007/BF00052777]

6 **Lega IC**, Lipscombe LL. Review: Diabetes, Obesity, and Cancer-Pathophysiology and Clinical Implications. *Endocr Rev* 2020; **41** [PMID: 31722374 DOI: 10.1210/endrev/bnz014]

7 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638 [PMID: 12711737 DOI: 10.1056/NEJMoa021423]

8 **Singh S**, Singh H, Singh PP, Murad MH, Limburg PJ. Antidiabetic medications and the risk of colorectal cancer in patients with diabetes mellitus: a systematic review and meta-analysis. *Cancer Epidemiol Biomarkers Prev* 2013; **22**: 2258-2268 [PMID: 24042261 DOI: 10.1158/1055-9965.EPI-13-0429]

9 **Osório-Costa F**, Rocha GZ, Dias MM, Carvalheira JB. Epidemiological and molecular mechanisms aspects linking obesity and cancer. *Arq Bras Endocrinol Metabol* 2009; **53**: 213-226 [PMID: 19466214]

10 **De Bruijn KM**, Arends LR, Hansen BE, Leeflang S, Ruiter R, van Eijck CH. Systematic review and meta-analysis of the association between diabetes mellitus and incidence and mortality in breast and colorectal cancer. *Br J Surg* 2013; **100**: 1421-1429 [PMID: 24037561 DOI: 10.1002/bjs.9229]

11 **Cunha Júnior AD**, Pericole FV, Carvalheira JBC. Metformin and blood cancers. *Clinics (Sao Paulo)* 2018; **73**: e412s [PMID: 30208162 DOI: 10.6061/clinics/2018/e412s]

12 **Ben Q**, Xu M, Ning X, Liu J, Hong S, Huang W, Zhang H, Li Z. Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. *Eur J Cancer* 2011; **47**: 1928-1937 [PMID: 21458985 DOI: 10.1016/j.ejca.2011.03.003]

13 **Luo W**, Cao Y, Liao C, Gao F. Diabetes mellitus and the incidence and mortality of colorectal cancer: a meta-analysis of 24 cohort studies. *Colorectal Dis* 2012; **14**: 1307-1312 [PMID: 23046351 DOI: 10.1111/j.1463-1318.2012.02875.x]

14 **Chen J**, Han Y, Xu C, Xiao T, Wang B. Effect of type 2 diabetes mellitus on the risk for hepatocellular carcinoma in chronic liver diseases: a meta-analysis of cohort studies. *Eur J Cancer Prev* 2015; **24**: 89-99 [PMID: 24809655 DOI: 10.1097/CEJ.0000000000000038]

15 **Singh PP**, Shi Q, Foster NR, Grothey A, Nair SG, Chan E, Shields AF, Goldberg RM, Gill S, Kahlenberg MS, Sinicrope FA, Sargent DJ, Alberts SR. Relationship Between Metformin Use and Recurrence and Survival in Patients With Resected Stage III Colon Cancer Receiving Adjuvant Chemotherapy: Results From North Central Cancer Treatment Group N0147 (Alliance). *Oncologist* 2016; **21**: 1509-1521 [PMID: 27881709 DOI: 10.1634/theoncologist.2016-0153]

16 **Bailey CJ**. Metformin: its botanical background. *Pract Diab Int* 2004; 21 [DOI: 10.1002/pdi.606]

17 **Evans JM**, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 2005; **330**: 1304-1305 [PMID: 15849206 DOI: 10.1136/bmj.38415.708634.F7]

18 **Decensi A**, Puntoni M, Goodwin P, Cazzaniga M, Gennari A, Bonanni B, Gandini S. Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. *Cancer Prev Res (Phila)* 2010; **3**: 1451-1461 [PMID: 20947488 DOI: 10.1158/1940-6207.CAPR-10-0157]

19 **Noto H**, Goto A, Tsujimoto T, Noda M. Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis. *PLoS One* 2012; **7**: e33411 [PMID: 22448244 DOI: 10.1371/journal.pone.0033411]

20 **Franciosi M**, Lucisano G, Lapice E, Strippoli GF, Pellegrini F, Nicolucci A. Metformin therapy and risk of cancer in patients with type 2 diabetes: systematic review. *PLoS One* 2013; **8**: e71583 [PMID: 23936520 DOI: 10.1371/journal.pone.0071583]

21 **Gandini S**, Puntoni M, Heckman-Stoddard BM, Dunn BK, Ford L, DeCensi A, Szabo E. Metformin and cancer risk and mortality: a systematic review and meta-analysis taking into account biases and confounders. *Cancer Prev Res (Phila)* 2014; **7**: 867-885 [PMID: 24985407 DOI: 10.1158/1940-6207.CAPR-13-0424]

22 **Chen G**, Xu S, Renko K, Derwahl M. Metformin inhibits growth of thyroid carcinoma cells, suppresses self-renewal of derived cancer stem cells, and potentiates the effect of chemotherapeutic agents. *J Clin Endocrinol Metab* 2012; **97**: E510-E520 [PMID: 22278418 DOI: 10.1210/jc.2011-1754]

23 **Bednar F**, Simeone DM. Metformin and cancer stem cells: old drug, new targets. *Cancer Prev Res (Phila)* 2012; **5**: 351-354 [PMID: 22389436 DOI: 10.1158/1940-6207.CAPR-12-0026]

24 **Gong J**, Kelekar G, Shen J, Kaur S, Mita M. The expanding role of metformin in cancer: an update on antitumor mechanisms and clinical development. *Target Oncol* 2016; **11**: 447-467 [PMID: 26864078 DOI: 10.1007/s11523-016-0423-z]

25 **Pollak MN**. Investigating metformin for cancer prevention and treatment: the end of the beginning. *Cancer Discov* 2012; **2**: 778-790 [PMID: 22926251 DOI: 10.1158/2159-8290.CD-12-0263]

26 **Algire C**, Amrein L, Zakikhani M, Panasci L, Pollak M. Metformin blocks the stimulative effect of a high-energy diet on colon carcinoma growth *in vivo* and is associated with reduced expression of fatty acid synthase. *Endocr Relat Cancer* 2010; **17**: 351-360 [PMID: 20228137 DOI: 10.1677/ERC-09-0252]

27 **Choi YK**, Park KG. Metabolic roles of AMPK and metformin in cancer cells. *Mol Cells* 2013; **36**: 279-287 [PMID: 23794020 DOI: 10.1007/s10059-013-0169-8]

28 **Zakikhani M**, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Res* 2006; **66**: 10269-10273 [PMID:

17062558 DOI: 10.1158/0008-5472.CAN-06-1500]
29 **Verdura S**, Cuyàs E, Martin-Castillo B, Menendez JA. Metformin as an archetype immuno-metabolic adjuvant for cancer immunotherapy. *Oncoimmunology* 2019; **8**: e1633235 [PMID: 31646077 DOI: 10.1080/2162402X.2019.1633235]
30 **Rogers CJ**, Prabhu KS, Vijay-Kumar M. The microbiome and obesity-an established risk for certain types of cancer. *Cancer J* 2014; **20**: 176-180 [PMID: 24855004 DOI: 10.1097/PPO.0000000000000049]
31 **Bryrup T**, Thomsen CW, Kern T, Allin KH, Brandslund I, Jørgensen NR, Vestergaard H, Hansen T, Hansen TH, Pedersen O, Nielsen T. Metformin-induced changes of the gut microbiota in healthy young men: results of a non-blinded, one-armed intervention study. *Diabetologia* 2019; **62**: 1024-1035 [PMID: 30904939 DOI: 10.1007/s00125-019-4848-7]
32 **Zhou G**, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 2001; **108**: 1167-1174 [PMID: 11602624 DOI: 10.1172/JCI13505]
33 **Hawley SA**, Gadalla AE, Olsen GS, Hardie DG. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade *via* an adenine nucleotide-independent mechanism. *Diabetes* 2002; **51**: 2420-2425 [PMID: 12145153]
34 **Pollak M**. The insulin and insulin-like growth factor receptor family in neoplasia: an update. *Nat Rev Cancer* 2012; **12**: 159-169 [PMID: 22337149 DOI: 10.1038/nrc3215]
35 **Martin M**, Marais R. Metformin: a diabetes drug for cancer, or a cancer drug for diabetics? *J Clin Oncol* 2012; **30**: 2698-2700 [PMID: 22565000 DOI: 10.1200/JCO.2012.42.1677]
36 **Zakikhani M**, Bazile M, Hashemi S, Javeshghani S, Avizonis D, St Pierre J, Pollak MN. Alterations in cellular energy metabolism associated with the antiproliferative effects of the ATM inhibitor KU-55933 and with metformin. *PLoS One* 2012; **7**: e49513 [PMID: 23185347 DOI: 10.1371/journal.pone.0049513]
37 **Janzer A**, German NJ, Gonzalez-Herrera KN, Asara JM, Haigis MC, Struhl K. Metformin and phenformin deplete tricarboxylic acid cycle and glycolytic intermediates during cell transformation and NTPs in cancer stem cells. *Proc Natl Acad Sci USA* 2014; **111**: 10574-10579 [PMID: 25002509 DOI: 10.1073/pnas.1409844111]
38 **Nair V**, Pathi S, Jutooru I, Sreevalsan S, Basha R, Abdelrahim M, Samudio I, Safe S. Metformin inhibits pancreatic cancer cell and tumor growth and downregulates Sp transcription factors. *Carcinogenesis* 2013; **34**: 2870-2879 [PMID: 23803693 DOI: 10.1093/carcin/bgt231]
39 **Nair V**, Sreevalsan S, Basha R, Abdelrahim M, Abudayyeh A, Rodrigues Hoffman A, Safe S. Mechanism of metformin-dependent inhibition of mammalian target of rapamycin (mTOR) and Ras activity in pancreatic cancer: role of specificity protein (Sp) transcription factors. *J Biol Chem* 2014; **289**: 27692-27701 [PMID: 25143389 DOI: 10.1074/jbc.M114.592576]
40 **Blandino G**, Valerio M, Cioce M, Mori F, Casadei L, Pulito C, Sacconi A, Biagioni F, Cortese G, Galanti S, Manetti C, Citro G, Muti P, Strano S. Metformin elicits anticancer effects through the sequential modulation of DICER and c-MYC. *Nat Commun* 2012; **3**: 865 [PMID: 22643892 DOI: 10.1038/ncomms1859]
41 **Li W**, Yuan Y, Huang L, Qiao M, Zhang Y. Metformin alters the expression profiles of microRNAs in human pancreatic cancer cells. *Diabetes Res Clin Pract* 2012; **96**: 187-195 [PMID: 22245693 DOI: 10.1016/j.diabres.2011.12.028]
42 **Algire C**, Moiseeva O, Deschênes-Simard X, Amrein L, Petruccelli L, Birman E, Viollet B, Ferbeyre G, Pollak MN. Metformin reduces endogenous reactive oxygen species and associated DNA damage. *Cancer Prev Res (Phila)* 2012; **5**: 536-543 [PMID: 22262811 DOI: 10.1158/1940-6207.CAPR-11-0536]
43 **Nakamura M**, Ogo A, Yamura M, Yamaguchi Y, Nakashima H. Metformin suppresses sonic hedgehog expression in pancreatic cancer cells. *Anticancer Res* 2014; **34**: 1765-1769 [PMID: 24692708]
44 **Rios A**, Hsu SH, Blanco A, Buryanek J, Day AL, McGuire MF, Brown RE. Durable response of glioblastoma to adjuvant therapy consisting of temozolomide and a weekly dose of AMD3100 (plerixafor), a CXCR4 inhibitor, together with lapatinib, metformin and niacinamide. *Oncoscience* 2016; **3**: 156-163 [PMID: 27489862 DOI: 10.18632/oncoscience.311]
45 **Dirat B**, Ader I, Golzio M, Massa F, Mettouchi A, Laurent K, Larbret F, Malavaud B, Cormont M, Lemichez E, Cuvillier O, Tanti JF, Bost F. Inhibition of the GTPase Rac1 mediates the antimigratory effects of metformin in prostate cancer cells. *Mol Cancer Ther* 2015; **14**: 586-596 [PMID: 25527635 DOI: 10.1158/1535-7163.MCT-14-0102]
46 **Zhou J**, Wulfkuhle J, Zhang H, Gu P, Yang Y, Deng J, Margolick JB, Liotta LA, Petricoin E 3rd, Zhang Y. Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. *Proc Natl Acad Sci USA* 2007; **104**: 16158-16163 [PMID: 17911267 DOI: 10.1073/pnas.0702596104]
47 **Korkaya H**, Paulson A, Charafe-Jauffret E, Ginestier C, Brown M, Dutcher J, Clouthier SG, Wicha MS. Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. *PLoS Biol* 2009; **7**: e1000121 [PMID: 19492080 DOI: 10.1371/journal.pbio.1000121]
48 **Dowling RJ**, Niraula S, Stambolic V, Goodwin PJ. Metformin in cancer: translational challenges. *J Mol Endocrinol* 2012; **48**: R31-R43 [PMID: 22355097 DOI: 10.1530/JME-12-0007]
49 **Memmott RM**, Mercado JR, Maier CR, Kawabata S, Fox SD, Dennis PA. Metformin prevents

tobacco carcinogen--induced lung tumorigenesis. *Cancer Prev Res (Phila)* 2010; **3**: 1066-1076 [PMID: 20810672 DOI: 10.1158/1940-6207.CAPR-10-0055]

50 **Sarfstein R**, Friedman Y, Attias-Geva Z, Fishman A, Bruchim I, Werner H. Metformin downregulates the insulin/IGF-I signaling pathway and inhibits different uterine serous carcinoma (USC) cells proliferation and migration in p53-dependent or -independent manners. *PLoS One* 2013; **8**: e61537 [PMID: 23620761 DOI: 10.1371/journal.pone.0061537]

51 **Zakikhani M**, Blouin MJ, Piura E, Pollak MN. Metformin and rapamycin have distinct effects on the AKT pathway and proliferation in breast cancer cells. *Breast Cancer Res Treat* 2010; **123**: 271-279 [PMID: 20135346 DOI: 10.1007/s10549-010-0763-9]

52 **El-Mir MY**, Nogueira V, Fontaine E, Avéret N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration *via* an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* 2000; **275**: 223-228 [PMID: 10617608]

53 **Inoki K**, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 2003; **115**: 577-590 [PMID: 14651849]

54 **Cerezo M**, Tichet M, Abbe P, Ohanna M, Lehraiki A, Rouaud F, Allegra M, Giacchero D, Bahadoran P, Bertolotto C, Tartare-Deckert S, Ballotti R, Rocchi S. Metformin blocks melanoma invasion and metastasis development in AMPK/p53-dependent manner. *Mol Cancer Ther* 2013; **12**: 1605-1615 [PMID: 23741061 DOI: 10.1158/1535-7163.MCT-12-1226-T]

55 **Ben Sahra I**, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P, Tanti JF, Giorgetti-Peraldi S, Bost F. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer Res* 2011; **71**: 4366-4372 [PMID: 21540236 DOI: 10.1158/0008-5472.CAN-10-1769]

56 **Hirsch HA**, Iliopoulos D, Struhl K. Metformin inhibits the inflammatory response associated with cellular transformation and cancer stem cell growth. *Proc Natl Acad Sci USA* 2013; **110**: 972-977 [PMID: 23277563 DOI: 10.1073/pnas.1221055110]

57 **Rattan R**, Giri S, Hartmann LC, Shridhar V. Metformin attenuates ovarian cancer cell growth in an AMP-kinase dispensable manner. *J Cell Mol Med* 2011; **15**: 166-178 [PMID: 19874425 DOI: 10.1111/j.1582-4934.2009.00954.x]

58 **Kalender A**, Selvaraj A, Kim SY, Gulati P, Brûlé S, Viollet B, Kemp BE, Bardeesy N, Dennis P, Schlager JJ, Marette A, Kozma SC, Thomas G. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab* 2010; **11**: 390-401 [PMID: 20444419 DOI: 10.1016/j.cmet.2010.03.014]

59 **Kim SH**, Kim SC, Ku JL. Metformin increases chemo-sensitivity *via* gene downregulation encoding DNA replication proteins in 5-Fu resistant colorectal cancer cells. *Oncotarget* 2017; **8**: 56546-56557 [PMID: 28915611 DOI: 10.18632/oncotarget.17798]

60 **Rocha GZ**, Dias MM, Ropelle ER, Osório-Costa F, Rossato FA, Vercesi AE, Saad MJ, Carvalheira JB. Metformin amplifies chemotherapy-induced AMPK activation and antitumoral growth. *Clin Cancer Res* 2011; **17**: 3993-4005 [PMID: 21543517 DOI: 10.1158/1078-0432.CCR-10-2243]

61 **Wheaton WW**, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, Glasauer A, Dufour E, Mutlu GM, Budigner GS, Chandel NS. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife* 2014; **3**: e02242 [PMID: 24843020 DOI: 10.7554/eLife.02242]

62 **Son J**, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, Perera RM, Ferrone CR, Mullarky E, Shyh-Chang N, Kang Y, Fleming JB, Bardeesy N, Asara JM, Haigis MC, DePinho RA, Cantley LC, Kimmelman AC. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* 2013; **496**: 101-105 [PMID: 23535601 DOI: 10.1038/nature12040]

63 **Eikawa S**, Nishida M, Mizukami S, Yamazaki C, Nakayama E, Udono H. Immune-mediated antitumor effect by type 2 diabetes drug, metformin. *Proc Natl Acad Sci USA* 2015; **112**: 1809-1814 [PMID: 25624476 DOI: 10.1073/pnas.1417636112]

64 **Pearce EL**, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, Jones RG, Choi Y. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 2009; **460**: 103-107 [PMID: 19494812 DOI: 10.1038/nature08097]

65 **Prlic M**, Williams MA, Bevan MJ. Requirements for CD8 T-cell priming, memory generation and maintenance. *Curr Opin Immunol* 2007; **19**: 315-319 [PMID: 17433873 DOI: 10.1016/j.coi.2007.04.010]

66 **Zhang JJ**, Zhang QS, Li ZQ, Zhou JW, Du J. Metformin attenuates PD-L1 expression through activating Hippo signaling pathway in colorectal cancer cells. *Am J Transl Res* 2019; **11**: 6965-6976 [PMID: 31814900]

67 **Bridgeman SC**, Ellison GC, Melton PE, Newsholme P, Mamotte CDS. Epigenetic effects of metformin: From molecular mechanisms to clinical implications. *Diabetes Obes Metab* 2018; **20**: 1553-1562 [PMID: 29457866 DOI: 10.1111/dom.13262]

68 **White-Al Habeeb NM**, Garcia J, Fleshner N, Bapat B. Metformin Elicits Antitumor Effects and Downregulates the Histone Methyltransferase Multiple Myeloma SET Domain (MMSET) in Prostate Cancer Cells. *Prostate* 2016; **76**: 1507-1518 [PMID: 27404348 DOI: 10.1002/pros.23235]

69 **Zhang E**, Guo Q, Gao H, Xu R, Teng S, Wu Y. Metformin and Resveratrol Inhibited High Glucose-Induced Metabolic Memory of Endothelial Senescence through SIRT1/p300/p53/p21 Pathway. *PLoS One* 2015; **10**: e0143814 [PMID: 26629991 DOI: 10.1371/journal.pone.0143814]

70 **DU Y**, Zheng H, Wang J, Ren Y, Li M, Gong C, Xu F, Yang C. Metformin inhibits histone H2B monoubiquitination and downstream gene transcription in human breast cancer cells. *Oncol Lett*

2014; **8**: 809-812 [PMID: 25009658 DOI: 10.3892/ol.2014.2158]
71 **Mogavero A**, Maiorana MV, Zanutto S, Varinelli L, Bozzi F, Belfiore A, Volpi CC, Gloghini A, Pierotti MA, Gariboldi M. Metformin transiently inhibits colorectal cancer cell proliferation as a result of either AMPK activation or increased ROS production. *Sci Rep* 2017; **7**: 15992 [PMID: 29167573 DOI: 10.1038/s41598-017-16149-z]
72 **Lan B**, Zhang J, Zhang P, Zhang W, Yang S, Lu D, Li W, Dai Q. Metformin suppresses CRC growth by inducing apoptosis *via* ADORA1. *Front Biosci (Landmark Ed)* 2017; **22**: 248-257 [PMID: 27814614 DOI: 10.2741/4484]
73 **Feng YH**, Wu CL, Shiau AL, Lee JC, Chang JG, Lu PJ, Tung CL, Feng LY, Huang WT, Tsao CJ. MicroRNA-21-mediated regulation of Sprouty2 protein expression enhances the cytotoxic effect of 5-fluorouracil and metformin in colon cancer cells. *Int J Mol Med* 2012; **29**: 920-926 [PMID: 22322462 DOI: 10.3892/ijmm.2012.910]
74 **Zhang Y**, Guan M, Zheng Z, Zhang Q, Gao F, Xue Y. Effects of metformin on CD133+ colorectal cancer cells in diabetic patients. *PLoS One* 2013; **8**: e81264 [PMID: 24278407 DOI: 10.1371/journal.pone.0081264]
75 **Jeong YK**, Kim MS, Lee JY, Kim EH, Ha H. Metformin Radiosensitizes p53-Deficient Colorectal Cancer Cells through Induction of G2/M Arrest and Inhibition of DNA Repair Proteins. *PLoS One* 2015; **10**: e0143596 [PMID: 26599019 DOI: 10.1371/journal.pone.0143596]
76 **Nangia-Makker P**, Yu Y, Vasudevan A, Farhana L, Rajendra SG, Levi E, Majumdar AP. Metformin: a potential therapeutic agent for recurrent colon cancer. *PLoS One* 2014; **9**: e84369 [PMID: 24465408 DOI: 10.1371/journal.pone.0084369]
77 **Dilman VM**. Metabolic immunodepression which increases the risk of cancer. *Lancet* 1977; **2**: 1207-1209 [PMID: 73905 DOI: 10.1016/s0140-6736(77)90442-1]
78 **Tomimoto A**, Endo H, Sugiyama M, Fujisawa T, Hosono K, Takahashi H, Nakajima N, Nagashima Y, Wada K, Nakagama H, Nakajima A. Metformin suppresses intestinal polyp growth in ApcMin/+ mice. *Cancer Sci* 2008; **99**: 2136-2141 [PMID: 18803638 DOI: 10.1111/j.1349-7006.2008.00933.x]
79 **Hosono K**, Endo H, Takahashi H, Sugiyama M, Uchiyama T, Suzuki K, Nozaki Y, Yoneda K, Fujita K, Yoneda M, Inamori M, Tomatsu A, Chihara T, Shimpo K, Nakagama H, Nakajima A. Metformin suppresses azoxymethane-induced colorectal aberrant crypt foci by activating AMP-activated protein kinase. *Mol Carcinog* 2010; **49**: 662-671 [PMID: 20564343 DOI: 10.1002/mc.20637]
80 **Li W**, Wang QL, Liu X, Dong SH, Li HX, Li CY, Guo LS, Gao JM, Berger NA, Li L, Ma L, Wu YJ. Combined use of vitamin D3 and metformin exhibits synergistic chemopreventive effects on colorectal neoplasia in rats and mice. *Cancer Prev Res (Phila)* 2015; **8**: 139-148 [PMID: 25416412 DOI: 10.1158/1940-6207.CAPR-14-0128]
81 **Mohamed Suhaimi NA**, Phyo WM, Yap HY, Choy SHY, Wei X, Choudhury Y, Tan WJ, Tan LAPY, Foo RSY, Tan SHS, Tiang Z, Wong CF, Koh PK, Tan MH. Metformin Inhibits Cellular Proliferation and Bioenergetics in Colorectal Cancer Patient-Derived Xenografts. *Mol Cancer Ther* 2017; **16**: 2035-2044 [PMID: 28533437 DOI: 10.1158/1535-7163.MCT-16-0793]
82 **Cardel M**, Jensen SM, Pottegård A, Jørgensen TL, Hallas J. Long-term use of metformin and colorectal cancer risk in type II diabetics: a population-based case-control study. *Cancer Med* 2014; **3**: 1458-1466 [PMID: 25091592 DOI: 10.1002/cam4.306]
83 **Lee MS**, Hsu CC, Wahlqvist ML, Tsai HN, Chang YH, Huang YC. Type 2 diabetes increases and metformin reduces total, colorectal, liver and pancreatic cancer incidences in Taiwanese: a representative population prospective cohort study of 800,000 individuals. *BMC Cancer* 2011; **11**: 20 [PMID: 21241523 DOI: 10.1186/1471-2407-11-20]
84 **Sehdev A**, Shih YC, Vekhter B, Bissonnette MB, Olopade OI, Polite BN. Metformin for primary colorectal cancer prevention in patients with diabetes: a case-control study in a US population. *Cancer* 2015; **121**: 1071-1078 [PMID: 25424411 DOI: 10.1002/cncr.29165]
85 **Tseng CH**. Diabetes, metformin use, and colon cancer: a population-based cohort study in Taiwan. *Eur J Endocrinol* 2012; **167**: 409-416 [PMID: 22778198 DOI: 10.1530/EJE-12-0369]
86 **Zhang ZJ**, Zheng ZJ, Kan H, Song Y, Cui W, Zhao G, Kip KE. Reduced risk of colorectal cancer with metformin therapy in patients with type 2 diabetes: a meta-analysis. *Diabetes Care* 2011; **34**: 2323-2328 [PMID: 21949223 DOI: 10.2337/dc11-0512]
87 **Kowall B**, Stang A, Rathmann W, Kostev K. No reduced risk of overall, colorectal, lung, breast, and prostate cancer with metformin therapy in diabetic patients: database analyses from Germany and the UK. *Pharmacoepidemiol Drug Saf* 2015; **24**: 865-874 [PMID: 26132313 DOI: 10.1002/pds.3823]
88 **Lin CM**, Huang HL, Chu FY, Fan HC, Chen HA, Chu DM, Wu LW, Wang CC, Chen WL, Lin SH, Ho SY. Association between Gastroenterological Malignancy and Diabetes Mellitus and Anti-Diabetic Therapy: A Nationwide, Population-Based Cohort Study. *PLoS One* 2015; **10**: e0125421 [PMID: 25978841 DOI: 10.1371/journal.pone.0125421]
89 **Smiechowski B**, Azoulay L, Yin H, Pollak MN, Suissa S. The use of metformin and colorectal cancer incidence in patients with type II diabetes mellitus. *Cancer Epidemiol Biomarkers Prev* 2013; **22**: 1877-1883 [PMID: 23966577 DOI: 10.1158/1055-9965.EPI-13-0196]
90 **Bodmer M**, Becker C, Meier C, Jick SS, Meier CR. Use of metformin is not associated with a decreased risk of colorectal cancer: a case-control analysis. *Cancer Epidemiol Biomarkers Prev* 2012; **21**: 280-286 [PMID: 22178569 DOI: 10.1158/1055-9965.EPI-11-0992-T]
91 **Knapen LM**, Dittrich ST, de Vries F, Starup-Linde J, Vestergaard P, Henry RM, Stolk LM, Neef C, Bazelier MT. Use of biguanides and the risk of colorectal cancer: a register-based cohort study. *Curr*

Drug Saf 2013; **8**: 349-356 [PMID: 24215310 DOI: 10.2174/15680266113136660068]
92 **Bradley MC**, Ferrara A, Achacoso N, Ehrlich SF, Quesenberry CP Jr, Habel LA. A Cohort Study of Metformin and Colorectal Cancer Risk among Patients with Diabetes Mellitus. *Cancer Epidemiol Biomarkers Prev* 2018; **27**: 525-530 [PMID: 29716927 DOI: 10.1158/1055-9965.EPI-17-0424]
93 **Liu F**, Yan L, Wang Z, Lu Y, Chu Y, Li X, Liu Y, Rui D, Nie S, Xiang H. Metformin therapy and risk of colorectal adenomas and colorectal cancer in type 2 diabetes mellitus patients: A systematic review and meta-analysis. *Oncotarget* 2017; **8**: 16017-16026 [PMID: 27926481 DOI: 10.18632/oncotarget.13762]
94 **Higurashi T**, Hosono K, Takahashi H, Komiya Y, Umezawa S, Sakai E, Uchiyama T, Taniguchi L, Hata Y, Uchiyama S, Hattori A, Nagase H, Kessoku T, Arimoto J, Matsuhashi N, Inayama Y, Yamanaka S, Taguri M, Nakajima A. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: a multicentre double-blind, placebo-controlled, randomised phase 3 trial. *Lancet Oncol* 2016; **17**: 475-483 [PMID: 26947328 DOI: 10.1016/S1470-2045(15)00565-3]
95 **Garrett CR**, Hassabo HM, Bhadkamkar NA, Wen S, Baladandayuthapani V, Kee BK, Eng C, Hassan MM. Survival advantage observed with the use of metformin in patients with type II diabetes and colorectal cancer. *Br J Cancer* 2012; **106**: 1374-1378 [PMID: 22421948 DOI: 10.1038/bjc.2012.71]
96 **Meng F**, Song L, Wang W. Metformin Improves Overall Survival of Colorectal Cancer Patients with Diabetes: A Meta-Analysis. *J Diabetes Res* 2017; **2017**: 5063239 [PMID: 28271076 DOI: 10.1155/2017/5063239]
97 **He XK**, Su TT, Si JM, Sun LM. Metformin Is Associated With Slightly Reduced Risk of Colorectal Cancer and Moderate Survival Benefits in Diabetes Mellitus: A Meta-Analysis. *Medicine (Baltimore)* 2016; **95**: e2749 [PMID: 26886616 DOI: 10.1097/MD.0000000000002749]
98 **Petrera M**, Paleari L, Clavarezza M, Puntoni M, Caviglia S, Briata IM, Oppezzi M, Mislej EM, Stabuc B, Gnant M, Bachleitner-Hofmann T, Roth W, Scherer D, Haefeli WE, Ulrich CM, DeCensi A. The ASAMET trial: a randomized, phase II, double-blind, placebo-controlled, multicenter, 2 × 2 factorial biomarker study of tertiary prevention with low-dose aspirin and metformin in stage I-III colorectal cancer patients. *BMC Cancer* 2018; **18**: 1210 [PMID: 30514262 DOI: 10.1186/s12885-018-5126-7]
99 **Ramjeesingh R**, Orr C, Bricks CS, Hopman WM, Hammad N. A retrospective study on the role of diabetes and metformin in colorectal cancer disease survival. *Curr Oncol* 2016; **23**: e116-e122 [PMID: 27122979 DOI: 10.3747/co.23.2809]
100 **Skinner HD**, Crane CH, Garrett CR, Eng C, Chang GJ, Skibber JM, Rodriguez-Bigas MA, Kelly P, Sandulache VC, Delclos ME, Krishnan S, Das P. Metformin use and improved response to therapy in rectal cancer. *Cancer Med* 2013; **2**: 99-107 [PMID: 24133632 DOI: 10.1002/cam4.54]
101 **Miranda VC**, Braghiroli MI, Faria LD, Bariani G, Alex A, Bezerra Neto JE, Capareli FC, Sabbaga J, Lobo Dos Santos JF, Hoff PM, Riechelmann RP. Phase 2 Trial of Metformin Combined With 5-Fluorouracil in Patients With Refractory Metastatic Colorectal Cancer. *Clin Colorectal Cancer* 2016; **15**: 321-328. e1 [PMID: 27262895 DOI: 10.1016/j.clcc.2016.04.011]
102 **Bragagnoli AC**, Araujo RLC, Ferraz MW, Dos Santos LV, Abdalla KC, Comar F, Santos FA, Oliveira MA, Carvalheira JBC, Cárcano FM, da Silveira Nogueira Lima JP. Metformin plus Irinotecan in patients with refractory colorectal cancer: a phase 2 clinical trial. *Br J Cancer* 2021 [PMID: 33398062 DOI: 10.1038/s41416-020-01208-6]
103 **El-Fatatry BM**, Ibrahim OM, Hussien FZ, Mostafa TM. Role of metformin in oxaliplatin-induced peripheral neuropathy in patients with stage III colorectal cancer: randomized, controlled study. *Int J Colorectal Dis* 2018; **33**: 1675-1683 [PMID: 29931409 DOI: 10.1007/s00384-018-3104-9]
104 **Yu G**, Fang W, Xia T, Chen Y, Gao Y, Jiao X, Huang S, Wang J, Li Z, Xie K. Metformin potentiates rapamycin and cisplatin in gastric cancer in mice. *Oncotarget* 2015; **6**: 12748-12762 [PMID: 25909163 DOI: 10.18632/oncotarget.3327]
105 **Wu X**. Effect of metformin combined with chemotherapeutic agents on gastric cancer cell line AGS. *Pak J Pharm Sci* 2017; **30**: 1833-1836 [PMID: 29084654]
106 **Lesan V**, Ghaffari SH, Salaramoli J, Heidari M, Rostami M, Alimoghaddam K, Ghavamzadeh A. Evaluation of antagonistic effects of metformin with Cisplatin in gastric cancer cells. *Int J Hematol Oncol Stem Cell Res* 2014; **8**: 12-19 [PMID: 25642303]
107 **Tseng CH**. Metformin reduces gastric cancer risk in patients with type 2 diabetes mellitus. *Aging (Albany NY)* 2016; **8**: 1636-1649 [PMID: 27587088 DOI: 10.18632/aging.101019]
108 **Dulskas A**, Patasius A, Kaceniene A, Linkeviciute-Ulinskiene D, Zabuliene L, Smailyte G. A Cohort Study of Antihyperglycemic Medication Exposure and Gastric Cancer Risk. *J Clin Med* 2020; **9** [PMID: 32033451 DOI: 10.3390/jcm9020435]
109 **Ruiter R**, Visser LE, van Herk-Sukel MP, Coebergh JW, Haak HR, Geelhoed-Duijvestijn PH, Straus SM, Herings RM, Stricker BH. Lower risk of cancer in patients on metformin in comparison with those on sulfonylurea derivatives: results from a large population-based follow-up study. *Diabetes Care* 2012; **35**: 119-124 [PMID: 22100960 DOI: 10.2337/dc11-0857]
110 **Kim YI**, Kim SY, Cho SJ, Park JH, Choi IJ, Lee YJ, Lee EK, Kook MC, Kim CG, Ryu KW, Kim YW. Long-term metformin use reduces gastric cancer risk in type 2 diabetics without insulin treatment: a nationwide cohort study. *Aliment Pharmacol Ther* 2014; **39**: 854-863 [PMID: 24612291 DOI: 10.1111/apt.12660]
111 **Valent F**. Diabetes mellitus and cancer of the digestive organs: An Italian population-based cohort

study. *J Diabetes Complications* 2015; **29**: 1056-1061 [PMID: 26275864 DOI: 10.1016/j.jdiacomp.2015.07.017]

112 **Cheung KS**, Chan EW, Wong AYS, Chen L, Seto WK, Wong ICK, Leung WK. Metformin Use and Gastric Cancer Risk in Diabetic Patients After Helicobacter pylori Eradication. *J Natl Cancer Inst* 2019; **111**: 484-489 [PMID: 30329127 DOI: 10.1093/jnci/djy144]

113 **Tsilidis KK**, Capothanassi D, Allen NE, Rizos EC, Lopez DS, van Veldhoven K, Sacerdote C, Ashby D, Vineis P, Tzoulaki I, Ioannidis JP. Metformin does not affect cancer risk: a cohort study in the U.K. Clinical Practice Research Datalink analyzed like an intention-to-treat trial. *Diabetes Care* 2014; **37**: 2522-2532 [PMID: 24898303 DOI: 10.2337/dc14-0584]

114 **de Jong RG**, Burden AM, de Kort S, van Herk-Sukel MP, Vissers PA, Janssen PK, Haak HR, Masclee AA, de Vries F, Janssen-Heijnen ML. No Decreased Risk of Gastrointestinal Cancers in Users of Metformin in The Netherlands; A Time-Varying Analysis of Metformin Exposure. *Cancer Prev Res (Phila)* 2017; **10**: 290-297 [PMID: 28274936 DOI: 10.1158/1940-6207.CAPR-16-0277]

115 **Zheng J**, Xie SH, Santoni G, Lagergren J. Metformin use and risk of gastric adenocarcinoma in a Swedish population-based cohort study. *Br J Cancer* 2019; **121**: 877-882 [PMID: 31591459 DOI: 10.1038/s41416-019-0598-z]

116 **Shuai Y**, Li C, Zhou X. The effect of metformin on gastric cancer in patients with type 2 diabetes: a systematic review and meta-analysis. *Clin Transl Oncol* 2020; **22**: 1580-1590 [PMID: 32060719 DOI: 10.1007/s12094-020-02304-y]

117 **Zhou XL**, Xue WH, Ding XF, Li LF, Dou MM, Zhang WJ, Lv Z, Fan ZR, Zhao J, Wang LX. Association between metformin and the risk of gastric cancer in patients with type 2 diabetes mellitus: a meta-analysis of cohort studies. *Oncotarget* 2017; **8**: 55622-55631 [PMID: 28903449 DOI: 10.18632/oncotarget.16973]

118 **Lee CK**, Jung M, Jung I, Heo SJ, Jeong YH, An JY, Kim HI, Cheong JH, Hyung WJ, Noh SH, Kim HS, Rha SY, Chung HC. Cumulative Metformin Use and Its Impact on Survival in Gastric Cancer Patients After Gastrectomy. *Ann Surg* 2016; **263**: 96-102 [PMID: 25575260 DOI: 10.1097/SLA.0000000000001086]

119 **Seo HS**, Jung YJ, Kim JH, Lee HH, Park CH. The Effect of Metformin on Prognosis in Patients With Locally Advanced Gastric Cancer Associated With Type 2 Diabetes Mellitus. *Am J Clin Oncol* 2019; **42**: 909-917 [PMID: 31693512 DOI: 10.1097/COC.0000000000000627]

120 **Lacroix O**, Couttenier A, Vaes E, Cardwell CR, De Schutter H, Robert A. Impact of metformin on gastric adenocarcinoma survival: A Belgian population based study. *Cancer Epidemiol* 2018; **53**: 149-155 [PMID: 29453032 DOI: 10.1016/j.canep.2018.02.001]

121 **Baglia ML**, Cui Y, Zheng T, Yang G, Li H, You M, Xu L, Murff H, Gao YT, Zheng W, Xiang YB, Shu XO. Diabetes Medication Use in Association with Survival among Patients of Breast, Colorectal, Lung, or Gastric Cancer. *Cancer Res Treat* 2019; **51**: 538-546 [PMID: 29986576 DOI: 10.4143/crt.2017.591]

122 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020; **70**: 7-30 [PMID: 31912902 DOI: 10.3322/caac.21590]

123 **Currie CJ**, Poole CD, Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia* 2009; **52**: 1766-1777 [PMID: 19572116 DOI: 10.1007/s00125-009-1440-6]

124 **Li D**, Yeung SC, Hassan MM, Konopleva M, Abbruzzese JL. Antidiabetic therapies affect risk of pancreatic cancer. *Gastroenterology* 2009; **137**: 482-488 [PMID: 19375425 DOI: 10.1053/j.gastro.2009.04.013]

125 **Zhang P**, Li H, Tan X, Chen L, Wang S. Association of metformin use with cancer incidence and mortality: a meta-analysis. *Cancer Epidemiol* 2013; **37**: 207-218 [PMID: 23352629 DOI: 10.1016/j.canep.2012.12.009]

126 **Soranna D**, Scotti L, Zambon A, Bosetti C, Grassi G, Catapano A, La Vecchia C, Mancia G, Corrao G. Cancer risk associated with use of metformin and sulfonylurea in type 2 diabetes: a meta-analysis. *Oncologist* 2012; **17**: 813-822 [PMID: 22643536 DOI: 10.1634/theoncologist.2011-0462]

127 **Yu H**, Zhong X, Gao P, Shi J, Wu Z, Guo Z, Wang Z, Song Y. The Potential Effect of Metformin on Cancer: An Umbrella Review. *Front Endocrinol (Lausanne)* 2019; **10**: 617 [PMID: 31620081 DOI: 10.3389/fendo.2019.00617]

128 **Sadeghi N**, Abbruzzese JL, Yeung SC, Hassan M, Li D. Metformin use is associated with better survival of diabetic patients with pancreatic cancer. *Clin Cancer Res* 2012; **18**: 2905-2912 [PMID: 22465831 DOI: 10.1158/1078-0432.CCR-11-2994]

129 **Chaiteerakij R**, Petersen GM, Bamlet WR, Chaffee KG, Zhen DB, Burch PA, Leof ER, Roberts LR, Oberg AL. Metformin Use and Survival of Patients With Pancreatic Cancer: A Cautionary Lesson. *J Clin Oncol* 2016; **34**: 1898-1904 [PMID: 27069086 DOI: 10.1200/JCO.2015.63.3511]

130 **Ambe CM**, Mahipal A, Fulp J, Chen L, Malafa MP. Effect of Metformin Use on Survival in Resectable Pancreatic Cancer: A Single-Institution Experience and Review of the Literature. *PLoS One* 2016; **11**: e0151632 [PMID: 26967162 DOI: 10.1371/journal.pone.0151632]

131 **Cerullo M**, Gani F, Chen SY, Canner J, Pawlik TM. Metformin Use Is Associated with Improved Survival in Patients Undergoing Resection for Pancreatic Cancer. *J Gastrointest Surg* 2016; **20**: 1572-1580 [PMID: 27255657 DOI: 10.1007/s11605-016-3173-4]

132 **Jang WI**, Kim MS, Kang SH, Jo AJ, Kim YJ, Tchoe HJ, Park CM, Kim HJ, Choi JA, Choi HJ, Paik EK, Seo YS, Yoo HJ, Kang JK, Han CJ, Kim SB, Ko MJ. Association between metformin use and mortality in patients with type 2 diabetes mellitus and localized resectable pancreatic cancer: a nationwide population-based study in korea. *Oncotarget* 2017; **8**: 9587-9596 [PMID: 28077783

DOI: 10.18632/oncotarget.14525]
133 **Lee SH**, Yoon SH, Lee HS, Chung MJ, Park JY, Park SW, Song SY, Chung JB, Bang S. Can metformin change the prognosis of pancreatic cancer? *Dig Liver Dis* 2016; **48**: 435-440 [PMID: 26775128 DOI: 10.1016/j.dld.2015.12.006]
134 **Choi Y**, Kim TY, Oh DY, Lee KH, Han SW, Im SA, Bang YJ. The Impact of Diabetes Mellitus and Metformin Treatment on Survival of Patients with Advanced Pancreatic Cancer Undergoing Chemotherapy. *Cancer Res Treat* 2016; **48**: 171-179 [PMID: 25779362 DOI: 10.4143/crt.2014.292]
135 **Hwang AL**, Haynes K, Hwang WT, Yang YX. Metformin and survival in pancreatic cancer: a retrospective cohort study. *Pancreas* 2013; **42**: 1054-1059 [PMID: 24051965 DOI: 10.1097/MPA.0b013e3182965a3c]
136 **Zhou DC**, Gong H, Tan CQ, Luo JQ. Prognostic significance of anti-diabetic medications in pancreatic cancer: A meta-analysis. *Oncotarget* 2017; **8**: 62349-62357 [PMID: 28977950 DOI: 10.18632/oncotarget.17728]
137 **Kordes S**, Pollak MN, Zwinderman AH, Mathôt RA, Weterman MJ, Beeker A, Punt CJ, Richel DJ, Wilmink JW. Metformin in patients with advanced pancreatic cancer: a double-blind, randomised, placebo-controlled phase 2 trial. *Lancet Oncol* 2015; **16**: 839-847 [PMID: 26067687 DOI: 10.1016/S1470-2045(15)00027-3]
138 **Reni M**, Dugnani E, Cereda S, Belli C, Balzano G, Nicoletti R, Liberati D, Pasquale V, Scavini M, Maggiora P, Sordi V, Lampasona V, Ceraulo D, Di Terlizzi G, Doglioni C, Falconi M, Piemonti L. (Ir)relevance of Metformin Treatment in Patients with Metastatic Pancreatic Cancer: An Open-Label, Randomized Phase II Trial. *Clin Cancer Res* 2016; **22**: 1076-1085 [PMID: 26459175 DOI: 10.1158/1078-0432.CCR-15-1722]
139 **Li X**, Li T, Liu Z, Gou S, Wang C. The effect of metformin on survival of patients with pancreatic cancer: a meta-analysis. *Sci Rep* 2017; **7**: 5825 [PMID: 28724893 DOI: 10.1038/s41598-017-06207-x]
140 **Wan G**, Sun X, Li F, Wang X, Li C, Li H, Yu X, Cao F. Survival Benefit of Metformin Adjuvant Treatment For Pancreatic Cancer Patients: a Systematic Review and Meta-Analysis. *Cell Physiol Biochem* 2018; **49**: 837-847 [PMID: 30184547 DOI: 10.1159/000493214]
141 **Braghiroli MI**, de Celis Ferrari AC, Pfiffer TE, Alex AK, Nebuloni D, Carneiro AS, Caparelli F, Senna L, Lobo J, Hoff PM, Riechelmann RP. Phase II trial of metformin and paclitaxel for patients with gemcitabine-refractory advanced adenocarcinoma of the pancreas. *Ecancermedicalscience* 2015; **9**: 563 [PMID: 26316884 DOI: 10.3332/ecancer.2015.563]
142 **Viale A**, Pettazzoni P, Lyssiotis CA, Ying H, Sánchez N, Marchesini M, Carugo A, Green T, Seth S, Giuliani V, Kost-Alimova M, Muller F, Colla S, Nezi L, Genovese G, Deem AK, Kapoor A, Yao W, Brunetto E, Kang Y, Yuan M, Asara JM, Wang YA, Heffernan TP, Kimmelman AC, Wang H, Fleming JB, Cantley LC, DePinho RA, Draetta GF. Oncogene ablation-resistant pancreatic cancer cells depend on mitochondrial function. *Nature* 2014; **514**: 628-632 [PMID: 25119024 DOI: 10.1038/nature13611]
143 **Ming M**, Sinnett-Smith J, Wang J, Soares HP, Young SH, Eibl G, Rozengurt E. Dose-Dependent AMPK-Dependent and Independent Mechanisms of Berberine and Metformin Inhibition of mTORC1, ERK, DNA Synthesis and Proliferation in Pancreatic Cancer Cells. *PLoS One* 2014; **9**: e114573 [PMID: 25493642 DOI: 10.1371/journal.pone.0114573]
144 **Kisfalvi K**, Eibl G, Sinnett-Smith J, Rozengurt E. Metformin disrupts crosstalk between G protein-coupled receptor and insulin receptor signaling systems and inhibits pancreatic cancer growth. *Cancer Res* 2009; **69**: 6539-6545 [PMID: 19679549 DOI: 10.1158/0008-5472.CAN-09-0418]
145 **Karnevi E**, Said K, Andersson R, Rosendahl AH. Metformin-mediated growth inhibition involves suppression of the IGF-I receptor signalling pathway in human pancreatic cancer cells. *BMC Cancer* 2013; **13**: 235 [PMID: 23663483 DOI: 10.1186/1471-2407-13-235]
146 **Moffitt RA**, Marayati R, Flate EL, Volmar KE, Loeza SG, Hoadley KA, Rashid NU, Williams LA, Eaton SC, Chung AH, Smyla JK, Anderson JM, Kim HJ, Bentrem DJ, Talamonti MS, Iacobuzio-Donahue CA, Hollingsworth MA, Yeh JJ. Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma. *Nat Genet* 2015; **47**: 1168-1178 [PMID: 26343385 DOI: 10.1038/ng.3398]
147 **Collisson EA**, Bailey P, Chang DK, Biankin AV. Molecular subtypes of pancreatic cancer. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 207-220 [PMID: 30718832 DOI: 10.1038/s41575-019-0109-y]
148 **Bailey P**, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, Miller DK, Christ AN, Bruxner TJ, Quinn MC, Nourse C, Murtaugh LC, Harliwong I, Idrisoglu S, Manning S, Nourbakhsh E, Wani S, Fink L, Holmes O, Chin V, Anderson MJ, Kazakoff S, Leonard C, Newell F, Waddell N, Wood S, Xu Q, Wilson PJ, Cloonan N, Kassahn KS, Taylor D, Quek K, Robertson A, Pantano L, Mincarelli L, Sanchez LN, Evers L, Wu J, Pinese M, Cowley MJ, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chantrill LA, Mawson A, Humphris J, Chou A, Pajic M, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Lovell JA, Merrett ND, Toon CW, Epari K, Nguyen NQ, Barbour A, Zeps N, Moran-Jones K, Jamieson NB, Graham JS, Duthie F, Oien K, Hair J, Grützmann R, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Rusev B, Capelli P, Salvia R, Tortora G, Mukhopadhyay D, Petersen GM; Australian Pancreatic Cancer Genome Initiative, Munzy DM, Fisher WE, Karim SA, Eshleman JR, Hruban RH, Pilarsky C, Morton JP, Sansom OJ, Scarpa A, Musgrove EA, Bailey UM, Hofmann O, Sutherland RL, Wheeler DA, Gill AJ, Gibbs RA, Pearson JV, Biankin AV, Grimmond SM. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* 2016; **531**: 47-52 [PMID: 26909576 DOI:

10.1038/nature16965]
149 **Vaccaro V**, Sperduti I, Vari S, Bria E, Melisi D, Garufi C, Nuzzo C, Scarpa A, Tortora G, Cognetti F, Reni M, Milella M. Metastatic pancreatic cancer: Is there a light at the end of the tunnel? *World J Gastroenterol* 2015; **21**: 4788-4801 [PMID: 25944992 DOI: 10.3748/wjg.v21.i16.4788]
150 **Reni M**, Cereda S, Milella M, Novarino A, Passardi A, Mambrini A, Di Lucca G, Aprile G, Belli C, Danova M, Bergamo F, Franceschi E, Fugazza C, Ceraulo D, Villa E. Maintenance sunitinib or observation in metastatic pancreatic adenocarcinoma: a phase II randomised trial. *Eur J Cancer* 2013; **49**: 3609-3615 [PMID: 23899530 DOI: 10.1016/j.ejca.2013.06.041]
151 **Hallet J**, Law CH, Cukier M, Saskin R, Liu N, Singh S. Exploring the rising incidence of neuroendocrine tumors: a population-based analysis of epidemiology, metastatic presentation, and outcomes. *Cancer* 2015; **121**: 589-597 [PMID: 25312765 DOI: 10.1002/cncr.29099]
152 **Pusceddu S**, Vernieri C, Prinzi N, Torchio M, Coppa J, Antista M, Niger M, Milione M, Giacomelli L, Corti F, Prisciandaro M, Monteleone M, Colombo E, Di Bartolomeo M, de Braud F. The potential role of metformin in the treatment of patients with pancreatic neuroendocrine tumors: a review of preclinical to clinical evidence. *Therap Adv Gastroenterol* 2020; **13**: 1756284820927271 [PMID: 32821286 DOI: 10.1177/1756284820927271]
153 **Pusceddu S**, Vernieri C, Di Maio M, Marconcini R, Spada F, Massironi S, Ibrahim T, Brizzi MP, Campana D, Faggiano A, Giuffrida D, Rinzivillo M, Cingarlini S, Aroldi F, Antonuzzo L, Berardi R, Catena L, De Divitiis C, Ermacora P, Perfetti V, Fontana A, Razzore P, Carnaghi C, Davì MV, Cauchi C, Duro M, Ricci S, Fazio N, Cavalcoli F, Bongiovanni A, La Salvia A, Brighi N, Colao A, Puliafito I, Panzuto F, Ortolani S, Zaniboni A, Di Costanzo F, Torniai M, Bajetta E, Tafuto S, Garattini SK, Femia D, Prinzi N, Concas L, Lo Russo G, Milione M, Giacomelli L, Buzzoni R, Delle Fave G, Mazzaferro V, de Braud F. Metformin Use Is Associated With Longer Progression-Free Survival of Patients With Diabetes and Pancreatic Neuroendocrine Tumors Receiving Everolimus and/or Somatostatin Analogues. *Gastroenterology* 2018; **155**: 479-489. e7 [PMID: 29655834 DOI: 10.1053/j.gastro.2018.04.010]
154 **Vlotides G**, Tanyeri A, Spampatti M, Zitzmann K, Chourdakis M, Spttl C, Maurer J, Nölting S, Göke B, Auernhammer CJ. Anticancer effects of metformin on neuroendocrine tumor cells in vitro. *Hormones (Athens)* 2014; **13**: 498-508 [PMID: 25402373 DOI: 10.14310/horm.2002.1517]
155 **Herrera-Martínez AD**, Pedraza-Arevalo S, L-López F, Gahete MD, Gálvez-Moreno MA, Castaño JP, Luque RM. Type 2 Diabetes in Neuroendocrine Tumors: Are Biguanides and Statins Part of the Solution? *J Clin Endocrinol Metab* 2019; **104**: 57-73 [PMID: 30265346 DOI: 10.1210/jc.2018-01455]
156 **Vitali E**, Boemi I, Tarantola G, Piccini S, Zerbi A, Veronesi G, Baldelli R, Mazziotti G, Smiroldo V, Lavezzi E, Spada A, Mantovani G, Lania AG. Metformin and Everolimus: A Promising Combination for Neuroendocrine Tumors Treatment. *Cancers (Basel)* 2020; **12** [PMID: 32748870 DOI: 10.3390/cancers12082143]
157 **McGlynn KA**, Petrick JL, El-Serag HB. Epidemiology of Hepatocellular Carcinoma. *Hepatology* 2021; **73** Suppl 1: 4-13 [PMID: 32319693 DOI: 10.1002/hep.31288]
158 **Hassan MM**, Curley SA, Li D, Kaseb A, Davila M, Abdalla EK, Javle M, Moghazy DM, Lozano RD, Abbruzzese JL, Vauthey JN. Association of diabetes duration and diabetes treatment with the risk of hepatocellular carcinoma. *Cancer* 2010; **116**: 1938-1946 [PMID: 20166205 DOI: 10.1002/cncr.24982]
159 **Donadon V**, Balbi M, Mas MD, Casarin P, Zanette G. Metformin and reduced risk of hepatocellular carcinoma in diabetic patients with chronic liver disease. *Liver Int* 2010; **30**: 750-758 [PMID: 20331505 DOI: 10.1111/j.1478-3231.2010.02223.x]
160 **Ma S**, Zheng Y, Xiao Y, Zhou P, Tan H. Meta-analysis of studies using metformin as a reducer for liver cancer risk in diabetic patients. *Medicine (Baltimore)* 2017; **96**: e6888 [PMID: 28489794 DOI: 10.1097/MD.0000000000006888]
161 **Cunha V**, Cotrim HP, Rocha R, Carvalho K, Lins-Kusterer L. Metformin in the prevention of hepatocellular carcinoma in diabetic patients: A systematic review. *Ann Hepatol* 2020; **19**: 232-237 [PMID: 31836424 DOI: 10.1016/j.aohep.2019.10.005]
162 **Home PD**, Kahn SE, Jones NP, Noronha D, Beck-Nielsen H, Viberti G; ADOPT Study Group; RECORD Steering Committee. Experience of malignancies with oral glucose-lowering drugs in the randomised controlled ADOPT (A Diabetes Outcome Progression Trial) and RECORD (Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycaemia in Diabetes) clinical trials. *Diabetologia* 2010; **53**: 1838-1845 [PMID: 20532476 DOI: 10.1007/s00125-010-1804-y]
163 **Chen TM**, Lin CC, Huang PT, Wen CF. Metformin associated with lower mortality in diabetic patients with early stage hepatocellular carcinoma after radiofrequency ablation. *J Gastroenterol Hepatol* 2011; **26**: 858-865 [PMID: 21251068 DOI: 10.1111/j.1440-1746.2011.06664.x]
164 **Ma SJ**, Zheng YX, Zhou PC, Xiao YN, Tan HZ. Metformin use improves survival of diabetic liver cancer patients: systematic review and meta-analysis. *Oncotarget* 2016; **7**: 66202-66211 [PMID: 27494848 DOI: 10.18632/oncotarget.11033]
165 **Zheng L**, Yang W, Wu F, Wang C, Yu L, Tang L, Qiu B, Li Y, Guo L, Wu M, Feng G, Zou D, Wang H. Prognostic significance of AMPK activation and therapeutic effects of metformin in hepatocellular carcinoma. *Clin Cancer Res* 2013; **19**: 5372-5380 [PMID: 23942093 DOI: 10.1158/1078-0432.CCR-13-0203]
166 **Cai X**, Hu X, Cai B, Wang Q, Li Y, Tan X, Hu H, Chen X, Huang J, Cheng J, Jing X. Metformin

suppresses hepatocellular carcinoma cell growth through induction of cell cycle G1/G0 phase arrest and p21CIP and p27KIP expression and downregulation of cyclin D1 *in vitro* and in vivo. *Oncol Rep* 2013; **30**: 2449-2457 [PMID: 24008375 DOI: 10.3892/or.2013.2718]

167 **Antwi SO**, Mousa OY, Patel T. Racial, Ethnic, and Age Disparities in Incidence and Survival of Intrahepatic Cholangiocarcinoma in the United States; 1995-2014. *Ann Hepatol* 2018; **17**: 604-614 [PMID: 29893702 DOI: 10.5604/01.3001.0012.0929]

168 **Chaiteerakij R**, Yang JD, Harmsen WS, Slettedahl SW, Mettler TA, Fredericksen ZS, Kim WR, Gores GJ, Roberts RO, Olson JE, Therneau TM, Roberts LR. Risk factors for intrahepatic cholangiocarcinoma: association between metformin use and reduced cancer risk. *Hepatology* 2013; **57**: 648-655 [PMID: 23055147 DOI: 10.1002/hep.26092]

169 **Yang Z**, Zhang X, Roberts RO, Roberts LR, Chaiteerakij R. Metformin does not improve survival of cholangiocarcinoma patients with diabetes. *Hepatology* 2016; **63**: 667-668 [PMID: 25833233 DOI: 10.1002/hep.27821]

170 **Hundal R**, Shaffer EA. Gallbladder cancer: epidemiology and outcome. *Clin Epidemiol* 2014; **6**: 99-109 [PMID: 24634588 DOI: 10.2147/CLEP.S37357]

171 **Ling S**, Feng T, Ke Q, Fan N, Li L, Li Z, Dong C, Wang C, Xu F, Li Y, Wang L. Metformin inhibits proliferation and enhances chemosensitivity of intrahepatic cholangiocarcinoma cell lines. *Oncol Rep* 2014; **31**: 2611-2618 [PMID: 24788596 DOI: 10.3892/or.2014.3151]

172 **Ling S**, Xie H, Yang F, Shan Q, Dai H, Zhuo J, Wei X, Song P, Zhou L, Xu X, Zheng S. Metformin potentiates the effect of arsenic trioxide suppressing intrahepatic cholangiocarcinoma: roles of p38 MAPK, ERK3, and mTORC1. *J Hematol Oncol* 2017; **10**: 59 [PMID: 28241849 DOI: 10.1186/s13045-017-0424-0]

173 **Fujimori T**, Kato K, Fujihara S, Iwama H, Yamashita T, Kobayashi K, Kamada H, Morishita A, Kobara H, Mori H, Okano K, Suzuki Y, Masaki T. Antitumor effect of metformin on cholangiocarcinoma: In vitro and *in vivo* studies. *Oncol Rep* 2015; **34**: 2987-2996 [PMID: 26398221 DOI: 10.3892/or.2015.4284]

174 **Zhu HQ**, Ma JB, Song X, Gao HJ, Ma CQ, Chang H, Li HG, Liu FF, Lu J, Zhou X. Metformin potentiates the anticancer activities of gemcitabine and cisplatin against cholangiocarcinoma cells *in vitro* and in vivo. *Oncol Rep* 2016; **36**: 3488-3496 [PMID: 27779693 DOI: 10.3892/or.2016.5187]

175 **Liu Y**, Wang Z, Li M, Ye Y, Xu Y, Zhang Y, Yuan R, Jin Y, Hao Y, Jiang L, Hu Y, Chen S, Liu F, Wu W, Liu Y. Chloride intracellular channel 1 regulates the antineoplastic effects of metformin in gallbladder cancer cells. *Cancer Sci* 2017; **108**: 1240-1252 [PMID: 28378944 DOI: 10.1111/cas.13248]

176 **Ye J**, Chen K, Qi L, Li R, Tang H, Zhou C, Zhai W. Metformin suppresses hypoxiainduced migration *via* the HIF1α/VEGF pathway in gallbladder cancer in vitro and in vivo. *Oncol Rep* 2018; **40**: 3501-3510 [PMID: 30272364 DOI: 10.3892/or.2018.6751]

177 **Yamashita T**, Kato K, Fujihara S, Iwama H, Morishita A, Yamana H, Kobayashi K, Kamada H, Chiyo T, Kobara H, Tsutsui K, Okano K, Suzuki Y, Masaki T. Anti-diabetic drug metformin inhibits cell proliferation and tumor growth in gallbladder cancer *via* G0/G1 cell cycle arrest. *Anticancer Drugs* 2020; **31**: 231-240 [PMID: 31815765 DOI: 10.1097/CAD.0000000000000870]

178 **Bi T**, Zhu A, Yang X, Qiao H, Tang J, Liu Y, Lv R. Metformin synergistically enhances antitumor activity of cisplatin in gallbladder cancer *via* the PI3K/AKT/ERK pathway. *Cytotechnology* 2018; **70**: 439-448 [PMID: 29110119 DOI: 10.1007/s10616-017-0160-x]

179 **Sung H**, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021 [PMID: 33538338 DOI: 10.3322/caac.21660]

180 **Lim SS**, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, Amann M, Anderson HR, Andrews KG, Aryee M, Atkinson C, Bacchus LJ, Bahalim AN, Balakrishnan K, Balmes J, Barker-Collo S, Baxter A, Bell ML, Blore JD, Blyth F, Bonner C, Borges G, Bourne R, Boussinesq M, Brauer M, Brooks P, Bruce NG, Brunekreef B, Bryan-Hancock C, Bucello C, Buchbinder R, Bull F, Burnett RT, Byers TE, Calabria B, Carapetis J, Carnahan E, Chafe Z, Charlson F, Chen H, Chen JS, Cheng AT, Child JC, Cohen A, Colson KE, Cowie BC, Darby S, Darling S, Davis A, Degenhardt L, Dentener F, Des Jarlais DC, Devries K, Dherani M, Ding EL, Dorsey ER, Driscoll T, Edmond K, Ali SE, Engell RE, Erwin PJ, Fahimi S, Falder G, Farzadfar F, Ferrari A, Finucane MM, Flaxman S, Fowkes FG, Freedman G, Freeman MK, Gakidou E, Ghosh S, Giovannucci E, Gmel G, Graham K, Grainger R, Grant B, Gunnell D, Gutierrez HR, Hall W, Hoek HW, Hogan A, Hosgood HD 3rd, Hoy D, Hu H, Hubbell BJ, Hutchings SJ, Ibeanusi SE, Jacklyn GL, Jasrasaria R, Jonas JB, Kan H, Kanis JA, Kassebaum N, Kawakami N, Khang YH, Khatibzadeh S, Khoo JP, Kok C, Laden F, Lalloo R, Lan Q, Lathlean T, Leasher JL, Leigh J, Li Y, Lin JK, Lipshultz SE, London S, Lozano R, Lu Y, Mak J, Malekzadeh R, Mallinger L, Marcenes W, March L, Marks R, Martin R, McGale P, McGrath J, Mehta S, Mensah GA, Merriman TR, Micha R, Michaud C, Mishra V, Mohd Hanafiah K, Mokdad AA, Morawska L, Mozaffarian D, Murphy T, Naghavi M, Neal B, Nelson PK, Nolla JM, Norman R, Olives C, Omer SB, Orchard J, Osborne R, Ostro B, Page A, Pandey KD, Parry CD, Passmore E, Patra J, Pearce N, Pelizzari PM, Petzold M, Phillips MR, Pope D, Pope CA 3rd, Powles J, Rao M, Razavi H, Rehfuess EA, Rehm JT, Ritz B, Rivara FP, Roberts T, Robinson C, Rodriguez-Portales JA, Romieu I, Room R, Rosenfeld LC, Roy A, Rushton L, Salomon JA, Sampson U, Sanchez-Riera L, Sanman E, Sapkota A, Seedat S, Shi P, Shield K, Shivakoti R, Singh GM, Sleet DA, Smith E, Smith KR, Stapelberg NJ, Steenland K, Stöckl H, Stovner LJ, Straif K, Straney L, Thurston GD, Tran JH, Van Dingenen R, van Donkelaar A, Veerman JL, Vijayakumar L, Weintraub R, Weissman

MM, White RA, Whiteford H, Wiersma ST, Wilkinson JD, Williams HC, Williams W, Wilson N, Woolf AD, Yip P, Zielinski JM, Lopez AD, Murray CJ, Ezzati M, AlMazroa MA, Memish ZA. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; **380**: 2224-2260 [PMID: 23245609 DOI: 10.1016/S0140-6736(12)61766-8]

181 **Laterre PF**, François B. Strengths and limitations of industry vs. academic randomized controlled trials. *Clin Microbiol Infect* 2015; **21**: 906-909 [PMID: 26183298 DOI: 10.1016/j.cmi.2015.07.004]

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REVIEW

Mesenchymal stromal cell secretome in liver failure: Perspectives on COVID-19 infection treatment

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Abstract

Due to their immunomodulatory potential and release of trophic factors that promote healing, mesenchymal stromal cells (MSCs) are considered important players in tissue homeostasis and regeneration. MSCs have been widely used in clinical trials to treat multiple conditions associated with inflammation and tissue damage. Recent evidence suggests that most of the MSC therapeutic effects are derived from their secretome, including the extracellular vesicles, representing a promising approach in regenerative medicine application to treat organ failure as a result of inflammation/fibrosis. The recent outbreak of respiratory syndrome coronavirus, caused by the newly identified agent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has forced scientists worldwide to use all available instruments to fight the infection, including the inflammatory cascade caused by this pandemic disease. The use of MSCs is a valid approach to combat organ inflammation in different compartments. In addition to the lungs, which are considered the main inflammatory target for this virus, other organs are compromised by the infection. In particular, the liver is involved in the inflammatory response to SARS-CoV-2 infection, which causes organ failure, leading to death in coronavirus disease 2019 (COVID-19) patients. We herein summarize the current implications derived from the use of MSCs and their soluble derivatives in COVID-19 treatment, and emphasize the potential of MSC-based therapy in this clinical setting.

Key Words: Mesenchymal stromal cell; COVID-19; SARS-CoV-2; Organ failure;

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Core Tip: The recent coronavirus disease 2019 (COVID-19) pandemic outbreak has forced scientists worldwide to use all available options to fight this disease, in particular the inflammatory cascade caused by this infection. Mesenchymal stromal cells, for their immunomodulatory potential, represent a valid approach to combat organ inflammation. The main targets for this virus are the lungs, while other organs such as the liver are compromised by the infection. Evaluation of the albumin role in COVID-19 patients, and the connection to the "capillary leak syndrome" have focused attention on liver dysfunction correlated with the infection.

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INTRODUCTION

The liver can be damaged by various factors, including cytotoxic molecules, ischemia, metabolic alterations, or viral infections[1], which result in inflammatory responses contributing to further liver damage[2]. If the inflammation persists, a transition from acute to chronic injury can occur, inducing hepatic fibrosis[2]. Therefore, therapies that can reduce liver inflammation/fibrosis are crucial in order to avoid organ failure and the need for transplantation.

In recent years, the use of mesenchymal stromal cells (MSCs) has been considered a promising therapeutic approach to treat liver injuries[3]. MSCs can be isolated from different compartments including adipose tissue[4], umbilical cord[5], bone marrow[6], or placenta[7,8]. These cells have been successfully used in different therapeutic applications aimed at reducing inflammatory responses[9]. Moreover, the infusion of MSCs immediately after liver transplantation promotes organ regeneration and prolonged recipient survival by reducing acute inflammation[10].

Despite their beneficial properties, there are several limitations to the use of MSCs for cellular therapies; for example, their plasticity causes the potential risk of differentiation into undesired tissues and the possibility of malignant transformation is under debate[11,12]. To overcome these issues, the use of cell-free therapy is gaining considerable attention as a treatment for liver injury, an alternative to conventional cell transplantation[13]. Indeed, the regenerative properties of the MSC secretome include immunomodulatory effects mediated by growth factors and cytokines, such as transforming growth factor beta (TGF-β), prostaglandin E2, indoleamine 2,3-dioxygenase, hepatocyte growth factor (HGF), interleukin-10 (IL-10), and tumor necrosis factor alpha (TNF-α)[14,15], which can also attenuate fibrogenesis. In addition, the MSC therapeutic effects could also result from the released extracellular vesicles (EVs). EVs include a highly heterogeneous group of vesicles of different size able to modulate the immune responses[16,17]. Indeed, MSC-derived EVs can be selectively enriched with anti-fibrotic[18] and anti-apoptotic[19] factors, as well as specific non-coding RNA with therapeutic potential[20].

In December 2019, several cases of death from pneumonia were reported in Wuhan, later related to a new coronavirus-related disease called coronavirus disease 2019 (COVID-19). Analysis of its genome revealed it to be phylogenetically related to severe acute respiratory syndrome coronavirus (SARS-CoV)[21], and for this reason it was named SARS-CoV-2 by the World Health Organization (WHO). Due to its worldwide spread, the WHO declared COVID-19 a pandemic in March 2020. Angiotensin-converting enzyme 2 receptor (ACE2), highly expressed in the respiratory tract, was considered the main SARS-CoV-2 viral attachment for animal cells. Most likely for this reason, the lungs are the principal target organs for SARS-CoV-2[22,23]. This virus triggers an exacerbated immune reaction because large amounts of different

inflammatory factors, including cytokines and chemokines, are produced by immune reactive cells.

It has been hypothesized that MSC-based therapy for COVID-19 patients can prevent the development of a cytokine storm by activating the immune system and promoting organ repair[24,25]. Intravenously injected MSCs reach the lungs, where they engraft and secrete a variety of soluble factors including anti-inflammatory factors, angiogenic factors, and EVs[26,27]. Studies aimed toward reversing COVID-19 side effects through MSC treatment are ongoing. In this review, we summarize the therapeutic potentials of the MSC secretome for treating liver injuries associated with COVID-19.

MSC SECRETOME AND EVS FOR ORGAN INJURY

The use of MSC-based therapy for regenerative medicine applications counts in the hundreds of registered clinical trials (www.clinicaltrial.gov) because of the ability of these cells to promote immunomodulation and organ regeneration[28]. The release of trophic factors has demonstrated that their action is in part attributable to their secretome and, in particular, to secreted EVs[29]. Because of their intrinsic therapeutic potential, EVs are a powerful tool of regenerative medicine for the treatment of a wide range of diseases[30]. Due to heterogeneity in size and contents, as well as lack of specific markers, distinguishing the various EV subtypes is an ongoing challenge. According to the International Society for Extracellular Vesicles (ISEV), the generic term EVs includes nano-sized particles naturally released into the extracellular space by all cell types; they are delimited by a lipid bilayer and cannot replicate[31]. The ISEV consensus suggests considering physical parameters (*e.g.*, size or density) to distinguish "small" EVs, often referred as "exosomes" (< 100-200 nm in diameter) from "medium/large" EVs or "microvesicles" (> 200 nm). EVs are replete with diverse proteins, lipids, carbohydrates, and nucleic acids, and exert many of their functions of intercellular communicators by transferring their cargo molecules among cells. The specific cargo composition of EVs is largely defined by the tissue/cell type from which they originate[32]. Similarly to EVs from other cell types, MSC-EVs can be characterized according to the guidelines indicated by the ISEV. The available data suggest that EVs may significantly contribute to the paracrine effects of MSCs on tissue regeneration[33]. Because of EVs' broad biological functions, as well as their ability to transfer molecules between cells, MSC-EV-based therapy represents an attractive alternative to cell-based therapy. Application of MSC-EVs as a cell-free therapy has several advantages over conventional cell therapy. Primarily, EV injection carries lower safety risks because of their minimal reactivity to the immune system, and seem to be generally well tolerated, even when used xenogenically[34]. Then, because of their small size compared to MSCs, the intravenous delivery of EVs presents lower risk of vascular obstructions. Finally, EVs can also be genetically manipulated to carry desired therapeutic cargo for a broad, expanding range of potential clinical applications. The number of studies demonstrating the therapeutic potential of MSC-EVs in different disease models is growing rapidly. The beneficial effects of MSC-EV-based treatment are evidenced especially in cardioprotection and angiogenesis[35].

Understanding the mechanisms of action behind the therapeutic effects of MSC-EVs are crucial in view of their future clinical applications. Despite increasing interest, this field is still in its infancy in identifying the relevant bioactive molecules released by MSC-EVs that play a role in tissue repair. Efforts to identify these molecules lead to the conclusion that MSC-EVs preferentially contain mRNAs and microRNAs (miRNAs) targeting genes that participate in several cellular pathways involved in tissue repair, such as angiogenesis, migration, proliferation, self-renewal, differentiation, cellular transport, and apoptosis[36,37]. The overexpression of certain miRNAs can contribute to enhancing the therapeutic efficacy of MSC-EVs. For example, MSC-EVs over-expressing miR-21 have neuroprotective effects by targeting several genes involved in the inhibition of cell apoptosis[38,39]. The list of miRNAs known to increase the therapeutic potential of MSC-EVs in numerous disease models is long, and their therapeutic effects range from tumor modulation, immune suppression, and angiogenesis to tissue regeneration[40].

In addition to miRNAs, the beneficial effect of EV-derived proteins has been explored in terms of tissue repair and anti-inflammatory effects as a treatment for liver fibrosis, ischemia, myocardial infarction, acute renal injury, neural regeneration, or in the context of bone and cartilage regeneration[40]. Proteins identified in MSC-EVs and

linked to tissue repair include glial-derived neurotrophic factor, vascular endothelial growth factor, fibroblast growth factor, HGF, and angiotensin 1[41].

Although the number of clinical studies is limited, growing evidence shows the beneficial effects of MSC-EVs on tissue injuries. The impact of MSC-EVs on tissue regeneration has been investigated in several animal models of neuronal, cardiac, bone, cartilage, kidney, muscle, wound healing, respiratory injury, and liver regeneration[41,42]. Interestingly, data from animal models indicate that MSC-EVs can exert therapeutic potential similar to their cellular origin[41,43-46]. The list of registered clinical trials (https://clinicaltrials.gov) reporting tissue injuries-treated with MSC-EVs is shown in Table 1.

MSC-EVs show great potential as a regenerative medicine treatment for liver diseases. The benefits of MSC-EVs in liver diseases are documented in animal models of both acute[20] and chronic[47] liver injuries. MSC-EVs exert a beneficial effect by alleviating fibrosis and improving regeneration of hepatocytes[46]. In particular, EVs from fetal MSCs promote hepatocyte proliferation and decrease hepatocyte apoptosis in liver injury induced by carbon tetrachloride[48], or ameliorate oxidative stress in ischemia reperfusion injury (IRI) models in rats[49] and mice[50]. Similarly, EVs of MSC-derived induced pluripotent stem cells have hepatoprotective effects on a rat model of IRI by inducing hepatocyte proliferation[51,52]. Finally, the anti-fibrotic effects of hydrogel-embedded MSC-EVs are documented in chronic liver failure[53]. The results from *in vivo* studies indicate EVs as essential contributors to MSC therapeutic efficacy, and suggest that MSC-EV-based therapy may be a successful alternative to cell-based treatments. Nevertheless, there are still many important questions to be answered before MSC-EVs can become a fully realized cell-free therapy. These challenges comprise studies establishing the exact contribution of EVs to MSC-based therapy, including the underlying molecule mechanisms, or identifying which EV population is the most therapeutically effective. In addition, a major ongoing debate in the field of MSC EV-based therapy concerns the purity of the obtained vesicles due to contamination of the samples with non-EV proteins, RNAs, and lipoproteins[41].

LIVER FAILURE IN COVID-19 PATIENTS

SARS-CoV-2 is the etiological agent of the pandemic COVID-19, characterized by respiratory distress and/or hypoxemia, fever, fatigue, dry cough and, in severe cases, septic shock, metabolic acidosis, and death[54]. SARS-CoV-2, as with other corona viruses, enters the host cells by binding to the ACE-2 receptor[55], while the serine protease transmembrane serine protease 2 is required for S protein priming[56]. Despite the higher tropism for the respiratory tract, SARS-CoV-2 also targets other tissues, given that the ACE2 receptor is widely distributed in other tissues[57-61]. To shed light on the SARS-CoV-2 tropism, Nardo *et al*[62] analyzed, on the Human Protein Atlas, the expression levels of two proteins, ACE-2 receptor and TMPRSS2, in different human tissues, thus revealing a higher expression in the intestine and gall bladder, but their absence in the liver. A single-cell analysis, performed on healthy human liver samples, showed that while ACE-2 expression level in cholangiocytes is comparable to that of alveolar cells in the lungs, it is barely detectable in hepatocytes[63]. Interestingly, the liver cell line HuH7 is an established permissive cell type for both SARS-CoV and SARS-CoV-2 infection, and has recently been extensively used as a model in SARS-CoV-2 studies[64,65]. In addition, an *in vitro* study found that SARS-CoV-2 infection leads to a decrease of cholangiocellular tight junction protein claudin 1 mRNA expression, implying a reduced barrier function of cholangiocytes[66]. The presence of SARS-CoV-2 receptors in the gastrointestinal (GI) tract suggests an important role of the hepatobiliary tract in viral replication and excretion[67]. In fact, the virus has also been isolated from stool samples[68]. The involvement of the GI tract in COVID-19 disease is confirmed by the GI symptoms occurring in more than 60% of infected patients, as la ack of appetite, loss of smell and taste, anorexia, diarrhea, abdominal pain, nausea, and vomiting[69-74]. Moreover, post-mortem biopsies of SARS-CoV-2-infected patients showed the presence of the viral genome in hepatocytes and the GI tract by reverse transcription polymerase chain reaction (RT-PCR)[75-77].

Though liver failure in COVID-19 patients has been considered marginal, the incidence of hepatic tissue injury in these patients ranges from 14.8% to 53%[78], while mortality ranges from 58.06% to 78%[79,80]. The liver is a key organ in nearly all metabolic processes, has immunologic functions, and is the main detoxifying organ.

Table 1 List of registered clinical trials on the use of mesenchymal stromal cell-derived extracellular vesicles for tissue injury

Tissue injury disease	Condition	Treatment	Trial ID	Status
Chronic lung disease	Pediatric bronchopulmonary dysplasia	BM-MSC-derived EVs	NCT03857841	Phase I
Lung disease	Pneumonia, COVID-19	BM-MSC-derived EVs	NCT04493242	Not yet recruiting
Lung disease	Pneumonia, COVID-19	Inhalation of mesenchymal stem cell exosomes	NCT04276987	Phase I
Multiple organ failure	Multiple organ dysfunction syndrome	MSC exosomes	NCT04356300	Not yet recruiting
Lung disease	Pulmonary infection	MSC exosomes	NCT04544215	Recruiting
Dry eye	GVHD	UC-MSC exosomes	NCT04213248	Recruiting
Cartilage injury	Osteoarthritis	Secretome or EVs from adipose MSCs	NCT04223622	Not yet recruiting
Skin disease	Dystrophic epidermolysis bullosa	BM-MSC EVs	NCT04173650	Phase II
Brain	Cerebrovascular disorders	Allogenic MSCs enriched with miR-124	NCT03384433	Phase II

BM: Bone marrow; COVID-19: Coronavirus disease 2019; EV: Extracellular vesicle; GVHD: Graft-*vs*-host disease; MSC: Mesenchymal stromal cell; UC: Umbilical cord.

Moreover, because of the production of albumin, acute phase reactants and coagulation factors, the liver can strongly affect the multisystem manifestations of COVID-19[62]. In fact, modified levels of hepatic function indicators such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, gamma-glutamyl transferase, and bilirubin have been observed in patients with COVID-19, and principally in severe diseases[59,81-84]. Many studies have shown that liver injury occurs in the early stage of the disease, with mild or moderate increase of ALT, AST, or bilirubin together with a decrease in albumin levels[79,85,86]. High AST levels have been associated with the highest mortality risk[57,87], while decreased albumin levels have been associated with severe infection and poor prognosis[88,89]. Since the specific pathogenetic mechanism by which the virus causes liver injury is still unclear, many hypotheses have been offered, including immune-mediated damage. The triggering of an exacerbated immune response to the viral infection leads to a massive release of cytokines and inflammation mediators known as cytokine storm, which is responsible for immune-mediated liver damage[89] (Figure 1).

High levels of cytokines and chemokines (*i.e.* IL-1β, Il-2, IL-6, IL-8, IL-10, Il-17, interferons [IFNs], IFN-induced protein 10, TNF-α, granulocyte-macrophage colony-stimulating factor [GM-CSF], monocyte chemoattractant protein-1, macrophage inflammatory protein-1α) and other inflammatory molecules (PCR, ferritin, lactate dehydrogenase, D-dimer) have been observed in severe SARS-CoV-2-infected patients[54,57,88,90-92]. This highly inflammatory milieu leads to multiorgan damage, including liver failure, and is strictly linked to poor prognosis and death in COVID-19 patients[88,90]. As confirmation, liver samples from COVID-19 patient autopsies have revealed micro-vesicular steatosis and inflammation[93-95]. In fact, SARS-CoV-2 infects both hepatic cells and bile duct epithelium, causing liver impairment by direct cytopathic effect, as demonstrated by high transaminase levels and post-mortem liver biopsy specimens showing moderate micro-vesicular steatosis and mild lobular and portal activity[79]. Furthermore, the presence of SARS-CoV-2 has been found in parenchymal cells and vascular endothelium of the liver in COVID-19 patients[76,77].

Additional causes of liver injury can include hypoxia, hypovolemia, and microvascular thrombosis. The hypoxic state associated with COVID-19 can induce ischemic/hypoxic liver injury[87-89]. Considering that COVID-19 patients suffer from severe hypoxia, with the induction of ACE2 receptor expression on hepatocytes[96], a direct infection of hepatocytes by SARS-CoV-2 in hypoxic conditions has been suggested[25]. Liver injury can also be drug-induced. Most of the drugs used against SARS-CoV-2 are potentially hepatotoxic: Antivirals (lopinavir/ritonavir, remdesivir, umifenovir), antibiotics (macrolides, quinolones), chloroquine, tocilizumab, and steroids as well as antipyretic drugs used for fever in COVID-19[79,90,97,98]. Moreover, it must be considered that the majority of COVID-19 patients developing liver complications have a pre-existing chronic liver disease, rendering them more susceptible to liver injury. Interestingly, it has been reported that liver fibrotic/cirrhotic conditions lead to an increase of ACE-2 receptor expression in hepatocytes[96], thus suggesting again a possible role of pre-existing pathological liver

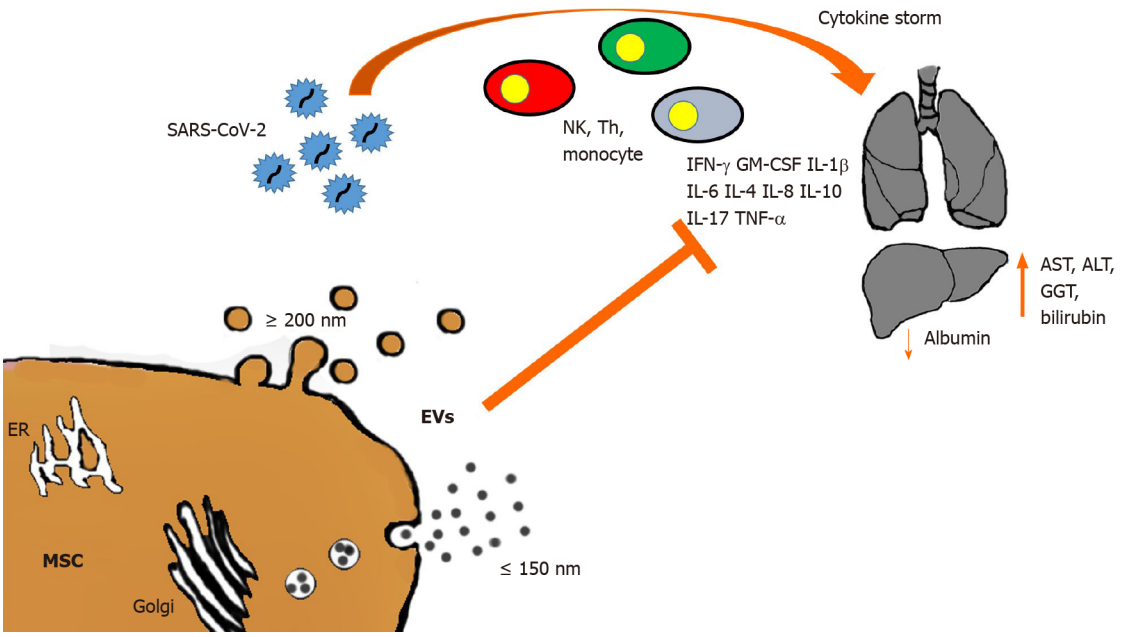


Figure 1 Schematic representation of severe acute respiratory syndrome coronavirus 2 impact on lungs and liver. Cytokine storm with the cascade triggered by natural killer (NK) cells, T helper (Th) cell and monocytes, and the production of inflammatory cytokines (interleukin 1 beta [IL-1b], Il-2, IL-6, IL-8, IL-10, Il-17, interferons [IFNs], IFN-induced protein 10, tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factor [GM-CSF]). The infection in the liver causes an increase in aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and bilirubin, and a decrease in albumin. Mesenchymal stromal cells (MSCs) can reduce the inflammatory response by extracellular vesicle (EV) release (large ≥ 200 nm and small ≤ 150 nm). ER: Endoplasmic reticulum; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

conditions in exacerbating SARS-CoV-2 hepatic tropism.

MSCS AND IMMUNOMODULATION IN COVID-19

The immunomodulatory properties of MSCs represent a promising therapeutic approach for the treatment of autoimmune and inflammatory diseases[99]. The anti-inflammatory and regenerative properties of MSCs have been established in numerous preclinical models of immune-related disorders including graft-*vs*-host disease, sepsis, inflammatory bowel disease, and allergic airway disease[100-103]. Recent phase I/II clinical trials have shown that the infusion of MSCs immediately after liver transplantation promoted organ regeneration and prolonged recipient survival by reducing acute inflammation, thus suggesting that MSCs can be a promising candidate for cell-based immunotherapy in solid organ transplantation[10,104]. In addition, murine models of liver fibrosis showed that human MSC-derived EVs are able to reduce hepatic inflammation and fibrosis through a decrease of TGF-β, IFN-γ, IL-1, IL-2 and TNF-α levels, an increase of Treg numbers, and a reduction of collagen deposition, all acting together to combat necrosis in the liver[105-107] (Figure 1). Among others, liver injury has been reported as a common complication in SARS-CoV-2 infection, with the degree of liver damage strictly related to the severity of COVID-19[92,108-110]. Although the exact mechanism of liver injury in COVID-19 patients is still unknown, it has been suggested that either the progression of pre-existing hepatic diseases or a direct damage of the liver can be associated with the systemic inflammation caused by SARS-CoV-2 infection, toxicity of anti-viral drugs, or hypoxia-reperfusion injury[109,110].

Pathogenic T cells are rapidly activated after SARS-CoV-2 infection, thus producing GM-CSF, IL-6, and other proinflammatory factors. GM-CSF will further activate inflammatory monocytes ($CD14^+CD16^+$), which in turn produce a larger amount of IL-6 and other pro-inflammatory factors, triggering the cytokine storm, which is the main cause of the organ damage, such as in the lungs, kidney, and liver[110]. Recently, the use of MSCs has been proposed as a promising therapeutic approach for COVID-19 patients. The effectiveness and safety of MSC-based treatment are supported by several clinical studies, suggesting that MSC therapy may improve the clinical outcomes of COVID-19 patients through immunomodulation, regulation of

inflammatory response, and promotion of tissue repair[111-115]. Moreover, a vast number of clinical trials that use MSCs to treat COVID-19 have already been registered (http://www.chictr.org.cn; https://clinicaltrials.gov). According to their immunomodulatory properties, the use of MSC-based therapies could be a novel strategy to counteract the harmful effects on the liver caused by SARS-CoV-2 infection.

DISCUSSION

Among the numerous drug treatments, which include antiviral therapy, cytokine inhibitors (*e.g.*, IL-6), and specific antibody treatment (serum/monoclonal)[116], MSCs represent a potential option for critical cases[117]. As discussed above, SARS-CoV-2 infection induces a cytokine storm, causing acute respiratory distress syndrome and multiple-organ failure. IL-6 inhibition by tocilizumab was positively tested in a randomized clinical trial (http://www.chictr.org.cn/showprojen.aspx?proj=49409). Likewise, in this inhibition MSCs can represent a valid alternative, and it has been shown that EV administration counteracts IL-6-induced acute liver injury (ALI) in rat models through the presence of miR-455-3p[118]. MSC treatment showed that the symptomatology of patients was relieved within 2-4 d after MSC infusion, with oxygen saturation increasing to 95% at rest[119]. Another study involved critically ill COVID-19 patients treated with an infusion of human umbilical cord MSCs. In this case, the patients were treated with three different infusions of cells at an interval of 3 d, displayed no observable side effects, and were able to walk within 4 d[115]. Leng *et al*[119] showed that after infusion of MSCs in COVID-19 patients, the number of peripheral lymphocytes increased, while the levels of C-reactive protein decreased. In addition, in MSC-treated COVID-19 patients compared with those treated with conventional therapy a clear reduction of the major pro-inflammatory cytokine TNF-α, and an increase of IL-10 concentration were observed[119]. Therefore, in an immune-mediated disease condition like COVID-19 infection, the anti-inflammatory activities of MSCs could contribute to improving the conditions of patients after their infusion.

Despite the limited published data, and based on various studies, it could be speculated that SARS-COV-2 induces ALI[79]. SARS-CoV-2 could insult the liver either directly, by the cytopathic effect of the virus after infections of the hepatocytes, or indirectly, by induction of uncontrolled immune reaction, oxidative stress, and/or by pharmacological treatments for COVID-19 that induce liver injury. However, the mechanisms underlying liver impairment in COVID-19 patients are still unknown. Tian *et al*[94] found sinusoidal dilatation and focal macrovesicular steatosis in liver biopsies obtained post-mortem from four patients with COVID-19 and, in one of these, SARS-CoV-2 RNA was isolated from liver tissue. Wang *et al*[73] found that four patients (2.9% of 138 patients hospitalized for COVID-19) had chronic liver disease. In another study, cases of ALI were reported in 13 of 274 patients (4.7%)[120]. Interestingly, Richardson *et al*[121] showed that, in a study including 5700 COVID-19 patients, 58.4% and 39% developed higher levels of ALT and AST, respectively. In addition, among these patients, 56 (1%) developed acute hepatic injury (32320003). Therefore, many COVID-19 patients showed higher levels of both ALT and AST, and mainly in patients with severe disease, liver impairment can occur[54,59,120].

The intravenous administration of MSCs lowered the elevated serum levels of AST and ALT, and increased the amount of HGF, resulting in reduction of ALI[122]. Moreover, in a rat model of ALI, MSCs inhibited neutrophil infiltration, oxidative stress, and hepatocyte apoptosis[123], showing that MSC treatment had significant systemic anti-inflammatory effects and reduction of hepatic inflammation. Moreover, MSCs can prevent lung damage not only directly, with anti-inflammatory activity, but also indirectly by supporting liver function in maintaining the plasma level of albumin (Figure 1). Johnson *et al*[124] recently underscored the interplay between albumin and SARS-CoV-2, while the importance of albumin in COVID-19 patients has also been strongly stressed by several research teams, who describe a "capillary leak syndrome" in infected patients. This extravascular leakage of intravascular fluids is induced by hypoglobulinemia[125]. A histological analysis of COVID-19 lungs in SARS-CoV-2-infected patients confirmed the presence of pulmonary vascular permeability where the endothelial cells appear swollen[126]. Hypoalbuminemia is an indication of liver dysfunction in the elderly, where it is, per se, an index of increased mortality[127]. The large amounts of extravascular fluid due to the resulting vascular permeability, require mechanical ventilation to overcome the problem.

CONCLUSION

At present, there is no standardized therapy for COVID-19 patients. Though many innovative treatments have been rapidly approved, additional experimental therapies are necessary to treat the worse cases of infection. Despite the fact that all MSC clinical trials for COVID-19 treatment are currently focused on lung/respiratory function, and some of the exclusion criteria are liver disease/insufficiency, we believe, on the basis of current studies, that MSC-based therapy can also help liver dysfunction correlated with SARS-CoV-2 infection.

REFERENCES

1 **Guicciardi ME**, Gores GJ. Apoptosis: a mechanism of acute and chronic liver injury. *Gut* 2005; **54**: 1024-1033 [PMID: 15951554 DOI: 10.1136/gut.2004.053850]
2 **Del Campo JA**, Gallego P, Grande L. Role of inflammatory response in liver diseases: Therapeutic strategies. *World J Hepatol* 2018; **10**: 1-7 [PMID: 29399273 DOI: 10.4254/wjh.v10.i1.1]
3 **Wang YH**, Wu DB, Chen B, Chen EQ, Tang H. Progress in mesenchymal stem cell-based therapy for acute liver failure. *Stem Cell Res Ther* 2018; **9**: 227 [PMID: 30143052 DOI: 10.1186/s13287-018-0972-4]
4 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13**: 4279-4295 [PMID: 12475952 DOI: 10.1091/mbc.e02-02-0105]
5 **Miranda JP**, Filipe E, Fernandes AS, Almeida JM, Martins JP, De la Fuente A, Abal M, Barcia RN, Cruz P, Cruz H, Castro M, Santos JM. The Human Umbilical Cord Tissue-Derived MSC Population UCX(®) Promotes Early Motogenic Effects on Keratinocytes and Fibroblasts and G-CSF-Mediated Mobilization of BM-MSCs When Transplanted In Vivo. *Cell Transplant* 2015; **24**: 865-877 [PMID: 24480602 DOI: 10.3727/096368913X676231]
6 **Colter DC**, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci USA* 2000; **97**: 3213-3218 [PMID: 10725391 DOI: 10.1073/pnas.070034097]
7 **Miceli V**, Pampalone M, Vella S, Carreca AP, Amico G, Conaldi PG. Comparison of Immunosuppressive and Angiogenic Properties of Human Amnion-Derived Mesenchymal Stem Cells between 2D and 3D Culture Systems. *Stem Cells Int* 2019; **2019**: 7486279 [PMID: 30911299 DOI: 10.1155/2019/7486279]
8 **Magatti M**, De Munari S, Vertua E, Gibelli L, Wengler GS, Parolini O. Human amnion mesenchyme harbors cells with allogeneic T-cell suppression and stimulation capabilities. *Stem Cells* 2008; **26**: 182-192 [PMID: 17901399 DOI: 10.1634/stemcells.2007-0491]
9 **Sargent A**, Miller RH. MSC Therapeutics in Chronic Inflammation. *Curr Stem Cell Rep* 2016; **2**: 168-173 [PMID: 28133600 DOI: 10.1007/s40778-016-0044-6]
10 **Wang W**, Du Z, Yan J, Ma D, Shi M, Zhang M, Peng C, Li H. Mesenchymal stem cells promote liver regeneration and prolong survival in small-for-size liver grafts: involvement of C-Jun N-terminal kinase, cyclin D1, and NF-κB. *PLoS One* 2014; **9**: e112532 [PMID: 25479410 DOI: 10.1371/journal.pone.0112532]
11 **Volarevic V**, Markovic BS, Gazdic M, Volarevic A, Jovicic N, Arsenijevic N, Armstrong L, Djonov V, Lako M, Stojkovic M. Ethical and Safety Issues of Stem Cell-Based Therapy. *Int J Med Sci* 2018; **15**: 36-45 [PMID: 29333086 DOI: 10.7150/ijms.21666]
12 **Herberts CA**, Kwa MS, Hermsen HP. Risk factors in the development of stem cell therapy. *J Transl Med* 2011; **9**: 29 [PMID: 21418664 DOI: 10.1186/1479-5876-9-29]
13 **Hu C**, Zhao L, Zhang L, Bao Q, Li L. Mesenchymal stem cell-based cell-free strategies: safe and effective treatments for liver injury. *Stem Cell Res Ther* 2020; **11**: 377 [PMID: 32883343 DOI: 10.1186/s13287-020-01895-1]
14 **Alfaifi M**, Eom YW, Newsome PN, Baik SK. Mesenchymal stromal cell therapy for liver diseases. *J Hepatol* 2018; **68**: 1272-1285 [PMID: 29425678 DOI: 10.1016/j.jhep.2018.01.030]
15 **Parekkadan B**, van Poll D, Megeed Z, Kobayashi N, Tilles AW, Berthiaume F, Yarmush ML. Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells. *Biochem Biophys Res Commun* 2007; **363**: 247-252 [PMID: 17869217 DOI: 10.1016/j.bbrc.2007.05.150]
16 **Bulati M**, Miceli V, Gallo A, Amico G, Carcione C, Pampalone M, Conaldi PG. The Immunomodulatory Properties of the Human Amnion-Derived Mesenchymal Stromal/Stem Cells Are Induced by INF-γ Produced by Activated Lymphomonocytes and Are Mediated by Cell-To-Cell Contact and Soluble Factors. *Front Immunol* 2020; **11**: 54 [PMID: 32117234 DOI: 10.3389/fimmu.2020.00054]
17 **Kordelas L**, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, Epple M, Horn PA, Beelen DW, Giebel B. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 2014; **28**: 970-973 [PMID: 24445866 DOI: 10.1038/leu.2014.41]
18 **Rong X**, Liu J, Yao X, Jiang T, Wang Y, Xie F. Human bone marrow mesenchymal stem cells-derived exosomes alleviate liver fibrosis through the Wnt/β-catenin pathway. *Stem Cell Res Ther* 2019; **10**: 98 [PMID: 30885249 DOI: 10.1186/s13287-019-1204-2]

19 **Lou G**, Chen Z, Zheng M, Liu Y. Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. *Exp Mol Med* 2017; **49**: e346 [PMID: 28620221 DOI: 10.1038/emm.2017.63]

20 **Haga H**, Yan IK, Takahashi K, Matsuda A, Patel T. Extracellular Vesicles from Bone Marrow-Derived Mesenchymal Stem Cells Improve Survival from Lethal Hepatic Failure in Mice. *Stem Cells Transl Med* 2017; **6**: 1262-1272 [PMID: 28213967 DOI: 10.1002/sctm.16-0226]

21 **Lu R**, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 2020; **395**: 565-574 [PMID: 32007145 DOI: 10.1016/S0140-6736(20)30251-8]

22 **Jia HP**, Look DC, Shi L, Hickey M, Pewe L, Netland J, Farzan M, Wohlford-Lenane C, Perlman S, McCray PB Jr. ACE2 receptor expression and severe acute respiratory syndrome coronavirus infection depend on differentiation of human airway epithelia. *J Virol* 2005; **79**: 14614-14621 [PMID: 16282461 DOI: 10.1128/JVI.79.23.14614-14621.2005]

23 **Liu Z**, Xiao X, Wei X, Li J, Yang J, Tan H, Zhu J, Zhang Q, Wu J, Liu L. Composition and divergence of coronavirus spike proteins and host ACE2 receptors predict potential intermediate hosts of SARS-CoV-2. *J Med Virol* 2020; **92**: 595-601 [PMID: 32100877 DOI: 10.1002/jmv.25726]

24 **Glenn JD**, Whartenby KA. Mesenchymal stem cells: Emerging mechanisms of immunomodulation and therapy. *World J Stem Cells* 2014; **6**: 526-539 [PMID: 25426250 DOI: 10.4252/wjsc.v6.i5.526]

25 **Lizardo-Thiebaud MJ**, Cervantes-Alvarez E, Limon-de la Rosa N, Tejeda-Dominguez F, Palacios-Jimenez M, Méndez-Guerrero O, Delaye-Martinez M, Rodriguez-Alvarez F, Romero-Morales B, Liu WH, Huang CA, Kershenobich D, Navarro-Alvarez N. Direct or Collateral Liver Damage in SARS-CoV-2-Infected Patients. *Semin Liver Dis* 2020; **40**: 321-330 [PMID: 32886936 DOI: 10.1055/s-0040-1715108]

26 **Lee RH**, Pulin AA, Seo MJ, Kota DJ, Ylostalo J, Larson BL, Semprun-Prieto L, Delafontaine P, Prockop DJ. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 2009; **5**: 54-63 [PMID: 19570514 DOI: 10.1016/j.stem.2009.05.003]

27 **Hu S**, Park J, Liu A, Lee J, Zhang X, Hao Q, Lee JW. Mesenchymal Stem Cell Microvesicles Restore Protein Permeability Across Primary Cultures of Injured Human Lung Microvascular Endothelial Cells. *Stem Cells Transl Med* 2018; **7**: 615-624 [PMID: 29737632 DOI: 10.1002/sctm.17-0278]

28 **Chinnici CM**, Pietrosi G, Iannolo G, Amico G, Cuscino N, Pagano V, Conaldi PG. Mesenchymal stromal cells isolated from human fetal liver release soluble factors with a potential role in liver tissue repair. *Differentiation* 2019; **105**: 14-26 [PMID: 30553176 DOI: 10.1016/j.diff.2018.12.001]

29 **Lai RC**, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010; **4**: 214-222 [PMID: 20138817 DOI: 10.1016/j.scr.2009.12.003]

30 **Tsiapalis D**, O'Driscoll L. Mesenchymal Stem Cell Derived Extracellular Vesicles for Tissue Engineering and Regenerative Medicine Applications. *Cells* 2020; **9** [PMID: 32316248 DOI: 10.3390/cells9040991]

31 **Théry C**, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina Zavec A, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, Borràs FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MÁ, Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Górecka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, Buzás EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado MF, D'Souza-Schorey C, Das S, Datta Chaudhuri A, de Candia P, De Santana EF, De Wever O, Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di Vizio D, Dieterich LC, Dolo V, Dominguez Rubio AP, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM, Ekström K, El Andaloussi S, Elie-Caille C, Erdbrügger U, Falcón-Pérez JM, Fatima F, Fish JE, Flores-Bellver M, Försönits A, Frelet-Barrand A, Fricke F, Fuhrmann G, Gabrielsson S, Gámez-Valero A, Gardiner C, Gärtner K, Gaudin R, Gho YS, Giebel B, Gilbert C, Gimona M, Giusti I, Goberdhan DC, Görgens A, Gorski SM, Greening DW, Gross JC, Gualerzi A, Gupta GN, Gustafson D, Handberg A, Haraszti RA, Harrison P, Hegyesi H, Hendrix A, Hill AF, Hochberg FH, Hoffmann KF, Holder B, Holthofer H, Hosseinkhani B, Hu G, Huang Y, Huber V, Hunt S, Ibrahim AG, Ikezu T, Inal JM, Isin M, Ivanova A, Jackson HK, Jacobsen S, Jay SM, Jayachandran M, Jenster G, Jiang L, Johnson SM, Jones JC, Jong A, Jovanovic-Talisman T, Jung S, Kalluri R, Kano SI, Kaur S, Kawamura Y, Keller ET, Khamari D, Khomyakova E, Khvorova A, Kierulf P, Kim KP, Kislinger T, Klingeborn M, Klinke DJ 2nd, Kornek M, Kosanović MM, Kovács ÁF, Krämer-Albers EM, Krasemann S, Krause M, Kurochkin IV, Kusuma GD, Kuypers S, Laitinen S, Langevin SM, Languino LR, Lannigan J, Lässer C, Laurent LC, Lavieu G, Lázaro-Ibáñez E, Le Lay S, Lee MS, Lee YXF, Lemos DS, Lenassi M, Leszczynska A, Li IT, Liao K, Libregts SF, Ligeti

E, Lim R, Lim SK, Linē A, Linnemannstöns K, Llorente A, Lombard CA, Lorenowicz MJ, Lörincz ÁM, Lötvall J, Lovett J, Lowry MC, Loyer X, Lu Q, Lukomska B, Lunavat TR, Maas SL, Malhi H, Marcilla A, Mariani J, Mariscal J, Martens-Uzunova ES, Martin-Jaular L, Martinez MC, Martins VR, Mathieu M, Mathivanan S, Maugeri M, McGinnis LK, McVey MJ, Meckes DG Jr, Meehan KL, Mertens I, Minciacchi VR, Möller A, Møller Jørgensen M, Morales-Kastresana A, Morhayim J, Mullier F, Muraca M, Musante L, Mussack V, Muth DC, Myburgh KH, Najrana T, Nawaz M, Nazarenko I, Nejsum P, Neri C, Neri T, Nieuwland R, Nimrichter L, Nolan JP, Nolte-'t Hoen EN, Noren Hooten N, O'Driscoll L, O'Grady T, O'Loghlen A, Ochiya T, Olivier M, Ortiz A, Ortiz LA, Osteikoetxea X, Østergaard O, Ostrowski M, Park J, Pegtel DM, Peinado H, Perut F, Pfaffl MW, Phinney DG, Pieters BC, Pink RC, Pisetsky DS, Pogge von Strandmann E, Polakovicova I, Poon IK, Powell BH, Prada I, Pulliam L, Quesenberry P, Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez MI, Raposo G, Rayyan MS, Regev-Rudzki N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC, Rohde E, Rome S, Rouschop KM, Rughetti A, Russell AE, Saá P, Sahoo S, Salas-Huenuleo E, Sánchez C, Saugstad JA, Saul MJ, Schiffelers RM, Schneider R, Schøyen TH, Scott A, Shahaj E, Sharma S, Shatnyeva O, Shekari F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM, Skowronek A, Snyder OL 2nd, Soares RP, Sódar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel W, Stott SL, Strasser EF, Swift S, Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh WS, Tomasini R, Torrecilhas AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van der Grein SG, Van Deun J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME, van Wijnen AJ, Vasconcelos MH, Vechetti IJ Jr, Veit TD, Vella LJ, Velot É, Verweij FJ, Vestad B, Viñas JL, Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP, Weber V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L, Wolfram J, Xagorari A, Xander P, Xu J, Yan X, Yáñez-Mó M, Yin H, Yuana Y, Zappulli V, Zarubova J, Žėkas V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco D, Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018; **7**: 1535750 [PMID: 30637094 DOI: 10.1080/20013078.2018.1535750]

32 **Doyle LM**, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 2019; **8** [PMID: 31311206 DOI: 10.3390/cells8070727]

33 **Zhang B**, Tian X, Hao J, Xu G, Zhang W. Mesenchymal Stem Cell-Derived Extracellular Vesicles in Tissue Regeneration. *Cell Transplant* 2020; **29**: 963689720908500 [PMID: 32207341 DOI: 10.1177/0963689720908500]

34 **Saleh AF**, Lázaro-Ibáñez E, Forsgard MA, Shatnyeva O, Osteikoetxea X, Karlsson F, Heath N, Ingelsten M, Rose J, Harris J, Mairesse M, Bates SM, Clausen M, Etal D, Leonard E, Fellows MD, Dekker N, Edmunds N. Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. *Nanoscale* 2019; **11**: 6990-7001 [PMID: 30916672 DOI: 10.1039/c8nr08720b]

35 **Amosse J**, Martinez MC, Le Lay S. Extracellular vesicles and cardiovascular disease therapy. *Stem Cell Investig* 2017; **4**: 102 [PMID: 29359141 DOI: 10.21037/sci.2017.11.07]

36 **Eirin A**, Riester SM, Zhu XY, Tang H, Evans JM, O'Brien D, van Wijnen AJ, Lerman LO. MicroRNA and mRNA cargo of extracellular vesicles from porcine adipose tissue-derived mesenchymal stem cells. *Gene* 2014; **551**: 55-64 [PMID: 25158130 DOI: 10.1016/j.gene.2014.08.041]

37 **Chinnici CM**, Amico G, Gallo A, Iannolo G, Cuscino N, Vella S, Carcione C, Nascari D, Conaldi PG. Small Extracellular Vesicles from Human Fetal Dermal Cells and Their MicroRNA Cargo: KEGG Signaling Pathways Associated with Angiogenesis and Wound Healing. *Stem Cells Int* 2020; **2020**: 8889379 [PMID: 32855639 DOI: 10.1155/2020/8889379]

38 **Zhang H**, Wang Y, Lv Q, Gao J, Hu L, He Z. MicroRNA-21 Overexpression Promotes the Neuroprotective Efficacy of Mesenchymal Stem Cells for Treatment of Intracerebral Hemorrhage. *Front Neurol* 2018; **9**: 931 [PMID: 30459705 DOI: 10.3389/fneur.2018.00931]

39 **Kang J**, Li Z, Zhi Z, Wang S, Xu G. MiR-21 derived from the exosomes of MSCs regulates the death and differentiation of neurons in patients with spinal cord injury. *Gene Ther* 2019; **26**: 491-503 [PMID: 31570818 DOI: 10.1038/s41434-019-0101-8]

40 **Park KS**, Bandeira E, Shelke GV, Lässer C, Lötvall J. Enhancement of therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. *Stem Cell Res Ther* 2019; **10**: 288 [PMID: 31547882 DOI: 10.1186/s13287-019-1398-3]

41 **Varderidou-Minasian S**, Lorenowicz MJ. Mesenchymal stromal/stem cell-derived extracellular vesicles in tissue repair: challenges and opportunities. *Theranostics* 2020; **10**: 5979-5997 [PMID: 32483432 DOI: 10.7150/thno.40122]

42 **Abraham A**, Krasnodembskaya A. Mesenchymal stem cell-derived extracellular vesicles for the treatment of acute respiratory distress syndrome. *Stem Cells Transl Med* 2020; **9**: 28-38 [PMID: 31647191 DOI: 10.1002/sctm.19-0205]

43 **Kim DK**, Nishida H, An SY, Shetty AK, Bartosh TJ, Prockop DJ. Chromatographically isolated CD63+CD81+ extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI. *Proc Natl Acad Sci USA* 2016; **113**: 170-175 [PMID: 26699510 DOI: 10.1073/pnas.1522297113]

44 **Bruno S**, Grange C, Deregibus MC, Calogero RA, Saviozzi S, Collino F, Morando L, Busca A, Falda M, Bussolati B, Tetta C, Camussi G. Mesenchymal stem cell-derived microvesicles protect

against acute tubular injury. *J Am Soc Nephrol* 2009; **20**: 1053-1067 [PMID: 19389847 DOI: 10.1681/ASN.2008070798]

45 **Stone ML**, Zhao Y, Robert Smith J, Weiss ML, Kron IL, Laubach VE, Sharma AK. Mesenchymal stromal cell-derived extracellular vesicles attenuate lung ischemia-reperfusion injury and enhance reconditioning of donor lungs after circulatory death. *Respir Res* 2017; **18**: 212 [PMID: 29268735 DOI: 10.1186/s12931-017-0704-9]

46 **Rostom DM**, Attia N, Khalifa HM, Abou Nazel MW, El Sabaawy EA. The Therapeutic Potential of Extracellular Vesicles Versus Mesenchymal Stem Cells in Liver Damage. *Tissue Eng Regen Med* 2020; **17**: 537-552 [PMID: 32506351 DOI: 10.1007/s13770-020-00267-3]

47 **Mardpour S**, Hassani SN, Mardpour S, Sayahpour F, Vosough M, Ai J, Aghdami N, Hamidieh AA, Baharvand H. Extracellular vesicles derived from human embryonic stem cell-MSCs ameliorate cirrhosis in thioacetamide-induced chronic liver injury. *J Cell Physiol* 2018; **233**: 9330-9344 [PMID: 29266258 DOI: 10.1002/jcp.26413]

48 **Tan CY**, Lai RC, Wong W, Dan YY, Lim SK, Ho HK. Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res Ther* 2014; **5**: 76 [PMID: 24915963 DOI: 10.1186/scrt465]

49 **Yao J**, Zheng J, Cai J, Zeng K, Zhou C, Zhang J, Li S, Li H, Chen L, He L, Chen H, Fu H, Zhang Q, Chen G, Yang Y, Zhang Y. Extracellular vesicles derived from human umbilical cord mesenchymal stem cells alleviate rat hepatic ischemia-reperfusion injury by suppressing oxidative stress and neutrophil inflammatory response. *FASEB J* 2019; **33**: 1695-1710 [PMID: 30226809 DOI: 10.1096/fj.201800131RR]

50 **Haga H**, Yan IK, Borrelli DA, Matsuda A, Parasramka M, Shukla N, Lee DD, Patel T. Extracellular vesicles from bone marrow-derived mesenchymal stem cells protect against murine hepatic ischemia/reperfusion injury. *Liver Transpl* 2017; **23**: 791-803 [PMID: 28407355 DOI: 10.1002/lt.24770]

51 **Nong K**, Wang W, Niu X, Hu B, Ma C, Bai Y, Wu B, Wang Y, Ai K. Hepatoprotective effect of exosomes from human-induced pluripotent stem cell-derived mesenchymal stromal cells against hepatic ischemia-reperfusion injury in rats. *Cytotherapy* 2016; **18**: 1548-1559 [PMID: 27592404 DOI: 10.1016/j.jcyt.2016.08.002]

52 **Du Y**, Li D, Han C, Wu H, Xu L, Zhang M, Zhang J, Chen X. Exosomes from Human-Induced Pluripotent Stem Cell-Derived Mesenchymal Stromal Cells (hiPSC-MSCs) Protect Liver against Hepatic Ischemia/ Reperfusion Injury *via* Activating Sphingosine Kinase and Sphingosine-1-Phosphate Signaling Pathway. *Cell Physiol Biochem* 2017; **43**: 611-625 [PMID: 28934733 DOI: 10.1159/000480533]

53 **Mardpour S**, Ghanian MH, Sadeghi-Abandansari H, Mardpour S, Nazari A, Shekari F, Baharvand H. Hydrogel-Mediated Sustained Systemic Delivery of Mesenchymal Stem Cell-Derived Extracellular Vesicles Improves Hepatic Regeneration in Chronic Liver Failure. *ACS Appl Mater Interfaces* 2019; **11**: 37421-37433 [PMID: 31525863 DOI: 10.1021/acsami.9b10126]

54 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

55 **Li W**, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003; **426**: 450-454 [PMID: 14647384 DOI: 10.1038/nature02145]

56 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280. e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]

57 **Zhou F**, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* 2020; **395**: 1054-1062 [PMID: 32171076 DOI: 10.1016/S0140-6736(20)30566-3]

58 **Hamming I**, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol* 2004; **203**: 631-637 [PMID: 15141377 DOI: 10.1002/path.1570]

59 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]

60 **Yan R**, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science* 2020; **367**: 1444-1448 [PMID: 32132184 DOI: 10.1126/science.abb2762]

61 **Xu H**, Zhong L, Deng J, Peng J, Dan H, Zeng X, Li T, Chen Q. High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. *Int J Oral Sci* 2020; **12**: 8 [PMID: 32094336 DOI: 10.1038/s41368-020-0074-x]

62 **Nardo AD**, Schneeweiss-Gleixner M, Bakail M, Dixon ED, Lax SF, Trauner M. Pathophysiological mechanisms of liver injury in COVID-19. *Liver Int* 2021; **41**: 20-32 [PMID: 33190346 DOI: 10.1111/liv.14730]

63 **Chai X**, Hu L, Zhang Y, Han W, Lu Z, Ke A, Zhou J, Shi G, Fang N, Fan J, Cai J, Lan F. Specific

ACE2 Expression in Cholangiocytes May Cause Liver Damage After 2019-nCoV Infection. 2020 Preprint. Available from: bioRxiv:2020.2002.2003.931766 [DOI: 10.1101/2020.02.03.931766]

64 **Chu H**, Chan JF, Yuen TT, Shuai H, Yuan S, Wang Y, Hu B, Yip CC, Tsang JO, Huang X, Chai Y, Yang D, Hou Y, Chik KK, Zhang X, Fung AY, Tsoi HW, Cai JP, Chan WM, Ip JD, Chu AW, Zhou J, Lung DC, Kok KH, To KK, Tsang OT, Chan KH, Yuen KY. Comparative tropism, replication kinetics, and cell damage profiling of SARS-CoV-2 and SARS-CoV with implications for clinical manifestations, transmissibility, and laboratory studies of COVID-19: an observational study. *Lancet Microbe* 2020; **1**: e14-e23 [PMID: 32835326 DOI: 10.1016/S2666-5247(20)30004-5]

65 **Yang L**, Han Y, Nilsson-Payant BE, Gupta V, Wang P, Duan X, Tang X, Zhu J, Zhao Z, Jaffré F, Zhang T, Kim TW, Harschnitz O, Redmond D, Houghton S, Liu C, Naji A, Ciceri G, Guttikonda S, Bram Y, Nguyen DT, Cioffi M, Chandar V, Hoagland DA, Huang Y, Xiang J, Wang H, Lyden D, Borczuk A, Chen HJ, Studer L, Pan FC, Ho DD, tenOever BR, Evans T, Schwartz RE, Chen S. A Human Pluripotent Stem Cell-based Platform to Study SARS-CoV-2 Tropism and Model Virus Infection in Human Cells and Organoids. *Cell Stem Cell* 2020; **27**: 125-136. e7 [PMID: 32579880 DOI: 10.1016/j.stem.2020.06.015]

66 **Zhao B**, Ni C, Gao R, Wang Y, Yang L, Wei J, Lv T, Liang J, Zhang Q, Xu W, Xie Y, Wang X, Yuan Z, Zhang R, Lin X. Recapitulation of SARS-CoV-2 infection and cholangiocyte damage with human liver ductal organoids. *Protein Cell* 2020; **11**: 771-775 [PMID: 32303993 DOI: 10.1007/s13238-020-00718-6]

67 **Xu Z**, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med* 2020; **8**: 420-422 [PMID: 32085846 DOI: 10.1016/S2213-2600(20)30076-X]

68 **Xiao F**, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for Gastrointestinal Infection of SARS-CoV-2. *Gastroenterology* 2020; **158**: 1831-1833. e3 [PMID: 32142773 DOI: 10.1053/j.gastro.2020.02.055]

69 **Luo S**, Zhang X, Xu H. Don't Overlook Digestive Symptoms in Patients With 2019 Novel Coronavirus Disease (COVID-19). *Clin Gastroenterol Hepatol* 2020; **18**: 1636-1637 [PMID: 32205220 DOI: 10.1016/j.cgh.2020.03.043]

70 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

71 **Zhou Z**, Zhao N, Shu Y, Han S, Chen B, Shu X. Effect of Gastrointestinal Symptoms in Patients With COVID-19. *Gastroenterology* 2020; **158**: 2294-2297 [PMID: 32199880 DOI: 10.1053/j.gastro.2020.03.020]

72 **Redd WD**, Zhou JC, Hathorn KE, McCarty TR, Bazarbashi AN, Thompson CC, Shen L, Chan WW. Prevalence and Characteristics of Gastrointestinal Symptoms in Patients With Severe Acute Respiratory Syndrome Coronavirus 2 Infection in the United States: A Multicenter Cohort Study. *Gastroenterology* 2020; **159**: 765-767. e2 [PMID: 32333911 DOI: 10.1053/j.gastro.2020.04.045]

73 **Wang D**, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 2020; **323**: 1061-1069 [PMID: 32031570 DOI: 10.1001/jama.2020.1585]

74 **Schmulson M**, Dávalos MF, Berumen J. Beware: Gastrointestinal symptoms can be a manifestation of COVID-19. *Rev Gastroenterol Mex* 2020; **85**: 282-287 [PMID: 32376072 DOI: 10.1016/j.rgmx.2020.04.001]

75 **Lin L**, Jiang X, Zhang Z, Huang S, Fang Z, Gu Z, Gao L, Shi H, Mai L, Liu Y, Lin X, Lai R, Yan Z, Li X, Shan H. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. *Gut* 2020; **69**: 997-1001 [PMID: 32241899 DOI: 10.1136/gutjnl-2020-321013]

76 **Ding Y**, He L, Zhang Q, Huang Z, Che X, Hou J, Wang H, Shen H, Qiu L, Li Z, Geng J, Cai J, Han H, Li X, Kang W, Weng D, Liang P, Jiang S. Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. *J Pathol* 2004; **203**: 622-630 [PMID: 15141376 DOI: 10.1002/path.1560]

77 **Farcas GA**, Poutanen SM, Mazzulli T, Willey BM, Butany J, Asa SL, Faure P, Akhavan P, Low DE, Kain KC. Fatal severe acute respiratory syndrome is associated with multiorgan involvement by coronavirus. *J Infect Dis* 2005; **191**: 193-197 [PMID: 15609228 DOI: 10.1086/426870]

78 **Zhang J**, Wang S, Xue Y. Fecal specimen diagnosis 2019 novel coronavirus-infected pneumonia. *J Med Virol* 2020; **92**: 680-682 [PMID: 32124995 DOI: 10.1002/jmv.25742]

79 **Xu L**, Liu J, Lu M, Yang D, Zheng X. Liver injury during highly pathogenic human coronavirus infections. *Liver Int* 2020; **40**: 998-1004 [PMID: 32170806 DOI: 10.1111/liv.14435]

80 **Zhang B**, Zhou X, Qiu Y, Song Y, Feng F, Feng J, Song Q, Jia Q, Wang J. Clinical characteristics of 82 cases of death from COVID-19. *PLoS One* 2020; **15**: e0235458 [PMID: 32645044 DOI: 10.1371/journal.pone.0235458]

81 **Kulkarni AV**, Kumar P, Tevethia HV, Premkumar M, Arab JP, Candia R, Talukdar R, Sharma M, Qi X, Rao PN, Reddy DN. Systematic review with meta-analysis: liver manifestations and outcomes

in COVID-19. *Aliment Pharmacol Ther* 2020; **52**: 584-599 [PMID: 32638436 DOI: 10.1111/apt.15916]

82 **Yadav DK**, Singh A, Zhang Q, Bai X, Zhang W, Yadav RK, Zhiwei L, Adhikari VP, Liang T. Involvement of liver in COVID-19: systematic review and meta-analysis. *Gut* 2021; **70**: 807-809 [PMID: 32669289 DOI: 10.1136/gutjnl-2020-322072]

83 **Kumar-M P**, Mishra S, Jha DK, Shukla J, Choudhury A, Mohindra R, Mandavdhare HS, Dutta U, Sharma V. Coronavirus disease (COVID-19) and the liver: a comprehensive systematic review and meta-analysis. *Hepatol Int* 2020; **14**: 711-722 [PMID: 32623633 DOI: 10.1007/s12072-020-10071-9]

84 **Liu C**, Jiang ZC, Shao CX, Zhang HG, Yue HM, Chen ZH, Ma BY, Liu WY, Huang HH, Yang J, Wang Y, Liu HY, Xu D, Wang JT, Yang JY, Pan HQ, Zou SQ, Li FJ, Lei JQ, Li X, He Q, Gu Y, Qi XL. [Preliminary study of the relationship between novel coronavirus pneumonia and liver function damage: a multicenter study]. *Zhonghua Gan Zang Bing Za Zhi* 2020; **28**: 107-111 [PMID: 32077660 DOI: 10.3760/cma.j.issn.1007-3418.2020.02.003]

85 **Duan ZP**, Chen Y, Zhang J, Zhao J, Lang ZW, Meng FK, Bao XL. [Clinical characteristics and mechanism of liver injury in patients with severe acute respiratory syndrome]. *Zhonghua Ganzang Bing Zazhi* 2003; **11**: 493-496 [PMID: 12939186]

86 **Duan XF**, Liu Z, Hao R, Luo L, Zhang YN. [The dynamic change of liver injury in patients with severe acute respiratory syndrome]. *Zhonghua Ganzang Bing Zazhi* 2004; **12**: 439 [PMID: 15268817]

87 **Wang Y**, Liu S, Liu H, Li W, Lin F, Jiang L, Li X, Xu P, Zhang L, Zhao L, Cao Y, Kang J, Yang J, Li L, Liu X, Li Y, Nie R, Mu J, Lu F, Zhao S, Lu J, Zhao J. SARS-CoV-2 infection of the liver directly contributes to hepatic impairment in patients with COVID-19. *J Hepatol* 2020; **73**: 807-816 [PMID: 32437830 DOI: 10.1016/j.jhep.2020.05.002]

88 **Zhong P**, Xu J, Yang D, Shen Y, Wang L, Feng Y, Du C, Song Y, Wu C, Hu X, Sun Y. COVID-19-associated gastrointestinal and liver injury: clinical features and potential mechanisms. *Signal Transduct Target Ther* 2020; **5**: 256 [PMID: 33139693 DOI: 10.1038/s41392-020-00373-7]

89 **Bangash MN**, Patel J, Parekh D. COVID-19 and the liver: little cause for concern. *Lancet Gastroenterol Hepatol* 2020; **5**: 529-530 [PMID: 32203680 DOI: 10.1016/S2468-1253(20)30084-4]

90 **Lozano-Sepulveda SA**, Galan-Huerta K, Martínez-Acuña N, Arellanos-Soto D, Rivas-Estilla AM. SARS-CoV-2 another kind of liver aggressor, how does it do that? *Ann Hepatol* 2020; **19**: 592-596 [PMID: 32858226 DOI: 10.1016/j.aohep.2020.08.062]

91 **Chen G**, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang M, Wu S, Song J, Chen T, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest* 2020; **130**: 2620-2629 [PMID: 32217835 DOI: 10.1172/JCI137244]

92 **Li J**, Fan JG. Characteristics and Mechanism of Liver Injury in 2019 Coronavirus Disease. *J Clin Transl Hepatol* 2020; **8**: 13-17 [PMID: 32274341 DOI: 10.14218/JCTH.2020.00019]

93 **Barton LM**, Duval EJ, Stroberg E, Ghosh S, Mukhopadhyay S. COVID-19 Autopsies, Oklahoma, USA. *Am J Clin Pathol* 2020; **153**: 725-733 [PMID: 32275742 DOI: 10.1093/ajcp/aqaa062]

94 **Tian S**, Xiong Y, Liu H, Niu L, Guo J, Liao M, Xiao SY. Pathological study of the 2019 novel coronavirus disease (COVID-19) through postmortem core biopsies. *Mod Pathol* 2020; **33**: 1007-1014 [PMID: 32291399 DOI: 10.1038/s41379-020-0536-x]

95 **Hanley B**, Lucas SB, Youd E, Swift B, Osborn M. Autopsy in suspected COVID-19 cases. *J Clin Pathol* 2020; **73**: 239-242 [PMID: 32198191 DOI: 10.1136/jclinpath-2020-206522]

96 **Paizis G**, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, Shaw T, Warner FJ, Zuilli A, Burrell LM, Angus PW. Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. *Gut* 2005; **54**: 1790-1796 [PMID: 16166274 DOI: 10.1136/gut.2004.062398]

97 **Lee WM**, Hynan LS, Rossaro L, Fontana RJ, Stravitz RT, Larson AM, Davern TJ 2nd, Murray NG, McCashland T, Reisch JS, Robuck PR; Acute Liver Failure Study Group. Intravenous N-acetylcysteine improves transplant-free survival in early stage non-acetaminophen acute liver failure. *Gastroenterology* 2009; **137**: 856-864, 864. e1 [PMID: 19524577 DOI: 10.1053/j.gastro.2009.06.006]

98 **Grein J**, Ohmagari N, Shin D, Diaz G, Asperges E, Castagna A, Feldt T, Green G, Green ML, Lescure FX, Nicastri E, Oda R, Yo K, Quiros-Roldan E, Studemeister A, Redinski J, Ahmed S, Bernett J, Chelliah D, Chen D, Chihara S, Cohen SH, Cunningham J, D'Arminio Monforte A, Ismail S, Kato H, Lapadula G, L'Her E, Maeno T, Majumder S, Massari M, Mora-Rillo M, Mutoh Y, Nguyen D, Verweij E, Zoufaly A, Osinusi AO, DeZure A, Zhao Y, Zhong L, Chokkalingam A, Elboudwarej E, Telep L, Timbs L, Henne I, Sellers S, Cao H, Tan SK, Winterbourne L, Desai P, Mera R, Gaggar A, Myers RP, Brainard DM, Childs R, Flanigan T. Compassionate Use of Remdesivir for Patients with Severe Covid-19. *N Engl J Med* 2020; **382**: 2327-2336 [PMID: 32275812 DOI: 10.1056/NEJMoa2007016]

99 **Rad F**, Ghorbani M, Mohammadi Roushandeh A, Habibi Roudkenar M. Mesenchymal stem cell-based therapy for autoimmune diseases: emerging roles of extracellular vesicles. *Mol Biol Rep* 2019; **46**: 1533-1549 [PMID: 30623280 DOI: 10.1007/s11033-019-04588-y]

100 **Polchert D**, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, Genrich K, Mehrotra S, Setty S, Smith B, Bartholomew A. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft *vs* host disease. *Eur J Immunol* 2008; **38**: 1745-1755 [PMID: 18493986 DOI:

10.1002/eji.200738129]
101 **Ren G**, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs *via* concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008; **2**: 141-150 [PMID: 18371435 DOI: 10.1016/j.stem.2007.11.014]
102 **Kavanagh H**, Mahon BP. Allogeneic mesenchymal stem cells prevent allergic airway inflammation by inducing murine regulatory T cells. *Allergy* 2011; **66**: 523-531 [PMID: 21091718 DOI: 10.1111/j.1398-9995.2010.02509.x]
103 **Akiyama K**, Chen C, Wang D, Xu X, Qu C, Yamaza T, Cai T, Chen W, Sun L, Shi S. Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. *Cell Stem Cell* 2012; **10**: 544-555 [PMID: 22542159 DOI: 10.1016/j.stem.2012.03.007]
104 **English K**, Wood KJ. Mesenchymal stromal cells in transplantation rejection and tolerance. *Cold Spring Harb Perspect Med* 2013; **3**: a015560 [PMID: 23637312 DOI: 10.1101/cshperspect.a015560]
105 **Tamura R**, Uemoto S, Tabata Y. Immunosuppressive effect of mesenchymal stem cell-derived exosomes on a concanavalin A-induced liver injury model. *Inflamm Regen* 2016; **36**: 26 [PMID: 29259699 DOI: 10.1186/s41232-016-0030-5]
106 **Li T**, Yan Y, Wang B, Qian H, Zhang X, Shen L, Wang M, Zhou Y, Zhu W, Li W, Xu W. Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev* 2013; **22**: 845-854 [PMID: 23002959 DOI: 10.1089/scd.2012.0395]
107 **Bulut Ö**, GÜrsel İ. Mesenchymal stem cell derived extracellular vesicles: promising immunomodulators against autoimmune, autoinflammatory disorders and SARS-CoV-2 infection. *Turk J Biol* 2020; **44**: 273-282 [PMID: 32595362 DOI: 10.3906/biy-2002-79]
108 **Portincasa P**, Krawczyk M, Machill A, Lammert F, Di Ciaula A. Hepatic consequences of COVID-19 infection. Lapping or biting? *Eur J Intern Med* 2020; **77**: 18-24 [PMID: 32507608 DOI: 10.1016/j.ejim.2020.05.035]
109 **Huang C**, Li Q, Xu W, Chen L. Molecular and cellular mechanisms of liver dysfunction in COVID-19. *Discov Med* 2020; **30**: 107-112 [PMID: 33382966]
110 **Zhao JN**, Fan Y, Wu SD. Liver injury in COVID-19: A minireview. *World J Clin Cases* 2020; **8**: 4303-4310 [PMID: 33083389 DOI: 10.12998/wjcc.v8.i19.4303]
111 **Golchin A**, Farahany TZ, Khojasteh A, Soleimanifar F, Ardeshirylajimi A. The Clinical Trials of Mesenchymal Stem Cell Therapy in Skin Diseases: An Update and Concise Review. *Curr Stem Cell Res Ther* 2019; **14**: 22-33 [PMID: 30210006 DOI: 10.2174/1574888X13666180913123424]
112 **Matthay MA**, Calfee CS, Zhuo H, Thompson BT, Wilson JG, Levitt JE, Rogers AJ, Gotts JE, Wiener-Kronish JP, Bajwa EK, Donahoe MP, McVerry BJ, Ortiz LA, Exline M, Christman JW, Abbott J, Delucchi KL, Caballero L, McMillan M, McKenna DH, Liu KD. Treatment with allogeneic mesenchymal stromal cells for moderate to severe acute respiratory distress syndrome (START study): a randomised phase 2a safety trial. *Lancet Respir Med* 2019; **7**: 154-162 [PMID: 30455077 DOI: 10.1016/S2213-2600(18)30418-1]
113 **Chen J**, Hu C, Chen L, Tang L, Zhu Y, Xu X, Gao H, Lu X, Yu L, Dai X, Xiang C, Li L. Clinical Study of Mesenchymal Stem Cell Treatment for Acute Respiratory Distress Syndrome Induced by Epidemic Influenza A (H7N9) Infection: A Hint for COVID-19 Treatment. *Engineering (Beijing)* 2020; **6**: 1153-1161 [PMID: 32292627 DOI: 10.1016/j.eng.2020.02.006]
114 **Namba F**. Mesenchymal stem cells for the prevention of bronchopulmonary dysplasia. *Pediatr Int* 2019; **61**: 945-950 [PMID: 31487104 DOI: 10.1111/ped.14001]
115 **Liang B**, Chen J, Li T, Wu H, Yang W, Li Y, Li J, Yu C, Nie F, Ma Z, Yang M, Xiao M, Nie P, Gao Y, Qian C, Hu M. Clinical remission of a critically ill COVID-19 patient treated by human umbilical cord mesenchymal stem cells: A case report. *Medicine (Baltimore)* 2020; **99**: e21429 [PMID: 32756149 DOI: 10.1097/MD.0000000000021429]
116 **Ye Q**, Wang B, Mao J. The pathogenesis and treatment of the `Cytokine Storm' in COVID-19. *J Infect* 2020; **80**: 607-613 [PMID: 32283152 DOI: 10.1016/j.jinf.2020.03.037]
117 **Shah TG**, Predescu D, Predescu S. Mesenchymal stem cells-derived extracellular vesicles in acute respiratory distress syndrome: a review of current literature and potential future treatment options. *Clin Transl Med* 2019; **8**: 25 [PMID: 31512000 DOI: 10.1186/s40169-019-0242-9]
118 **Shao M**, Xu Q, Wu Z, Chen Y, Shu Y, Cao X, Chen M, Zhang B, Zhou Y, Yao R, Shi Y, Bu H. Exosomes derived from human umbilical cord mesenchymal stem cells ameliorate IL-6-induced acute liver injury through miR-455-3p. *Stem Cell Res Ther* 2020; **11**: 37 [PMID: 31973730 DOI: 10.1186/s13287-020-1550-0]
119 **Leng Z**, Zhu R, Hou W, Feng Y, Yang Y, Han Q, Shan G, Meng F, Du D, Wang S, Fan J, Wang W, Deng L, Shi H, Li H, Hu Z, Zhang F, Gao J, Liu H, Li X, Zhao Y, Yin K, He X, Gao Z, Wang Y, Yang B, Jin R, Stambler I, Lim LW, Su H, Moskalev A, Cano A, Chakrabarti S, Min KJ, Ellison-Hughes G, Caruso C, Jin K, Zhao RC. Transplantation of ACE2^{-} Mesenchymal Stem Cells Improves the Outcome of Patients with COVID-19 Pneumonia. *Aging Dis* 2020; **11**: 216-228 [PMID: 32257537 DOI: 10.14336/AD.2020.0228]
120 **Chen T**, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, Ding C, Zhang X, Huang J, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ* 2020; **368**: m1091 [PMID: 32217556 DOI: 10.1136/bmj.m1091]
121 **Richardson S**, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW; the Northwell COVID-19 Research Consortium, Barnaby DP, Becker LB, Chelico JD, Cohen SL, Cookingham J, Coppa K, Diefenbach MA, Dominello AJ, Duer-Hefele J, Falzon L, Gitlin J, Hajizadeh N, Harvin

TG, Hirschwerk DA, Kim EJ, Kozel ZM, Marrast LM, Mogavero JN, Osorio GA, Qiu M, Zanos TP. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. *JAMA* 2020; **323**: 2052-2059 [PMID: 32320003 DOI: 10.1001/jama.2020.6775]

122 **Hwang Y**, Kim JC, Tae G. Significantly enhanced recovery of acute liver failure by liver targeted delivery of stem cells *via* heparin functionalization. *Biomaterials* 2019; **209**: 67-78 [PMID: 31026612 DOI: 10.1016/j.biomaterials.2019.04.019]

123 **Wang Y**, Wang JL, Ma HC, Tang ZT, Ding HR, Shi XL. Mesenchymal stem cells increase heme oxygenase 1-activated autophagy in treatment of acute liver failure. *Biochem Biophys Res Commun* 2019; **508**: 682-689 [PMID: 30528392 DOI: 10.1016/j.bbrc.2018.11.146]

124 **Johnson AS**, Fatemi R, Winlow W. SARS-CoV-2 Bound Human Serum Albumin and Systemic Septic Shock. *Front Cardiovasc Med* 2020; **7**: 153 [PMID: 33088822 DOI: 10.3389/fcvm.2020.00153]

125 **Wu MA**, Fossali T, Pandolfi L, Carsana L, Ottolina D, Frangipane V, Rech R, Tosoni A, Lopez G, Agarossi A, Cogliati C, Meloni F, Marchini B, Nebuloni M, Catena E, Colombo R. Hypoalbuminemia in COVID-19: assessing the hypothesis for underlying pulmonary capillary leakage. *J Intern Med* 2021 [PMID: 33411411 DOI: 10.1111/joim.13208]

126 **Bahloul M**, Ketata W, Lahyeni D, Mayoufi H, Kotti A, Smaoui F, Kallel N, Daoud E, Bouaziz M, Kammoun S. Pulmonary capillary leak syndrome following COVID-19 virus infection. *J Med Virol* 2021; **93**: 94-96 [PMID: 32519786 DOI: 10.1002/jmv.26152]

127 **Corti MC**, Guralnik JM, Salive ME, Sorkin JD. Serum albumin level and physical disability as predictors of mortality in older persons. *JAMA* 1994; **272**: 1036-1042 [PMID: 8089886]

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MINIREVIEWS

Artificial intelligence applications in inflammatory bowel disease: Emerging technologies and future directions

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Abstract

Inflammatory bowel disease (IBD) is a complex and multifaceted disorder of the gastrointestinal tract that is increasing in incidence worldwide and associated with significant morbidity. The rapid accumulation of large datasets from electronic health records, high-definition multi-omics (including genomics, proteomics, transcriptomics, and metagenomics), and imaging modalities (endoscopy and endomicroscopy) have provided powerful tools to unravel novel mechanistic insights and help address unmet clinical needs in IBD. Although the application of artificial intelligence (AI) methods has facilitated the analysis, integration, and interpretation of large datasets in IBD, significant heterogeneity in AI methods, datasets, and clinical outcomes and the need for unbiased prospective validations studies are current barriers to incorporation of AI into clinical practice. The purpose of this review is to summarize the most recent advances in the application of AI and machine learning technologies in the diagnosis and risk prediction, assessment of disease severity, and prediction of clinical outcomes in patients with IBD.

Key Words: Artificial intelligence; Machine learning; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Clinical outcomes

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Core Tip: The application of artificial intelligence (AI) in the field of inflammatory bowel disease (IBD) has grown significantly in the past decade. AI has been used to analyze genomic datasets, construct IBD risk prediction models, and increase IBD diagnosis precision. Machine learning has been used to analyze endoscopic images to

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improve disease severity grading. AI has enabled the integration of large clinical and laboratory datasets with gene expression profiles to predict clinical outcomes such as therapy response. Future studies will need to validate these findings in independent cohorts and determine whether applying these AI-derived prediction models improves clinical outcomes in IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disorder of the gastrointestinal tract. IBD has emerged as a global disease with increasing incidence worldwide and associated with significant healthcare utilization[1,2]. The pathogenesis of IBD is complex and is thought to involve an interplay between loss of tolerance to commensal gut bacteria, intestinal epithelial barrier dysfunction, and immune dysregula-tion[3-7]. The diagnosis of IBD is based on a combination of factors including clinical data (*e.g.*, chronicity of gastrointestinal symptoms), laboratory values (elevated inflammatory markers such as C-reactive protein and fecal calprotectin), imaging, endoscopy, and histology (gastrointestinal inflammation with architectural distortion)[8]. Although treatment algorithms based on clinical trials and experience have been developed to inform clinical management in IBD[9], there is significant heterogeneity among patients with IBD with regards to presentation, response to therapy, and long-term clinical outcomes such development of strictures and need for surgery[10,11]. There is a great need for precision medicine strategies to improve diagno-stic and therapeutic approaches in IBD.

Precision medicine efforts in IBD have led to more in-depth phenotyping of patients with IBD using large scale databases from clinical trials and cohort studies, deep immunophenotyping using whole genome gene expression datasets, proteomics, transcriptomics, and metagenomics of gut microbiota, and complex predictive models incorporating computer-assisted analysis of endoscopic images and histology[12-14]. This has inevitably led to vast arrays of high dimensional data that pose significant challenges with traditional statistical and computational methods[15]. Technological advances in artificial intelligence (AI) have revolutionized the ability of clinicians and researchers to process, analyze, and interpret high dimensional data and large datasets.

AI is a broad and multidisciplinary field incorporating concepts from computer science, engineering, philosophy, and linguistics aimed at understanding and designing systems that display or mimic human intelligence. The term was first coined in 1965 by McCarthy J[16,17]. Machine learning (ML) is a subdiscipline of AI where computer algorithms apply statistical models to learn associations of predictive power from examples in provided datasets (*e.g.*, Dragon dictation, SPAM, Netflix). ML may be programmed through supervised learning or unsupervised learning. In supervising learning, computer programs are trained to learn associations between inputs and outputs in data through analysis of predefined outputs of interest (by human operator). Once associations have been learned using existing data, supervised ML classifiers could then be used to predict future examples using different datasets. Examples of supervised ML include random forest (RF) and support vector machines (SVM). In unsupervised learning, computer programs learn associations in data without external definitions of associations of interest. This method allows for the identification of previously undiscovered predictors. Deep learning, commonly known as neural networks, includes newer techniques that are based on models with fewer assumptions, rely on multiple layers of representation of the data with successive transformations that amplify aspects of the input which improves discrimination power and thus able to handle more complex data (*e.g.*, Facebook face recognition, credit card fraud)[17]. There has been increased interest in use of AI in IBD in recent

years with many prior groups applying ML methods to identify meaningful insights in diagnostics and prediction models in IBD. The purpose of this review is to provide a comprehensive summary of advances in the application of AI and ML technologies in the diagnosis and risk prediction, assessment of disease severity, and prediction of clinical outcomes in patients with IBD.

LITERATURE SEARCH

We performed a literature review using PubMed (MEDLINE) from inception to December 15, 2020 of studies applying AI in IBD. Our search strategy included the following combinations: ((((((((inflammatory bowel disease[Title])) OR (ulcerative colitis[Title])) OR (Crohn's disease[Title])) AND (artificial intelligence[Title])) OR (computer-assisted[Title])) OR (computer-aided[Title])) OR (neural network[Title])) OR (machine learning[Title])) OR (deep learning[Title]). We included studies that used AI in the (1) diagnosis or risk prediction of IBD, (2) assessment of disease severity in IBD, and (3) prediction of therapy response and clinical outcomes in IBD. We excluded reviews, studies with non-human subjects (animal models), or studies that did not provide objective measures of the efficacy of AI applications (*e.g.*, measures of precision, accuracy, area under the curve (AUC), sensitivity, specificity, *etc.*).

RESULTS

Our search strategy yielded 98 studies evaluating AI in IBD of which 58 studies[18-74] met inclusion criteria and were included in the final review. About 86.2% (50/58) of studies were published within the past 5 years (2015 and later). There were 23 studies[18-39] that focused on IBD diagnosis and risk prediction, 19 studies[40-58] which evaluated disease activity, and 17 studies[45,59-74] which predicted IBD clinical outcomes (response to therapy, colonic neoplasia, post-surgical complications, quality of life, IBD well-being and emotional content). There were 22 studies with combined IBD cohorts (CD and UC), 16 studies with UC patients only, 18 studies with CD only, and 5 pediatric IBD cohorts. The most common AI classifications used were neural networks (convolutional and deep) at 32.7% (19/58 studies), RF at 29.3% (17/58 studies), and SVM at 29.3% (17/58 studies).

AI in diagnosis and risk prediction of IBD

Table 1 summarizes studies included which applied AI in the diagnosis and risk prediction of IBD. There were 17 studies focused on IBD diagnosis, whereas 5 studies focused on predicting risk of IBD. Data modalities included genetic/genomic datasets (n = 16 studies), imaging and endoscopic datasets (n = 4), and protein expression/proteomics (n = 2 studies). Some groups have used ML to develop IBD risk prediction models based on gene expression datasets. In a cross-sectional study of 180 CD patients, 149 UC patients and 90 healthy controls by Isakov *et al*[21], RF and SVM used microarray and RNA-seq data sets to classify a list of 16390 genes. Their combined IBD risk prediction model demonstrated an AUC, sensitivity, specificity, and accuracy values of 0.829, 0.577, 0.880, and 0.808, respectively. In another cross-sectional study of 18227 CD patients and 34050 healthy controls, Romagnoni *et al*[20] used gradient boosted trees and artificial neural networks to analyze gene expression profiles. Using single nucleotide polymorphisms, their final predictive model for CD achieved AUC of 0.80. Likewise, a cross-sectional study of 20 UC patients and 20 healthy controls by Duttagupta *et al*[33] used SVM to analyze microRNA profiles. Their SVM classifier measurements revealed a predictive score accuracy of 92.8%, specificity of 96.2%, and sensitivity of 89.5% in distinguishing UC patients from normal individuals.

A major challenge in IBD diagnosis is the distinction between CD and UC which is based on clinical features such as the distribution of inflammation along the gastrointestinal tract. The misdiagnosis of IBD subtype is not uncommon[74]. Distinguishing between CD and UC is clinically important as IBD subtype informs clinical management. AI has been employed to analyze molecular data to distinguish between CD and UC. In a cross-sectional study of 59 CD patients, 26 UC patients, and 42 healthy controls applying deep belief networks (DBNs) and SVM to gene expression datasets, Smolander *et al*[25] explored the diagnosis UC from CD. Using DBN only, the accuracy for diagnosis of UC was 97.06% and CD was 97.07%. Using both DBN and SVM, accuracy for diagnosis of UC was 97.06% and CD was 97.03%. In

Table 1 Artificial intelligence in diagnosis and risk prediction of inflammatory bowel disease

Ref.	AI classifier *vs* comparator	IBD type	Study design and sample size	Modality	Outcome	Study results/validation cohort
Mossotto *et al* [18], 2017	Support vector machines (SVM) *vs* linear discriminant	Peds CD/UC	Prospective cohort, 287 IBD patients	Endoscopic and histologic inflammation	Diagnosis of IBD	Diagnostic accuracy of 82.7% with an AUC of 0.87 in diagnosing Crohn's disease or ulcerative colitis. Validation cohort included
Wei *et al*[19], 2013	SVM with gradient boosted trees (GBT) *vs* simple log odds method	CD/UC	Cross-sectional, 30000 IBD patients, 22000 healthy controls	Genetics, ImmunoChip	Risk of IBD	The SVM demonstrated very comparable performance (AUC 0.862 and 0.826 for CD and UC, respectively), whereas GBT showed inferior performance (AUC 0.802 and0.782 for CD and UC, respectively. Validation cohort included
Romagnoni *et al*[20], 2019	Artificial neural networks (ANNs) *vs* penalized logistic regression (LR), and GBT	CD	Cross-sectional, 18227 CD patients, 34050 healthy controls	Genetics, ImmunoChip	Risk of IBD	Using single nucleotide polymorphisms (SNPs), final predictive model achieved AUC of 0.80. Validation cohort included
Isakov *et al*[21], 2017	Random forest (RF), SVM with svmPoly), extreme gradient boosting *vs* elastic net regularized generalized linear model (glmnet)	CD/UC	Cross-sectional, 180 CD patients, 149 UC patients, 90 healthy controls	Expression data (microarray and RNA-seq)	Risk of IBD	The method was used to classify a list of 16390 genes. Each gene received a score that was used to prioritize it according to its predicted association to IBD. The combined model demonstrated AUC, sensitivity, specificity, and accuracy values of 0.829, 0.577, 0.88, and 0.808, respectively. Validation cohort included
Yuan *et al*[22], 2017	Sequential minimal optimization *vs* DisGeNET (Version 4.0)	CD/UC	Cross-sectional, 59 CD patients, 26 UC patients, 42 healthy controls	Gene Expression datasets	Risk of IBD	By analyzing the gene expression profiles using minimum redundancy maximum relevance and incremental feature selection, 21 genes were obtained that could effectively distinguish samples from IBD and the non-IBD samples. Highest total prediction accuracy was 97.64% using the 1170th feature set. Validation cohort included
Hübenthal *et al*[23], 2015	SVM *vs* RF	CD/UC	Cross-sectional, 40 CD patients, 36 UC patients, 38 healthy controls	MicroRNAs	Diagnosis of IBD	Measured by the AUC the corresponding median holdout-validated accuracy was estimated as ranging from 0.75 to 1.00 and 0.89 to 0.98, respectively. In combination, the corresponding models provide tools for the distinction of CD and UC as well as CD, UC and healthy control with expected classification error rates of 3.1 and 3.3%, respectively. Validation cohort included
Tong *et al*[24], 2020	RF *vs* convolutional neural network (CNN)	CD/UC	Retrospective Cohort, 875 CD patients, 5128 UC patients	Colonoscopy Endoscopic Images	Diagnosis of IBD	RF sensitivities/specificities of UC/CD were 0.89/0.84, 0.83/0.82, and 0.72/0.77, respectively, while the values for the CNN of CD was 0.90/0.77. The precisions/recalls of UC-CD when employing RF were 0.97/0.97, 0.65/0.53, respectively, and when employing the CNN were 0.99/0.97 and 0.87/0.83, respectively. Validation cohort included
Smolander *et al*[25], 2019	Deep belief networks (DBNs) *vs* SVM	CD/UC	Cross-sectional, 59 CD patients, 26 UC patients, 42 healthy controls	Gene Expression datasets	Diagnosis of IBD	Using DBN only, accuracy for diagnosis of UC was 97.06% and CD was 97.07%. Using both DBN and SVM, accuracy for diagnosis of UC was 97.06% and CD was 97.03%. Validation cohort included
Abbas *et al*[26], 2019	RF *vs* network-based biomarker discovery	Peds CD/UC	Cross-sectional, 657 IBD patients, 316 healthy controls	Large dataset of new-onset pediatric IBD metagenomics biopsy samples	Diagnosis of IBD	For the diagnosis of IBD, highest AUC attained by top Random Forest classifiers was 0.77. No validation cohort included
Khorasani *et al*[27], 2020	SVM *vs* recently developed feature selection algorithm (robustness-performance tradeoff, RPT)	UC	Cross-sectional, 146 UC patients, 60 healthy controls	Gene Expression dataset	Diagnosis of IBD	Our model perfectly detected all active cases and had an average precision of 0.62 in the inactive cases. Validation cohort included
Rubin *et al*[28], 2019	CITRUS supervised machine learning algorithm. No comparator	CD/UC	Cross-sectional, 68 IBD patients	Peripheral blood mononuclear cells and intestinal biopsies mass cytometry	Diagnosis of IBD	An 8-parameter immune signature distinguished Crohn's disease from ulcerative colitis with an AUC = 0.845 (95%CI: 0.742-0.948). No validation cohort included

Pal *et al*[29], 2017	Naïve Bayes and with a consensus machine learning method *vs* Critical Assessment of Genome Interpretation (CAGI) 4 method	CD	Cross-sectional, 64 CD patients, 47 healthy controls	Genotypes from Exome Sequencing Data	Risk of IBD	The AUC for predicting risk of Crohn's disease using the SNP model was 0.72. No validation cohort included
Aoki *et al*[30], 2019	Deep CNN. No comparator	CD	Retrospective Cohort, 115 IBD patients	Wireless capsule endoscopy images	Diagnosis of IBD	The AUC for the detection of erosions and ulcerations was 0.958 (95%CI: 0.947-0.968). The sensitivity, specificity, and accuracy of the CNN were 88.2% (95%CI: 84.8-91.0), 90.9% (95%CI: 90.3-91.4), and 90.8% (95%CI: 90.2-91.3), respectively. Validation cohort included
Bielecki *et al*[31], 2012	SVM *vs* human reader (pathologist)	CD/UC	Cross-sectional, 14 CD patients, 13 UC patients, 11 healthy controls	Raman spectroscopic imaging of epithelium cells	Diagnosis of IBD	Raman maps of human colon tissue sections were analyzed by utilizing innovative chemometric approaches. Using SVM, it was possible to separate between healthy control patients, patients with Crohn's Disease, and patients with ulcerative colitis with an accuracy of 98.90%. No validation cohort included
Cui *et al*[32], 2013	Recursive SVM *vs* unsupervised learning strategy	CD/UC	Cross-sectional, 124 IBD patients, 99 healthy controls	16S rRNA gene analysis	Diagnosis of IBD	Selection level of 200 features results in the best leave-one-out cross-validation result (accuracy = 88%, sensitivity = 92%, specificity = 84%). Validation cohort included
Duttagupta *et al*[33], 2012	SVM. No comparator	UC	Cross-sectional, 20 UC patients, 20 healthy controls	MicroRNAs	Diagnosis of IBD	SVM classifier measurements revealed a predictive score of 92.8% accuracy, 96.2% specificity and 89.5% sensitivity in distinguishing ulcerative colitis patients from normal individuals. Validation cohort included
Daneshjou *et al*[34], 2017	Naïve bayes, neural networks, random forests *vs* CAGI methods	CD	Cross-sectional, 64 ICD patients, 47 healthy controls	Exome Sequencing	Diagnosis of IBD	In CAGI4, 111 exomes were derived from a mix of 64 Crohn's disease patients. Top performing methods had an AUC of 0.87. Validation cohort included
Geurts *et al* [35], 2005	RF *vs* SVM	CD/UC	Prospective cohort, 30 CD patients, 30 CD patients	Proteomic Mass Spectrometry	Diagnosis of IBD	Random forest model to diagnosis IBD had a sensitivity of 81.67%, specificity of 81.17%. Support vector machine model to diagnosis IBD had a sensitivity of 87.92%, specificity of 87.87%. Validation cohort included
Li *et al*[36], 2020	RF *vs* ANN	UC	Cross-sectional, 193 UC patients, 21 healthy controls	Gene Expression Profiles	Diagnosis of IBD	The random forest algorithm was introduced to determine 1 downregulated and 29 upregulated differentially expressed genes contributing highest to ulcerative colitis occurrence. ANN was developed to calculate differentially expressed genes weights to ulcerative colitis. Prediction results agreed with that of an independent data set (AUC = 0.9506/PR-AUC = 0.9747). Validation cohort included
Wingfield *et al*[37], 2019	RF *vs* SVM	CD	Cross-sectional, 668 CD patients	Metagenomic Data	Diagnosis of IBD	Highest RPT measure for Crohn's disease was random forest 0.60 and SVM 0.58. For ulcerative colitis, RPT was random forest 0.70 and SVM 0.48. Validation cohort included
Han *et al*[38], 2018	RF *vs* LR, CORG	CD/UC	Cross-sectional, 24 CD patients, 59 UC patients, 76 healthy controls	Gene Expression Profiles	Diagnosis of IBD	The gene-based feature sets had median AUC on the validation sets ranging from 0.6 to 0.76). Validation cohort included
Wang *et al*[39], 2019	AVADx (Analysis of Variation for Association with Disease) *vs* two GWAS-based CD evaluation methods	CD	Cross-sectional, 64 CD patients, 47 healthy controls	Whole Exome or Genome Sequencing Data	Diagnosis of IBD	AVADx highlighted known CD genes including NOD2and new potential CD genes. AVADx identified 16% (at strict cutoff) of CD patients at 99% precision and 58% of the patients (at default cutoff) with 82% precision in over 3000 individuals from separately sequenced panels. Validation cohort included

AI: Artificial intelligence; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AUC: Area under the curve.

a cross-sectional study of 68 IBD patients using a CITRUS supervised ML algorithm to analyze single cell immunophenotyping of peripheral blood mononuclear cells by mass cytometry, Rubin *et al*[28] demonstrated that an 8-parameter immune signature distinguished CD from UC with an AUC = 0.845 [95% confidence interval (CI): 0.742-0.948]. ML algorithms have also been applied to analyze large arrays of endoscopic images to differentiate between UC and CD. In a recent retrospective cohort study of 875 CD patients and 5128 UC patients by Tong *et al*[24] using RF and convolutional neural networks (CNNs) on endoscopic images, the precision of diagnosing UC/CD with RF and CNNs were 0.97/0.65 and 0.99/0.87, respectively. Taken together, these studies suggest that AI classifiers have high performance in diagnosing or predicting risk of IBD but have some variability with type of AI classifier and modality of data (molecular *vs* endoscopic).

AI in assessment of disease severity in IBD

The assessment of disease activity and grading of severity in IBD could be accomplished using validated clinical symptom scores (*e.g.*, Harvey Bradshaw Index for CD, Mayo Score for UC)[75,76], biomarkers of inflammation (*e.g.*, C-reactive protein, fecal calprotectin)[77,78], endoscopic inflammation indices (*e.g.*, Mayo endoscopic score, simple endoscopic score)[79,80], and histologic scoring systems (*e.g.*, Geboes Score, Robarts Histopathology Index)[81,82]. However, these systems may be subject to recall bias, heterogeneity in patient clinical presentation, and intraobserver and interobserver variability[83]. AI has been applied to these existing systems to improve precision and accuracy of quantifying disease severity in IBD.

Table 2 summarizes studies included which applied AI in the assessment of disease severity in IBD. There were 2 studies that assessed clinical disease activity, 2 studies that assessed disease activity by biomarker (C-reactive protein), 13 studies that focused on endoscopic inflammation, and 3 studies that focused on histologic inflammation. Data modalities included electronic health records (n = 2), molecular datasets (n = 3), endoscopic datasets (n = 11 studies), and histologic datasets *via* endomicroscopy/endocytoscopy (n = 2). Using RF to integrate and analyze clinical and laboratory data from publicly available clinical trials (UNITI-1, UNITI-2, and IM-UNITI) data consisting of 401 CD patients, Waljee *et al*[42] constructed a CD remission prediction model using the week 6 albumin to C-reactive protein ratio with an AUC of 0.76 (95%CI: 0.71-0.82). Reddy *et al*[44] applied gradient boosting machines to electronic health records and predicted inflammation severity in a retrospective cohort of 3335 CD patients with a very high accuracy (AUC) = 92.82%. In a CNN analysis of colonoscopy images from a retrospective cohort of 841 UC patients by Ozawa *et al*[55], the CNN-based computer aided diagnostic system showed a high level of performance with AUC of 0.86 and 0.98 to identify Mayo 0 and 0-1, respectively. The performance of the CNN was better for the rectum than for the right side and left side of the colon when identifying Mayo 0 (AUC = 0.92, 0.83, and 0.83, respectively). Likewise, in an ordinal CNN analysis of wireless capsule endoscopy images in a retrospective cohort of 49 CD patients by Barash *et al*[50], the classification accuracy of the algorithm was 0.91 for grade 1 *vs* grade 3 ulcers, 0.78 for grade 2 *vs* grade 3, and 0.624 for grade 1 *vs* grade 2. The role of AI in grading severity of histologic inflammation in IBD has also been explored. For example, in a retrospective cohort study of 187 UC patients by Maeda *et al*[46], application of SVM to data derived from endocytoscopy to assess histologic inflammation provided diagnostic sensitivity, specificity, and accuracy of 74% (95%CI: 65-81), 97% (95%CI: 95-99), and 91% (95%CI: 83-95), respectively. These examples highlight the clinical utility, versatility, and performance of AI classifiers in grading the disease activity of IBD patients at the clinical, endoscopic, and histologic level. AI performance may be affected by location of inflammation and may be limited by ability to discriminate between subtle differences.

AI in prediction of therapy response and clinical outcomes in IBD

The armamentarium of therapies in IBD have expanded significantly in recent years with diverse mechanisms of action ranging from biologics that inhibit proinflammatory cytokines (anti-tumor necrosis factor-α, anti-interleukin-12/23) and leukocyte trafficking to the gut (anti-α4β7) to small molecule inhibitors of the JAK-STAT signaling pathway[84-86]. Despite several IBD treatment options available to clinicians, there are no effective biomarkers or tools to predict response to therapy or to guide selection of alternative therapies after a failed response. Likewise, there is also an unmet clinical need to predict long term clinical outcomes in IBD such as colon cancer. To address these challenges, several groups have applied AI and ML algorithms to existing clinical and molecular datasets.

Table 2 Artificial Intelligence in assessment of disease severity in inflammatory bowel disease

Ref.	AI classifier *vs* comparator	IBD type	Study design and sample size	Modality	Outcomes	Study results/validation cohort
Kumar *et al*[40], 2012	Support vector machines (SVM) *vs* human observers	CD	Cross-sectional, 50000 images (number of patients not given)	Small bowel capsule endoscopy	Endoscopic Inflammation	Database of 47 studies including 50000 capsule endoscopy images evaluating severity of small bowel lesions. Method had good precision (> 90% for lesion detection) and recall (> 90%) for lesions of varying severity. Validation cohort included
Biasci *et al*[41], 2019	Logistic regression with an adaptive Elastic-Net penalty. No comparator	CD/UC	Prospective cohort, 118 IBD patients	Transcriptomics from purified CD8 T cells and/or whole blood	Disease severity, medication escalation	A 17-gene qPCR-based classifier stratified patients into two distinct subgroups. IBDhi patients experienced significantly more aggressive disease than IBDlo patients (analogous to IBD2), with earlier need for treatment escalation [HR 2.65 (CD), 3.12 (UC)] and more escalations over time [for multiple escalations within 18 months: sensitivity=72.7% (CD), 100% (UC); negative predictive value = 90.9% (CD), 100% (UC)]. Validation cohort included
Waljee *et al*[42], 2019	RF. No comparator	CD	Post-hoc analysis of prospective clinical trials, 401 CD patients	Clinical and laboratory data from publicly available clinical trials (UNITI-1, UNITI-2, and IM-UNITI)	Crohn's disease remission, C-reactive protein < 5 mg/L	A prediction model using the week-6 albumin to C-reactive protein ratio had an AUC of 0.76 [95% confidence interval (CI): 0.71-0.82]. Validation cohort included
Mahapatra *et al*[43], 2016	RF. No comparator	CD	Cross-sectional, 35 CD patients	Abdominal magnetic resonance imaging	Segmentation of diseased colon (intestinal inflammation)	Model segmentation accuracy ranged from 82.7% to 92.2%. Validation cohort included
Reddy *et al*[44], 2019	Gradient boosting machines *vs* logistic regression	CD	Retrospective, 3335 CD patients	Electronic medical record	Severity of intestinal inflammation (by C-reactive protein)	Machine-learning-based analytic methods such as gradient boosting machines can predict the inflammation severity with a very high accuracy (AUC) = 92.82%. Validation cohort included
Douglas *et al*[45], 2018	RF. No comparator	Peds CD	Cross-sectional, 20 CD patients, 20 healthy controls	Shotgun metagenomics (MGS), 16S rRNA gene sequencing	Disease State (Relapse/Remission)	MGS modules significantly classified samples by disease state (accuracy = 68.4%, P = 0.043 and accuracy = 65.8%, P = 0.03, respectively), 16S datasets had a maximum accuracy of 68.4% and P = 0.016 based on strain level for disease state. Validation cohort included
Maeda *et al*[46], 2019	SVM *vs* human reader	UC	Retrospective cohort, 187 UC patients	Endocytoscopy	Histologic inflammation	Computer aided diagnosis (CAD) of histologic inflammation provided diagnostic sensitivity, specificity, and accuracy as follows: 74% (95%CI: 65-81), 97% (95%CI: 95-99), and 91% (95%CI: 83-95), respectively. Its reproducibility was perfect (k = 1). Validation cohort included
Charisis *et al*[47], 2016	SVM *vs* human reader	CD	Retrospective cohort, 13 CD patients	Wireless capsule endoscopy (WCE) images	Endoscopic Inflammation	Experimental results, along with comparison with other related efforts, have shown that the hybrid adaptive filtering [HAF-Differential Lacunarity (DLac) analysis (HAF-DLac)] *via* SVM approach evidently outperforms them in the field of WCE image analysis for automated lesion detection, providing higher classification results, up to 93.8% (accuracy), 95.2% (sensitivity), 92.4% (specificity) and 92.6% (precision). Validation cohort included
Klang *et al*[48], 2020	Convolutional neural network (CNN) *vs* human reader	CD	Retrospective cohort, 49 CD patients	WCE images	Endoscopic Inflammation	Dataset included 17640 CE images from 49 patients: 7391 images with mucosal ulcers and 10249 images of normal mucosa. For randomly split images results, AUC was 0.99 with accuracies ranging from 95.4% to 96.7%. For individual patient-level experiments, the AUCs were 0.94-0.99. Validation cohort included
Ungaro *et al*[49], 2021	Random survival forest. No comparator	Peds CD	Retrospective case-control, 265 peds CD patients	Protein biomarkers using a proximity extension assay (Olink Proteomics)	Penetrating and stricturing complications	A model with 5 protein markers predicted penetrating complications with an AUC of 0.79 (95%CI: 0.76-0.82) compared to 0.69 (95%CI: 0.66-0.72) for serologies and 0.74 (95%CI: 0.71-0.77) for clinical variables. A model with 4 protein markers predicted structuring complications with an AUC of 0.68 (95%CI: 0.65-0.71) compared to 0.62 (95%CI: 0.59-0.65) for serologies and 0.52 (95%CI: 0.50-0.55) for clinical variables. Validation cohort included

Barash *et al*[50], 2021	Ordinal CNN. No comparator	CD	Retrospective cohort, 49 CD patients	WCE images	Ulcer Severity Grading	The classification accuracy of the algorithm was 0.91 (95%CI: 0.867-0.954) for grade 1 *vs* grade 3 ulcers, 0.78 (95%CI: 0.716-0.844) for grade 2 *vs* grade 3, and 0.624 (95%CI: 0.547-0.701) for grade 1 *vs* grade 2. Validation cohort included
Lamash *et al*[51], 2019	CNN *vs* semi-supervised and active learning models	CD	Retrospective cohort, 23 CD patients	Magnetic resonance imaging	Active Crohn's Disease	CNN exhibited Dice similarity coefficient of 75% ± 18%, 81% ± 8%, and 97% ± 2% for the lumen, wall, and background, respectively. The extracted markers of wall thickness at the location of min radius (P = 0.0013) and the median value of relative contrast enhancement (P = 0.0033) could differentiate active and nonactive disease segments. Other extracted markers could differentiate between segments with strictures and segments without strictures (P < 0.05). Validation cohort included
Takenaka *et al*[52], 2020	Deep neural networks *vs* human reader (endoscopist)	UC	Prospective cohort, 2012 UC patients	Colonoscopy images	Endoscopic inflammation	Deep neural network identified patients with endoscopic remission with 90.1% accuracy (95%CI: 89.2-90.9) and a kappa coefficient of 0.798 (95%CI: 0.780-0.814), using findings reported by endoscopists as the reference standard. Validation cohort included
Bossuyt *et al* [53], 2020	Computer algorithm based on red density (RD) *vs* blinded central readers	UC	Prospective cohort, 29 UC patients, 6 healthy controls	Colonoscopy Images	Endoscopic and histologic inflammation	In the construction cohort, RD correlated with rhi (r = 0.74, P < 0.0001), Mayo endoscopic subscores (r = 0.76, P < 0.0001) and Endoscopic index of severity scores (r = 0.74, P < 0.0001). The RD sensitivity to change had a standardized effect size of 1.16. in the validation set, RD correlated with rhi (r = 0.65, P = 0.00002). Validation cohort included
Bhambhvani *et al*[54], 2021	CNN *vs* human reader (endoscopist)	UC	Retrospective cohort, 777 UC patients	Colonoscopy images	Mayo Endoscopic Scores (MES)	The final model classified MES 3 disease with an AUC of 0.96, MES 2 disease with an AUC of 0.86, and MES 1 disease with an AUC 0.89. Overall accuracy was 77.2%. Across MES 1, 2, and 3, average specificity was 85.7%, average sensitivity was 72.4%, average PPV was 77.7%, and the average NPV was 87.0%. Validation cohort included
Ozawa *et al* [55], 2019	CNN *vs* human reader (endoscopist)	UC	Retrospective cohort, 841 UC patients	Colonoscopy images	MES	The CNN-based CAD system showed a high level of performance with AUC of 0.86 and 0.98 to identify Mayo 0 and 0-1, respectively. The performance of the CNN was better for the rectum than for the right side and left side of the colon when identifying Mayo 0 (AUC = 0.92, 0.83, and 0.83, respectively). Validation cohort included
Bossuyt *et al*[56], 2021	Automated CAD Algorithm *vs* human reader	UC	Prospective cohort, 48 UC patients	Colonoscopy images with confocal laser endomicroscopy	Histologic Remission	The current automated CAD algorithm detects histologic remission with a high performance (sensitivity of 0.79 and specificity of 0.90) compared with the UCEIS (sensitivity of 0.95 and specificity of 0.69) and MES (sensitivity of 0.98 and specificity of 0.61). No validation cohort included
Stidham *et al*[57], 2019	CNN *vs* human reader	UC	Retrospective cohort, 3082 UC patients	Colonoscopy images	Endoscopy severity	The CNN was excellent for distinguishing endoscopic remission from moderate-to-severe disease with an AUC of 0.966 (95%CI: 0.967-0.972); a PPV of 0.87 (95%CI: 0.85-0.88) with a sensitivity of 83.0% (95%CI: 80.8-85.4) and specificity of96.0% (95%CI: 95.1-97.1); and NPV of 0.94 (95%CI: 0.93-0.95). No validation cohort included
Gottlieb *et al*[58], 2021	Neural network *vs* human central reader	UC	Prospective cohort, 249 UC patients	Colonoscopy images	Endoscopy severity	The model's agreement metric was excellent, with a quadratic weighted kappa of 0.844 (95%CI: 0.787-0.901) for endoscopic Mayo Score and 0.855 (95%CI: 0.80-0.91) for UCEIS. No validation cohort included

AI: Artificial intelligence; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AUC: Area under the curve; NPV: Negative predictive value; PPV: Positive predictive value; qPCR: Quantitative real-time polymerase chain reaction; HR: Hazard ratio.

Table 3 summarizes studies included which applied AI in the prediction of therapy response and clinical outcomes in IBD. There were 9 studies that predicted therapy response, 2 studies that predicted presence of extraintestinal manifestations of IBD, 1 study predicting colonic neoplasia, and 1 study predicting post-surgical complications after colectomy. Data modalities included electronic health records (n = 11), molecular

Table 3 Artificial intelligence in prediction of therapy response and clinical outcomes in inflammatory bowel disease

Ref.	AI classifier *vs* comparator	IBD type	Study design and sample size	Modality	Outcomes	Study results/validation cohort
Waljee *et al*[59], 2018	Random forest (RF). No comparator	CD/UC	Post-hoc analysis of prospective clinical trial, 594 CD patients	Veteran's Health Administration Electronic Health Record (EHR)	Outpatient corticosteroids prescribed for IBD and inpatient hospitalizations associated with a diagnosis of IBD	AUC for the RF longitudinal model was 0.85 [95% confidence interval (CI): 0.84–0.85]. AUC for the RF longitudinal model using previous hospitalization or steroid use was 0.87 (95%CI: 0.87-0.88). Validation cohort included
Uttam *et al*[60], 2019	Support vector machines (SVM) *vs* nanoscale nuclear architecture mapping (NanoNAM)	CD/UC	Prospective cohort, 103 IBD patients	3-dimensional NanoNAM of normal-appearing rectal biopsies	Colonic neoplasia	NanoNAM detects colonic neoplasia with an AUC of 0.87 ± 0.04, sensitivity of 0.81 ± 0.09, and specificity of 0.82 ± 0.07 in the independent validation set. Validation cohort included
Waljee *et al*[61], 2017	RF. No comparator	CD/UC	Retrospective cohort, 1080 IBD patients	EHR, lab values	Remission and clinical outcomes with thiopurines	AUC for algorithm-predicted remission in the validation set was 0.79 *vs* 0.49 for 6-TGN. The mean number of clinical events per year in patients with sustained algorithm-predicted remission (APR) was 1.08 *vs* 3.95 in those that did not have sustained APR ($P < 1 \times 10^{-5}$). Validation cohort included
Popa *et al*[62], 2020	Neural network model. No comparator	UC	Prospective cohort, 55 UC patients	Clinical and biological parameters and the endoscopic Mayo score	Disease activity after one year of anti-TNF treatment	The classifier achieved an excellent performance predicting the disease activity at one year with an accuracy of 90% and AUC 0.92 on the test set and an accuracy of 100% and an AUC of 1 on the validation set. Validation cohort included
Douglas *et al*[45], 2018	RF. No comparator	Peds CD	Cross-sectional, 20 CD patients, 20 healthy controls	Shotgun metagenomics (MGS), 16S rRNA gene sequencing	Response to induction therapy	16S genera were again the top dataset (accuracy = 77.8%; $P = 0.008$) for predicting response to therapy. MGS strain ($P = 0.029$), genus ($P = 0.013$), and KEGG pathway ($P = 0.018$) datasets could also classify patients according to therapy response with accuracy = 72.2% for all three. Validation cohort included
Waljee *et al*[63], 2010	RF *vs* boosted trees, RuleFit	CD/UC	Cross-sectional, 774 IBD patients	EHR, lab values (thiopurine metabolites)	Response to thiopurine therapy	A RF algorithm using laboratory values and patient age differentiated clinical response from nonresponse in the model validation data set with an AUC of 0.856 (95%CI: 0.793-0.919). Validation cohort included
Menti *et al*[64], 2016	Naïve bayes *vs* Bayesian additive regression trees *vs* Bayesian networks	CD/UC	Retrospective cohort, 152 CD patients	Genomic DNA, genetic polymorphism	Presence of extra-intestinal manifestations in IBD patients	Bayesian networks achieved accuracy of 82% when considering only clinical factors and 89% when considering also genetic information, outperforming the other techniques. Validation cohort included
Waljee *et al*[65], 2017	RF *vs* baseline regression model	CD/UC	Retrospective cohort, 20368 IBD patients	EHR, lab values	Corticosteroid-free biologic remission with vedolizumab	The AUC for corticosteroid-free biologic remission at week 52 using baseline data was only 0.65 (95%CI: 0.53-0.77), but was 0.75 (95%CI: 0.64-0.86) with data through week 6 of vedolizumab. Validation cohort included
Morilla *et al*[66], 2019	Deep neural networks. No comparator	UC	Retrospective cohort, 47 UC patients	Colonic microrna profiles	Responses to therapy	A deep neural network-based classifier identified 9 microRNAs plus 5 clinical factors, routinely recorded at time of hospital admission, that were associated with responses of patients to treatment. This panel discriminated responders to steroids from non-responders with 93% accuracy (AUC, 0.91). Three algorithms, based on microRNA levels, identified responders to infliximab *vs* non-responders (84% accuracy, AUC 0.82) and responders to cyclosporine *vs* non-responders (80% accuracy, AUC 0.79). Validation cohort included
Wang *et al*[67], 2020	Back-propagation neural network (BPNN), SVM *vs* logistic regression	CD	Cross-sectional, 446 CD patients	EHR	Medication nonadherence to maintenance therapy	The average classification accuracy and AUC of the three models were 85.9% and 0.912 for BPNN, and 87.7% and 0.930 for SVM, respectively. Validation cohort included
Bottigliengo	Bayesian machine	CD/UC	Retrospective cohort,	EHR, genetic	Presence of extra-intestinal	BMLTs had an AUC of 0.50 for classifying the presence of extra-intestinal manifestations. Validation

et al[68], 2019	learning techniques (BMLTs) *vs* logistic regression		142 IBD patients	polymorphisms	manifestations in IBD patients	cohort included
Ghoshal *et al*[69], 2020	Nonlinear artificial neural network (ANN) *vs* multivariate linear PCA	UC	Prospective cohort, 263 UC patients	EHR	Responses to therapy	The multilayer perceptron neural network was trained by back-propagation algorithm (10 networks retained out of 16 tested). The classification accuracy rate was 73% in correctly classifying response to medical treatment in UC patients. No validation cohort included
Sofo *et al*[70], 2020	SVM leave-one-out cross-validation. No comparator	UC	Retrospective cohort, 32 UC patients	EHR	Post-surgical complications after colectomy	Evaluating only preoperative features, machine learning algorithms were able to predict minor postoperative complications with a high strike rate (84.3%), high sensitivity (87.5%) and high specificity (83.3%) during the testing phase. Validation cohort included
Kang *et al*[71], 2017	ANN *vs* logistic regression	UC	Cross-sectional, 24 UC patients	Gene expression profiles	Response to anti-TNF	Balanced accuracy in cross validation test for predicting response to anti-TNF therapy in ulcerative colitis patient was 82%. Validation cohort included
Babic *et al*[72], 1997	CART *vs* back propagation neural network (BPNN)	CD/UC	Cross-sectional, 200 IBD patients	EHR	Quality of life	Best reached classification accuracy did not exceed 80% in any case. Other classifiers namely, K-nearest-neighbor, learning vector quantization and BPNN confirmed that outcome. Validation cohort included
Dong *et al*[73], 2019	RF, SVM, ANN *vs* logistic regression	CD	Retrospective cohort, 239 CD patients	EHR, laboratory tests	Crohn's related surgery	The results revealed that RF predictive model performed better than LR model in terms of accuracy (93.11% *vs* 91.15%), precision (53.42% *vs* 44.81%), F1 score (0.6016 *vs* 0.5763), TN rate (95.08% *vs* 92.00%), and the AUC (0.8926 *vs* 0.8809). The AUCs were excellent at 0.9864 in RF,0.9538 in LR, 0.8809 in DT, 0.9497 in SVM, and 0.9059 in ANN, respectively. Validation cohort included
Lerrigo *et al*[74], 2019	Latent Dirichlet allocation, unsupervised machine learning algorithm. No comparator	CD/UC	Retrospective cohort, 28623 IBD patients	Online posts from the Crohn's and colitis foundation community forum	Impact of online community forums on well-being and their emotional content	10702 (20.8%) posts were identified expressing: gratitude (40%), anxiety/fear (20.8%), empathy (18.2%), anger/frustration (13.4%), hope (13.2%), happiness (10.0%), sadness/depression (5.8%), shame/guilt (2.5%), and/or loneliness (2.5%). A common subtheme was the importance of fostering social support. No validation cohort included

AI: Artificial intelligence; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AUC: Area under the curve; TNF: Tumor necrosis factor.

datasets (n = 4), and histologic data (n = 1). Waljee *et al*[59,65] and Popa *et al*[62] have previously applied RF classifiers to clinical data from electronic health records and laboratory values to predict response to various IBD therapies. In one study using data from a prospective clinical trial consisting of 594 CD patients[59], the AUC for a RF longitudinal model for predicting inpatient hospitalizations in IBD patients prescribed outpatient corticosteroids was 0.85 (95%CI: 0.84-0.85). Using a similar RF approach for predicting remission with thiopurine therapy in a prospective cohort of 55 UC patients yielded an AUC of 0.79[62]. Applying RF to data from a retrospective cohort of 20368 IBD patients with vedolizumab use yielded an AUC of 0.65 (95%CI: 0.53-0.77) for corticosteroid-free vedolizumab remission at week 52 using baseline data and an AUC of 0.75 (95%CI: 0.64-0.86) with data through week 6 of vedolizumab[65]. Molecular datasets have also been used to differentiate between responders and non-responders to various IBD therapies. For example, Morilla *et al*[66] used a deep neural network classifier to construct a predictive panel of colonic microRNAs for IBD therapies in a retrospective cohort of 47 UC patients. Their panel discriminated responders to steroids from non-responders with 93% accuracy (AUC, 0.91). In addition, three

algorithms, based on microRNA levels, identified responders to infliximab *vs* non-responders (84% accuracy, AUC 0.82) and responders to cyclosporine *vs* non-responders (80% accuracy, AUC 0.79). A more recent prospective cohort study of 55 UC patients by Popa *et al*[62] integrated clinical, laboratory, and endoscopic (Mayo scores) datasets using a neural network classifier to predict disease activity after one year of anti-tumor necrosis factor therapy in patients with UC. This classifier achieved an AUC of 0.92 for predicting the disease activity at one year on the test set and an AUC of 1.00 on the validation set. These studies suggest that AI classifiers may play a role in predicting clinical outcomes and response to specific therapies in patients with IBD. However, future clinical trials are needed to compare the efficacy of AI applications in IBD clinical management *vs* standard of care before incorporation into real life clinical practice.

Finally, AI algorithms have been previously applied to enhance the detection of colonic polyps[87] and distinguish among subtypes of neoplastic colorectal lesions[88] in the general population. Although patients with IBD who have extensive colitis have a significantly greater risk of colorectal cancer compared to the general population [89,90], there have been limited studies applying AI technologies to improve colorectal cancer surveillance or develop prediction risk models in patients with IBD. Most studies evaluating polyp detection have excluded IBD patients[91-93]. Our literature search yielded only one study applying AI for the detection of colonic neoplasia in IBD. Uttam *et al*[60] employed support SVM to analyze 3-dimensional nanoscale nuclear architecture mapping (NanoNAM) of normal-appearing rectal biopsies in a prospective cohort of 103 IBD patients. In their study, NanoNAM detected colonic neoplasia with an AUC of 0.87 ± 0.04, sensitivity of 0.81 ± 0.09, and specificity of 0.82 ± 0.07 in the independent validation set. Further studies should focus on determining the clinical utility of incorporating AI methods to enhance standard of cancer surveillance in patients with IBD such as chromoendoscopy[94] and to develop predictive models for risks of colorectal malignancy in IBD patient populations.

CONCLUSION

In conclusion, our literature review has revealed that the applications of AI in IBD have significantly increased in recent years. Our review also highlighted that various AI classifiers may be applied to analyze and integrate large datasets ranging from clinical data from electronic health records, molecular data including gene expression and protein-based studies to a wide array of datasets consisting of endoscopic and histologic images. The application of AI has the potential to improve the accuracy and precision of predicting risk and diagnosis of IBD, assessing disease severity, and predicting outcomes with various IBD therapies. Currently, the application of AI methods in IBD has been limited to the research setting and has not yet been adopted in real life clinical practice. Furthermore, studies applying AI in the context of colorectal cancer surveillance or prediction in IBD are much needed. Given the current status of the field of AI in IBD, future directions should include: (1) Prospective validation of AI applications in IBD in independent cohorts as there is a risk of bias from internal training cohorts and potential limitations with generalizability; (2) Standardization of AI methods and comparative studies evaluating effect of heterogeneity from using different types of datasets on outcomes of interest; (3) Randomized controlled trials to determine whether application of AI in the clinical management of IBD improves clinical outcomes and could be translated into clinical practice; and (4) Randomized controlled trials to determine whether application of AI leads to greater clinical efficacy and cost-effectiveness compared to standard of care in IBD.

REFERENCES

1 **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 2017; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]

2 **Click B**, Ramos Rivers C, Koutroubakis IE, Babichenko D, Anderson AM, Hashash JG, Dunn MA, Schwartz M, Swoger J, Baidoo L, Barrie A 3rd, Regueiro M, Binion DG. Demographic and Clinical Predictors of High Healthcare Use in Patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 1442-1449 [PMID: 26950309 DOI: 10.1097/MIB.0000000000000763]

3 **Ananthakrishnan AN**, Bernstein CN, Iliopoulos D, Macpherson A, Neurath MF, Ali RAR, Vavricka SR, Fiocchi C. Environmental triggers in IBD: a review of progress and evidence. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 39-49 [PMID: 29018271 DOI: 10.1038/nrgastro.2017.136]

4 **de Souza HSP**, Fiocchi C, Iliopoulos D. The IBD interactome: an integrated view of aetiology, pathogenesis and therapy. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 739-749 [PMID: 28831186 DOI: 10.1038/nrgastro.2017.110]

5 **Shouval DS**, Rufo PA. The Role of Environmental Factors in the Pathogenesis of Inflammatory Bowel Diseases: A Review. *JAMA Pediatr* 2017; **171**: 999-1005 [PMID: 28846760 DOI: 10.1001/jamapediatrics.2017.2571]

6 **van der Sloot KWJ**, Amini M, Peters V, Dijkstra G, Alizadeh BZ. Inflammatory Bowel Diseases: Review of Known Environmental Protective and Risk Factors Involved. *Inflamm Bowel Dis* 2017; **23**: 1499-1509 [PMID: 28777099 DOI: 10.1097/MIB.0000000000001217]

7 **Danese S**, Sans M, Fiocchi C. Inflammatory bowel disease: the role of environmental factors. *Autoimmun Rev* 2004; **3**: 394-400 [PMID: 15288007 DOI: 10.1016/j.autrev.2004.03.002]

8 **Tontini GE**, Vecchi M, Pastorelli L, Neurath MF, Neumann H. Differential diagnosis in inflammatory bowel disease colitis: state of the art and future perspectives. *World J Gastroenterol* 2015; **21**: 21-46 [PMID: 25574078 DOI: 10.3748/wjg.v21.i1.21]

9 **Panaccione R**, Rutgeerts P, Sandborn WJ, Feagan B, Schreiber S, Ghosh S. Review article: treatment algorithms to maximize remission and minimize corticosteroid dependence in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2008; **28**: 674-688 [PMID: 18532990 DOI: 10.1111/j.1365-2036.2008.03753.x]

10 **Bettenworth D**, Lopez R, Hindryckx P, Levesque BG, Rieder F. Heterogeneity in endoscopic treatment of Crohn's disease-associated strictures: An international inflammatory bowel disease specialist survey. *J Gastroenterol* 2016; **51**: 939-948 [PMID: 26831355 DOI: 10.1007/s00535-016-1172-6]

11 **Ananthakrishnan AN**, Shi HY, Tang W, Law CC, Sung JJ, Chan FK, Ng SC. Systematic Review and Meta-analysis: Phenotype and Clinical Outcomes of Older-onset Inflammatory Bowel Disease. *J Crohns Colitis* 2016; **10**: 1224-1236 [PMID: 26928965 DOI: 10.1093/ecco-jcc/jjw054]

12 **Anderson AJ**, Click B, Ramos-Rivers C, Babichenko D, Koutroubakis IE, Hartman DJ, Hashash JG, Schwartz M, Swoger J, Barrie AM 3rd, Dunn MA, Regueiro M, Binion DG. Development of an Inflammatory Bowel Disease Research Registry Derived from Observational Electronic Health Record Data for Comprehensive Clinical Phenotyping. *Dig Dis Sci* 2016; **61**: 3236-3245 [PMID: 27619390 DOI: 10.1007/s10620-016-4278-z]

13 **Weersma RK**, Xavier RJ; IBD Multi Omics Consortium, Vermeire S, Barrett JC. Multiomics Analyses to Deliver the Most Effective Treatment to Every Patient With Inflammatory Bowel Disease. *Gastroenterology* 2018; **155**: e1-e4 [PMID: 30077628 DOI: 10.1053/j.gastro.2018.07.039]

14 **Syed S**, Stidham RW. Potential for Standardization and Automation for Pathology and Endoscopy in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2020; **26**: 1490-1497 [PMID: 32869844 DOI: 10.1093/ibd/izaa211]

15 **Johnstone IM**, Titterington DM. Statistical challenges of high-dimensional data. *Philos Trans A Math Phys Eng Sci* 2009; **367**: 4237-4253 [PMID: 19805443 DOI: 10.1098/rsta.2009.0159]

16 **Wang W**, Siau K. Artificial intelligence, machine learning, automation, robotics, future of work and future of humanity: a review and research agenda. *J Database Manag* 2019; **30**: 61-79 [DOI: 10.4018/JDM.2019010104]

17 **Panch T**, Szolovits P, Atun R. Artificial intelligence, machine learning and health systems. *J Glob Health* 2018; **8**: 020303 [PMID: 30405904 DOI: 10.7189/jogh.08.020303]

18 **Mossotto E**, Ashton JJ, Coelho T, Beattie RM, MacArthur BD, Ennis S. Classification of Paediatric Inflammatory Bowel Disease using Machine Learning. *Sci Rep* 2017; **7**: 2427 [PMID: 28546534 DOI: 10.1038/s41598-017-02606-2]

19 **Wei Z**, Wang W, Bradfield J, Li J, Cardinale C, Frackelton E, Kim C, Mentch F, Van Steen K, Visscher PM, Baldassano RN, Hakonarson H; International IBD Genetics Consortium. Large sample size, wide variant spectrum, and advanced machine-learning technique boost risk prediction for inflammatory bowel disease. *Am J Hum Genet* 2013; **92**: 1008-1012 [PMID: 23731541 DOI: 10.1016/j.ajhg.2013.05.002]

20 **Romagnoni A**, Jégou S, Van Steen K, Wainrib G, Hugot JP; International Inflammatory Bowel Disease Genetics Consortium (IIBDGC). Comparative performances of machine learning methods for classifying Crohn Disease patients using genome-wide genotyping data. *Sci Rep* 2019; **9**: 10351 [PMID: 31316157 DOI: 10.1038/s41598-019-46649-z]

21 **Isakov O**, Dotan I, Ben-Shachar S. Machine Learning-Based Gene Prioritization Identifies Novel Candidate Risk Genes for Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2017; **23**: 1516-1523 [PMID: 28795970 DOI: 10.1097/MIB.0000000000001222]

22 **Yuan F**, Zhang YH, Kong XY, Cai YD. Identification of Candidate Genes Related to Inflammatory Bowel Disease Using Minimum Redundancy Maximum Relevance, Incremental Feature Selection, and the Shortest-Path Approach. *Biomed Res Int* 2017; **2017**: 5741948 [PMID: 28293637 DOI: 10.1155/2017/5741948]

23 **Hübenthal M**, Hemmrich-Stanisak G, Degenhardt F, Szymczak S, Du Z, Elsharawy A, Keller A, Schreiber S, Franke A. Sparse Modeling Reveals miRNA Signatures for Diagnostics of Inflammatory Bowel Disease. *PLoS One* 2015; **10**: e0140155 [PMID: 26466382 DOI: 10.1371/journal.pone.0140155]

24 **Tong Y**, Lu K, Yang Y, Li J, Lin Y, Wu D, Yang A, Li Y, Yu S, Qian J. Can natural language processing help differentiate inflammatory intestinal diseases in China? *BMC Med Inform Decis Mak* 2020; **20**: 248 [PMID: 32993636 DOI: 10.1186/s12911-020-01277-w]

25 **Smolander J**, Dehmer M, Emmert-Streib F. Comparing deep belief networks with support vector machines for classifying gene expression data from complex disorders. *FEBS Open Bio* 2019; **9**: 1232-1248 [PMID: 31074948 DOI: 10.1002/2211-5463.12652]

26 **Abbas M**, Matta J, Le T, Bensmail H, Obafemi-Ajayi T, Honavar V, El-Manzalawy Y. Biomarker discovery in inflammatory bowel diseases using network-based feature selection. *PLoS One* 2019; **14**: e0225382 [PMID: 31756219 DOI: 10.1371/journal.pone.0225382]

27 **Khorasani HM**, Usefi H, Peña-Castillo L. Detecting ulcerative colitis from colon samples using efficient feature selection and machine learning. *Sci Rep* 2020; **10**: 13744 [PMID: 32792678 DOI: 10.1038/s41598-020-70583-0]

28 **Rubin SJS**, Bai L, Haileselassie Y, Garay G, Yun C, Becker L, Streett SE, Sinha SR, Habtezion A. Mass cytometry reveals systemic and local immune signatures that distinguish inflammatory bowel diseases. *Nat Commun* 2019; **10**: 2686 [PMID: 31217423 DOI: 10.1038/s41467-019-10387-7]

29 **Pal LR**, Kundu K, Yin Y, Moult J. CAGI4 Crohn's exome challenge: Marker SNP *vs* exome variant models for assigning risk of Crohn disease. *Hum Mutat* 2017; **38**: 1225-1234 [PMID: 28512778 DOI: 10.1002/humu.23256]

30 **Aoki T**, Yamada A, Aoyama K, Saito H, Tsuboi A, Nakada A, Niikura R, Fujishiro M, Oka S, Ishihara S, Matsuda T, Tanaka S, Koike K, Tada T. Automatic detection of erosions and ulcerations in wireless capsule endoscopy images based on a deep convolutional neural network. *Gastrointest Endosc* 2019; **89**: 357-363. e2 [PMID: 30670179 DOI: 10.1016/j.gie.2018.10.027]

31 **Bielecki C**, Bocklitz TW, Schmitt M, Krafft C, Marquardt C, Gharbi A, Knösel T, Stallmach A, Popp J. Classification of inflammatory bowel diseases by means of Raman spectroscopic imaging of epithelium cells. *J Biomed Opt* 2012; **17**: 076030 [PMID: 22894513 DOI: 10.1117/1.JBO.17.7.076030]

32 **Cui H**, Zhang X. Alignment-free supervised classification of metagenomes by recursive SVM. *BMC Genomics* 2013; **14**: 641 [PMID: 24053649 DOI: 10.1186/1471-2164-14-641]

33 **Duttagupta R**, DiRienzo S, Jiang R, Bowers J, Gollub J, Kao J, Kearney K, Rudolph D, Dawany NB, Showe MK, Stamato T, Getts RC, Jones KW. Genome-wide maps of circulating miRNA biomarkers for ulcerative colitis. *PLoS One* 2012; **7**: e31241 [PMID: 22359580 DOI: 10.1371/journal.pone.0031241]

34 **Daneshjou R**, Wang Y, Bromberg Y, Bovo S, Martelli PL, Babbi G, Lena PD, Casadio R, Edwards M, Gifford D, Jones DT, Sundaram L, Bhat RR, Li X, Pal LR, Kundu K, Yin Y, Moult J, Jiang Y, Pejaver V, Pagel KA, Li B, Mooney SD, Radivojac P, Shah S, Carraro M, Gasparini A, Leonardi E, Giollo M, Ferrari C, Tosatto SCE, Bachar E, Azaria JR, Ofran Y, Unger R, Niroula A, Vihinen M, Chang B, Wang MH, Franke A, Petersen BS, Pirooznia M, Zandi P, McCombie R, Potash JB, Altman RB, Klein TE, Hoskins RA, Repo S, Brenner SE, Morgan AA. Working toward precision medicine: Predicting phenotypes from exomes in the Critical Assessment of Genome Interpretation (CAGI) challenges. *Hum Mutat* 2017; **38**: 1182-1192 [PMID: 28634997 DOI: 10.1002/humu.23280]

35 **Geurts P**, Fillet M, de Seny D, Meuwis MA, Malaise M, Merville MP, Wehenkel L. Proteomic mass spectra classification using decision tree based ensemble methods. *Bioinformatics* 2005; **21**: 3138-3145 [PMID: 15890743 DOI: 10.1093/bioinformatics/bti494]

36 **Li H**, Lai L, Shen J. Development of a susceptibility gene based novel predictive model for the diagnosis of ulcerative colitis using random forest and artificial neural network. *Aging (Albany NY)* 2020; **12**: 20471-20482 [PMID: 33099536 DOI: 10.18632/aging.103861]

37 **Wingfield B**, Coleman S, McGinnity TM, Bjourson AJ. Robust Microbial Markers for Non-Invasive Inflammatory Bowel Disease Identification. *IEEE/ACM Trans Comput Biol Bioinform* 2019; **16**: 2078-2088 [PMID: 29994028 DOI: 10.1109/TCBB.2018.2831212]

38 **Han L**, Maciejewski M, Brockel C, Gordon W, Snapper SB, Korzenik JR, Afzelius L, Altman RB. A probabilistic pathway score (PROPS) for classification with applications to inflammatory bowel disease. *Bioinformatics* 2018; **34**: 985-993 [PMID: 29048458 DOI: 10.1093/bioinformatics/btx651]

39 **Wang Y**, Miller M, Astrakhan Y, Petersen BS, Schreiber S, Franke A, Bromberg Y. Identifying Crohn's disease signal from variome analysis. *Genome Med* 2019; **11**: 59 [PMID: 31564248 DOI: 10.1186/s13073-019-0670-6]

40 **Kumar R**, Zhao Q, Seshamani S, Mullin G, Hager G, Dassopoulos T. Assessment of Crohn's disease lesions in wireless capsule endoscopy images. *IEEE Trans Biomed Eng* 2012; **59**: 355-362 [PMID: 22020661 DOI: 10.1109/TBME.2011.2172438]

41 **Biasci D**, Lee JC, Noor NM, Pombal DR, Hou M, Lewis N, Ahmad T, Hart A, Parkes M, McKinney EF, Lyons PA, Smith KGC. A blood-based prognostic biomarker in IBD. *Gut* 2019; **68**: 1386-1395 [PMID: 31030191 DOI: 10.1136/gutjnl-2019-318343]

42 **Waljee AK**, Wallace BI, Cohen-Mekelburg S, Liu Y, Liu B, Sauder K, Stidham RW, Zhu J, Higgins PDR. Development and Validation of Machine Learning Models in Prediction of Remission in Patients With Moderate to Severe Crohn Disease. *JAMA Netw Open* 2019; **2**: e193721 [PMID: 31074823 DOI: 10.1001/jamanetworkopen.2019.3721]

43 **Mahapatra D**, Vos FM, Buhmann JM. Active learning based segmentation of Crohns disease from abdominal MRI. *Comput Methods Programs Biomed* 2016; **128**: 75-85 [PMID: 27040833 DOI: 10.1016/j.cmpb.2016.01.014]

44 **Reddy BK**, Delen D, Agrawal RK. Predicting and explaining inflammation in Crohn's disease

patients using predictive analytics methods and electronic medical record data. *Health Informatics J* 2019; **25**: 1201-1218 [PMID: 29320910 DOI: 10.1177/1460458217751015]

45 **Douglas GM**, Hansen R, Jones CMA, Dunn KA, Comeau AM, Bielawski JP, Tayler R, El-Omar EM, Russell RK, Hold GL, Langille MGI, Van Limbergen J. Multi-omics differentially classify disease state and treatment outcome in pediatric Crohn's disease. *Microbiome* 2018; **6**: 13 [PMID: 29335008 DOI: 10.1186/s40168-018-0398-3]

46 **Maeda Y**, Kudo SE, Mori Y, Misawa M, Ogata N, Sasanuma S, Wakamura K, Oda M, Mori K, Ohtsuka K. Fully automated diagnostic system with artificial intelligence using endocytoscopy to identify the presence of histologic inflammation associated with ulcerative colitis (with video). *Gastrointest Endosc* 2019; **89**: 408-415 [PMID: 30268542 DOI: 10.1016/j.gie.2018.09.024]

47 **Charisis VS**, Hadjileontiadis LJ. Potential of hybrid adaptive filtering in inflammatory lesion detection from capsule endoscopy images. *World J Gastroenterol* 2016; **22**: 8641-8657 [PMID: 27818583 DOI: 10.3748/wjg.v22.i39.8641]

48 **Klang E**, Barash Y, Margalit RY, Soffer S, Shimon O, Albshesh A, Ben-Horin S, Amitai MM, Eliakim R, Kopylov U. Deep learning algorithms for automated detection of Crohn's disease ulcers by video capsule endoscopy. *Gastrointest Endosc* 2020; **91**: 606-613. e2 [PMID: 31743689 DOI: 10.1016/j.gie.2019.11.012]

49 **Ungaro RC**, Hu L, Ji J, Nayar S, Kugathasan S, Denson LA, Hyams J, Dubinsky MC, Sands BE, Cho JH. Machine learning identifies novel blood protein predictors of penetrating and stricturing complications in newly diagnosed paediatric Crohn's disease. *Aliment Pharmacol Ther* 2021; **53**: 281-290 [PMID: 33131065]

50 **Barash Y**, Azaria L, Soffer S, Margalit Yehuda R, Shlomi O, Ben-Horin S, Eliakim R, Klang E, Kopylov U. Ulcer severity grading in video capsule images of patients with Crohn's disease: an ordinal neural network solution. *Gastrointest Endosc* 2021; **93**: 187-192 [PMID: 32535191 DOI: 10.1016/j.gie.2020.05.066]

51 **Lamash Y**, Kurugol S, Freiman M, Perez-Rossello JM, Callahan MJ, Bousvaros A, Warfield SK. Curved planar reformatting and convolutional neural network-based segmentation of the small bowel for visualization and quantitative assessment of pediatric Crohn's disease from MRI. *J Magn Reson Imaging* 2019; **49**: 1565-1576 [PMID: 30353957 DOI: 10.1002/jmri.26330]

52 **Takenaka K**, Ohtsuka K, Fujii T, Negi M, Suzuki K, Shimizu H, Oshima S, Akiyama S, Motobayashi M, Nagahori M, Saito E, Matsuoka K, Watanabe M. Development and Validation of a Deep Neural Network for Accurate Evaluation of Endoscopic Images From Patients With Ulcerative Colitis. *Gastroenterology* 2020; **158**: 2150-2157 [PMID: 32060000 DOI: 10.1053/j.gastro.2020.02.012]

53 **Bossuyt P**, Nakase H, Vermeire S, de Hertogh G, Eelbode T, Ferrante M, Hasegawa T, Willekens H, Ikemoto Y, Makino T, Bisschops R. Automatic, computer-aided determination of endoscopic and histological inflammation in patients with mild to moderate ulcerative colitis based on red density. *Gut* 2020; **69**: 1778-1786 [PMID: 31915237 DOI: 10.1136/gutjnl-2019-320056]

54 **Bhambhvani HP**, Zamora A. Deep learning enabled classification of Mayo endoscopic subscore in patients with ulcerative colitis. *Eur J Gastroenterol Hepatol* 2021; **33**: 645-649 [PMID: 33079775 DOI: 10.1097/MEG.0000000000001952]

55 **Ozawa T**, Ishihara S, Fujishiro M, Saito H, Kumagai Y, Shichijo S, Aoyama K, Tada T. Novel computer-assisted diagnosis system for endoscopic disease activity in patients with ulcerative colitis. *Gastrointest Endosc* 2019; **89**: 416-421. e1 [PMID: 30367878 DOI: 10.1016/j.gie.2018.10.020]

56 **Bossuyt P**, De Hertogh G, Eelbode T, Vermeire S, Bisschops R. Computer-Aided Diagnosis With Monochromatic Light Endoscopy for Scoring Histologic Remission in Ulcerative Colitis. *Gastroenterology* 2021; **160**: 23-25 [PMID: 33058863 DOI: 10.1053/j.gastro.2020.09.053]

57 **Stidham RW**, Liu W, Bishu S, Rice MD, Higgins PDR, Zhu J, Nallamothu BK, Waljee AK. Performance of a Deep Learning Model *vs* Human Reviewers in Grading Endoscopic Disease Severity of Patients With Ulcerative Colitis. *JAMA Netw Open* 2019; **2**: e193963 [PMID: 31099869 DOI: 10.1001/jamanetworkopen.2019.3963]

58 **Gottlieb K**, Requa J, Karnes W, Chandra Gudivada R, Shen J, Rael E, Arora V, Dao T, Ninh A, McGill J. Central Reading of Ulcerative Colitis Clinical Trial Videos Using Neural Networks. *Gastroenterology* 2021; **160**: 710-719. e2 [PMID: 33098883 DOI: 10.1053/j.gastro.2020.10.024]

59 **Waljee AK**, Liu B, Sauder K, Zhu J, Govani SM, Stidham RW, Higgins PDR. Predicting Corticosteroid-Free Biologic Remission with Vedolizumab in Crohn's Disease. *Inflamm Bowel Dis* 2018; **24**: 1185-1192 [PMID: 29668915 DOI: 10.1093/ibd/izy031]

60 **Uttam S**, Hashash JG, LaFace J, Binion D, Regueiro M, Hartman DJ, Brand RE, Liu Y. Three-Dimensional Nanoscale Nuclear Architecture Mapping of Rectal Biopsies Detects Colorectal Neoplasia in Patients with Inflammatory Bowel Disease. *Cancer Prev Res (Phila)* 2019; **12**: 527-538 [PMID: 31164345 DOI: 10.1158/1940-6207.CAPR-19-0024]

61 **Waljee AK**, Sauder K, Patel A, Segar S, Liu B, Zhang Y, Zhu J, Stidham RW, Balis U, Higgins PDR. Machine Learning Algorithms for Objective Remission and Clinical Outcomes with Thiopurines. *J Crohns Colitis* 2017; **11**: 801-810 [PMID: 28333183 DOI: 10.1093/ecco-jcc/jjx014]

62 **Popa IV**, Burlacu A, Mihai C, Prelipcean CC. A Machine Learning Model Accurately Predicts Ulcerative Colitis Activity at One Year in Patients Treated with Anti-Tumour Necrosis Factor α Agents. *Medicina (Kaunas)* 2020; **56** [PMID: 33233514 DOI: 10.3390/medicina56110628]

63 **Waljee AK**, Joyce JC, Wang S, Saxena A, Hart M, Zhu J, Higgins PD. Algorithms outperform metabolite tests in predicting response of patients with inflammatory bowel disease to thiopurines.

Clin Gastroenterol Hepatol 2010; **8**: 143-150 [PMID: 19835986 DOI: 10.1016/j.cgh.2009.09.031]

64 **Menti E**, Lanera C, Lorenzoni G, Giachino DF, Marchi M, Gregori D, Berchialla P; Piedmont Study Group on the Genetics of IBD. Bayesian Machine Learning Techniques for revealing complex interactions among genetic and clinical factors in association with extra-intestinal Manifestations in IBD patients. *AMIA Annu Symp Proc* 2016; **2016**: 884-893 [PMID: 28269885]

65 **Waljee AK**, Lipson R, Wiitala WL, Zhang Y, Liu B, Zhu J, Wallace B, Govani SM, Stidham RW, Hayward R, Higgins PDR. Predicting Hospitalization and Outpatient Corticosteroid Use in Inflammatory Bowel Disease Patients Using Machine Learning. *Inflamm Bowel Dis* 2017; **24**: 45-53 [PMID: 29272474 DOI: 10.1093/ibd/izx007]

66 **Morilla I**, Uzzan M, Laharie D, Cazals-Hatem D, Denost Q, Daniel F, Belleannee G, Bouhnik Y, Wainrib G, Panis Y, Ogier-Denis E, Treton X. Colonic MicroRNA Profiles, Identified by a Deep Learning Algorithm, That Predict Responses to Therapy of Patients With Acute Severe Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2019; **17**: 905-913 [PMID: 30223112 DOI: 10.1016/j.cgh.2018.08.068]

67 **Wang L**, Fan R, Zhang C, Hong L, Zhang T, Chen Y, Liu K, Wang Z, Zhong J. Applying Machine Learning Models to Predict Medication Nonadherence in Crohn's Disease Maintenance Therapy. *Patient Prefer Adherence* 2020; **14**: 917-926 [PMID: 32581518 DOI: 10.2147/PPA.S253732]

68 **Bottigliengo D**, Berchialla P, Lanera C, Azzolina D, Lorenzoni G, Martinato M, Giachino D, Baldi I, Gregori D. The Role of Genetic Factors in Characterizing Extra-Intestinal Manifestations in Crohn's Disease Patients: Are Bayesian Machine Learning Methods Improving Outcome Predictions? *J Clin Med* 2019; **8** [PMID: 31212952 DOI: 10.3390/jcm8060865]

69 **Ghoshal UC**, Rai S, Kulkarni A, Gupta A. Prediction of outcome of treatment of acute severe ulcerative colitis using principal component analysis and artificial intelligence. *JGH Open* 2020; **4**: 889-897 [PMID: 33102760 DOI: 10.1002/jgh3.12342]

70 **Sofo L**, Caprino P, Schena CA, Sacchetti F, Potenza AE, Ciociola A. New perspectives in the prediction of postoperative complications for high-risk ulcerative colitis patients: machine learning preliminary approach. *Eur Rev Med Pharmacol Sci* 2020; **24**: 12781-12787 [PMID: 33378027 DOI: 10.26355/eurrev_202012_24178]

71 **Kang T**, Ding W, Zhang L, Ziemek D, Zarringhalam K. A biological network-based regularized artificial neural network model for robust phenotype prediction from gene expression data. *BMC Bioinformatics* 2017; **18**: 565 [PMID: 29258445 DOI: 10.1186/s12859-017-1984-2]

72 **Babic A**, Ster B, Pavesic N, Wigertz O. Machine learning for the quality of life in inflammatory bowel disease. *Stud Health Technol Inform* 1997; **43** Pt B: 661-665 [PMID: 10179749]

73 **Dong Y**, Xu L, Fan Y, Xiang P, Gao X, Chen Y, Zhang W, Ge Q. A novel surgical predictive model for Chinese Crohn's disease patients. *Medicine (Baltimore)* 2019; **98**: e17510 [PMID: 31725605 DOI: 10.1097/MD.0000000000017510]

74 **Lerrigo R**, Coffey JT, Kravitz JL, Jadhav P, Nikfarjam A, Shah NH, Jurafsky D, Sinha SR. The Emotional Toll of Inflammatory Bowel Disease: Using Machine Learning to Analyze Online Community Forum Discourse. *Crohn's & Colitis 360* 2019; **1**: otz011 [DOI: 10.1093/crocol/otz011]

75 **Papadakis KA**, Tabibzadeh S. Diagnosis and misdiagnosis of inflammatory bowel disease. *Gastrointest Endosc Clin N Am* 2002; **12**: 433-449 [PMID: 12486937 DOI: 10.1016/s1052-5157(02)00005-3]

76 **Best WR**. Predicting the Crohn's disease activity index from the Harvey-Bradshaw Index. *Inflamm Bowel Dis* 2006; **12**: 304-310 [PMID: 16633052 DOI: 10.1097/01.MIB.0000215091.77492.2a]

77 **Lewis JD**, Chuai S, Nessel L, Lichtenstein GR, Aberra FN, Ellenberg JH. Use of the noninvasive components of the Mayo score to assess clinical response in ulcerative colitis. *Inflamm Bowel Dis* 2008; **14**: 1660-1666 [PMID: 18623174 DOI: 10.1002/ibd.20520]

78 **Jones J**, Loftus EV Jr, Panaccione R, Chen LS, Peterson S, McConnell J, Baudhuin L, Hanson K, Feagan BG, Harmsen SW, Zinsmeister AR, Helou E, Sandborn WJ. Relationships between disease activity and serum and fecal biomarkers in patients with Crohn's disease. *Clin Gastroenterol Hepatol* 2008; **6**: 1218-1224 [PMID: 18799360 DOI: 10.1016/j.cgh.2008.06.010]

79 **Schoepfer AM**, Beglinger C, Straumann A, Safroneeva E, Romero Y, Armstrong D, Schmidt C, Trummler M, Pittet V, Vavricka SR. Fecal calprotectin more accurately reflects endoscopic activity of ulcerative colitis than the Lichtiger Index, C-reactive protein, platelets, hemoglobin, and blood leukocytes. *Inflamm Bowel Dis* 2013; **19**: 332-341 [PMID: 23328771 DOI: 10.1097/MIB.0b013e3182810066]

80 **Xie T**, Zhang T, Ding C, Dai X, Li Y, Guo Z, Wei Y, Gong J, Zhu W, Li J. Ulcerative Colitis Endoscopic Index of Severity (UCEIS) *vs* Mayo Endoscopic Score (MES) in guiding the need for colectomy in patients with acute severe colitis. *Gastroenterol Rep (Oxf)* 2018; **6**: 38-44 [PMID: 29479441 DOI: 10.1093/gastro/gox016]

81 **Daperno M**, D'Haens G, Van Assche G, Baert F, Bulois P, Maunoury V, Sostegni R, Rocca R, Pera A, Gevers A, Mary JY, Colombel JF, Rutgeerts P. Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. *Gastrointest Endosc* 2004; **60**: 505-512 [PMID: 15472670 DOI: 10.1016/s0016-5107(04)01878-4]

82 **Novak G**, Parker CE, Pai RK, MacDonald JK, Feagan BG, Sandborn WJ, D'Haens G, Jairath V, Khanna R. Histologic scoring indices for evaluation of disease activity in Crohn's disease. *Cochrane Database Syst Rev* 2017; **7**: CD012351 [PMID: 28731502 DOI: 10.1002/14651858.CD012351.pub2]

83 **Mosli MH**, Feagan BG, Sandborn WJ, D'haens G, Behling C, Kaplan K, Driman DK, Shackelton LM, Baker KA, Macdonald JK, Vandervoort MK, Geboes K, Levesque BG. Histologic evaluation of

ulcerative colitis: a systematic review of disease activity indices. *Inflamm Bowel Dis* 2014; **20**: 564-575 [PMID: 24412993 DOI: 10.1097/01.MIB.0000437986.00190.71]

84 **Falvey JD**, Hoskin T, Meijer B, Ashcroft A, Walmsley R, Day AS, Gearry RB. Disease activity assessment in IBD: clinical indices and biomarkers fail to predict endoscopic remission. *Inflamm Bowel Dis* 2015; **21**: 824-831 [PMID: 25738372 DOI: 10.1097/MIB.0000000000000341]

85 **Neurath MF**. Current and emerging therapeutic targets for IBD. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 269-278 [PMID: 28144028 DOI: 10.1038/nrgastro.2016.208]

86 **Weisshof R**, El Jurdi K, Zmeter N, Rubin DT. Emerging Therapies for Inflammatory Bowel Disease. *Adv Ther* 2018; **35**: 1746-1762 [PMID: 30374806 DOI: 10.1007/s12325-018-0795-9]

87 **Luo Y**, Zhang Y, Liu M, Lai Y, Liu P, Wang Z, Xing T, Huang Y, Li Y, Li A, Wang Y, Luo X, Liu S, Han Z. Artificial Intelligence-Assisted Colonoscopy for Detection of Colon Polyps: a Prospective, Randomized Cohort Study. *J Gastrointest Surg* 2020 [PMID: 32968933 DOI: 10.1007/s11605-020-04802-4]

88 **Selaru FM**, Xu Y, Yin J, Zou T, Liu TC, Mori Y, Abraham JM, Sato F, Wang S, Twigg C, Olaru A, Shustova V, Leytin A, Hytiroglou P, Shibata D, Harpaz N, Meltzer SJ. Artificial neural networks distinguish among subtypes of neoplastic colorectal lesions. *Gastroenterology* 2002; **122**: 606-613 [PMID: 11874992 DOI: 10.1053/gast.2002.31904]

89 **Gillen CD**, Walmsley RS, Prior P, Andrews HA, Allan RN. Ulcerative colitis and Crohn's disease: a comparison of the colorectal cancer risk in extensive colitis. *Gut* 1994; **35**: 1590-1592 [PMID: 7828978 DOI: 10.1136/gut.35.11.1590]

90 **Bernstein CN**, Blanchard JF, Kliewer E, Wajda A. Cancer risk in patients with inflammatory bowel disease: a population-based study. *Cancer* 2001; **91**: 854-862 [PMID: 11241255 DOI: 10.1002/1097-0142(20010215)91:4<854::aid-cncr1073>3.0.co;2-z]

91 **Misawa M**, Kudo SE, Mori Y, Cho T, Kataoka S, Yamauchi A, Ogawa Y, Maeda Y, Takeda K, Ichimasa K, Nakamura H, Yagawa Y, Toyoshima N, Ogata N, Kudo T, Hisayuki T, Hayashi T, Wakamura K, Baba T, Ishida F, Itoh H, Roth H, Oda M, Mori K. Artificial Intelligence-Assisted Polyp Detection for Colonoscopy: Initial Experience. *Gastroenterology* 2018; **154**: 2027-2029. e3 [PMID: 29653147 DOI: 10.1053/j.gastro.2018.04.003]

92 **Wang P**, Berzin TM, Glissen Brown JR, Bharadwaj S, Becq A, Xiao X, Liu P, Li L, Song Y, Zhang D, Li Y, Xu G, Tu M, Liu X. Real-time automatic detection system increases colonoscopic polyp and adenoma detection rates: a prospective randomised controlled study. *Gut* 2019; **68**: 1813-1819 [PMID: 30814121 DOI: 10.1136/gutjnl-2018-317500]

93 **Wang P**, Liu X, Berzin TM, Glissen Brown JR, Liu P, Zhou C, Lei L, Li L, Guo Z, Lei S, Xiong F, Wang H, Song Y, Pan Y, Zhou G. Effect of a deep-learning computer-aided detection system on adenoma detection during colonoscopy (CADe-DB trial): a double-blind randomised study. *Lancet Gastroenterol Hepatol* 2020; **5**: 343-351 [PMID: 31981517 DOI: 10.1016/S2468-1253(19)30411-X]

94 **Ogawa R**, Nishikawa J, Hideura E, Goto A, Koto Y, Ito S, Unno M, Yamaoka Y, Kawasato R, Hashimoto S, Okamoto T, Ogihara H, Hamamoto Y, Sakaida I. Objective Assessment of the Utility of Chromoendoscopy with a Support Vector Machine. *J Gastrointest Cancer* 2019; **50**: 386-391 [PMID: 29504086 DOI: 10.1007/s12029-018-0083-6]

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MINIREVIEWS

Post-pancreatitis diabetes mellitus and excess intra-pancreatic fat deposition as harbingers of pancreatic cancer

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Abstract

Progress in the fight against pancreatic cancer has been hampered by many factors. One of them is the inability to detect the disease early in overwhelming majority of patients. The present paper outlines a novel way in which progress could be accelerated. This includes a focus on two harbingers—post-pancreatitis diabetes mellitus and excess intra-pancreatic fat deposition—that converge at affecting the tumor macroenvironment and microenvironment specifically in the pancreas, not other organs. The two entities have the potential to be incorporated into future screening strategies with a view to early detecting of pancreatic cancer.

Key Words: Pancreatic cancer; Post-pancreatitis diabetes mellitus; Intra-pancreatic fat; Pancreatitis; Early detection; Screening

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Core Tip: Identification of harbingers of pancreatic cancer that are specifically related to the pancreas is necessary to enable cost-effective and achievement-appropriate screening for this disease. Post-pancreatitis diabetes mellitus and excess intra-pancreatic fat deposition are positioned well to serve the purpose.

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INTRODUCTION

Pancreatic cancer incidence rates are on the rise since the 1990s[1]. Given that around 90% of pancreatic cancer is unresectable at the time of diagnosis, early detection of pancreatic cancer is critical with a view to lessening the burden of this disease[2]. The development of an accurate test for early detection of sporadic pancreatic cancer would considerably improve the survival of these patients[3]. However, while more than 2500 putative biomarkers (genomics, transcriptomics, proteomics) were found to be overexpressed at the messenger RNA or protein level in pancreatic cancer[4], translating biomarker discoveries into clinical applications has been a litany of failures in the setting of pancreatic cancer. Identification of harbingers of sporadic pancreatic cancer and development of screening policies based on them is another avenue towards lessening the burden of this disease. New-onset diabetes and obesity became recognized as one of the most prominent risk factors for pancreatic cancer[5-7]. The present review focuses on how that knowledge has crystallized over the past 5 years or so to consider post-pancreatitis diabetes mellitus (PPDM) and excess intra-pancreatic fat deposition (IPFD) as the specific harbingers of pancreatic cancer that are superior to general risk factors such as new-onset diabetes mellitus and obesity.

PERSISTENT HYPERGLYCEMIA

A 2011 meta-analysis of 35 cohort studies showed that people with prevalent diabetes had a 1.9-times higher risk for pancreatic cancer as compared with those without diabetes (Figure 1)[8]. Later, a 2018 study from the Mayo clinic demonstrated that not any diabetes but only incident diabetes holds promise as a harbinger of pancreatic cancer[9]. A 60-mo temporal fasting plasma glucose profile was constructed for patients diagnosed with pancreatic cancer (as well as matched controls). The authors showed that hyperglycemia first occurred 30-36 mo prior to pancreatic cancer diagnosis and reached the diabetes threshold 6-12 mo prior to cancer diagnosis. Moreover, fasting plasma glucose concentrations increased with tumor volume, with the smallest tumor volume associated with hyperglycemia being 1.1-2.0 mL (which is considerably smaller than the average tumor volume of 11.5 mL at diagnosis of pancreatic cancer)[9]. In theory, diagnosing pancreatic cancer when it is that small could markedly increase cure rates and long-term survival. In practice, however, hyperglycemia alone cannot be implemented as a cost-effective screening strategy because pancreatic cancer is rare whereas hyperglycemia is very common. Further, hyperglycemia in the context of pancreatic cancer represents a paraneoplastic syndrome and therefore is not specific. For example, data from two large prospective cohorts (494078 person-years of follow-up) in the United States published in 2020 confirmed that incident diabetes is a significant risk factor for pancreatic cancer (adjusted hazard ratio 2.07; 95% confidence interval 1.70 to 2.52)[10]. However, the study also showed that incident diabetes is a significant risk factor for cancer in 7 other organs (breast, large intestine, endome-trium, esophagus, liver, lung, and thyroid). Combining incident diabetes with weight loss was shown to increase the ability to predict the occurrence of pancreatic cancer in a 2018 retrospective study[11]. However, weight loss is another non-specific symptom and therefore is unlikely to be much more useful in determination of pancreatic cancer risk (as compared with cancer in the 7 organs mentioned above).

It is conceivable that accurate determination of pancreatic cancer risk among people with persistent hyperglycemia can only be achieved when factors related specifically to the pancreas are considered. One such factor is inflammation of the pancreas prior to new-onset diabetes (*i.e.*, PPDM). PPDM is a sub-type of diabetes of the exocrine pancreas and is caused by acute pancreatitis in four out of five people and chronic pancreatitis in one out of five people[12]. Its epidemiology, risk factors, pathogenesis, and management were comprehensively reviewed elsewhere[12]. A large 2020 cohort study by Cho *et al*[13] compared the risks of developing pancreatic cancer in PPDM *vs* type 2 diabetes mellitus without history of pancreatitis and showed that PPDM was associated with a 7-times significantly higher risk for pancreatic cancer (adjusted hazard ratio 6.94; 95% confidence interval 4.09 to 11.77). This held true after adjustment for age, sex, ethnicity, social deprivation index, alcohol abuse, tobacco smoking, history of gallstones, cholecystectomy, and Charlson comorbidity index. When a 12-mo lag period between diabetes diagnosis and pancreatic cancer diagnosis was introduced (to minimize the possibility of reverse causality), the results did not change materially (adjusted hazard ratio 7.93; 95% confidence interval 3.53 to 17.81).

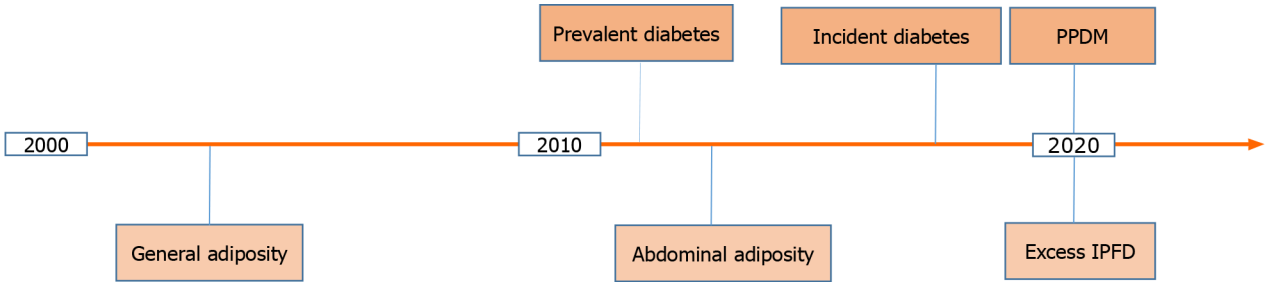


Figure 1 A timeline of the major developments in regards to persistent hyperglycemia and excess body fat as harbingers of pancreatic cancer in the 21st century to date. IPFD: Intra-pancreatic fat deposition; PPDM: Post-pancreatitis diabetes mellitus.

Also, people with history of pancreatitis (without diabetes mellitus) had a 4.8-times significantly higher risk of pancreatic cancer (95% confidence interval 3.38 to 6.99) than those with type 2 diabetes mellitus without history of pancreatitis[13]. This suggests that diabetes mellitus without history of pancreatitis is not a major risk factor for pancreatic cancer; rather it is pancreatitis that is a major risk factor for pancreatic cancer in individuals with diabetes. Moreover, the study showed that an attack of pancreatitis in individuals with diabetes had a differential effect on the subsequent risk of pancreatic cancer depending on whether it occurred before or after diabetes. Specifically, it found that people with PPDM had a 2.3-times significantly higher risk of pancreatic cancer (95% confidence interval 1.12 to 4.93) than those with type 2 diabetes mellitus that precedes pancreatitis, after adjustment for the above-mentioned covariates[13]. This suggests that the increased risk of pancreatic cancer in individuals with PPDM is not due to merely the effect of pancreatitis as a comorbidity in individuals with type 2 diabetes mellitus but rather pancreatitis exerts an effect beyond being a comorbidity in individuals with PPDM.

The 2018 Mayo clinic study[9] and the 2020 COSMOS study[13] are highly complemen-tary in nature, paving the way to identification of population at high risk of pancreatic cancer within a cohort of people with diabetes, which has the potential to enrich the cohort for pancreatic cancer. The 3-year incidence of pancreatic cancer in the Mayo clinic study was 1.0% among individuals with diabetes, which is in line with the 0.7% estimate in individuals with diabetes in the entire cohort of the COSMOS study. The Mayo clinic developed a model using the data of 1516 individuals with first diagnosis of diabetes (based on fasting blood glucose and/or estimated average glucose) and the incidence of pancreatic cancer increased to 3.6% after applying the model[11]. The model requires five variables: age at first diagnosis of diabetes, blood glucose levels at two time points (approximately 12 mo prior to and at first diagnosis of diabetes), and weight at two time points (approximately 12 mo prior to and at first diagnosis of diabetes). The COMSOS study of 139843 individuals offered a complementary non-overlapping approach, in which the consideration of history of pancreatitis prior to first diagnosis of diabetes (regardless of changes in glycemia and weight prior to diabetes) enabled the enrichment of the cohort of people with diabetes for pancreatic cancer to the extent the Mayo clinic study did (from 0.7% to 3.1% in the COSMOS study as compared with from 1.0% to 3.6% in the Mayo clinic study)[13]. Interestingly, the COSMOS study found that resected pancreatic cancer yielded the highest risk (hazard ratio 16.2) in individuals with PPDM[13]. This likely reflects the higher likelihood of detection of pancreatic cancer at earlier stages in individuals with PPDM *vs* type 2 diabetes mellitus, which may be attributable to the fact that individuals with PPDM are more likely to undergo more intensive work-up during hospitalization for pancreatitis (*e.g.*, earlier abdominal imaging and carbohydrate antigen 19-9, possibly resulting in a lead time) and are more closely monitored after hospital discharge. This is not dissimilar to the notion of 'incidentaloma'—incidental abnormal finding from imaging test. Based on the above findings, it is reasonable to suggest that taking into account history of pancreatitis (in addition to age at diabetes diagnosis and changes in glycemia and body composition prior to diabetes) will further enrich cohorts of people with diabetes for pancreatic cancer. Purposely-designed studies are warranted to operationalize the combined approach. But, in principal, it could be applied to all middle-aged and older adults after an attack of pancreatitis who develop new-onset diabetes and unintentional changes in body composition during follow-up. This might ultimately make screening for pancreatic cancer cost-effective and achievement-appropriate.

EXCESS BODY FAT

In a 2003 prospective cohort study of more than 900000 adults, the relative risk of pancreatic cancer for people with morbid obesity (body mass index > 40 kg/m^2) was 2.76 (95% confidence interval 1.74 to 4.36) for women and 2.61 (95% confidence interval 1.30 to 5.40) for men (Figure 1)[14]. A 2009 prospective cohort study of more than 450416 adults estimated that general overweight or obesity (body mass index ≥ 25 kg/m^2) explained 8% of the population attributable risk for pancreatic cancer, which made it the second largest population attributable risk (following tobacco smoking) among all the modifiable factors studied[15]. Later, visceral adiposity (as evidenced by waist circumference) became acknowledged as a more accurate measure of excess body fat (Figure 1). Several prospective studies showed a significant association between risk of pancreatic cancer and visceral adiposity. These studies (encompassing 787356 adults) were meta-analyzed in 2012 and the risk of pancreatic cancer was estimated to increase 1.1-times (95% confidence interval 1.05 to 1.18) with every 10-cm increase in waist circumference[16]. Based on the best available evidence in regards to both body mass index and waist circumference, the World Cancer Research Fund and the American Institute for Cancer Research concluded that the association between excess adiposity and pancreatic cancer is causal[17]. However, the causality was also postulated in relation to excess adiposity and cancer in several other organs (esophagus, liver, colorectum, breast, endometrium, kidney). Given that both general adiposity and abdominal adiposity have a low specificity, these are not useful specifically for the purpose of early detection of pancreatic cancer.

More recently, local fat contained within the pancreas—termed IPFD—has emerged as an early specific factor contributing to the formation of pancreatic tumorigenesis (Figure 1). The relationship between IPFD and pancreatic cancer or premalignant lesions had been investigated in several studies that were systematically reviewed in a 2020 systematic review and meta-analysis by Sreedhar *et al*[18]. A total of 13 retrospective studies (encompassing 2178 individuals) were included. The pooled prevalence of fatty pancreas disease in individuals with pancreatic cancer was 52% (95% confidence interval 38 to 66%). Further, there was a 2.8-times higher prevalence of fatty pancreas disease among individuals with pancreatic cancer or pre-malignant lesions compared with controls (risk ratio 2.78; 95% confidence interval 1.56 to 4.94)[18]. High IPFD was also associated with dissemination and increased mortality of the disease in two single-center studies[19,20]. Besides, there was an evidence of a consistent association between the presence of pancreatic pre-malignant lesions and high IPFD, independent of fatty liver disease, abdominal adiposity, and general adiposity. In particular, one study showed a significantly increased IPFD in individuals with intraductal papillary mucinous neoplasm (n = 85), as compared with age-, sex-, and diabetes status-matched individuals with no pancreatic cyst (n = 85)[21]. Taking into account that two types of pancreatic cancer can develop in individuals with intraductal papillary mucinous neoplasm (invasive carcinoma within the index lesion and concomitant pancreatic ductal adenocarcinoma arising at a site other than intraductal papillary mucinous neoplasm) and taking into account that progression to high-grade dysplasia within the index lesion is relatively easy to detect and follow up[22,23], an increased IPFD during follow-up could be particularly helpful in identifying individuals with intraductal papillary mucinous neoplasm who harbor concomitant pancreatic ductal adenocarcinoma.

IPFD was also investigated in the setting of pancreatitis—a major risk factor for pancreatic cancer[13,24-27]. A cross-sectional study by Stuart *et al*[28] investigated 119 individuals after an attack of pancreatitis and 38 healthy volunteers. It found that IPFD (determined with the use of chemical shift-encoded magnetic resonance imaging) was significantly greater in individuals after an attack of pancreatitis (both acute and chronic) than healthy volunteers, in both crude analysis and after adjustment for age, sex, ethnicity, visceral-to-subcutaneous fat volume ratio, glycated hemoglobin, triglycerides. Notably, two other common ectopic fat phenotypes—liver fat and skeletal muscle fat deposition—did not differ significantly between the groups[28]. Several other cross-sectional studies showed that excess IPFD is associated with worse outcomes during hospitalization for acute pancreatitis[29-31]. Individuals with chronic pancreatitis alone (n = 58) had a significantly greater IPFD in comparison with controls (n = 60) in a cross-sectional study from the United States (determined with the use of chemical shift-encoded magnetic resonance imaging)[32]. Also, the severity of pancreatic ductal changes (based on the Cambridge classification) in individuals with chronic pancreatitis was not associated with IPFD[32]. It is worth noting that the study groups were compared in crude analysis only in that study, despite the fact that there were significant differences between the groups in terms of age, body composition,

alcohol consumption, and tobacco smoking. An earlier study from the United States found that individuals with chronic pancreatitis alone (n = 35) had a significantly greater IPFD in comparison with controls (n = 50) in a post-hoc analysis constrained to non-obese people only (body mass index < 30 kg/m^2)[33]. A longitudinal study from Japan sought to investigate the temporal relationship between IPFD and chronic pancreatitis[34]. A total of 9933 individuals without pancreatitis were examined in 2008 and followed up for 4 years as part of their medical check-up. The presence of fatty pancreas disease at baseline was associated with a 3.9-times higher risk of incident pancreatitis during follow-up (odds ratio 3.9; 95% confidence interval 2.0 to 7.7), after adjustment for age, sex, body mass index, glycated hemoglobin, systolic blood pressure, alcohol abuse, tobacco smoking, and other covariates[34]. However, it is worth noting that transabdominal ultrasound was used in this study, which is suboptimal for diagnosing of both chronic pancreatitis and fatty pancreas disease.

Beyond people with pancreatic premalignant lesions or history of pancreatitis, it is tempting to speculate that people with incidentally found fatty pancreas disease could benefit from a regular follow-up with a view to early detecting of pancreatic cancer. However, given that fatty pancreas disease is very common in the general population (prevalence 16.1%; 95% confidence interval 13.3 to 18.8)[35] and taking into account that the state-of-the-art sequential assessment of the pancreas (*i.e.*, the use of magnetic resonance imaging) is costly[36], screening of unselected people with fatty pancreas disease for pancreatic cancer is unlikely to reach current cost-effectiveness standards. However, it is envisaged that future studies will identify a subgroup of people with fatty pancreas disease in the general population that is at high risk for sporadic pancreatic cancer.

CONCLUSION

The complex nature and the relative rarity of pancreatic cancer make it challenging to implement screening in people with no family history of the disease[37,38]. In fact, a 2019 evidence-based report by the United States Preventive Services Task Force deemed screening for pancreatic cancer in asymptomatic adults not to be cost-effective[39]. However, to date, the cost-effectiveness of only conventional non-specific risk factors has been considered. A 2021 microsimulation screening analysis model investigated the impact of relevant uncertainties on the effectiveness of pancreatic cancer screening and showed that test specificity had higher influence than sensitivity[40]. Growing evidence compels a consideration of middle-aged and older adults with PPDM and/or incidentally found fatty pancreas disease as specific populations at very high risk of developing pancreatic cancer. Comprehensive understanding of the intricate relationship between PPDM and IPFD will offer actionable insights into early detection of pancreatic cancer.

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REFERENCES

1 **Gordon-Dseagu VL**, Devesa SS, Goggins M, Stolzenberg-Solomon R. Pancreatic cancer incidence trends: Evidence from the Surveillance, Epidemiology and End Results (SEER) population-based data. *Int J Epidemiol* 2018; **47**: 427-439 [PMID: 29149259 DOI: 10.1093/ije/dyx232]

2 **McGuigan A**, Kelly P, Turkington RC, Jones C, Coleman HG, McCain RS. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J Gastroenterol* 2018; **24**: 4846-4861 [PMID: 30487695 DOI: 10.3748/wjg.v24.i43.4846]

3 **Nannini G**, Meoni G, Amedei A, Tenori L. Metabolomics profile in gastrointestinal cancers: Update and future perspectives. *World J Gastroenterol* 2020; **26**: 2514-2532 [PMID: 32523308 DOI: 10.3748/wjg.v26.i20.2514]

4 **Harsha HC**, Kandasamy K, Ranganathan P, Rani S, Ramabadran S, Gollapudi S, Balakrishnan L, Dwivedi SB, Telikicherla D, Selvan LD, Goel R, Mathivanan S, Marimuthu A, Kashyap M, Vizza RF, Mayer RJ, Decaprio JA, Srivastava S, Hanash SM, Hruban RH, Pandey A. A compendium of potential biomarkers of pancreatic cancer. *PLoS Med* 2009; **6**: e1000046 [PMID: 19360088 DOI:

10.1371/journal.pmed.1000046]
5 **Molina-Montes E**, Coscia C, Gómez-Rubio P, Fernández A, Boenink R, Rava M, Márquez M, Molero X, Löhr M, Sharp L, Michalski CW, Farré A, Perea J, O'Rorke M, Greenhalf W, Iglesias M, Tardón A, Gress TM, Barberá VM, Crnogorac-Jurcevic T, Muñoz-Bellvís L, Dominguez-Muñoz JE, Renz H, Balcells J, Costello E, Ilzarbe L, Kleeff J, Kong B, Mora J, O'Driscoll D, Poves I, Scarpa A, Yu J, Hidalgo M, Lawlor RT, Ye W, Carrato A, Real FX, Malats N; PanGenEU Study Investigators. Deciphering the complex interplay between pancreatic cancer, diabetes mellitus subtypes and obesity/BMI through causal inference and mediation analyses. *Gut* 2021; **70**: 319-329 [PMID: 32409590 DOI: 10.1136/gutjnl-2019-319990]
6 **Pothuraju R**, Rachagani S, Junker WM, Chaudhary S, Saraswathi V, Kaur S, Batra SK. Pancreatic cancer associated with obesity and diabetes: an alternative approach for its targeting. *J Exp Clin Cancer Res* 2018; **37**: 319 [PMID: 30567565 DOI: 10.1186/s13046-018-0963-4]
7 **Paternoster S**, Falasca M. The intricate relationship between diabetes, obesity and pancreatic cancer. *Biochim Biophys Acta Rev Cancer* 2020; **1873**: 188326 [PMID: 31707038 DOI: 10.1016/j.bbcan.2019.188326]
8 **Ben Q**, Xu M, Ning X, Liu J, Hong S, Huang W, Zhang H, Li Z. Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. *Eur J Cancer* 2011; **47**: 1928-1937 [PMID: 21458985 DOI: 10.1016/j.ejca.2011.03.003]
9 **Sharma A**, Smyrk TC, Levy MJ, Topazian MA, Chari ST. Fasting blood glucose levels provide estimate of duration and progression of pancreatic cancer before diagnosis. *Gastroenterology* 2018; **155**: 490-500. e2 [PMID: 29723506 DOI: 10.1053/j.gastro.2018.04.025]
10 **Hu Y**, Zhang X, Ma Y, Yuan C, Wang M, Wu K, Tabung FK, Tobias D, Hu FB, Giovannucci E, Song M. Incident type 2 diabetes duration and cancer risk: a prospective study in two us cohorts. *J Natl Cancer Inst* 2021; **113**: 381-389 [PMID: 33225344 DOI: 10.1093/jnci/djaa141]
11 **Sharma A**, Kandlakunta H, Nagpal SJS, Feng Z, Hoos W, Petersen GM, Chari ST. Model to determine risk of pancreatic cancer in patients with new-onset diabetes. *Gastroenterology* 2018; **155**: 730-739. e3 [PMID: 29775599 DOI: 10.1053/j.gastro.2018.05.023]
12 **Petrov MS**. Post-pancreatitis diabetes mellitus: prime time for secondary disease. *Eur J Endocrinol* 2021; **184**: R137-R149 [PMID: 33460393 DOI: 10.1530/EJE-20-0468]
13 **Cho J**, Scragg R, Petrov MS. Postpancreatitis diabetes confers higher risk for pancreatic cancer than type 2 diabetes: results from a nationwide cancer registry. *Diabetes Care* 2020; **43**: 2106-2112 [PMID: 32616613 DOI: 10.2337/dc20-0207]
14 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638 [PMID: 12711737 DOI: 10.1056/NEJMoa021423]
15 **Jiao L**, Mitrou PN, Reedy J, Graubard BI, Hollenbeck AR, Schatzkin A, Stolzenberg-Solomon R. A combined healthy lifestyle score and risk of pancreatic cancer in a large cohort study. *Arch Intern Med* 2009; **169**: 764-770 [PMID: 19398688 DOI: 10.1001/archinternmed.2009.46]
16 **Aune D**, Greenwood DC, Chan DS, Vieira R, Vieira AR, Navarro Rosenblatt DA, Cade JE, Burley VJ, Norat T. Body mass index, abdominal fatness and pancreatic cancer risk: A systematic review and non-linear dose-response meta-analysis of prospective studies. *Ann Oncol* 2012; **23**: 843-852 [PMID: 21890910 DOI: 10.1093/annonc/mdr398]
17 **World Cancer Research Fund/American Institute for Cancer Research**. Body fatness & weight gain. Continuous update project expert report 2020. [cited 1 March 2021]. In: World Cancer Research Fund International [Internet]. Available from: https://www.wcrf.org/dietandcancer/exposures/body-fatness
18 **Sreedhar UL**, DeSouza SV, Park B, Petrov MS. A systematic review of intra-pancreatic fat deposition and pancreatic carcinogenesis. *J Gastrointest Surg* 2020; **24**: 2560-2569 [PMID: 31749093 DOI: 10.1007/s11605-019-04417-4]
19 **Mathur A**, Zyromski NJ, Pitt HA, Al-Azzawi H, Walker JJ, Saxena R, Lillemoe KD. Pancreatic steatosis promotes dissemination and lethality of pancreatic cancer. *J Am Coll Surg* 2009; **208**: 989-94; discussion 994 [PMID: 19476877 DOI: 10.1016/j.jamcollsurg.2008.12.026]
20 **Mathur A**, Hernandez J, Shaheen F, Shroff M, Dahal S, Morton C, Farrior T, Kedar R, Rosemurgy A. Preoperative computed tomography measurements of pancreatic steatosis and visceral fat: prognostic markers for dissemination and lethality of pancreatic adenocarcinoma. *HPB (Oxford)* 2011; **13**: 404-410 [PMID: 21609373 DOI: 10.1111/j.1477-2574.2011.00304.x]
21 **Kashiwagi K**, Seino T, Fukuhara S, Minami K, Horibe M, Iwasaki E, Takaishi H, Itoh K, Sugino Y, Inoue N, Iwao Y, Kanai T. Pancreatic fat content detected by computed tomography and its significant relationship with intraductal papillary mucinous neoplasm. *Pancreas* 2018; **47**: 1087-1092 [PMID: 30028443 DOI: 10.1097/MPA.0000000000001103]
22 **Tanaka M**, Fernández-Del Castillo C, Kamisawa T, Jang JY, Levy P, Ohtsuka T, Salvia R, Shimizu Y, Tada M, Wolfgang CL. Revisions of international consensus Fukuoka guidelines for the management of IPMN of the pancreas. *Pancreatology* 2017; **17**: 738-753 [PMID: 28735806 DOI: 10.1016/j.pan.2017.07.007]
23 **Kanno A**, Masamune A, Hanada K, Maguchi H, Shimizu Y, Ueki T, Hasebe O, Ohtsuka T, Nakamura M, Takenaka M, Kitano M, Kikuyama M, Gabata T, Yoshida K, Sasaki T, Serikawa M, Furukawa T, Yanagisawa A, Shimosegawa T; Japan Study Group on the Early Detection of Pancreatic Cancer (JEDPAC). Multicenter study of early pancreatic cancer in Japan. *Pancreatology* 2018; **18**: 61-67 [PMID: 29170051 DOI: 10.1016/j.pan.2017.11.007]

24 **Feng Q**, Li C, Zhang S, Tan CL, Mai G, Liu XB, Chen YH. Recurrence and survival after surgery for pancreatic cancer with or without acute pancreatitis. *World J Gastroenterol* 2019; **25**: 6006-6015 [PMID: 31660036 DOI: 10.3748/wjg.v25.i39.6006]

25 **Syed A**, Babich O, Thakkar P, Patel A, Abdul-Baki H, Farah K, Morrissey S, Mitre M, Dhawan M, Kochhar G, Kulkarni A, Thakkar S. Defining pancreatitis as a risk factor for pancreatic cancer: the role, incidence, and timeline of development. *Pancreas* 2019; **48**: 1098-1101 [PMID: 31404017 DOI: 10.1097/MPA.0000000000001367]

26 **Gayam V**, Sidhu JS, Mandal A, Garlapati P, Adapa S, Konala VM, Naramala S, Then EO, Maddika S, Gaduputi V. National trends and hospitalizations related to pancreatic cancer in acute pancreatitis patients: a nationwide inpatient sample study. *Cureus* 2019; **11**: e5155 [PMID: 31523582 DOI: 10.7759/cureus.5155]

27 **Sadr-Azodi O**, Oskarsson V, Discacciati A, Videhult P, Askling J, Ekbom A. Pancreatic cancer following acute pancreatitis: A population-based matched cohort study. *Am J Gastroenterol* 2018; **113**: 1711-1719 [PMID: 30315287 DOI: 10.1038/s41395-018-0255-9]

28 **Stuart CE**, Ko J, Modesto AE, Alarcon Ramos GC, Bharmal SH, Cho J, Singh RG, Petrov MS. Implications of tobacco smoking and alcohol consumption on ectopic fat deposition in individuals after pancreatitis. *Pancreas* 2020; **49**: 924-934 [PMID: 32658076 DOI: 10.1097/MPA.0000000000001600]

29 **Navina S**, Acharya C, DeLany JP, Orlichenko LS, Baty CJ, Shiva SS, Durgampudi C, Karlsson JM, Lee K, Bae KT, Furlan A, Behari J, Liu S, McHale T, Nichols L, Papachristou GI, Yadav D, Singh VP. Lipotoxicity causes multisystem organ failure and exacerbates acute pancreatitis in obesity. *Sci Transl Med* 2011; **3**: 107ra110 [PMID: 22049070 DOI: 10.1126/scitranslmed.3002573]

30 **Durgampudi C**, Noel P, Patel K, Cline R, Trivedi RN, DeLany JP, Yadav D, Papachristou GI, Lee K, Acharya C, Jaligama D, Navina S, Murad F, Singh VP. Acute lipotoxicity regulates severity of biliary acute pancreatitis without affecting its initiation. *Am J Pathol* 2014; **184**: 1773-1784 [PMID: 24854864 DOI: 10.1016/j.ajpath.2014.02.015]

31 **Xie J**, Xu L, Pan Y, Li P, Liu Y, Shan K, Zhang Y. Nonalcoholic fatty pancreas disease is related independently to the severity of acute pancreatitis. *Eur J Gastroenterol Hepatol* 2019; **31**: 973-978 [PMID: 31233410 DOI: 10.1097/MEG.0000000000001477]

32 **Tirkes T**, Jeon CY, Li L, Joon AY, Seltman TA, Sankar M, Persohn SA, Territo PR. Association of pancreatic steatosis with chronic pancreatitis, obesity, and type 2 diabetes mellitus. *Pancreas* 2019; **48**: 420-426 [PMID: 30747825 DOI: 10.1097/MPA.0000000000001252]

33 **Acharya C**, Cline RA, Jaligama D, Noel P, Delany JP, Bae K, Furlan A, Baty CJ, Karlsson JM, Rosario BL, Patel K, Mishra V, Dugampudi C, Yadav D, Navina S, Singh VP. Fibrosis reduces severity of acute-on-chronic pancreatitis in humans. *Gastroenterology* 2013; **145**: 466-475 [PMID: 23684709 DOI: 10.1053/j.gastro.2013.05.012]

34 **Fujii M**, Ohno Y, Yamada M, Kamada Y, Miyoshi E. Impact of fatty pancreas and lifestyle on the development of subclinical chronic pancreatitis in healthy people undergoing a medical checkup. *Environ Health Prev Med* 2019; **24**: 10 [PMID: 30732577 DOI: 10.1186/s12199-019-0763-2]

35 **Wong VW**, Wong GL, Yeung DK, Abrigo JM, Kong AP, Chan RS, Chim AM, Shen J, Ho CS, Woo J, Chu WC, Chan HL. Fatty pancreas, insulin resistance, and β-cell function: a population study using fat-water magnetic resonance imaging. *Am J Gastroenterol* 2014; **109**: 589-597 [PMID: 24492753 DOI: 10.1038/ajg.2014.1]

36 **Siddiqui N**, Vendrami CL, Chatterjee A, Miller FH. Advanced MR imaging techniques for pancreas imaging. *Magn Reson Imaging Clin N Am* 2018; **26**: 323-344 [PMID: 30376973 DOI: 10.1016/j.mric.2018.03.002]

37 **Chhoda A**, Lu L, Clerkin BM, Risch H, Farrell JJ. Current approaches to pancreatic cancer screening. *Am J Pathol* 2019; **189**: 22-35 [PMID: 30558719 DOI: 10.1016/j.ajpath.2018.09.013]

38 **Das KK**, Early D. Pancreatic cancer screening. *Curr Treat Options Gastroenterol* 2017; **15**: 562-575 [PMID: 28879469 DOI: 10.1007/s11938-017-0149-8]

39 **Henrikson NB**, Aiello Bowles EJ, Blasi PR, Morrison CC, Nguyen M, Pillarisetty VG, Lin JS. Screening for pancreatic cancer: Updated evidence report and systematic review for the us preventive services task force. *JAMA* 2019; **322**: 445-454 [PMID: 31386140 DOI: 10.1001/jama.2019.6190]

40 **Koopmann BD**, Harinck F, Kroep S, Konings IC, Naber SK, Lansdorp-Vogelaar I, Fockens P, van Hooft JE, Cahen DL, van Ballegooijen M, Bruno MJ, de Kok IM. Identifying key factors for the effectiveness of pancreatic cancer screening: A model-based analysis. *Int J Cancer* 2021 [PMID: 33644856 DOI: 10.1002/ijc.33540]

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MINIREVIEWS

BRCA mutated pancreatic cancer: A change is coming

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Abstract

Pancreatic cancer remains a leading cause of cancer-related death with few available therapies for advanced disease. Recently, patients with germline *BRCA* mutations have received increased attention due to advances in the management of *BRCA* mutated ovarian and breast tumors. Germline *BRCA* mutations significantly increase risk of developing pancreatic cancer and can be found in up to 8% of patients with sporadic pancreatic cancer. In patients with germline *BRCA* mutations, platinum-based chemotherapies and poly (ADP-ribose) polymerase inhibitors are effective treatment options which may offer survival benefits. This review will focus on the molecular biology, epidemiology, and management of *BRCA*-mutated pancreatic cancer. Further-more, we will discuss future directions for this area of research and promising active areas of research.

Key Words: Pancreatic cancer; Systemic therapy; Platinum chemotherapy; *BRCA*; Deoxyribonucleic acid repair; Poly (ADP-ribose) polymerase inhibitors

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Core Tip: Recent advances in the field of *BRCA*-mutated pancreatic cancer suggest that these patients benefit from platinum-based chemotherapy regimens. In light of new findings from the Pancreas Cancer Olaparib Ongoing trial, patients with germline *BRCA* mutations may benefit from maintenance treatment with olaparib, a Poly (ADP-ribose) polymerase inhibitors following response to platinum-based chemotherapy. Based on these important findings, all pancreatic cancer patients should be offered early access to genetic screening in order to identify patients who will benefit from these therapies.

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INTRODUCTION

Pancreatic cancer (PC) remains one of the most aggressive malignancies, with a 5-year survival rate of 8%[1,2]. Incidence of PC has increased over the past 4 decades, making it a leading cause of cancer-related mortality in North America[1-3]. The vast majority of pancreatic cancers are ductal adenocarcinomas (PDAC) of the exocrine pancreatic glands, occurring most commonly in the head of the pancreas[4]. Most cases of PDAC are considered sporadic, however 5%-10% are estimated to be familial with patients having a family history of PDAC[5]. Several genetic syndromes are known to cause familial PDAC including mutations of deoxyribonucleic acid (DNA) mismatch repair genes (Lynch syndrome), *BRCA1* and *BRCA2* (hereditary breast cancer syndrome); however, in the vast majority of cases a genetic cause cannot be identified[5-7].

Currently, the only potentially curative treatment for PC is surgical resection which is only possible in the early stages of the disease (locoregional) and highly dependent on the degree of invasion of surrounding critical structures such as vessels and bile ducts. Unfortunately, only 15%-20% of PDAC cases are considered resectable, and of these, over 75% will have recurrence within 5 years of their resection[4]. Recent data suggests that in patients with good performance status, treatment with a combination regimen of fluorouracil, oxaliplatin, leucovorin and irinotecan (FOLFIRINOX) is the optimal adjuvant therapy following resection[8]. Because early stage PC is usually asymptomatic, the vast majority of patients present with either locally advanced (involvement of local vasculature) or metastatic disease[4]. In these patients chemotherapy and occasionally radiotherapy form the backbone of treatment and are used to relieve symptoms and modestly prolong life.

In the advanced setting of disease, the two standard of care palliative chemotherapy options include gemcitabine plus albumin-bound paclitaxel (nab-paclitaxel) and FOLFIRINOX. In the first-line setting, both have been shown to prolong overall survival (OS) relative to gemcitabine monotherapy in prospective, randomized clinical trials[9,10]. Even with these treatments, 2-year survival remains at 10% and median OS ranges from 8-11 mo[4].

Recent genomic evidence suggests that PDAC is a genetically heterogenous disease with different molecular subtypes, potentially explaining the failure of many novel therapies when trialled in unselected populations[11,12]. Currently, efforts are ongoing to identify select PDAC patient populations who would benefit from targeted therapies. A patient group which has garnered much interest are those with mutations of *BRCA1* and *BRCA2*. These genes are important players in the homologous DNA repair (HR) pathway and mutations of both genes are strong risk factors for the development of several cancers including, breast, ovarian, prostate and pancreatic cancer[13,14]. Importantly, *BRCA* mutations also have implications for treatment as they may increase tumor susceptibility to both DNA-damaging chemotherapies such as platinum chemotherapy (PtCh), as well as poly (ADP-ribose) polymerase (PARP) inhibitors in breast and ovarian cancers. More recently, work has been done to determine if these clinical features translate to BRCA-mutated pancreatic cancer. This review will discuss the biology, epidemiology and clinical implications of *BRCA* mutations in PDAC, and will discuss future directions for this area of research.

MOLECULAR BIOLOGY OF HOMOLOGOUS REPAIR

Several reviews have previously described the biology of the HR system and the specific roles of *BRCA1/2*[15,16]. Briefly, DNA damage can occur as either a single-stranded DNA break (SSB) or double-stranded DNA break (DSB). HR along with non-homologous end joining (NHEJ) are the two major pathways that respond to DSB. HR has the highest fidelity and precision of the DSB repair pathways, therefore defects in this pathway (homologous repair deficiency, HRD) lead to error-prone repair and genomic instability, increasing cancer risk. Important proteins in the HR system include BRCA1, BRCA2, PALB2, ATM and RAD51[15]. Following DSB, BRCA1

negatively regulates factors involved in the NHEJ pathway (53BP1) and promotes end resection, an important first step in the HR pathway. BRCA1 directly interacts with PALB2 to bind BRCA2 which facilitates formation of RAD51 filaments later in the pathway[15]. RAD51 filament form along ssDNA created earlier by BRCA1-mediated end resection, allowing formation of homologous DNA and repair of the DSB (Figure 1)[15]. Notably, other proteins involved in the HR pathways such as PALB2 and ATM are also mutated in PC, highlighting the importance of HR pathway integrity in determining PDAC risk[11,17].

While *BRCA* mutations confer increased cancer risk, emerging evidence suggests they also may be important markers for personalized medicine. *In vitro* and *in vivo* evidence suggests that both platinum-based chemotherapies and PARP inhibitors are more effective in patients harboring *BRCA* mutations[11].

EPIDEMIOLOGY AND DIAGNOSIS OF *BRCA*-MUTATED PDAC

Incidence of pathogenic BRCA mutations in sporadic and familial PDAC

Mutations of the *BRCA1* and *BRCA2* genes were first identified as breast and ovarian cancer risk factors in the mid-1990s during studies aimed at characterizing the genes responsible for familial clustering of breast and ovarian cancers[18,19]. Early studies by the Breast Cancer Linkage Consortium identified a 2.3-fold and 3.5-fold increased risk of PC in carriers of *BRCA1* and *BRCA2* gene mutations, respectively[13,14]. In the general population, germline *BRCA* mutations occur at a rate between 1/300 and 1/800[20]. However, incidence varies based on population as certain ethnic groups harbor founder mutations, increasing the incidence of *BRCA* mutations in these subgroups. The strongest example of the founder effect in *BRCA* is the Ashkenazi Jewish (AJ) population, where the presence of 3 founder mutations have increased rates of *BRCA* mutation to 1/40[21]. Other groups with founder *BRCA* mutations who are therefore at increased risk include Dutch, Norwegian and French-Canadian populations[22].

Among unselected PC patient cohorts, multiple studies have aimed to estimate the incidence of germline pathogenic *BRCA* mutations. Prevalence estimates ranged from 0.7%-5.7% for *BRCA2* and 0.3%-2.3% for BRCA1 (Summarized in Table 1)[6,23-26]. Notably, the cohorts in these studies varied widely based on several factors which could influence estimates of prevalence, including, number of AJ PC patients included, the number of patients with family histories of cancer, and median patient age[23]. For example, in AJ PDAC patients, studies have found that up to 19% of patients harbour germline *BRCA* mutations[23,27,28].

In familial PC, *BRCA* mutations, especially *BRCA2* are also at increased frequency. In the case of *BRCA2* mutations, studies have found germline mutations in 3.7%-19% of patients with strong familial histories of PDAC[29-32]. This range in estimates is likely a result of different criteria for familial pancreatic cancer (FPC), and different studies methodologies. Studies finding higher rates of *BRCA2* mutation tended to have smaller sample sizes and included patients with three or more first- or second-degree relatives with PC, therefore included higher risk patients. Conversely, more recent studies have included larger sample sizes of patients, who met the more moderate FPC case definition (two first- or second- degree relatives with PC), finding more conservative estimates of prevalence (3.7% and 6%)[31,32]. Therefore, in patients with a stronger family history of PC, *BRCA* carrier status is more likely. The incidence of *BRCA1* mutations in FPC has not been studied as well as *BRCA2*, however a recent study by Zhen *et al*[31] found that germline *BRCA1* mutations were present in 1.2% of patients with FPC.

Diagnosis of BRCA-mutated PDAC and screening guidelines

While the identification of patients carrying *BRCA* mutations has been important in determining cancer risk, the discovery of personalized medicine options for this population has increased the clinical importance of identifying *BRCA* carriers. Genetic testing guidelines vary by region however, are primarily based on cancer phenotype which includes family history of breast, ovarian, prostate and pancreatic cancer, AJ ancestry and clinical presentation. Recently, genetic testing guidelines are being increasingly questioned as evidence accumulates to suggest that they would miss a large proportion of patients harboring *BRCA* mutations who may benefit from PARP inhibitors or platinum chemotherapies. In 2007, a Norwegian study tested breast and ovarian cancer patients for germline mutations in *BRCA1* and *BRCA2* and identified that 50% of patients with germline *BRCA* mutations do not have family histories of

Table 1 Summary of studies of incidence of germline *BRCA* mutations in unselected pancreatic cancer cohorts

Ref.	Year	Population	Cohort size (Number AJ)	Germline *BRCA1* pathogenic mutation incidence (%)	Germline *BRCA2* pathogenic mutation incidence (%)	Combined germline *BRCA* mutation Incidence
Holter *et al*[23]	2015	North American	306 (33)	1.0%	3.6%	4.6%
Brand *et al*[24]	2018	North American	298 (26)	1.3%	1.3%	2.6%
Mizukami *et al*[25]	2020	Japanese	1005 (-)	1.7%	2.5%	4.2%
Grant *et al*[6]	2015	North American	290 (13)	0.3%	0.7%	1%
Lowery *et al*[26]	2018	North American	615 (111)	2.3%	5.7%	8%

AJ: Ashkenazi Jewish; *BRCA:* Breast cancer susceptibility gene.

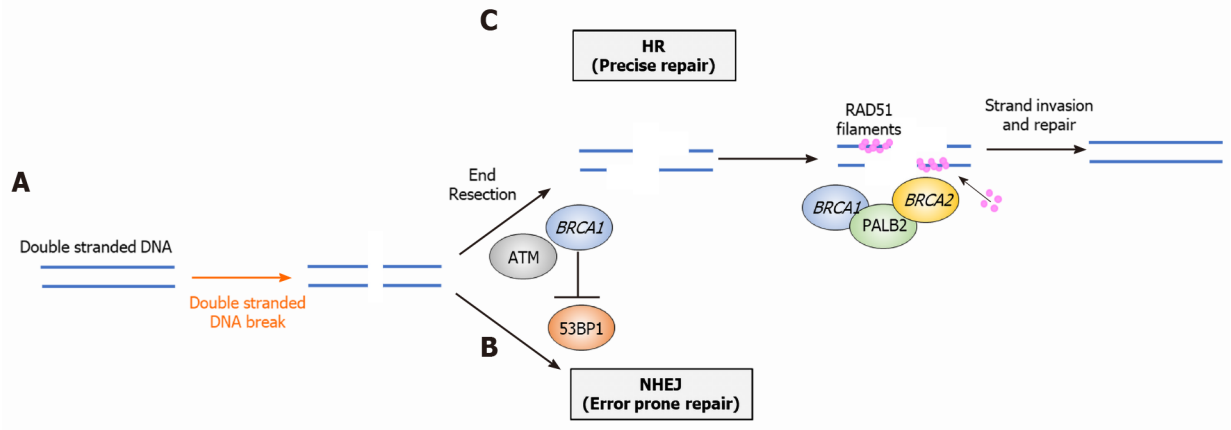


Figure 1 Overview of the homologous repair pathway and roles of key proteins. A: Following double strand break, BRCA 1 binds to the site of damage, mediating end resection and initiating homologous repair. This prevents repair *via* non-homologous end joining; B: *BRCA1* binds with PALB2 and *BRCA2* which facilitates assembly of RAD51 filaments; and C: RAD51 filaments form along ssDNA, subsequently leading to strand invasion and repair. DSB: Double strand break; HR: Homologous repair; NHEJ: Non-homologous end joining; BRCA: Breast cancer susceptibility gene.

BRCA-associated cancers[33]. Since then, multiple studies in different populations including patients with PDAC have confirmed these findings, showing poor associations between presence of *BRCA* mutations and expected family histories [23,34-38]. Furthermore, a recent study using data from 23&Me, a direct-to-consumer genetic test identified that 20% of carriers of the AJ founder variants don't identify as AJ, and therefore would be excluded from screening criteria that include AJ ancestry[39]. They also found that of 393 *BRCA* mutation carriers with available data on family cancer history, 44% had no family history of *BRCA*-associated cancers, and therefore, given a diagnosis of PDAC, would not meet screening requirements. The recent IMPACT trial by the Memorial Sloan-Kettering Cancer Centre provided strong evidence in favour of increased testing access. Investigators tested 1040 patients (176 PDAC) with advanced cancer and identified germline mutations in 21.5% of the PDAC patients. Notably, they found that across all cancers, 55% of clinically actionable mutations would not have been detected under current phenotype-based screening guidelines[40]. Together, this evidence strongly supports calls for increased access to genetic testing for PC patients. In early 2020, the National Comprehensive Cancer Network updated their recommenda-tions to suggest universal genetic testing for all PC patients as early as possible due to the rapid progression of the disease, and potential for early personalized therapy[41].

CLINICAL FEATURES OF *BRCA*-MUTATED PDAC AND PROGNOSTIC IMPLICATIONS

While the ability of *BRCA* mutations to increase risk of PDAC is well established, their impact on the clinical features of the disease is less clear. Multiple cohort studies have shown in PDAC patients with germline mutations including *BRCA1, BRCA2, PALB2, CDKN2A* and *ATM*, are diagnosed earlier with PDAC than PDAC patients without germline mutations[31,42]. Conversely, a 2009 study comparing Jewish PDAC patients with and without germline *BRCA* mutations found no significant differences between age at diagnosis or any other clinicopathologic feature studied[28]. From a prognostic perspective, studies have shown mixed results. The largest cohort study to date including 71 *BRCA*-positive PDAC patients found a median OS of 14 mo for the whole cohort and 12 mo for patients with stage 3/4 disease. At time of publication, the median OS for early stage disease had not been reached as 52% of patients were still alive at 60 mo[43]. These findings suggest that *BRCA*-mutated PDAC patients may have a considerably better prognosis than the general PDAC population. On the contrary, more recent case-control studies by Blair *et al*[44] compared PDAC patients with *BRCA1* and *BRCA2* mutations to age-matched controls and showed that both OS and disease-free survival (DFS) were lower in carriers than controls. Another case-control study comparing *BRCA* mutation-positive, early-stage PDAC patients undergoing surgical resection to age-matched *BRCA*-wildtype controls found no significant differences in median OS or DFS between the groups and concluded that *BRCA* mutations were not prognostic in early PDAC[45]. Authors have suggested that early findings of improved prognosis in this population may have been a result of ascertainment bias as patients surviving longer were more likely to receive genetic testing and participate in the study. Another factor that may lead to improved prognosis in this patient population is increased susceptibility to treatments such as PtCh. Most recently, a study using data from the Know Your Tumor program aimed to assess whether mutations of HRD and other DNA-damage response (DDR) genes conferred a survival benefit or whether observed benefits were a result of increased PtCh-sensitivity[46]. The authors found that patients with advanced PDAC and HR/DDR mutations had improved survival but only if treated with PtCh. In PtCh-naïve patients, there was no survival benefit in this patient population[46].

Overall, identifying clinical differences between *BRCA*-mutated PDAC and wildtype PDAC has been difficult due to the relative rarity of these patients. Furthermore, the increasing use of personalized therapies (PARP inhibitors and platinum chemotherapy) in this population will make determining the prognostic implications of *BRCA* mutations more challenging.

MANAGEMENT OF BRCA-MUTATED PDAC: SYSTEMIC THERAPY

Platinum chemotherapy

While both FOLFIRINOX and gemcitabine/nab-paclitaxel chemotherapy regimens are more effective than gemcitabine monotherapy, there is yet to be a comparative randomized clinical trial to provide data on which regimen is more effective. In the locally advanced setting, a recent case series of 485 consecutive patients suggested that FOLFIRINOX was associated with a higher response rate (19% *vs* 6%, $P = 0.001$), however OS was not different with either treatment[47]. Retrospective studies in metastatic PDAC are inconclusive, with some studies reporting survival improvement on FOLFIRINOX while others report no difference between the two regimens[47,48]. Given the increased toxicity associated with FOLFIRINOX and potential survival benefits, identifying subsets of patients who are more likely to benefit from this regimen will be an important advancement in PC management.

The HRD phenotype of *BRCA*-mutated cancers appears to render them more sensitive to chemotherapies that induce DNA damage, such as PtCh. Early studies found that cells lacking *BRCA1* are more sensitive to treatment with cisplatin[49]. In the presence of HRD, these cells are unable to appropriately repair the DNA damage, leading to genomic instability and cell death[50]. Clinical studies in breast cancer have found that platinum-chemotherapy improves objective response rates (ORRs) for metastatic breast cancer patients only in *BRCA*-mutated cancers. Based on genomic studies in PDAC, it appears that tumors with *BRCA*-mutations have "unstable" molecular phenotypes and are more likely to be sensitive to genotoxic therapies such as PtCh[11].

In PC, several large retrospective studies have investigated the efficacy of PtCh such as FOLFIRINOX in patients with *BRCA* mutations or other genetic mutations leading to HRD (Table 2). To date, the largest cohort study was conducted by Golan *et al*[43] This multi-institution cohort study included 71 PC patients with germline *BRCA* mutations and found that among patients with advanced PDAC, OS was significantly longer in patients treated with PtCh (22 *vs* 9 mo). Since this study, several other retrospective cohort studies have reported improved outcomes [ORR, progression free survival (PFS)] in patients with germline mutations to HR-related genes who were treated with PtCh in both resectable and non-resectable PDAC[35,44,51,52]. For example, Blair *et al*[44] showed that median survival was significantly improved in resected PDAC patients with germline *BRCA* mutations who were treated with adjuvant PtCh compared to non-PtCh (31.0 *vs* 17.8 mo). Reiss *et al*[52] showed significant improvement in mOS in patients with unresectable PDAC and mutations in *BRCA1*, *BRCA2* or *PALB2* who were treated with PtCh compared to patients treated with non-PtCh (median follow-up of 20.1 mo *vs* mOS of 15.5 mo). Several studies have also compared the effectiveness of PtCh between patients with and without HRD mutations. In a cohort study of platinum-treated PDAC patients, patients found to have tumor-level mutations to 12 HR-related genes (including *BRCA1*, *BRCA2*, *ATM* and *PALB2*) had significantly improved median PFS compared to platinum-treated patient without HR-related gene mutations[35]. Similarly, two recent case-control studies reported improved PFS and ORR in platinum-treated patients who carried mutations to *BRCA1*, *BRCA2* and *PALB2*[53,54]. Wattenberg *et al*[53] showed an ORR of 58% in mutation carriers treated with PtCh compared to 21% non-mutated PDAC patients. In resected PDAC treated with perioperative PtCh, Yu *et al*[54] reported that mutation carriers had significantly greater survival (mOS not met *vs* 23.1 mo, HR = 0.12).

While these studies are promising, the retrospective nature introduces several limitations. Firstly, outcomes are widely subdivided as PtCh *vs* non-PtCh, however the PtCh groups generally include a variety of regimens such as gemcitabine + cisplatin, gemcitabine + oxaliplatin, FOLFOX and FOLFIRINOX. Seeing as oxaliplatin and cisplatin exert DNA damage through different mechanisms of action, it is unclear how well these findings will translate to modern clinics where patients are typically treated with FOLFIRINOX as a first-line therapy[52]. One study reported that there was no significant difference in survival for mutation-positive patients on different PtCh regimens, however in the mutation-negative group, patients only responded to FOLFIRINOX[53]. This suggests that there is potentially a role for PtCh regimens in *BRCA*-mutated patients that did not show benefit when tested in unselected PDAC populations, in situations when FOLFIRINOX cannot be tolerated. Another limitation is the current practices with respect to treatment selection. Because of the toxicity associated with PtCh such as FOLFIRINOX, these regimens are generally used in younger patients with better performance status. Therefore, in retrospective analyses of *BRCA*-mutated PDAC cohorts, it is unclear whether survival benefits seen are because of increased activity of PtCh in this patient population or because the patients treated with PtCh are younger and have better performance status. Few studies have reported data on patient age in these analyses and none have reported patient performance status. In light of this, these retrospective analyses are difficult to interpret. Lastly, retrospective studies may be affected by survival bias. Most studies compared confirmed mutation carriers to untested cohorts. It is possible that patients who survive longer are more likely to undergo genetic testing and be classified as carriers. In light of these limitations, a recent meta-analysis concluded that the current available evidence suggests PtCh is more effective in *BRCA*-mutated patients, however the quality of evidence is low[55].

To date, there have been few prospective studies assessing the effectiveness of platinum-chemotherapies in this population. A recent phase II randomized controlled trial investigated cisplatin and gemcitabine with or without Veliparib, a PARP inhibitor in patients with untreated advanced PDAC and a germline mutation of *BRCA* or *PALB2*[56]. While the primary endpoint (response rate) was not significantly different with Veliparib, the authors reported unprecedented survival rates, with a 2-year survival rate of 30.6% and a 3-year survival rate of 17.8%[56]. Response rates were also high for both arms of the study (74% with Veliparib, 65.2% without veliparib)[56]. While this data provides compelling evidence for the use of PtCh in this patient population, the study lacks a control group treated with non-PtCh for comparison. This study adds to the literature as all patients were on the same PtCh regimen (gemcitabine + cisplatin) which showed impressive responses and survival rates. Notably, the patients included in this study all had a good performance status (ECOG 0-1) and therefore these results may not translate as well to real-world PDAC patients

Table 2 Retrospective studies of platinum-chemotherapies in *BRCA*-mutated pancreatic ductal adenocarcinoma

Ref.	Year	Study design	Patient population	Findings
Golan *et al*[43]	2014	Multi-institution cohort study	71 patients with germline *BRCA* mutations (21 *BRCA1*, 49 *BRCA2*, 1 both)	Superior mOS in stage 3/4 patients treated with platinum compared to non-platinum chemotherapy (22 *vs* 9 mo, P = 0.039)
Vyas *et al*[51]	2015	Cohort study	10 patients with *BRCA2* mutation and known PDAC	Duration of response on platinum agents ranged from 8-32 wk, mean of 19.3 wk
Blair *et al*[44]	2018	Combined case control cohort study	22 patients with resected sporadic PDAC and germline *BRCA* mutations (1 *BRCA1*, 18 *BRCA2*)	Improved OS in *BRCA*-mutated patients treated with adjuvant PtCh compared to patients treated with alternative chemotherapies or no adjuvant therapy (31.0 *vs* 17.8 *vs* 9.3 mo, P < 0.001)
Reiss *et al*[52]	2018	Cohort study	29 patients with unresectable PDAC and germline mutations of *BRCA1*, *BRCA2* or *PALB2*(12 *BRCA1*, 15 *BRCA2*, 2 *PALB2*)	Superior mOS in platinum-treated patients (undefined mOS (median follow up 21 mo) *vs* 15.5 mo, P = 0.02)
Kondo *et al*[35]	2018	Cohort study	28 patients with advanced PDAC (13 had HR-related gene mutations, 15 without mutations to HR-related genes)	Superior median PFS in HR-mutated PDAC patients treated with platinum chemotherapy compared to PDAC patients without HR mutations treated with platinum therapy (20.8 mo *vs* 1.7 mo, P = 0.049)
Yu *et al*[54]	2019	Case control study	32 resected PC patients with germline *BRCA1*, *BRCA2*, or *PALB2* mutation, 64 resected PC patient controls without germline mutations	With peri-operative platinum exposure, mOS was longer in mutation-positive group that mutation negative group (mOS not yet met *vs* 23.1 mo, HR= 0.12)
Wattenberg *et al*[53]	2020	Case control study	26 platinum-treated patients with advanced stage PDAC and mutations of *BRCA1, BRCA2* or *PALB2*, 52 platinum-treated, wildtype, age-matched controls	Improved ORR in patients with mutations compared to controls (58% *vs* 21%, P = 0.0022). Improved real world PFS in mutation carriers (10.1 mo *vs* 6.9 mo, HR = 0.43, P = 0.0068)

HR: Homologous Repair; mOS: Median overall survival; ORR: Objective response rate; PDAC: Pancreatic adenocarcinoma; PtCh: Platinum chemotherapy; *BRCA:* Breast cancer susceptibility gene.

where performance status may be lower.

Overall, there is evidence in favour of the use of PtCh as a first-line treatment for *BRCA*-mutated PDAC, however, most data is retrospective and the quality of the evidence in favour of this treatment is low. There is yet to be a randomized controlled trial confirming the observations that PtCh is preferable to other chemotherapy regimens in this population, however enrollment to such a study may be difficult due to current management practice. Furthermore, it is unclear whether or not FOLFIRINOX or gemcitabine plus cisplatin should be used for this patient population.

PARP inhibitors

The sensitivity of BRCA-deficient cancers to PARP inhibition was first reported in 2005, in which researchers identified that loss of function of both *BRCA* and PARP is synthetically lethal[57,58]. PARP is an important family of enzymes involved in responding to SSB the other prominent form of DNA damage other than DSB. This combined loss of SSB repair in HRD cells is thought to lead to synthetic lethality (Figure 2). While the exact mechanism of action is still unclear, the earliest theory was that PARP inhibition prevents the repair of single-stranded DNA breaks (SSBs), leading to accumulation of replication-associated DSBs[59]. In HRD cells which have defective DSB repair, DSBs are repaired *via* error-prone NHEJ, leading to genomic instability and cell death. More recent evidence suggests that the biology of BRCA and PARP deficient synthetic lethality is more complex, however the detailed mechanisms are outside the scope of this review[60].

Therapeutic inhibitors of this pathway were evaluated in a phase I study of olaparib and confirmed activity in several different tumor types harboring *BRCA* mutations[61]. In ovarian cancer, PARP inhibitors are FDA-approved for use as a maintenance therapy in patients with recurrent ovarian cancer who demonstrated a complete or partial response to PtCh, regardless of HRD biomarker status[62]. This approval came following three phase III trials which demonstrated significant improvements in PFS in patients treated with oral PARP inhibitors as maintenance therapy following chemotherapy[63-65]. More recently, emerging data from several randomized clinical trials reporting efficacy of PARP inhibitors as a front-line treatment for newly diagnosed ovarian cancer[62]. In advanced breast cancer, PARP inhibitors have demonstrated improvements in PFS relative to chemotherapy in patients with HER2-negative, *BRCA*-mutation positive tumors[66,67]. However, there

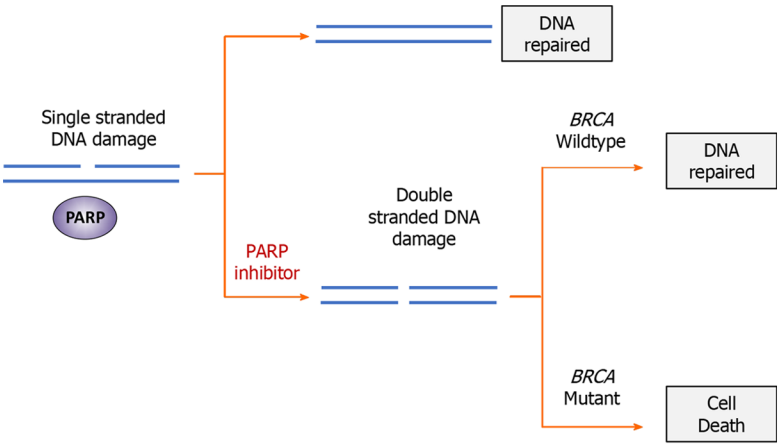


Figure 2 Mechanism of synthetic lethality in *BRCA*-mutated cells treated with poly (ADP-ribose) polymerase inhibitors. While neither a breast cancer susceptibility (*BRCA*) mutation or treatment with Poly (ADP-ribose) polymerase (PARP) inhibitors alone is lethal to cancer cells, dual-inhibition of both systems through mutation and pharmacological inhibition is incompatible with survival. Following PARP inhibition, single-stranded deoxyribonucleic acid (DNA) breaks are unable to be repaired. During replication, replication forks stall at unrepaired DNA damage, resulting in formation of double-stranded DNA break. In cells with defective homologous repair (*BRCA* mutations), double-stranded damage is repaired through non-homologous end joining, resulting in genomic instability and cell death. Poly (ADP-Ribose) Polymerase. PARP: Poly (ADP-ribose) polymerase; *BRCA*: Breast cancer susceptibility gene.

is yet to be a clinical trial demonstrating improvements in OS with PARP inhibitor use in advanced breast cancer[68]. Recently, PARP inhibitors have also demonstrated effectiveness in metastatic prostate cancer[69].

With the success of PARP inhibitors in other *BRCA*-associated cancers, focus has shifted to translating these findings to *BRCA*-associated PDAC. To date multiple phase II studies have evaluated the efficacy of PARP inhibitors in PDAC patients with germline *BRCA* mutations[56,70,71]. In a phase II study by Kaufman *et al*[71], 298 patients with advanced cancer (23 with pancreas cancer) and germline *BRCA1/2* mutations were treated with oral olaparib. The response rate among PC patients was 21.7% in patients who had received two prior lines of chemotherapy[71]. Conversely, another phase II study evaluated the efficacy of Veliparib in 16 advanced PDAC patients with known germline mutations of *BRCA1/2* or *PALB2* who had undergone 1-2 previous lines of treatment, finding no objective responses[70]. Authors suggested potential differences between olaparib and veliparib as a potential explanation for the difference in response rates between the two trials. Furthermore, the high rates of pre-treatment with PtCh (88% of study population) coupled with a high disease progression rate (64% of those on PtCh) may indicate a high-level of platinum-resistance in this study population, which may in turn lead to PARP inhibitor resistance[70]. This is a plausible explanation given the known association between platinum-sensitivity and PARP inhibitor sensitivity seen in ovarian cancer. Due to the tendency of cancers to develop resistance to PARP inhibitors, another approach that has been tried is combination regimens involving chemotherapy and PARP inhibitors. A recent phase II trial compared a combination regimen of gemcitabine plus cisplatin with or without veliparib as first line therapy for advanced PDAC patients with germline mutations of *BRCA1/2* or *PALB2*[56]. Veliparib did not improve response rates over gemcitabine plus cisplatin alone (74.1% *vs* 65.2%, P = 0.55), however as discussed earlier, the response rates in both arms both exceeded pre-study thresholds of efficacy and therefore, the high response rate to gemcitabine plus cisplatin may have obscured any signal of benefit from veliparib.

With the relative success of combination chemotherapy regimens in PDAC (FOLFIRINOX, Gemcitabine-Abraxane), focus has been placed on the development of maintenance therapies which can prolong PFS and improve quality of life (QOL) in responders. Most recently, data from the Pancreas Cancer Olaparib Ongoing (POLO) trial has supported the use of PARP inhibitors as a maintenance therapy in this patient population following response to platinum-chemotherapy[72]. The POLO trial was an international phase III, double-blind, placebo-controlled randomized clinical trial investigating oral olaparib maintenance therapy in metastatic PDAC patients with germline *BRCA1/2* mutations who had not progressed during first-line PtCh (minimum of 16 wk of chemotherapy). Patients were randomized to either olaparib or placebo maintenance therapy. PFS was significantly longer in the olaparib group (7.4 *vs* 3.8 mo). At the time of publication, data on OS was not yet mature but preliminary

results indicated no significant difference in OS between the two groups (18.9 *vs* 18.1 mo)[72]. 18 patients (20%) in the olaparib and 6 patients (11%) in the placebo group achieved a tumor response, and the median duration of responses were 24.9 mo and 3.7 mo, respectively. Other evidence for maintenance therapy comes from the phase II study by O'Reilly *et al*[56] who reported exploratory analyses for 10 patients with germline *BRCA* or *PALB2* mutations who underwent at least 4 mo of PtCh without progression and subsequently were switched to a PARP inhibitor as maintenance therapy, finding a median PFS of 23.4 mo in this subset of patients.

In the context of maintenance therapy, preservation of quality of life and minimization of adverse effects are important goals of treatment. In the POLO trial, Grade ≥ 3 adverse events occurred in 40% of the olaparib group and 23% of the placebo group[43]. The most frequently reported adverse events in the treatment group were fatigue or anesthesia, nausea and anemia, with the majority of these cases being low grade. Only 15% and 5% of patients on olaparib underwent dose reductions or discontinued treatment because of adverse events, respectively. More recently, secondary outcomes of health-related QOL were reported, showing that olaparib treatment did not lead to a reduction in quality of life scores, a concern in the context of maintenance therapy meant to preserve functioning and QOL[73].

In light of these findings, the FDA has approved olaparib for maintenance therapy in patients with metastatic PDAC patients with germline mutations of *BRCA1/2* who have not progressed on at least 16 wk of first-line PtCh. This approval is not without controversy as there are several criticisms of the POLO trial and unanswered questions in regards to this therapy[74]. For example, the lack of improvement in OS puts the validity of the finding of improved PFS into question[74]. However, this may be because of the high rates of therapy in the placebo group following disease progression, including 15% of the patients who received a PARP inhibitor. In addition, it should be stated that the OS results were from an interim analysis with only 46% data maturity. Furthermore, concern has been raised that the discontinuation of PtCh after 16 wk in patients who were responding is incongruent with clinical practice guidelines for first-line platinum chemotherapy[74]. However, in the POLO trial, the majority of patients received FOLFIRINOX (> 80%) with a median duration of first line PtCh of 5 mo and 33% of patients receiving > 6 mo prior to randomization[72]. In addition, the PRODIGE 4/ACCORD 11 trial recommended a total of 6 mo of palliative chemotherapy[10], therefore, the duration of therapy of 1st line PtCh may not be out of keeping with other clinical trials in this setting of disease. Furthermore, use of placebo alone in the control group has come under criticism as evidence has emerged in favour of the continuation of 5-FU as maintenance therapy in patients who respond to FOLFIRINOX[75]. That being said, the accumulating side effects of > 4 mo of FOLFIRINOX may justify a treatment break, especially if there is no evidence of progression on imaging. Lastly, POLO only included patients with germline mutations of *BRCA1/BRCA2*, therefore it remains unclear if there is a broader population of PDAC patients who would benefit from olaparib as well, such as patients with germline mutations to other components of the HR system (*PALB2*, *ATM*) or patients with other positive biomarkers of HRD.

Immunotherapies

While immunotherapies such as checkpoint inhibitors (anti-PD1/PDL1 and CTLA-4) have revolutionized the management of many cancers, they have had limited efficacy in PDAC. The genomic instability and increased total mutational load of *BRCA*-mutated and other HRD tumors results in neoantigens which may increase efficacy of immunotherapy in these tumors[11]. Recent translational studies have showed that specifically *BRCA2*-mutated tumors show increased sensitivity to immune checkpoint blockade as a result of their effect on the tumor immune microenvironment[76]. This is in line with previous findings of associations between *BRCA* mutations and PD-L1 expression in PDAC, a predictive marker for immunotherapy[77,78].

An emerging strategy for *BRCA*-mutated cancers is combination therapy with immune check point inhibitors and PARP inhibitors[79]. Given that treatment with PARP inhibitors also increases expression of PD-L1 and total mutational burden (potential biomarkers of response), combining these two therapies may act synergistically against HRD tumors[79]. In *BRCA*-mutated ovarian and breast cancers, several clinical trials are currently exploring the clinical efficacy of PARP inhibitor/immune checkpoint blockade combination therapy with early trials showing promising results[80]. In the maintenance setting, the ATHENA trial is currently testing a combination therapy consisting of rucaparib with nivolumab as a therapy for ovarian cancer following response to PtCh (NCT03522246). In PDAC, there are several ongoing Phase II trials investigating combination regimens involving PARP inhibitors

and immune checkpoint inhibitors (Table 3). The PARPVAX study is investigating combination therapy of niraparib + either ipilimumab or nivolumab as maintenance therapy following response to PtCh (NCT03404960). Another phase II study is investigating combination therapy regimens including olaparib plus durvalumab in PDAC with a primary outcomes of changes in genomic and immune markers (NCT03851614). Most recently, a study has been initiated comparing olaparib with and without pembrolizumab as maintenance therapy for *BRCA1/BRCA2* mutated-PDAC patients who responded to first-line PtCh (NCT04548752). Given the recent evidence for PARP inhibitors in PDAC, the use of immune checkpoint blockade for PDAC remains an active field of research.

BIOMARKERS OF HRD

In the context of both PtCh and PARP inhibitors, the development of biomarkers for HRD will be an important step in implementing these therapies broadly in clinical practice. While most research to date has focused on germline mutations of *BRCA1/2* and *PALB2*, combined these represent less than 10% of all PDAC cases. While this is an important mechanism of HRD, HRD can also arise through somatic mutations or epigenetic modification of DDR genes potentially resulting in sensitivity to PtCh and PARP inhibitors. Therefore, relying solely on germline mutations of these three genes for treatment selection will likely miss patients who would otherwise benefit from targeted therapy. For example, in advanced pancreatic cancer, tumor-level mutations to HRR genes such as *BRCA1/2, ATM, PALB2, RAD51* were highly predictive of response to PtCh[35]. Recently a meta-analysis compared outcomes (ORR, survival) in PARP inhibitor trials and found that similar outcomes between patients with germline and patients with somatic *BRCA* mutations[81]. Interestingly, out of 99 studies of PARP inhibitors screened, only 18 included patients with somatic mutations, indicating that this is an understudied area of research[81]. Specifically in PDAC, only two studies investigated PARP inhibitors in patients with somatic *BRCA* mutations and both reported a non-significant increase in response rate in patients with somatic mutations, relative to germline[81]. No trials to date have evaluated the efficacy of maintenance olaparib, the only FDA-approved PARP inhibitor indication in PDAC in patients with somatic HR mutations. Two active trials of olaparib in PDAC are including patients with BRCA-associated family history or somatic HRD mutations, but explicitly excluding patients with germline *BRCA* mutations (NCT02677038, NCT02511223). However, these trials are not using olaparib in the maintenance setting. Given the efficacy of PARP inhibitors and PtCh in somatic *BRCA*-mutated ovarian cancer[63,82] this is an important area for future investigation in PDAC.

In addition to mutations of *BRCA* and other HR-related genes, genomic signatures of HRD have emerged as a promising biomarker of the HRD phenotype and subsequent treatment response[11]. These biomarkers will allow the identification sub-populations of PDAC patients who would benefit from PtCh or PARP inhibitors, and therefore expand the scope of use for these agents in PDAC. Multiple commercial assays now exist which can assess tumor tissues and assign an HRD score[62]. Examples of these assays include MyChoice CDx Assay (Myriad Genetics) and the FoundationOne CDx (Foundation Medicine) which are both FDA-approved for the evaluation of HRD. These tests combine loss-of heterozygosity scores with other markers of genomic instability (telomeric-allelic imbalance, large-scale transition) in order to quantify HRD and identify patients who would benefit from HRD-targeting therapies. These assays have been used in several clinical trials in breast and ovarian cancer and have been validated as useful biomarkers for response to PARP inhibitors[64,83,84]. Confirmation of HRD by assay is now an FDA-approved biomarker for the use of several treatment regimens including combined olaparib with bevacizumab for ovarian cancer. Furthermore, olaparib was recently approved for metastatic prostate cancer in patients with BRCA mutations or HRD. Investigating these biomarkers in PDAC will aid in identifying *BRCA*-wildtype patients who may benefit from PARP inhibitors and PtCh, an important prospect considering the poor prognosis in advanced PC.

CONCLUSION

The field of HRD in PDAC is in its infancy relative to ovarian and breast cancers, however promising advances have been made in recent years. Currently, the available

Table 3 Ongoing phase II clinical trials investigating poly (ADP-ribose) polymerase inhibitor/Immune Checkpoint blockade combination therapy in pancreatic ductal adenocarcinoma

Study identifier	Patient population	Immunotherapy	PARP inhibitor	Phase and design	Estimated completion date
NCT03404960	Advanced PDAC patients who did not progress on PtCh	Nivolumab or Ipilimumab	Niraparib	Phase Ib/II trial evaluating effectiveness of olaparib with either nivolumab or ipilimumab	June 2021
NCT03851614	Advanced PDAC, leiomyosarcoma or mismatch repair-proficient colorectal cancer	Durvalumab	Olaparib	Phase II trial evaluating impact of combination therapy on genomic and immune biomarkers	March 2022
NCT04493060	Metastatic PDAC with mutations of *BRCA1/2* or *PALB2*, previously treated with 1-2 lines of chemotherapy including a PtCh agent	Dostarlimab	Niraparib	Phase II, evaluating the disease control rate at 12 weeks (DCR12) with combination therapy	December 2022
NCT04548752	Metastatic PDAC with germline *BRCA1* or *BRCA2* mutation treated with first-line PtCh	Pembrolizumab	Olaparib	Phase II trial comparing combination therapy to olaparib alone as maintenance therapy	March 2025

PDAC: Pancreatic adenocarcinoma; PtCh: Platinum chemotherapy; *BRCA*: Breast cancer susceptibility gene.

data from retrospective studies suggests that first-line PtCh is preferred however the PtCh regimen is yet to be defined. Olaparib maintenance therapy is a standard of care option in patients with *BRCA1/2* mutations and offers the benefit of ongoing anti-cancer therapy without traditional cytotoxic therapy toxicities. Important next steps include investigating these PtCh regimens and PARP inhibitors in the neoadjuvant setting, and determining if patients with somatic HR mutations or HRD as detected by genomic assays will also benefit from these treatments.

REFERENCES

1 **Canadian Cancer Society**. Canadian Cancer Statistics 2019 [cited 1 March 2021]. Available from: https://www.cancer.ca/~/media/cancer.ca/CW/cancer%20information/cancer%20101/Canadian%20cancer%20statistics/Canadian-Cancer-Statistics-2019-EN.pdf?la=en

2 **American Cancer Society**. Cancer Facts and Figures 2020 [cited 1 March 2021]. Available from: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2020/cancer-facts-and-figures-2020.pdf

3 **Saad AM**, Turk T, Al-Husseini MJ, Abdel-Rahman O. Trends in pancreatic adenocarcinoma incidence and mortality in the United States in the last four decades; a SEER-based study. *BMC Cancer* 2018; **18**: 688 [PMID: 29940910 DOI: 10.1186/s12885-018-4610-4]

4 **Ryan DP**, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med* 2014; **371**: 1039-1049 [PMID: 25207767 DOI: 10.1056/NEJMra1404198]

5 **Petersen GM**. Familial pancreatic cancer. *Semin Oncol* 2016; **43**: 548-553 [PMID: 27899186 DOI: 10.1053/j.seminoncol.2016.09.002]

6 **Grant RC**, Selander I, Connor AA, Selvarajah S, Borgida A, Briollais L, Petersen GM, Lerner-Ellis J, Holter S, Gallinger S. Prevalence of germline mutations in cancer predisposition genes in patients with pancreatic cancer. *Gastroenterology* 2015; **148**: 556-564 [PMID: 25479140 DOI: 10.1053/j.gastro.2014.11.042]

7 **Klein AP**, Brune KA, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJ, Griffin C, Cameron JL, Yeo CJ, Kern S, Hruban RH. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. *Cancer Res* 2004; **64**: 2634-2638 [PMID: 15059921 DOI: 10.1158/0008-5472.can-03-3823]

8 **Conroy T**, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, Choné L, Francois E, Artru P, Biagi JJ, Lecomte T, Assenat E, Faroux R, Ychou M, Volet J, Sauvanet A, Breysacher G, Di Fiore F, Cripps C, Kavan P, Texereau P, Bouhier-Leporrier K, Khemissa-Akouz F, Legoux JL, Juzyna B, Gourgou S, O'Callaghan CJ, Jouffroy-Zeller C, Rat P, Malka D, Castan F, Bachet JB; Canadian Cancer Trials Group and the Unicancer-GI–PRODIGE Group. FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer. *N Engl J Med* 2018; **379**: 2395-2406 [PMID: 30575490 DOI: 10.1056/NEJMoa1809775]

9 **Von Hoff DD**, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* 2013; **369**: 1691-1703 [PMID: 24131140 DOI: 10.1056/NEJMoa1304369]

10 **Conroy T**, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup. FOLFIRINOX *vs* gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817-1825 [PMID: 21561347 DOI: 10.1056/NEJMoa1011923]

11 **Waddell N**, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, Johns AL, Miller D, Nones K, Quek K, Quinn MC, Robertson AJ, Fadlullah MZ, Bruxner TJ, Christ AN, Harliwong I, Idrisoglu S, Manning S, Nourse C, Nourbakhsh E, Wani S, Wilson PJ, Markham E, Cloonan N, Anderson MJ, Fink JL, Holmes O, Kazakoff SH, Leonard C, Newell F, Poudel B, Song S, Taylor D, Waddell N, Wood S, Xu Q, Wu J, Pinese M, Cowley MJ, Lee HC, Jones MD, Nagrial AM, Humphris J, Chantrill LA, Chin V, Steinmann AM, Mawson A, Humphrey ES, Colvin EK, Chou A, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Pettitt JA, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N, Jamieson NB, Graham JS, Niclou SP, Bjerkvig R, Grützmann R, Aust D, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Falconi M, Zamboni G, Tortora G, Tempero MA; Australian Pancreatic Cancer Genome Initiative, Gill AJ, Eshleman JR, Pilarsky C, Scarpa A, Musgrove EA, Pearson JV, Biankin AV, Grimmond SM. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 2015; **518**: 495-501 [PMID: 25719666 DOI: 10.1038/nature14169]

12 **Hall BR**, Cannon A, Atri P, Wichman CS, Smith LM, Ganti AK, Are C, Sasson AR, Kumar S, Batra SK. Advanced pancreatic cancer: a meta-analysis of clinical trials over thirty years. *Oncotarget* 2018; **9**: 19396-19405 [PMID: 29721211 DOI: 10.18632/oncotarget.25036]

13 **Breast Cancer Linkage Consortium**. Cancer risks in BRCA2 mutation carriers. *J Natl Cancer Inst* 1999; **91**: 1310-1316 [PMID: 10433620 DOI: 10.1093/jnci/91.15.1310]

14 **Thompson D**, Easton DF; Breast Cancer Linkage Consortium. Cancer Incidence in BRCA1 mutation carriers. *J Natl Cancer Inst* 2002; **94**: 1358-1365 [PMID: 12237281 DOI: 10.1093/jnci/94.18.1358]

15 **Chen CC**, Feng W, Lim PX, Kass EM, Jasin M. Homology-Directed Repair and the Role of BRCA1, BRCA2, and Related Proteins in Genome Integrity and Cancer. *Annu Rev Cancer Biol* 2018; **2**: 313-336 [PMID: 30345412 DOI: 10.1146/annurev-cancerbio-030617-050502]

16 **Prakash R**, Zhang Y, Feng W, Jasin M. Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. *Cold Spring Harb Perspect Biol* 2015; **7**: a016600 [PMID: 25833843 DOI: 10.1101/cshperspect.a016600]

17 **Hu C**, Hart SN, Polley EC, Gnanaolivu R, Shimelis H, Lee KY, Lilyquist J, Na J, Moore R, Antwi SO, Bamlet WR, Chaffee KG, DiCarlo J, Wu Z, Samara R, Kasi PM, McWilliams RR, Petersen GM, Couch FJ. Association Between Inherited Germline Mutations in Cancer Predisposition Genes and Risk of Pancreatic Cancer. *JAMA* 2018; **319**: 2401-2409 [PMID: 29922827 DOI: 10.1001/jama.2018.6228]

18 **Miki Y**, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994; **266**: 66-71 [PMID: 7545954 DOI: 10.1126/science.7545954]

19 **Wooster R**, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G. Identification of the breast cancer susceptibility gene BRCA2. *Nature* 1995; **378**: 789-792 [PMID: 8524414 DOI: 10.1038/378789a0]

20 **Paluch-Shimon S**, Cardoso F, Sessa C, Balmana J, Cardoso MJ, Gilbert F, Senkus E; ESMO Guidelines Committee. Prevention and screening in BRCA mutation carriers and other breast/ovarian hereditary cancer syndromes: ESMO Clinical Practice Guidelines for cancer prevention and screening. *Ann Oncol* 2016; **27**: v103-v110 [PMID: 27664246 DOI: 10.1093/annonc/mdw327]

21 **Roa BB**, Boyd AA, Volcik K, Richards CS. Ashkenazi Jewish population frequencies for common mutations in BRCA1 and BRCA2. *Nat Genet* 1996; **14**: 185-187 [PMID: 8841191 DOI: 10.1038/ng1096-185]

22 **Janavičius R**. Founder BRCA1/2 mutations in the Europe: implications for hereditary breast-ovarian cancer prevention and control. *EPMA J* 2010; **1**: 397-412 [PMID: 23199084 DOI: 10.1007/s13167-010-0037-y]

23 **Holter S**, Borgida A, Dodd A, Grant R, Semotiuk K, Hedley D, Dhani N, Narod S, Akbari M, Moore M, Gallinger S. Germline BRCA Mutations in a Large Clinic-Based Cohort of Patients With Pancreatic Adenocarcinoma. *J Clin Oncol* 2015; **33**: 3124-3129 [PMID: 25940717 DOI: 10.1200/JCO.2014.59.7401]

24 **Brand R**, Borazanci E, Speare V, Dudley B, Karloski E, Peters MLB, Stobie L, Bahary N, Zeh H, Zureikat A, Hogg M, Lee K, Tsung A, Rhee J, Ohr J, Sun W, Lee J, Moser AJ, DeLeonardis K, Krejdovsky J, Dalton E, LaDuca H, Dolinsky J, Colvin A, Lim C, Black MH, Tung N. Prospective study of germline genetic testing in incident cases of pancreatic adenocarcinoma. *Cancer* 2018; **124**: 3520-3527 [PMID: 30067863 DOI: 10.1002/cncr.31628]

25 **Mizukami K**, Iwasaki Y, Kawakami E, Hirata M, Kamatani Y, Matsuda K, Endo M, Sugano K, Yoshida T, Murakami Y, Nakagawa H, Spurdle AB, Momozawa Y. Genetic characterization of pancreatic cancer patients and prediction of carrier status of germline pathogenic variants in cancer-predisposing genes. *EBioMedicine* 2020; **60**: 103033 [PMID: 32980694 DOI: 10.1016/j.ebiom.2020.103033]

26 **Lowery MA**, Wong W, Jordan EJ, Lee JW, Kemel Y, Vijai J, Mandelker D, Zehir A, Capanu M, Salo-Mullen E, Arnold AG, Yu KH, Varghese AM, Kelsen DP, Brenner R, Kaufmann E,

Ravichandran V, Mukherjee S, Berger MF, Hyman DM, Klimstra DS, Abou-Alfa GK, Tjan C, Covington C, Maynard H, Allen PJ, Askan G, Leach SD, Iacobuzio-Donahue CA, Robson ME, Offit K, Stadler ZK, O'Reilly EM. Prospective Evaluation of Germline Alterations in Patients With Exocrine Pancreatic Neoplasms. *J Natl Cancer Inst* 2018; **110**: 1067-1074 [PMID: 29506128 DOI: 10.1093/jnci/djy024]

27 **Lucas AL**, Shakya R, Lipsyc MD, Mitchel EB, Kumar S, Hwang C, Deng L, Devoe C, Chabot JA, Szabolcs M, Ludwig T, Chung WK, Frucht H. High prevalence of BRCA1 and BRCA2 germline mutations with loss of heterozygosity in a series of resected pancreatic adenocarcinoma and other neoplastic lesions. *Clin Cancer Res* 2013; **19**: 3396-3403 [PMID: 23658460 DOI: 10.1158/1078-0432.CCR-12-3020]

28 **Ferrone CR**, Levine DA, Tang LH, Allen PJ, Jarnagin W, Brennan MF, Offit K, Robson ME. BRCA germline mutations in Jewish patients with pancreatic adenocarcinoma. *J Clin Oncol* 2009; **27**: 433-438 [PMID: 19064968 DOI: 10.1200/JCO.2008.18.5546]

29 **Murphy KM**, Brune KA, Griffin C, Sollenberger JE, Petersen GM, Bansal R, Hruban RH, Kern SE. Evaluation of candidate genes MAP2K4, MADH4, ACVR1B, and BRCA2 in familial pancreatic cancer: deleterious BRCA2 mutations in 17%. *Cancer Res* 2002; **62**: 3789-3793 [PMID: 12097290]

30 **Hahn SA**, Greenhalf B, Ellis I, Sina-Frey M, Rieder H, Korte B, Gerdes B, Kress R, Ziegler A, Raeburn JA, Campra D, Grützmann R, Rehder H, Rothmund M, Schmiegel W, Neoptolemos JP, Bartsch DK. BRCA2 germline mutations in familial pancreatic carcinoma. *J Natl Cancer Inst* 2003; **95**: 214-221 [PMID: 12569143 DOI: 10.1093/jnci/95.3.214]

31 **Zhen DB**, Rabe KG, Gallinger S, Syngal S, Schwartz AG, Goggins MG, Hruban RH, Cote ML, McWilliams RR, Roberts NJ, Cannon-Albright LA, Li D, Moyes K, Wenstrup RJ, Hartman AR, Seminara D, Klein AP, Petersen GM. BRCA1, BRCA2, PALB2, and CDKN2A mutations in familial pancreatic cancer: a PACGENE study. *Genet Med* 2015; **17**: 569-577 [PMID: 25356972 DOI: 10.1038/gim.2014.153]

32 **Couch FJ**, Johnson MR, Rabe KG, Brune K, de Andrade M, Goggins M, Rothenmund H, Gallinger S, Klein A, Petersen GM, Hruban RH. The prevalence of BRCA2 mutations in familial pancreatic cancer. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 342-346 [PMID: 17301269 DOI: 10.1158/1055-9965.EPI-06-0783]

33 **Møller P**, Hagen AI, Apold J, Maehle L, Clark N, Fiane B, Løvslett K, Hovig E, Vabø A. Genetic epidemiology of BRCA mutations--family history detects less than 50% of the mutation carriers. *Eur J Cancer* 2007; **43**: 1713-1717 [PMID: 17574839 DOI: 10.1016/j.ejca.2007.04.023]

34 **Shindo K**, Yu J, Suenaga M, Fesharakizadeh S, Cho C, Macgregor-Das A, Siddiqui A, Witmer PD, Tamura K, Song TJ, Navarro Almario JA, Brant A, Borges M, Ford M, Barkley T, He J, Weiss MJ, Wolfgang CL, Roberts NJ, Hruban RH, Klein AP, Goggins M. Deleterious Germline Mutations in Patients With Apparently Sporadic Pancreatic Adenocarcinoma. *J Clin Oncol* 2017; **35**: 3382-3390 [PMID: 28767289 DOI: 10.1200/JCO.2017.72.3502]

35 **Kondo T**, Kanai M, Kou T, Sakuma T, Mochizuki H, Kamada M, Nakatsui M, Uza N, Kodama Y, Masui T, Takaori K, Matsumoto S, Miyake H, Okuno Y, Muto M. Association between homologous recombination repair gene mutations and response to oxaliplatin in pancreatic cancer. *Oncotarget* 2018; **9**: 19817-19825 [PMID: 29731985 DOI: 10.18632/oncotarget.24865]

36 **Gabai-Kapara E**, Lahad A, Kaufman B, Friedman E, Segev S, Renbaum P, Beeri R, Gal M, Grinshpun-Cohen J, Djemal K, Mandell JB, Lee MK, Beller U, Catane R, King MC, Levy-Lahad E. Population-based screening for breast and ovarian cancer risk due to BRCA1 and BRCA2. *Proc Natl Acad Sci USA* 2014; **111**: 14205-14210 [PMID: 25192939 DOI: 10.1073/pnas.1415979111]

37 **Manchanda R**, Loggenberg K, Sanderson S, Burnell M, Wardle J, Gessler S, Side L, Balogun N, Desai R, Kumar A, Dorkins H, Wallis Y, Chapman C, Taylor R, Jacobs C, Tomlinson I, McGuire A, Beller U, Menon U, Jacobs I. Population testing for cancer predisposing BRCA1/BRCA2 mutations in the Ashkenazi-Jewish community: a randomized controlled trial. *J Natl Cancer Inst* 2015; **107**: 379 [PMID: 25435541 DOI: 10.1093/jnci/dju379]

38 **Metcalfe KA**, Poll A, Royer R, Llacuachaqui M, Tulman A, Sun P, Narod SA. Screening for founder mutations in BRCA1 and BRCA2 in unselected Jewish women. *J Clin Oncol* 2010; **28**: 387-391 [PMID: 20008623 DOI: 10.1200/JCO.2009.25.0712]

39 **Tennen RI**, Laskey SB, Koelsch BL, McIntyre MH, Tung JY. Identifying Ashkenazi Jewish BRCA1/2 founder variants in individuals who do not self-report Jewish ancestry. *Sci Rep* 2020; **10**: 7669 [PMID: 32376921 DOI: 10.1038/s41598-020-63466-x]

40 **Mandelker D**, Zhang L, Kemel Y, Stadler ZK, Joseph V, Zehir A, Pradhan N, Arnold A, Walsh MF, Li Y, Balakrishnan AR, Syed A, Prasad M, Nafa K, Carlo MI, Cadoo KA, Sheehan M, Fleischut MH, Salo-Mullen E, Trottier M, Lipkin SM, Lincoln A, Mukherjee S, Ravichandran V, Cambria R, Galle J, Abida W, Arcila ME, Benayed R, Shah R, Yu K, Bajorin DF, Coleman JA, Leach SD, Lowery MA, Garcia-Aguilar J, Kantoff PW, Sawyers CL, Dickler MN, Saltz L, Motzer RJ, O'Reilly EM, Scher HI, Baselga J, Klimstra DS, Solit DB, Hyman DM, Berger MF, Ladanyi M, Robson ME, Offit K. Mutation Detection in Patients With Advanced Cancer by Universal Sequencing of Cancer-Related Genes in Tumor and Normal DNA *vs* Guideline-Based Germline Testing. *JAMA* 2017; **318**: 825-835 [PMID: 28873162 DOI: 10.1001/jama.2017.11137]

41 **Daly MB**, Pilarski R, Yurgelun MB, Berry MP, Buys SS, Dickson P, Domchek SM, Elkhanany A, Friedman S, Garber JE, Goggins M, Hutton ML, Khan S, Klein C, Kohlmann W, Kurian AW, Laronga C, Litton JK, Mak JS, Menendez CS, Merajver SD, Norquist BS, Offit K, Pal T, Pederson HJ, Reiser G, Shannon KM, Visvanathan K, Weitzel JN, Wick MJ, Wisinski KB, Dwyer MA, Darlow

SD. NCCN Guidelines Insights: Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic, Version 1.2020. *J Natl Compr Canc Netw* 2020; **18**: 380-391 [PMID: 32259785 DOI: 10.6004/jnccn.2020.0017]

42 **Smith AL**, Wong C, Cuggia A, Borgida A, Holter S, Hall A, Connor AA, Bascuñana C, Asselah J, Bouganim N, Poulin V, Jolivet J, Vafiadis P, Le P, Martel G, Lemay F, Beaudoin A, Rafatzand K, Chaudhury P, Barkun J, Metrakos P, Marcus V, Omeroglu A, Chong G, Akbari MR, Foulkes WD, Gallinger S, Zogopoulos G. Reflex Testing for Germline BRCA1 , BRCA2 , PALB2 , and ATM Mutations in Pancreatic Cancer: Mutation Prevalence and Clinical Outcomes From Two Canadian Research Registries. *JCO Precis Oncol* 2018; **2**: 1-16 [DOI: 10.1200/PO.17.00098]

43 **Golan T**, Kanji ZS, Epelbaum R, Devaud N, Dagan E, Holter S, Aderka D, Paluch-Shimon S, Kaufman B, Gershoni-Baruch R, Hedley D, Moore MJ, Friedman E, Gallinger S. Overall survival and clinical characteristics of pancreatic cancer in BRCA mutation carriers. *Br J Cancer* 2014; **111**: 1132-1138 [PMID: 25072261 DOI: 10.1038/bjc.2014.418]

44 **Blair AB**, Groot VP, Gemenetzis G, Wei J, Cameron JL, Weiss MJ, Goggins M, Wolfgang CL, Yu J, He J. BRCA1/BRCA2 Germline Mutation Carriers and Sporadic Pancreatic Ductal Adenocarcinoma. *J Am Coll Surg* 2018; **226**: 630-637. e1 [PMID: 29309945 DOI: 10.1016/j.jamcollsurg.2017.12.021]

45 **Golan T**, Sella T, O'Reilly EM, Katz MH, Epelbaum R, Kelsen DP, Borgida A, Maynard H, Kindler H, Friedmen E, Javle M, Gallinger S. Overall survival and clinical characteristics of BRCA mutation carriers with stage I/II pancreatic cancer. *Br J Cancer* 2017; **116**: 697-702 [PMID: 28183138 DOI: 10.1038/bjc.2017.19]

46 **Pishvaian MJ**, Blais EM, Brody JR, Lyons E, DeArbeloa P, Hendifar A, Mikhail S, Chung V, Sahai V, Sohal DPS, Bellakbira S, Thach D, Rahib L, Madhavan S, Matrisian LM, Petricoin EF 3rd. Overall survival in patients with pancreatic cancer receiving matched therapies following molecular profiling: a retrospective analysis of the Know Your Tumor registry trial. *Lancet Oncol* 2020; **21**: 508-518 [PMID: 32135080 DOI: 10.1016/S1470-2045(20)30074-7]

47 **Perri G**, Prakash L, Qiao W, Varadhachary GR, Wolff R, Fogelman D, Overman M, Pant S, Javle M, Koay EJ, Herman J, Kim M, Ikoma N, Tzeng CW, Lee JE, Katz MHG. Response and Survival Associated With First-line FOLFIRINOX *vs* Gemcitabine and nab-Paclitaxel Chemotherapy for Localized Pancreatic Ductal Adenocarcinoma. *JAMA Surg* 2020; **155**: 832-839 [PMID: 32667641 DOI: 10.1001/jamasurg.2020.2286]

48 **Williet N**, Saint A, Pointet AL, Tougeron D, Pernot S, Pozet A, Bechade D, Trouilloud I, Lourenco N, Hautefeuille V, Locher C, Desrame J, Artru P, Thirot Bidault A, Le Roy B, Pezet D, Phelip JM, Taieb J. Folfirinox *versus* gemcitabine/nab-paclitaxel as first-line therapy in patients with metastatic pancreatic cancer: a comparative propensity score study. *Therap Adv Gastroenterol* 2019; **12**: 1756284819878660 [PMID: 31598136 DOI: 10.1177/1756284819878660]

49 **Bhattacharyya A**, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J Biol Chem* 2000; **275**: 23899-23903 [PMID: 10843985 DOI: 10.1074/jbc.C000276200]

50 **Mylavarapu S**, Das A, Roy M. Role of BRCA Mutations in the Modulation of Response to Platinum Therapy. *Front Oncol* 2018; **8**: 16 [PMID: 29459887 DOI: 10.3389/fonc.2018.00016]

51 **Vyas O**, Leung K, Ledbetter L, Kaley K, Rodriguez T, Garcon MC, Saif MW. Clinical outcomes in pancreatic adenocarcinoma associated with BRCA-2 mutation. *Anticancer Drugs* 2015; **26**: 224-226 [PMID: 25304989 DOI: 10.1097/CAD.0000000000000178]

52 **Reiss KA**, Yu S, Judy R, Symecko H, Nathanson KL, Domchek SM. Retrospective Survival Analysis of Patients With Advanced Pancreatic Ductal Adenocarcinoma and Germline BRCA or PALB2 Mutations. *JCO Precis Oncol* 2018; **2**: 1-9 [DOI: 10.1200/PO.17.00152]

53 **Wattenberg MM**, Asch D, Yu S, O'Dwyer PJ, Domchek SM, Nathanson KL, Rosen MA, Beatty GL, Siegelman ES, Reiss KA. Platinum response characteristics of patients with pancreatic ductal adenocarcinoma and a germline BRCA1, BRCA2 or PALB2 mutation. *Br J Cancer* 2020; **122**: 333-339 [PMID: 31787751 DOI: 10.1038/s41416-019-0582-7]

54 **Yu S**, Agarwal P, Mamtani R, Symecko H, Spielman K, O'Hara M, O'Dwyer PJ, Schneider C, Teitelbaum U, Nathanson KL, Domchek SM, Reiss KA. Retrospective Survival Analysis of Patients With Resected Pancreatic Ductal Adenocarcinoma and a Germline BRCA or PALB2 Mutation. *JCO Precis Oncol* 2019; **2**: 1-11 [DOI: 10.1200/PO.18.00271]

55 **Rebelatto TF**, Falavigna M, Pozzari M, Spada F, Cella CA, Laffi A, Pellicori S, Fazio N. Should platinum-based chemotherapy be preferred for germline BReast CAncer genes (BRCA) 1 and 2-mutated pancreatic ductal adenocarcinoma (PDAC) patients? *Cancer Treat Rev* 2019; **80**: 101895 [PMID: 31542591 DOI: 10.1016/j.ctrv.2019.101895]

56 **O'Reilly EM**, Lee JW, Zalupski M, Capanu M, Park J, Golan T, Tahover E, Lowery MA, Chou JF, Sahai V, Brenner R, Kindler HL, Yu KH, Zervoudakis A, Vemuri S, Stadler ZK, Do RKG, Dhani N, Chen AP, Kelsen DP. Randomized, Multicenter, Phase II Trial of Gemcitabine and Cisplatin With or Without Veliparib in Patients With Pancreas Adenocarcinoma and a Germline *BRCA/PALB2* Mutation. *J Clin Oncol* 2020; **38**: 1378-1388 [PMID: 31976786 DOI: 10.1200/JCO.19.02931]

57 **Bryant HE**, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, Kyle S, Meuth M, Curtin NJ, Helleday T. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 2005; **434**: 913-917 [PMID: 15829966 DOI: 10.1038/nature03443]

58 **Farmer H**, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, Ashworth A. Targeting the DNA repair

defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005; **434**: 917-921 [PMID: 15829967 DOI: 10.1038/nature03445]

59 **Keung MYT**, Wu Y, Vadgama JV. PARP Inhibitors as a Therapeutic Agent for Homologous Recombination Deficiency in Breast Cancers. *J Clin Med* 2019; **8** [PMID: 30934991 DOI: 10.3390/jcm8040435]

60 **Helleday T**. The underlying mechanism for the PARP and BRCA synthetic lethality: clearing up the misunderstandings. *Mol Oncol* 2011; **5**: 387-393 [PMID: 21821475 DOI: 10.1016/j.molonc.2011.07.001]

61 **Fong PC**, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, Mortimer P, Swaisland H, Lau A, O'Connor MJ, Ashworth A, Carmichael J, Kaye SB, Schellens JH, de Bono JS. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* 2009; **361**: 123-134 [PMID: 19553641 DOI: 10.1056/NEJMoa0900212]

62 **Mirza MR**, Coleman RL, González-Martín A, Moore KN, Colombo N, Ray-Coquard I, Pignata S. The forefront of ovarian cancer therapy: update on PARP inhibitors. *Ann Oncol* 2020; **31**: 1148-1159 [PMID: 32569725 DOI: 10.1016/j.annonc.2020.06.004]

63 **Mirza MR**, Monk BJ, Herrstedt J, Oza AM, Mahner S, Redondo A, Fabbro M, Ledermann JA, Lorusso D, Vergote I, Ben-Baruch NE, Marth C, Mądry R, Christensen RD, Berek JS, Dørum A, Tinker AV, du Bois A, González-Martín A, Follana P, Benigno B, Rosenberg P, Gilbert L, Rimel BJ, Buscema J, Balser JP, Agarwal S, Matulonis UA; ENGOT-OV16/NOVA Investigators. Niraparib Maintenance Therapy in Platinum-Sensitive, Recurrent Ovarian Cancer. *N Engl J Med* 2016; **375**: 2154-2164 [PMID: 27717299 DOI: 10.1056/nejmoa1611310]

64 **Coleman RL**, Oza AM, Lorusso D, Aghajanian C, Oaknin A, Dean A, Colombo N, Weberpals JI, Clamp A, Scambia G, Leary A, Holloway RW, Gancedo MA, Fong PC, Goh JC, O'Malley DM, Armstrong DK, Garcia-Donas J, Swisher EM, Floquet A, Konecny GE, McNeish IA, Scott CL, Cameron T, Maloney L, Isaacson J, Goble S, Grace C, Harding TC, Raponi M, Sun J, Lin KK, Giordano H, Ledermann JA; ARIEL3 investigators. Rucaparib maintenance treatment for recurrent ovarian carcinoma after response to platinum therapy (ARIEL3): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **390**: 1949-1961 [PMID: 28916367 DOI: 10.1016/S0140-6736(17)32440-6]

65 **Pujade-Lauraine E**, Ledermann JA, Selle F, Gebski V, Penson RT, Oza AM, Korach J, Huzarski T, Poveda A, Pignata S, Friedlander M, Colombo N, Harter P, Fujiwara K, Ray-Coquard I, Banerjee S, Liu J, Lowe ES, Bloomfield R, Pautier P; SOLO2/ENGOT-Ov21 investigators. Olaparib tablets as maintenance therapy in patients with platinum-sensitive, relapsed ovarian cancer and a BRCA1/2 mutation (SOLO2/ENGOT-Ov21): a double-blind, randomised, placebo-controlled, phase 3 trial. *Lancet Oncol* 2017; **18**: 1274-1284 [PMID: 28754483 DOI: 10.1016/S1470-2045(17)30469-2]

66 **Robson M**, Im SA, Senkus E, Xu B, Domchek SM, Masuda N, Delaloge S, Li W, Tung N, Armstrong A, Wu W, Goessl C, Runswick S, Conte P. Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation. *N Engl J Med* 2017; **377**: 523-533 [PMID: 28578601 DOI: 10.1056/NEJMoa1706450]

67 **Litton JK**, Rugo HS, Ettl J, Hurvitz SA, Gonçalves A, Lee KH, Fehrenbacher L, Yerushalmi R, Mina LA, Martin M, Roché H, Im YH, Quek RGW, Markova D, Tudor IC, Hannah AL, Eiermann W, Blum JL. Talazoparib in Patients with Advanced Breast Cancer and a Germline BRCA Mutation. *N Engl J Med* 2018; **379**: 753-763 [PMID: 30110579 DOI: 10.1056/NEJMoa1802905]

68 **Robson ME**, Tung N, Conte P, Im SA, Senkus E, Xu B, Masuda N, Delaloge S, Li W, Armstrong A, Wu W, Goessl C, Runswick S, Domchek SM. OlympiAD final overall survival and tolerability results: Olaparib *vs* chemotherapy treatment of physician's choice in patients with a germline BRCA mutation and HER2-negative metastatic breast cancer. *Ann Oncol* 2019; **30**: 558-566 [PMID: 30689707 DOI: 10.1093/annonc/mdz012]

69 **de Bono J**, Mateo J, Fizazi K, Saad F, Shore N, Sandhu S, Chi KN, Sartor O, Agarwal N, Olmos D, Thiery-Vuillemin A, Twardowski P, Mehra N, Goessl C, Kang J, Burgents J, Wu W, Kohlmann A, Adelman CA, Hussain M. Olaparib for Metastatic Castration-Resistant Prostate Cancer. *N Engl J Med* 2020; **382**: 2091-2102 [PMID: 32343890 DOI: 10.1056/NEJMoa1911440]

70 **Lowery MA**, Kelsen DP, Capanu M, Smith SC, Lee JW, Stadler ZK, Moore MJ, Kindler HL, Golan T, Segal A, Maynard H, Hollywood E, Moynahan M, Salo-Mullen EE, Do RKG, Chen AP, Yu KH, Tang LH, O'Reilly EM. Phase II trial of veliparib in patients with previously treated BRCA-mutated pancreas ductal adenocarcinoma. *Eur J Cancer* 2018; **89**: 19-26 [PMID: 29223478 DOI: 10.1016/j.ejca.2017.11.004]

71 **Kaufman B**, Shapira-Frommer R, Schmutzler RK, Audeh MW, Friedlander M, Balmaña J, Mitchell G, Fried G, Stemmer SM, Hubert A, Rosengarten O, Steiner M, Loman N, Bowen K, Fielding A, Domchek SM. Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. *J Clin Oncol* 2015; **33**: 244-250 [PMID: 25366685 DOI: 10.1200/JCO.2014.56.2728]

72 **Golan T**, Hammel P, Reni M, Van Cutsem E, Macarulla T, Hall MJ, Park JO, Hochhauser D, Arnold D, Oh DY, Reinacher-Schick A, Tortora G, Algül H, O'Reilly EM, McGuinness D, Cui KY, Schlienger K, Locker GY, Kindler HL. Maintenance Olaparib for Germline *BRCA*-Mutated Metastatic Pancreatic Cancer. *N Engl J Med* 2019; **381**: 317-327 [PMID: 31157963 DOI: 10.1056/NEJMoa1903387]

73 **Hammel P**, Kindler HL, Reni M, Van Cutsem E, Macarulla T, Hall MJ, Park JO, Hochhauser D, Arnold D, Oh DY, Reinacher-Schick A, Tortora G, Algül H, O'Reilly EM, McGuinness D, Cui KY, Joo S, Yoo HK, Patel N, Golan T; POLO Investigators. Health-related quality of life in patients with

a germline BRCA mutation and metastatic pancreatic cancer receiving maintenance olaparib. *Ann Oncol* 2019; **30**: 1959-1968 [PMID: 31562758 DOI: 10.1093/annonc/mdz406]

74 **Nishikawa G**, Booth C, Prasad V. Olaparib for BRCA mutant pancreas cancer: Should the POLO trial change clinical practice? *Cancer* 2020; **126**: 4087-4088 [PMID: 32459369 DOI: 10.1002/cncr.32979]

75 **Hammel P**, Vitellius C, Boisteau É, Wisniewski M, Colle E, Hilmi M, Dengremont C, Granier S, Turpin A, de Mestier L, Neuzillet C. Maintenance therapies in metastatic pancreatic cancer: present and future with a focus on PARP inhibitors. *Ther Adv Med Oncol* 2020; **12**: 1758835920937949 [PMID: 32695234 DOI: 10.1177/1758835920937949]

76 **Samstein RM**, Krishna C, Ma X, Pei X, Lee KW, Makarov V, Kuo F, Chung J, Srivastava RM, Purohit TA, Hoen DR, Mandal R, Setton J, Wu W, Shah R, Qeriqi B, Chang Q, Kendall S, Braunstein L, Weigelt B, Blecua Carrillo Albornoz P, Morris LGT, Mandelker DL, Reis-Filho JS, de Stanchina E, Powell SN, Chan TA, Riaz N. Mutations in BRCA1 and BRCA2 differentially affect the tumor microenvironment and response to checkpoint blockade immunotherapy. *Nat Cancer* 2020; **1**: 1188-1203 [DOI: 10.1038/s43018-020-00139-8]

77 **Sato H**, Niimi A, Yasuhara T, Permata TBM, Hagiwara Y, Isono M, Nuryadi E, Sekine R, Oike T, Kakoti S, Yoshimoto Y, Held KD, Suzuki Y, Kono K, Miyagawa K, Nakano T, Shibata A. DNA double-strand break repair pathway regulates PD-L1 expression in cancer cells. *Nat Commun* 2017; **8**: 1751 [PMID: 29170499 DOI: 10.1038/s41467-017-01883-9]

78 **Seeber A**, Zimmer K, Kocher F, Puccini A, Xiu J, Nabhan C, Elliott A, Goldberg RM, Grothey A, Shields AF, Battaglin F, El-Deiry WS, Philip PA, Marshall JL, Hall M, Korn WM, Lenz HJ, Wolf D, Feistritzer C, Spizzo G. Molecular characteristics of *BRCA1/2* and *PALB2* mutations in pancreatic ductal adenocarcinoma. *ESMO Open* 2020; **5**: e000942 [PMID: 33229504 DOI: 10.1136/esmoopen-2020-000942]

79 **Peyraud F**, Italiano A. Combined PARP Inhibition and Immune Checkpoint Therapy in Solid Tumors. *Cancers (Basel)* 2020; **12** [PMID: 32526888 DOI: 10.3390/cancers12061502]

80 **Vikas P**, Borcherding N, Chennamadhavuni A, Garje R. Therapeutic Potential of Combining PARP Inhibitor and Immunotherapy in Solid Tumors. *Front Oncol* 2020; **10**: 570 [PMID: 32457830 DOI: 10.3389/fonc.2020.00570]

81 **Mohyuddin GR**, Aziz M, Britt A, Wade L, Sun W, Baranda J, Al-Rajabi R, Saeed A, Kasi A. Similar response rates and survival with PARP inhibitors for patients with solid tumors harboring somatic *vs* Germline BRCA mutations: a Meta-analysis and systematic review. *BMC Cancer* 2020; **20**: 507 [PMID: 32493233 DOI: 10.1186/s12885-020-06948-5]

82 **Pennington KP**, Walsh T, Harrell MI, Lee MK, Pennil CC, Rendi MH, Thornton A, Norquist BM, Casadei S, Nord AS, Agnew KJ, Pritchard CC, Scroggins S, Garcia RL, King MC, Swisher EM. Germline and somatic mutations in homologous recombination genes predict platinum response and survival in ovarian, fallopian tube, and peritoneal carcinomas. *Clin Cancer Res* 2014; **20**: 764-775 [PMID: 24240112 DOI: 10.1158/1078-0432.CCR-13-2287]

83 **Hoppe MM**, Sundar R, Tan DSP, Jeyasekharan AD. Biomarkers for Homologous Recombination Deficiency in Cancer. *J Natl Cancer Inst* 2018; **110**: 704-713 [PMID: 29788099 DOI: 10.1093/jnci/djy085]

84 **Ray-Coquard I**, Pautier P, Pignata S, Pérol D, González-Martín A, Berger R, Fujiwara K, Vergote I, Colombo N, Mäenpää J, Selle F, Sehouli J, Lorusso D, Guerra Alía EM, Reinthaller A, Nagao S, Lefeuvre-Plesse C, Canzler U, Scambia G, Lortholary A, Marmé F, Combe P, de Gregorio N, Rodrigues M, Buderath P, Dubot C, Burges A, You B, Pujade-Lauraine E, Harter P; PAOLA-1 Investigators. Olaparib plus Bevacizumab as First-Line Maintenance in Ovarian Cancer. *N Engl J Med* 2019; **381**: 2416-2428 [PMID: 31851799 DOI: 10.1056/NEJMoa1911361]

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MINIREVIEWS

Metabolic and cardiovascular complications after virological cure in hepatitis C: What awaits beyond

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Abstract

The association between chronic hepatitis C (CHC) infection and extrahepatic manifestations (EHMs), particularly cardiometabolic diseases, has been extensively examined. However, there has still been insufficient evaluation for these EHMs after virological cure. Several multidirectional mechanisms have been proposed explaining the ability of hepatitis C virus (HCV) developing EHMs, cardiometabolic ones, as well as the effect of antiviral therapy to resolve these EHMs. Data on these manifestations after achieving sustained virologic response (SVR) are still conflicting. However, current evidence suggests that reversal of hepatic steatosis and its coexistent hypocholesterolemia after successful viral eradication led to unfavorable lipid profile, which increases cardiovascular disease (CVD) risk. Additionally, most observations showed that metabolic alterations, such as insulin resistance and diabetes mellitus (DM), undergo some degree of reduction after viral clearance. These changes seem HCV-genotype dependent. Interferon-based antiviral therapy and direct acting antiviral drugs were shown to minimize incidence of DM. Large epidemiological studies that investigated the effect of SVR on CVD showed great discrepancies in terms of results, with predominant findings indicating that CVD events decreased in patients with SVR compared to non-responders or untreated ones. In this review, we present a summary of the current knowledge regarding extrahepatic sequelae of CHC following SVR, which may have an impact on healthcare providers' clinical practice.

Key Words: Chronic hepatitis C; Sustained virologic response; Hepatic steatosis; Diabetes mellitus; Cardiovascular disease

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Core Tip: The implementation of direct acting antiviral drugs has dramatically changed the landscape of hepatitis C virus (HCV) treatment, with over 95% of patients achieving sustained virologic response (SVR). Although consistent evidence demonstrated better outcomes for both hepatic and extrahepatic complications after viral clearance, data on cardiometabolic manifestation showed inconsistent results. In this review, we are shading light on the latest findings about cardiometabolic extrahepatic manifestations post-SVR. These updates may guide clinicians engaged in HCV care to integrate in their management post-viral eradication risks and subsequent long-term care.

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INTRODUCTION

Chronic hepatitis C (CHC) infection caused by hepatitis C virus (HCV) is associated with substantial morbidity and mortality globally, affecting approximately 2.5% individuals (equivalent to 177.5 million) worldwide[1]. It is currently one of the leading etiologies for hepatocellular carcinoma (HCC) and decompensated cirrhosis requiring liver transplantation in Western countries[2,3]. The primary goal of treatment is to achieve the cure of the infection or sustained virologic response (SVR), defined as undetectable HCV RNA in the serum 12 or 24 wk after the end of treatment[4,5]. Since the introduction of first-generation direct acting antiviral agents (DAAs), boceprevir and telaprevir, in 2011, there has been a rapidly expanding population of CHC patients achieving SVR[6]. Viral eradication has been associated with marked reduction in the risk of end-stage liver disease, need for liver transplantation, and decrease in both liver-related and overall mortality[7].

Although HCV is a hepatotropic virus, for two decades several studies described the association between HCV and a heterogeneous array of extrahepatic manifestations (EHMs)[8-10] (Figure 1). Yet, the mechanism by which the virus evokes the systemic diseases remain to be elucidated. Endocrine-metabolic alterations, which are most frequently found in CHC patients, are thought to be caused by direct and indirect effects of disturbing host lipid and glucose metabolism[11,12], as well as alteration in adipocytokines released from adipose tissue[13,14]. Likewise, CHC infection has also been identified as an independent predictor for cardiovascular events such as carotid artery atherosclerosis, stroke, myocardial ischemia and heart failure, all of which are linked with poor outcomes[12]. Nevertheless, the impact on cardiovascular disease (CVD) is not fully established[15]. In clinical settings, the prognosis of CHC is not only depending on liver-related outcomes but also on extrahepatic sequelae.

Recently, much attention is drawn toward EHMs that occur following viral cure. Nonetheless, whether the development of such manifestations is a long-term consequence of the viral infection itself or an effect of HCV medications remains unknown. In this context, various reports have shown ample evidence for high prevalence of CVD and metabolic alterations such as dyslipidemia, hepatic steatosis, insulin resistance (IR), obesity and diabetes mellitus (DM)[16-19] (Table 1). While the impact of CHC infection on liver-related outcomes pre- and post-treatment has been well studied, extrahepatic sequelae, especially in post-SVR setting, are less well known. This review article highlights the current knowledge regarding the effect of SVR on EHMs.

Table 1 Summary of studies investigated cardiometabolic manifestations of chronic hepatitis C after viral cure

Ref.	Antiviral regimen	The studied HCV-associated cardiometabolic manifestations	Post SVR outcomes
Fernández-Rodríguez *et al*[28], 2006	NA[1]	Lipid disturbances. Hepatic steatosis	Hypercholesterolemia in patients with genotype 3
			No change in genotype 3 non-responders and in patients with genotype 1 regardless of response
			Decrease in steatosis
Giordanino *et al*[71], 2008	IFN monotherapy or Peg-IFN + RBV (24-48 wk)	Glucose abnormalities (IFG or DM)	No significant reduction in the risk of glucose intolerance in long-term responders and non-responders
Arase *et al*[70], 2009	IFN monotherapy or IFN + RBV[2]	DM	Decreased incidence of DM in sustained responders. However, its development is associated with advanced liver disease
Corey *et al*[18], 2009	NA[1]	Lipid abnormalities	Increased LDL and total cholesterol from baseline compared to non-responders
		Risk of CVD	Increased CVD risk profile
Conjeevaram *et al*[67], 2011	Peg-IFN + RBV (24-48 wk)	IR	Decreased IR
		Obesity	Decreased in BMI
Kuo *et al*[27], 2011	Peg-IFN + RBV (24 wk)	Change in serum lipid	Total cholesterol and triglycerides levels significantly increased
			No evident change in lipid profile occurred in non-SVR group
Aghemo *et al*[68], 2012	Peg-IFN + RBV[2]	IR in non-diabetic CHC patients	Baseline and posttreatment HOMA-IR values were similar in SVR patients
			Significant increase in HOMA-IR was noted in non-SVR patients
Clark *et al*[25], 2012	Albinterferon α-2b + RBV	Lipid abnormalities in genotypes 2,3	Hypercholesterolemia
Thompson *et al*[66], 2012	Albinterferon α-2b *vs* Peg-IFN + RBV (24-48 wk)	IR in genotypes 1,2,3	Reduced IR in genotype 1 responders
			No change in genotype 1 non-responders and genotype 2 and 3 regardless of the response
Chang *et al*[29], 2014	eg-IFN + RBV (24/48 wk)	Lipids and IR in genotypes 2, 3	Increased total cholesterol and triglycerides in sustained responders
			Decreased HOMA-IR in patients with SVR and baseline IR
			High HOMA-IR was found in patients without baseline IR (only in genotype 1)
Hsu *et al*[88], 2015	Peg-IFN + RBV (16-48 wk)	Acute coronary syndrome and ischemic stroke	Improvement in both studied circulatory outcomes
Innes *et al*[89], 2015	NA[1]	CVD	Reduced hazard and absolute risk for CVD
Meissner *et al*[24], 2015	SOF + RBV (24 wk)	Lipid disturbances in genotype 1	Increased LDL level and particle size and decreased triglycerides concentration and VLDL particle size irrespective to treatment response
			Increased intrahepatic lipid-related genes in sustained responders
Leone *et al*[72], 2016	IFN-based regimen	DM and CVD	No significant risk reduction in DM and CVD in SVR group as opposed to non SVR
Yair-Sabag *et al*[39], 2016	Peg-IFN + RBV (24-48 wk)	IFG and DM. Triglycerides. Hepatic steatosis	Lower IFG and DM, and higher triglycerides in sustained responders
			Improvement in hepatic steatosis
Chang *et al*[16], 2017	NA[1]	Cardiovascular complications	An increased adipokine PAI-1 in SVR group, which accelerates cardiovascular risk, especially in vulnerable cases
Mahale *et al*[69], 2018	IFN-based regimen[2]	DM and CVD	Antiviral therapy associated with lower risk of DM and stroke whereas no significant effect on CVD

Nahon *et al*[90], 2017	Peg-IFN + RBV (16-48 wk) or combination therapies[3]	CVD	Lower risk of CVD in SVR subjects in comparison to non SVR
Stine *et al*[74], 2017	DAAs[2,3]	DM in genotypes 1, 2, 3	Glycosylated hemoglobin was not affected in known diabetic patients 1/3 of patients required escalation of anti-diabetic therapy during antiviral treatment
Carvalho *et al*[11], 2018	SOF + LDV ± RBV (group 1) *vs* Peg-IFN + RBV (group 2)	Lipid levels. Serum glucose. IR	While total cholesterol increased in both groups, triglycerides levels decreased in group 1 and increased in group 2 LDL elevated in group 1 and No change in group 2 No significant variation in serum glucose Significant increase in HOMA-IR only in group 2
Kawagishi *et al*[17], 2018	DAAs[3]	Hepatic steatosis. Lipid abnormalities	Decrease in CAP and LDL in patients with high baseline values Elevated sdLDL in patients who had dyslipidemia and hepatic steatosis at 24 wk
Li *et al*[73], 2018	DAAs[4]	DM	Lower risk of DM in SVR patients than in treatment failure group
Noureddin *et al*[46], 2018	DAAs[3]	Hepatic steatosis and fibrosis	High prevalence of fatty liver Although fibrosis has been reduced in patients with and without steatosis compared to baseline, patients with steatosis continued to have clinically significant liver stiffness
Li *et al*[10], 2019	IFN + RBV (48 wk)	Serum glucose level and IR	Reduced glucose level Improved IR
Butt *et al*[87], 2019	IFN + RBV[2,3].DAAs[2,3]	CVD	Lower incidence in treatment group, compared to controls DAAs showed greater risk reduction than interferon-based regimen SVR associated with decreased CVD risk
Abdo *et al*[75], 2020	SOF + DCV (12-24 wk)	Glycemic status, IR, and lipid profile in CHC patients with DM	Improvement of glycemic state and HOMA-IR Global worsening of lipid profile
Graf *et al*[45], 2020	DAAs[3]	IR, lipid perturbations, body weight changes, and hepatic steatosis	Lower HOMA-IR compared to baseline Higher total cholesterol, LDL, and HDL Higher CAP relative to baseline BMI did not significantly change over time
Huang *et al*[31], 2020	DAAs[4]	Lipids and cardiovascular events	Increased total cholesterol and LDL Higher cardio-cerebral diseases

[1]No data available on antiviral therapy.
[2]Data on course of treatment is not available.
[3]Various regimens were used.
[4]No data available neither on types of direct acting antiviral agents nor on treatment duration. BMI: Body mass index; CAP: Controlled attenuation parameter; CHC: Chronic hepatitis C; CVD: Cardiovascular disease; DAAs: Direct acting antiviral agents; DCV: Daclatasvir; DM: Diabetes mellitus; HDL: High density lipoprotein cholesterol; HOMA-IR: Homeostatic model assessment of insulin resistance; IFG: Impaired fasting glucose; IFN: Interferon; IR: Insulin resistance; LDL: Low-density lipoprotein cholesterol; LDV: Ledipasvir; NA: Not available; PAI-1: Plasminogen activator inhibitor-1; Peg-IFN: Pegylated interferon; RBV: Ribavirin; sdLDL: Small-dense low-density lipoprotein; SOF: Sofosbuvir; SVR: Sustained virologic response; VLDL: Very low-density lipoprotein cholesterol; HCV: Hepatitis C virus.

DYSLIPIDEMIA

It has been known that HCV possesses a mutual relationship with host lipids and lipoproteins metabolisms, which the virus uses for multiple key steps in its life cycle[20,21]. HCV circulates as a lipid-rich particle, utilizing lipoprotein cell receptors to gain entry into the hepatocyte[22,23]. Within hepatocytes, it influences three mechanisms in lipid metabolism: It upregulates lipid biosynthesis, impairs

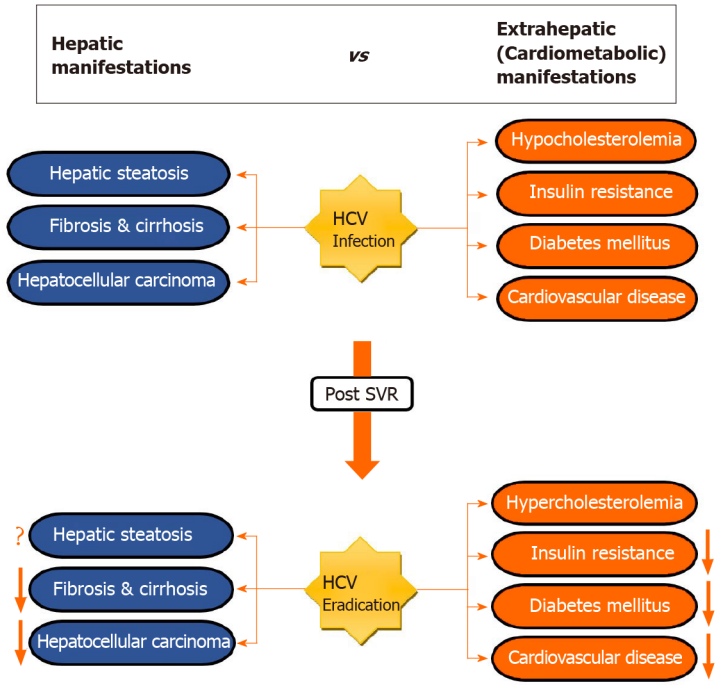


Figure 1 Hepatic and extrahepatic (cardiometabolic) manifestations associated with hepatitis C virus infection and the effect of sustained virologic response on the cardiometabolic manifestations based on most of the evidence. HCV: Hepatitis C virus; SVR: Sustained virologic response.

mitochondrial β-oxidation and thus lipid degradation, and reduces apolipoprotein exportation, in particular very low-density lipoprotein cholesterol (LDL), resulting in significant intracellular lipid accumulation and circulating hypocholesterolemia and hypolipoproteinemia[13].

Several studies have linked successful HCV eradication with rebound rise in lipid levels. Meissner *et al*[24], who investigated the influence of DAAs, sofosbuvir and ribavirin, on serum lipid profiles and intrahepatic lipid-related genes expression in patients with genotype 1 CHC, reported that serum LDL level and molecular size increased early in therapy, whereas triglycerides concentration and very low-density lipoprotein cholesterol (VLDL) particle size decreased concomitantly, irrespective of treatment outcome. This observation likely reflects a direct effect on lipid metabolism associated with the inhibition of HCV replication[24]. This notion was further supported in several reports. Clark *et al*[25], used cholesterol metabolites as an indicator to evaluate the impact of HCV on lipid metabolism. In this study, genotype 3 but not genotype 2 showed a selected interference with late cholesterol synthesis pathway, resulting in hypocholesterolemia. However, this interference was resolved after SVR. Another Japanese study that included 100 subjects showed early rebound (within 28 d) in LDL level in CHC patients who underwent interferon-free DAA treatment. However, the elevation was regimen-specific, more prominent in the group who received daclatasvir and asunaprevir for 24 wk than in those received ledipasvir and sofosbuvir for 12 wk[26]. In addition, many reports correlated the rebound in lipid profile with treatment response status[27-29]. This was clearly demonstrated by Corey *et al*[18], which conducted a 2 steps study to evaluate the relationship between CHC infection and its treatment with lipid levels. After confirming that HCV infection is associated with significantly lower LDL concentrations in the first step, they found that remarkable hyperlipidemia was developed in patients who achieved viral clearance, compared to non-responders or those who relapsed. In the same context, some studies have further investigated the role HCV genotype on post-SVR hypercholesterolemia. In a study that included 215 patients, Fernández-Rodríguez *et al*[28] observed that increased serum cholesterol levels were associated with genotype 3 in patients who achieved SVR. In contrast, un-changed serum cholesterol figures were noted in genotype 3 non-responders and genotype 1 regardless of response[28]. Although the reversal of both hepatic steatosis and hypolipidemia has been reported only in genotype 3 in this study, there is accumulating evidence demonstrating that the reversal of hypolipidemia is not HCV-genotype specific[29,30].

Many reports proposed that atherosclerotic CVD risk increases after successful eradication of HCV due to the unfavorable lipid profile, which is a result of reversed hypolipidemia, represented in high serum LDL and small dense LDL. The latter has greater atherogenic potential and is a better marker for prediction of CVD than LDL[31,32]. The important question at this point is whether these patients require lipid-lowering treatment. According to the National Cholesterol Education Program Adult Treatment Plan Guideline III, patients should be put on lipid lowering agents for: (1) an LDL >100 mg/dL, if they have coronary heart disease or its equivalents[1]; (2) an LDL >130 mg/dL, if they have two or more major coronary heart disease risk factors[2]; and (3) an LDL >190 mg/dL, with none or one major risk factor[33]. Corey and his colleagues have found that 13% of their studied cohort had post-SVR LDL levels requiring lipid lowering therapy as these patients had values > 130 mg/dL plus presence of two or more major coronary heart disease risk factors. Nonetheless, before antiviral therapy, none of these patients had LDL readings requiring medications. Post-treatment lipid profile deterioration may reach clinically meaningful level requiring the consideration for cholesterol lowering therapy.

HEPATIC STEATOSIS

Hepatic steatosis is a frequent histological liver finding in patients with CHC[34]. Since HCV is known to hijack lipid metabolic pathways for virion maturation and secretion, several possible mechanisms of HCV-induced liver steatosis have been suggested. HCV induces lipogenesis by increasing intrahepatic fat milieu through sterol regulatory element binding protein 1c, which is a protein that overexpresses LDL receptors which in turn facilitates fatty acid uptake by hepatocytes, leading to higher intrahepatic fat content[35]. In contrast, HCV inhibits lipolysis by disturbing mitochondrial β-oxidation[36], either directly by the virus itself or indirectly *via* downregulation of the enzyme carnitine palmitoyltransferase-1, which regulates fatty acids oxidation[37,38]. These two mechanisms further potentiated by HCV-induced IR[39]. Moreover, HCV core protein suppresses the activity of microsomal triacylglycerol transfer protein, which is used for the assembly and secretion of VLDL, resulting in increased intracytoplasmic lipid droplets and therefore steatosis[40]. Miyoshi *et al*[41], who studied the role of HCV core protein in development of steatosis in HCV genotype 2, revealed that core protein activates the enzyme δ-9 desaturase, fatty acid metabolizing enzyme, and therefore leads to accumulation of triglycerides. This lipid metabolism disorder was also associated with mitochondrial dysfunction[41].

Hepatic steatosis is commonly reported among patients with HCV genotype 1 and genotype 3. Its occurrence in the latter has been correlated mainly to the previously mentioned mechanisms. Therefore, resolution of steatosis observed after successful viral eradication suggests a direct steatogenic pathway for HCV genotype 3[42]. This hypothesis was backed in a study of patients treated with interferon-based regimen, in which 91% of genotype 3 patients and only 43% of other genotypes have had their steatosis improved after viral cure[43]. Kumar *et al*[44], have also observed similar findings when steatosis was profoundly reduced in genotype 3 patients post-SVR, while no change irrespective of the treatment response occurred in genotype 1. Although development of fatty liver was associated with viral characteristics in genotype 3 (viral steatosis), the condition in genotype 1 corresponded to metabolic features such as glucose level and IR (metabolic steatosis). This observation suggests that in patients with genotype 1, factors other than the viral features play an essential role in the development of hepatic steatosis[28].

After achieving SVR with antiviral therapy, reversal of steatosis is the most common reported outcome, which was seen in several studies[17,27,28,39]. However, recent reviews showed contradictory findings[45,46]. In a prospective study that investigated the prevalence of hepatic steatosis and fibrosis in patients with CHC post-SVR, steatosis prevalence found to be 47.5%, almost as same as the pre-treatment figure (50%). Besides, overall average fibrosis score was reduced after viral clearance. Nevertheless, patients who had steatosis have maintained clinically significant fibrosis scores, compared to those without fatty liver[46]. In another study included 49 patients aimed to evaluate the impact of DAAs on glucose and lipid homeostasis, controlled attenuation parameter values were markedly increased at the end of follow up compared to baseline. More importantly, this finding was independent of weight gain, since no change in body mass index (BMI) was observed over time[45].

Patients with CHC and viral-induced hepatic steatosis have been shown to have worse hepatic outcomes in pre-treatment setting[47]. Nonetheless, a recent study depicts that presence of post-SVR steatosis does not carry a better risk profile[48]. In this study, which aimed to assess the effect of steatosis on HCC and all-cause mortality in CHC patients post-SVR, presence of fatty liver was associated with a considerable 7.5-fold increase in both primary endpoints[48]. Furthermore, there is also a substantially higher risk of EHMs, particularly CVD, after amelioration of steatosis post-SVR[17]. These findings combined highlight the importance of hepatic steatosis as a major risk factor for poor outcome and warrant a special consideration of screening and follow-up in this population.

INSULIN RESISTANCE & DIABETES MELLITUS

Based on multiple epidemiological studies, metabolic alterations such as IR, DM, and metabolic syndrome are frequent comorbidities in patients with CHC, as opposed to controls[19,49]. The rationale behind this association is still not completely understood but it could be attributed to the presence of liver disease, metabolic characteristics such as obesity, or the inflammatory process induced by HCV infection. HCV has been found to modulate insulin signaling pathways although the precise molecular mechanism of HCV-mediated IR is not fully understood. In two mouse-model experimental studies, HCV core protein was found to play a major role in the development of IR[50,51] particularly through *PA28γ* gene-dependent pathway[50]. HCV genotypes 1, 2 and 4[52] and genotypes 1 and 4[53] were noticed to have higher IR compared to genotype 3[52] and genotypes 2 and 3[53], respectively. Oxidative stress and proinflammatory cytokines were also found to play a role in *de novo* IR[54,55]. The disruption in glucose and lipid metabolism associated with IR[56] leads to evolution of hepatic steatosis and development of DM. Among subjects with chronic liver disease, the prevalence of DM in CHC patients prior to treatment varies from 13.6% to 67.4%, which is higher than that reported in individuals with other etiologies, such as chronic hepatitis B[57]. Furthermore, a case-control study demonstrated that the presence of CHC was associated with an over 11-fold increase in risk of developing DM over a follow-up period of 9 years[58]. DM seems to have a bidirectional relationship with HCV, in which the latter causes IR while DM is linked with more aggressive course of HCV-related outcomes such as progressive fibrosis[49,59,60], and increased risk of cirrhosis and HCC[61,62]. All the above conditions make patients with CHC more susceptible to have metabolic syndrome[63]. However, due to the hypolipidemia caused by HCV infection[64], which does not fit the traditional diagnostic criteria, a peculiar type of metabolic syndrome known as hepatitis C-associated dysmetabolic syndrome has been defined[63,65].

There is frequent evidence that have showed a beneficial effect of antiviral therapy using interferon-based regimens on IR in long-term HCV responders. Thompson *et al*[66], who studied 1038 non-diabetic patients, concluded that IR was substantially decreased in HCV-genotype 1 responders but not in genotype 1 non-responders or those with genotype 2 or 3 irrespective of treatment outcome. This finding was independent of any changes in BMI. Similar findings were also reported in a prospective study[29]. In the Virahep-C, a prospective multicenter study, an improvement in the homeostatic model assessment for IR (HOMA-IR) was observed 24 wk after treatment completion among HCV genotype 1 patients who had IR prior to therapy[67]. Nonetheless, Aghemo *et al*[68], who enrolled 384 non-diabetic patients with HCV genotypes 1 and 4 failed to display any differences in HOMA-IR values between baseline and 24 wk post-SVR. All the above findings indicate that longer follow-up may be needed to better assess glucose metabolism disturbances after HCV viral clearance with interferon-based regimens, especially in HCV genotype 1 patients. Paradoxically, in a head-to-head comparison of 178 subjects with HCV genotype 1 and 4 between interferon-based antiviral therapy and DAAs to assess metabolic outcomes, there was a significant elevation in HOMA-IR in those who have taken interferon-based regimen[11].

In addition to its effect on IR, antiviral therapy has been thought to decrease incidence of post-SVR hyperglycemia and DM. Interferon-based regimens have been studied extensively and they are usually associated with a decreased incidence of DM in non-diabetic patients with CHC after elimination of HCV[69]. However, several studies have emphasized the beneficial role of attaining SVR, which lessens glucose metabolism abnormalities induced by HCV infection[10,39,70]. Other studies could not detect any significant differences between treatment responders and non-

responders[71,72]. Despite these conflicting results, a meta-analysis that included seven studies aiming to investigate the correlation between HCV clearance *via* interferon-based regimens and the incidence of hyperglycemia demonstrated that SVR is associated with lower risk of hyperglycemia [odds ratio 0.49, 95% confidence interval (CI): 0.42-0.58]. Heterogeneity between studies was minimal, indicating a reliable result. On the other hand, the use of DAAs for viral eradication was not investigated thoroughly. Studies on incidence of DM in non-diabetic patients demonstrated less glucose disturbance in long-term responders[10,73]. In a retrospective study conducted in United States, 5127 non-diabetic subjects with HCV were enrolled to investigate how the response to HCV treatment impacted the risk of subsequent DM. The authors found that those who achieved SVR had markedly lower risk of developing DM, compared to those with treatment failure[10]. Two studies investigated the effect of DAAs on previously known diabetic patients[74,75]. One illustrated an improvement in glycemic status after viral cure while in the other there was no difference in glycosylated hemoglobin between pretreatment and post-treatment values. Importantly, one-third of patients in the latter study required escalation of anti-diabetic therapy during antiviral treatment. Further long-term prospective studies are still needed to resolve the current dilemma of changes on IR related to antiviral treatment.

CARDIOVASCULAR DISEASE

CHC infection has been linked to an array of EHMs, including an increased risk of CVD[76-79]. Several direct and indirect HCV pro-atherogenic mechanisms have been postulated. HCV is assumed to play a direct role in the development of arterial atherosclerosis by inducing endothelial dysfunction, likely through interleukin 1β[80], a pro-inflammatory cytokine. Likewise, it has been observed that HCV has the ability to live and replicate inside carotid plaques[81], which further supports an immediate pro-atherogenic effect. Moreover, chronic inflammation and oxidative stress that are caused by structural and non-structural viral proteins have also been shown to trigger plaque formation[80]. In a multicenter Italian study that evaluated the effect of attaining SVR using DAAs on subclinical carotid arteriosclerosis compared to an untreated cohort, ultrasonographic carotid measurements showed a significant reduction in mean carotid intima-media thickness in treatment group at the end of follow-up compared to baseline (from 0.94 mm to 0.81 mm, $P < 0.001$). No significant changes in the intima-media thickness were found in the control group. The BMI of these patients did not change during follow-up, while a significant increase in serum cholesterol levels was observed. The study concluded that eradication of HCV by DAAs led to an amelioration in carotid atherosclerosis, particularly intima-media thickness. Furthermore, HCV can also induce atherosclerosis indirectly since it is associated with an increased risk of metabolic syndrome components, including IR, DM, and hepatic steatosis, which are well-known predisposing factors for CVD[82-84]. On the other hand, some studies have failed to show any significant association between HCV and cardiovascular events[85,86].

Several studies have shown that either antiviral therapy or the attainment of SVR minimize CVD risk[87-90]. However, the results are rather controversial. Butt *et al*[87], who studied the effect of antiviral therapy, interferon- or DAAs-based regimens, on CVD risk found that the incidence of CVD in treatment arm was 7.2% in comparison with 13% in control group, regardless of the antiviral regimen. Treatment with DAAs was superior to interferon-based regimen, with a hazard ratio (HR) of 0.57 (95%CI: 0.51-0.65) and HR 0.78 (95%CI: 0.71-0.85), respectively. SVR was also associated with lower risk of incident CVD events HR 0.87 (95%CI: 0.77-0.98). In a nation-wide cohort study on Taiwanese residents with HCV who had received interferon-based regimens compared to an untreated cohort, antiviral therapy was associated with lower risks of acute coronary syndrome and ischemic stroke, with HR 0.77 (95%CI: 0.62-0.97) and HR 0.62 (95%CI: 0.46-0.83), respectively. This risk reduction was not observed in subject who had insufficient treatment course (< 16 wk)[88]. Further supporting data was observed in a study comprising 3385 HCV patients, which found that SVR was associated with a lower relative hazard reduction and absolute risk reduction for CVD[89]. However, some epidemiological studies have found contradictory findings. A large retrospective cohort study which enrolled 160875 subjects was aimed to investigate the impact of successful viral eradication on a variety of EHMs. In terms of CVD risk, the study concluded that SVR was associated with a diminished risk for stroke HR 0.84 (95%CI: 0.74-0.94), but not for CVD aHR 1.12 (95%CI: 0.81-1.56), when

compared to the untreated cohort[69]. From the same perspective, a negative result was also reported by Leone *et al*[72], who studied the influence of SVR on EHMs. The researchers did not find any significant cardiovascular risk reduction in SVR group compared to non-SVR, with HR 1.14 (95%CI: 0.57-2.3). Despite disparities in the findings across individual studies, a meta-analysis including 53841 patients demonstrated that SVR significantly reduces CVD risk, with a pooled of HR 0.76 (95%CI: 0.61-0.94)[91].

Apart from the direct treatment effect on CVD risk, therapeutic changes on other EHMs may also play role in the development of atherosclerotic events. Deteriorated lipid profile after HCV clearance has been shown to predispose patients to an elevated risk of CVD[31]. In a study of 617 patients with a mean follow-up of 26.8 mo, Huang *et al*[31] investigated whether deterioration of lipid profile post-SVR increased the risk of cardio-cerebral disease. Five patients developed cardio-cerebrovascular events (3 CVD and 2 cerebrovascular disease) over 1376 person-years. An LDL surge >40% was found to be the only predictor of these vascular events, with a HR of 15.44 (95%CI: 1.73-138.20)[31]. Evidence on risk of CVD in CHC pre- or post-treatment remains contro-versial. Nonetheless, most of the literature indicates that achieving SVR *via* antiviral therapy is associated with a significant risk reduction.

CONCLUSION

EHMs including cardiometabolic conditions are commonly seen among patients with CHC infection. Data on these conditions after elimination of HCV is inconsistent. However, the predominant evidence in the literature suggests that viral clearance using antiviral therapy leads to deterioration in lipid profile, reduction in the incidence of metabolic alteration such as IR, DM, and hepatic steatosis, and improvement in CVD risk. To determine more robust level of association between SVR and EHMs and to understand the exact mechanisms of how antiviral therapies act on these EHMs, large prospective studies with long-term follow-up are needed.

REFERENCES

1 **Petruzziello A**, Marigliano S, Loquercio G, Cozzolino A, Cacciapuoti C. Global epidemiology of hepatitis C virus infection: An up-date of the distribution and circulation of hepatitis C virus genotypes. *World J Gastroenterol* 2016; **22**: 7824-7840 [PMID: 27678366 DOI: 10.3748/wjg.v22.i34.7824]

2 **Perz JF**, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538 [PMID: 16879891 DOI: 10.1016/j.jhep.2006.05.013]

3 **Chayama K**, Takahashi S, Toyota J, Karino Y, Ikeda K, Ishikawa H, Watanabe H, McPhee F, Hughes E, Kumada H. Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. *Hepatology* 2012; **55**: 742-748 [PMID: 21987462 DOI: 10.1002/hep.24724]

4 **Osinubi A**, Harris AM, Vellozzi C, Lom J, Miller L, Millman AJ. Evaluation of the Performance of Algorithms That Use Serial Hepatitis C RNA Tests to Predict Treatment Initiation and Sustained Virological Response Among Patients Infected With Hepatitis C Virus. *Am J Epidemiol* 2019; **188**: 555-561 [PMID: 30535062 DOI: 10.1093/aje/kwy270]

5 **Swain MG**, Lai MY, Shiffman ML, Cooksley WG, Zeuzem S, Dieterich DT, Abergel A, Pessôa MG, Lin A, Tietz A, Connell EV, Diago M. A sustained virologic response is durable in patients with chronic hepatitis C treated with peginterferon alfa-2a and ribavirin. *Gastroenterology* 2010; **139**: 1593-1601 [PMID: 20637202 DOI: 10.1053/j.gastro.2010.07.009]

6 **Sáez-González E**, Vinaixa C, San Juan F, Hontangas V, Benlloch S, Aguilera V, Rubín A, García M, Prieto M, López-Andujar R, Berenguer M. Impact of hepatitis C virus (HCV) antiviral treatment on the need for liver transplantation (LT). *Liver Int* 2018; **38**: 1022-1027 [PMID: 29105320 DOI: 10.1111/liv.13618]

7 **Durand F**, Francoz C. The future of liver transplantation for viral hepatitis. *Liver Int* 2017; **37** Suppl 1: 130-135 [PMID: 28052618 DOI: 10.1111/liv.13310]

8 **Sherman AC**, Sherman KE. Extrahepatic manifestations of hepatitis C infection: navigating CHASM. *Curr HIV/AIDS Rep* 2015; **12**: 353-361 [PMID: 26208812 DOI: 10.1007/s11904-015-0274-8]

9 **Wu ZY**, Li JR, Huang MH, Cheng JJ, Li H, Chen JH, Lv XQ, Peng ZG, Jiang JD. Internal driving factors leading to extrahepatic manifestation of the hepatitis C virus infection. *Int J Mol Med* 2017; **40**: 1792-1802 [PMID: 29039494 DOI: 10.3892/ijmm.2017.3175]

10 **Li Y**, Wang X, Yu G, Sun H, Lv J, Chi X, Wu R, Gao X, Niu J. The association of hepatitis c virus

infection status with serum glucose levels. *BMC Gastroenterol* 2019; **19**: 86 [PMID: 31195990 DOI: 10.1186/s12876-019-1003-3]

11 **Carvalho JR**, Velosa J, Serejo F. Lipids, glucose and iron metabolic alterations in chronic hepatitis C after viral eradication - comparison of the new direct-acting antiviral agents with the old regimens. *Scand J Gastroenterol* 2018; **53**: 857-863 [PMID: 29779403 DOI: 10.1080/00365521.2018.1473486]

12 **Flores-Chávez A**, Carrion JA, Forns X, Ramos-Casals M. Extrahepatic manifestations associated with Chronic Hepatitis C Virus Infection. *Rev Esp Sanid Penit* 2017; **19**: 87-97 [PMID: 29364334 DOI: 10.4321/S1575-06202017000300004]

13 **Chang ML**. Metabolic alterations and hepatitis C: From bench to bedside. *World J Gastroenterol* 2016; **22**: 1461-1476 [PMID: 26819514 DOI: 10.3748/wjg.v22.i4.1461]

14 **Chang ML**, Hsu CM, Lin CH, Lin CY, Kuo CJ, Huang SW, Chen CW, Cheng HT, Yeh CT, Chiu CT. The Evolving Interplay among Abundant Adipokines in Patients with Hepatitis C during Viral Clearance. *Nutrients* 2017; **9** [PMID: 28574439 DOI: 10.3390/nu9060570]

15 **Forde KA**, Haynes K, Troxel AB, Trooskin S, Osterman MT, Kimmel SE, Lewis JD, Lo Re V 3rd. Risk of myocardial infarction associated with chronic hepatitis C virus infection: a population-based cohort study. *J Viral Hepat* 2012; **19**: 271-277 [PMID: 22404725 DOI: 10.1111/j.1365-2893.2011.01545.x]

16 **Chang ML**, Lin YS, Pao LH, Huang HC, Chiu CT. Link between plasminogen activator inhibitor-1 and cardiovascular risk in chronic hepatitis C after viral clearance. *Sci Rep* 2017; **7**: 42503 [PMID: 28211910 DOI: 10.1038/srep42503]

17 **Kawagishi N**, Suda G, Nakamura A, Kimura M, Maehara O, Suzuki K, Ohara M, Izumi T, Umemura M, Nakai M, Sho T, Natsuizaka M, Morikawa K, Ogawa K, Kudo Y, Nishida M, Miyoshi H, Sakamoto N. Liver steatosis and dyslipidemia after HCV eradication by direct acting antiviral agents are synergistic risks of atherosclerosis. *PLoS One* 2018; **13**: e0209615 [PMID: 30576386 DOI: 10.1371/journal.pone.0209615]

18 **Corey KE**, Kane E, Munroe C, Barlow LL, Zheng H, Chung RT. Hepatitis C virus infection and its clearance alter circulating lipids: implications for long-term follow-up. *Hepatology* 2009; **50**: 1030-1037 [PMID: 19787818 DOI: 10.1002/hep.23219]

19 **Mostafa A**, Mohamed MK, Saeed M, Hasan A, Fontanet A, Godsland I, Coady E, Esmat G, El-Hoseiny M, Abdul-Hamid M, Hughes A, Chaturvedi N. Hepatitis C infection and clearance: impact on atherosclerosis and cardiometabolic risk factors. *Gut* 2010; **59**: 1135-1140 [PMID: 20584782 DOI: 10.1136/gut.2009.202317]

20 **Wakita T**, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791-796 [PMID: 15951748 DOI: 10.1038/nm1268]

21 **Lindenbach BD**. Measuring HCV infectivity produced in cell culture and in vivo. *Methods Mol Biol* 2009; **510**: 329-336 [PMID: 19009272 DOI: 10.1007/978-1-59745-394-3_24]

22 **Aizawa Y**, Seki N, Nagano T, Abe H. Chronic hepatitis C virus infection and lipoprotein metabolism. *World J Gastroenterol* 2015; **21**: 10299-10313 [PMID: 26420957 DOI: 10.3748/wjg.v21.i36.10299]

23 **Ploss A**, Dubuisson J. New advances in the molecular biology of hepatitis C virus infection: towards the identification of new treatment targets. *Gut* 2012; **61** Suppl 1: i25-i35 [PMID: 22504917 DOI: 10.1136/gutjnl-2012-302048]

24 **Meissner EG**, Lee YJ, Osinusi A, Sims Z, Qin J, Sturdevant D, McHutchison J, Subramanian M, Sampson M, Naggie S, Patel K, Remaley AT, Masur H, Kottilil S. Effect of sofosbuvir and ribavirin treatment on peripheral and hepatic lipid metabolism in chronic hepatitis C virus, genotype 1-infected patients. *Hepatology* 2015; **61**: 790-801 [PMID: 25203718 DOI: 10.1002/hep.27424]

25 **Clark PJ**, Thompson AJ, Vock DM, Kratz LE, Tolun AA, Muir AJ, McHutchison JG, Subramanian M, Millington DM, Kelley RI, Patel K. Hepatitis C virus selectively perturbs the distal cholesterol synthesis pathway in a genotype-specific manner. *Hepatology* 2012; **56**: 49-56 [PMID: 22318926 DOI: 10.1002/hep.25631]

26 **Hashimoto S**, Yatsuhashi H, Abiru S, Yamasaki K, Komori A, Nagaoka S, Saeki A, Uchida S, Bekki S, Kugiyama Y, Nagata K, Nakamura M, Migita K, Nakao K. Rapid Increase in Serum Low-Density Lipoprotein Cholesterol Concentration during Hepatitis C Interferon-Free Treatment. *PLoS One* 2016; **11**: e0163644 [PMID: 27680885 DOI: 10.1371/journal.pone.0163644]

27 **Kuo YH**, Chuang TW, Hung CH, Chen CH, Wang JH, Hu TH, Lu SN, Lee CM. Reversal of hypolipidemia in chronic hepatitis C patients after successful antiviral therapy. *J Formos Med Assoc* 2011; **110**: 363-371 [PMID: 21741004 DOI: 10.1016/S0929-6646(11)60054-5]

28 **Fernández-Rodríguez CM**, López-Serrano P, Alonso S, Gutiérrez ML, Lledó JL, Pérez-Calle JL, Temiño R, Cacho G, Nevado M, Casas ML, Gasalla JM, Bonet B. Long-term reversal of hypocholesterolaemia in patients with chronic hepatitis C is related to sustained viral response and viral genotype. *Aliment Pharmacol Ther* 2006; **24**: 507-512 [PMID: 16886916 DOI: 10.1111/j.1365-2036.2006.03000.x]

29 **Chang ML**, Tsou YK, Hu TH, Lin CH, Lin WR, Sung CM, Chen TH, Cheng ML, Chang KC, Chiu CT, Yeh CT, Pang JH, Shiao MS. Distinct patterns of the lipid alterations between genotype 1 and 2 chronic hepatitis C patients after viral clearance. *PLoS One* 2014; **9**: e104783 [PMID: 25122116 DOI: 10.1371/journal.pone.0104783]

30 **Ramcharran D**, Wahed AS, Conjeevaram HS, Evans RW, Wang T, Belle SH, Yee LJ; Virahep-C Study Group. Associations between serum lipids and hepatitis C antiviral treatment efficacy.

Hepatology 2010; **52**: 854-863 [PMID: 20690192 DOI: 10.1002/hep.23796]

31 **Huang CF**, Dai CY, Yeh ML, Huang CI, Lee HC, Lai WT, Liang PC, Lin YH, Hsieh MY, Hou NJ, Lin ZY, Chen SC, Huang JF, Chuang WL, Yu ML. Cure or curd: Modification of lipid profiles and cardio-cerebrovascular events after hepatitis C virus eradication. *Kaohsiung J Med Sci* 2020; **36**: 920-928 [PMID: 32643842 DOI: 10.1002/kjm2.12275]

32 **Hoogeveen RC**, Gaubatz JW, Sun W, Dodge RC, Crosby JR, Jiang J, Couper D, Virani SS, Kathiresan S, Boerwinkle E, Ballantyne CM. Small dense low-density lipoprotein-cholesterol concentrations predict risk for coronary heart disease: the Atherosclerosis Risk In Communities (ARIC) study. *Arterioscler Thromb Vasc Biol* 2014; **34**: 1069-1077 [PMID: 24558110 DOI: 10.1161/ATVBAHA.114.303284]

33 **Grundy SM**, Cleeman JI, Merz CN, Brewer HB Jr, Clark LT, Hunninghake DB, Pasternak RC, Smith SC Jr, Stone NJ; National Heart, Lung, and Blood Institute; American College of Cardiology Foundation; American Heart Association. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation* 2004; **110**: 227-239 [PMID: 15249516 DOI: 10.1161/01.Cir.0000133317.49796.0e]

34 **Czaja AJ**, Carpenter HA, Santrach PJ, Moore SB. Host- and disease-specific factors affecting steatosis in chronic hepatitis C. *J Hepatol* 1998; **29**: 198-206 [PMID: 9722200 DOI: 10.1016/s0168-8278(98)80004-4]

35 **Syed GH**, Tang H, Khan M, Hassanein T, Liu J, Siddiqui A. Hepatitis C virus stimulates low-density lipoprotein receptor expression to facilitate viral propagation. *J Virol* 2014; **88**: 2519-2529 [PMID: 24352472 DOI: 10.1128/JVI.02727-13]

36 **Korenaga M**, Wang T, Li Y, Showalter LA, Chan T, Sun J, Weinman SA. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005; **280**: 37481-37488 [PMID: 16150732 DOI: 10.1074/jbc.M506412200]

37 **Cheng Y**, Dharancy S, Malapel M, Desreumaux P. Hepatitis C virus infection down-regulates the expression of peroxisome proliferator-activated receptor alpha and carnitine palmitoyl acyl-CoA transferase 1A. *World J Gastroenterol* 2005; **11**: 7591-7596 [PMID: 16437683 DOI: 10.3748/wjg.v11.i48.7591]

38 **Tsukuda Y**, Suda G, Tsunematsu S, Ito J, Sato F, Terashita K, Nakai M, Sho T, Maehara O, Shimazaki T, Kimura M, Morikawa K, Natsuizaka M, Ogawa K, Ohnishi S, Chuma M, Sakamoto N. Anti-adipogenic and antiviral effects of l-carnitine on hepatitis C virus infection. *J Med Virol* 2017; **89**: 857-866 [PMID: 27664407 DOI: 10.1002/jmv.24692]

39 **Yair-Sabag S**, Nussinson E, Ben-Assuli O, Shibli F, Shahbari A, Zelber-Sagi S. Retrospective study of the associations between hepatitis C virus infection and metabolic factors. *World J Hepatol* 2016; **8**: 1269-1278 [PMID: 27843537 DOI: 10.4254/wjh.v8.i30.1269]

40 **Perlemuter G**, Sabile A, Letteron P, Vona G, Topilco A, Chrétien Y, Koike K, Pessayre D, Chapman J, Barba G, Bréchot C. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J* 2002; **16**: 185-194 [PMID: 11818366 DOI: 10.1096/fj.01-0396com]

41 **Miyoshi H**, Moriya K, Tsutsumi T, Shinzawa S, Fujie H, Shintani Y, Fujinaga H, Goto K, Todoroki T, Suzuki T, Miyamura T, Matsuura Y, Yotsuyanagi H, Koike K. Pathogenesis of lipid metabolism disorder in hepatitis C: polyunsaturated fatty acids counteract lipid alterations induced by the core protein. *J Hepatol* 2011; **54**: 432-438 [PMID: 21093950 DOI: 10.1016/j.jhep.2010.07.039]

42 **Lonardo A**, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP. Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease. *Gastroenterology* 2004; **126**: 586-597 [PMID: 14762795 DOI: 10.1053/j.gastro.2003.11.020]

43 **Castéra L**, Hézode C, Roudot-Thoraval F, Lonjon I, Zafrani ES, Pawlotsky JM, Dhumeaux D. Effect of antiviral treatment on evolution of liver steatosis in patients with chronic hepatitis C: indirect evidence of a role of hepatitis C virus genotype 3 in steatosis. *Gut* 2004; **53**: 420-424 [PMID: 14960527 DOI: 10.1136/gut.2002.009936]

44 **Kumar D**, Farrell GC, Fung C, George J. Hepatitis C virus genotype 3 is cytopathic to hepatocytes: Reversal of hepatic steatosis after sustained therapeutic response. *Hepatology* 2002; **36**: 1266-1272 [PMID: 12395339 DOI: 10.1053/jhep.2002.36370]

45 **Graf C**, Welzel T, Bogdanou D, Vermehren J, Beckel A, Bojunga J, Friedrich-Rust M, Dietz J, Kubesch A, Mondorf A, Fischer S, Lutz T, Stoffers P, Herrmann E, Poynard T, Zeuzem S, Dultz G, Mihm U. Hepatitis C Clearance by Direct-Acting Antivirals Impacts Glucose and Lipid Homeostasis. *J Clin Med* 2020; **9** [PMID: 32825571 DOI: 10.3390/jcm9092702]

46 **Noureddin M**, Wong MM, Todo T, Lu SC, Sanyal AJ, Mena EA. Fatty liver in hepatitis C patients post-sustained virological response with direct-acting antivirals. *World J Gastroenterol* 2018; **24**: 1269-1277 [PMID: 29568207 DOI: 10.3748/wjg.v24.i11.1269]

47 **Leandro G**, Mangia A, Hui J, Fabris P, Rubbia-Brandt L, Colloredo G, Adinolfi LE, Asselah T, Jonsson JR, Smedile A, Terrault N, Pazienza V, Giordani MT, Giostra E, Sonzogni A, Ruggiero G, Marcellin P, Powell EE, George J, Negro F; HCV Meta-Analysis (on) Individual Patients' Data Study Group. Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. *Gastroenterology* 2006; **130**: 1636-1642 [PMID: 16697727 DOI: 10.1053/j.gastro.2006.03.014]

48 **Peleg N**, Issachar A, Sneh Arbib O, Cohen-Naftaly M, Harif Y, Oxtrud E, Braun M, Leshno M, Barsheshet A, Shlomai A. Liver steatosis is a major predictor of poor outcomes in chronic hepatitis C patients with sustained virological response. *J Viral Hepat* 2019; **26**: 1257-1265 [PMID: 31243878

DOI: 10.1111/jvh.13167]
49 **Moucari R**, Asselah T, Cazals-Hatem D, Voitot H, Boyer N, Ripault MP, Sobesky R, Martinot-Peignoux M, Maylin S, Nicolas-Chanoine MH, Paradis V, Vidaud M, Valla D, Bedossa P, Marcellin P. Insulin resistance in chronic hepatitis C: association with genotypes 1 and 4, serum HCV RNA level, and liver fibrosis. *Gastroenterology* 2008; **134**: 416-423 [PMID: 18164296 DOI: 10.1053/j.gastro.2007.11.010]
50 **Miyamoto H**, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T, Miyamura T, Koike K, Matsuura Y. Involvement of the PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein. *J Virol* 2007; **81**: 1727-1735 [PMID: 17135326 DOI: 10.1128/jvi.01683-06]
51 **Shintani Y**, Fujie H, Miyoshi H, Tsutsumi T, Tsukamoto K, Kimura S, Moriya K, Koike K. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; **126**: 840-848 [PMID: 14988838 DOI: 10.1053/j.gastro.2003.11.056]
52 **Mihm S**. Hepatitis C virus, diabetes and steatosis: clinical evidence in favor of a linkage and role of genotypes. *Dig Dis* 2010; **28**: 280-284 [PMID: 20460924 DOI: 10.1159/000282103]
53 **Sersté T**, Nkuize M, Moucari R, Van Gossum M, Reynders M, Scheen R, Vertongen F, Buset M, Mulkay JP, Marcellin P. Metabolic disorders associated with chronic hepatitis C: impact of genotype and ethnicity. *Liver Int* 2010; **30**: 1131-1136 [PMID: 20536721 DOI: 10.1111/j.1478-3231.2010.02291.x]
54 **Sheikh MY**, Choi J, Qadri I, Friedman JE, Sanyal AJ. Hepatitis C virus infection: molecular pathways to metabolic syndrome. *Hepatology* 2008; **47**: 2127-2133 [PMID: 18446789 DOI: 10.1002/hep.22269]
55 **Kawaguchi T**, Yoshida T, Harada M, Hisamoto T, Nagao Y, Ide T, Taniguchi E, Kumemura H, Hanada S, Maeyama M, Baba S, Koga H, Kumashiro R, Ueno T, Ogata H, Yoshimura A, Sata M. Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. *Am J Pathol* 2004; **165**: 1499-1508 [PMID: 15509521 DOI: 10.1016/s0002-9440(10)63408-6]
56 **Kawaguchi Y**, Mizuta T. Interaction between hepatitis C virus and metabolic factors. *World J Gastroenterol* 2014; **20**: 2888-2901 [PMID: 24659880 DOI: 10.3748/wjg.v20.i11.2888]
57 **Serfaty L**. Metabolic Manifestations of Hepatitis C Virus: Diabetes Mellitus, Dyslipidemia. *Clin Liver Dis* 2017; **21**: 475-486 [PMID: 28689587 DOI: 10.1016/j.cld.2017.03.004]
58 **Mehta SH**, Brancati FL, Strathdee SA, Pankow JS, Netski D, Coresh J, Szklo M, Thomas DL. Hepatitis C virus infection and incident type 2 diabetes. *Hepatology* 2003; **38**: 50-56 [PMID: 12829986 DOI: 10.1053/jhep.2003.50291]
59 **Hui JM**, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, McCaughan GW, George J. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]. *Gastroenterology* 2003; **125**: 1695-1704 [PMID: 14724822 DOI: 10.1053/j.gastro.2003.08.032]
60 **Cua IH**, Hui JM, Bandara P, Kench JG, Farrell GC, McCaughan GW, George J. Insulin resistance and liver injury in hepatitis C is not associated with virus-specific changes in adipocytokines. *Hepatology* 2007; **46**: 66-73 [PMID: 17596870 DOI: 10.1002/hep.21703]
61 **Veldt BJ**, Chen W, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, de Knegt RJ, Zeuzem S, Manns MP, Hansen BE, Schalm SW, Janssen HL. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. *Hepatology* 2008; **47**: 1856-1862 [PMID: 18506898 DOI: 10.1002/hep.22251]
62 **Chen CL**, Yang HI, Yang WS, Liu CJ, Chen PJ, You SL, Wang LY, Sun CA, Lu SN, Chen DS, Chen CJ. Metabolic factors and risk of hepatocellular carcinoma by chronic hepatitis B/C infection: a follow-up study in Taiwan. *Gastroenterology* 2008; **135**: 111-121 [PMID: 18505690 DOI: 10.1053/j.gastro.2008.03.073]
63 **Adinolfi LE**, Restivo L, Zampino R, Lonardo A, Loria P. Metabolic alterations and chronic hepatitis C: treatment strategies. *Expert Opin Pharmacother* 2011; **12**: 2215-2234 [PMID: 21883025 DOI: 10.1517/14656566.2011.597742]
64 **Negro F**. HCV infection and metabolic syndrome: which is the chicken and which is the egg? *Gastroenterology* 2012; **142**: 1288-1292 [PMID: 22537435 DOI: 10.1053/j.gastro.2011.12.063]
65 **Adinolfi LE**, Restivo L, Marrone A. The predictive value of steatosis in hepatitis C virus infection. *Expert Rev Gastroenterol Hepatol* 2013; **7**: 205-213 [PMID: 23445230 DOI: 10.1586/egh.13.7]
66 **Thompson AJ**, Patel K, Chuang WL, Lawitz EJ, Rodriguez-Torres M, Rustgi VK, Flisiak R, Pianko S, Diago M, Arora S, Foster GR, Torbenson M, Benhamou Y, Nelson DR, Sulkowski MS, Zeuzem S, Pulkstenis E, Subramanian GM, McHutchison JG; ACHIEVE-1 and ACHIEVE-2/3 Study Teams. Viral clearance is associated with improved insulin resistance in genotype 1 chronic hepatitis C but not genotype 2/3. *Gut* 2012; **61**: 128-134 [PMID: 21873466 DOI: 10.1136/gut.2010.236158]
67 **Conjeevaram HS**, Wahed AS, Afdhal N, Howell CD, Everhart JE, Hoofnagle JH; Virahep-C Study Group. Changes in insulin sensitivity and body weight during and after peginterferon and ribavirin therapy for hepatitis C. *Gastroenterology* 2011; **140**: 469-477 [PMID: 21070775 DOI: 10.1053/j.gastro.2010.11.002]
68 **Aghemo A**, Prati GM, Rumi MG, Soffredini R, D'Ambrosio R, Orsi E, De Nicola S, Degasperi E, Grancini V, Colombo M. Sustained virological response prevents the development of insulin resistance in patients with chronic hepatitis C. *Hepatology* 2012; **56**: 1681-1687 [PMID: 22619107 DOI: 10.1002/hep.25867]

69 **Mahale P**, Engels EA, Li R, Torres HA, Hwang LY, Brown EL, Kramer JR. The effect of sustained virological response on the risk of extrahepatic manifestations of hepatitis C virus infection. *Gut* 2018; **67**: 553-561 [PMID: 28634198 DOI: 10.1136/gutjnl-2017-313983]

70 **Arase Y**, Suzuki F, Suzuki Y, Akuta N, Kobayashi M, Kawamura Y, Yatsuji H, Sezaki H, Hosaka T, Hirakawa M, Ikeda K, Kumada H. Sustained virological response reduces incidence of onset of type 2 diabetes in chronic hepatitis C. *Hepatology* 2009; **49**: 739-744 [PMID: 19127513 DOI: 10.1002/hep.22703]

71 **Giordanino C**, Bugianesi E, Smedile A, Ciancio A, Abate ML, Olivero A, Pellicano R, Cassader M, Gambino R, Bo S, Ciccone G, Rizzetto M, Saracco G. Incidence of type 2 diabetes mellitus and glucose abnormalities in patients with chronic hepatitis C infection by response to treatment: results of a cohort study. *Am J Gastroenterol* 2008; **103**: 2481-2487 [PMID: 18702647 DOI: 10.1111/j.1572-0241.2008.02002.x]

72 **Leone S**, Prosperi M, Costarelli S, Nasta P, Maggiolo F, Di Giambenedetto S, Saracino A, Di Pietro M, Gori A. Incidence and predictors of cardiovascular disease, chronic kidney disease, and diabetes in HIV/HCV-coinfected patients who achieved sustained virological response. *Eur J Clin Microbiol Infect Dis* 2016; **35**: 1511-1520 [PMID: 27272121 DOI: 10.1007/s10096-016-2692-y]

73 **Li J**, Zhang T, Gordon SC, Rupp LB, Trudeau S, Holmberg SD, Moorman AC, Spradling PR, Teshale EH, Boscarino JA, Schmidt MA, Daida YG, Lu M; CHeCS Investigators. Impact of sustained virologic response on risk of type 2 diabetes among hepatitis C patients in the United States. *J Viral Hepat* 2018; **25**: 952-958 [PMID: 29478263 DOI: 10.1111/jvh.12887]

74 **Stine JG**, Wynter JA, Niccum B, Kelly V, Caldwell SH, Shah NL. Effect of Treatment with Direct Acting Antiviral on Glycemic Control in Patients with Diabetes Mellitus and Chronic Hepatitis C. *Ann Hepatol* 2017; **16**: 215-220 [PMID: 31153414 DOI: 10.5604/16652681.1231581]

75 **Abdo M**, Rabiee A, Abdellatif Z, Abdel Alem S, Moustafa A. Impact of sustained virological response on metabolic disorders in diabetic chronic hepatitis C virus patients after treatment with generic sofosbuvir and daclatasvir. *Eur J Gastroenterol Hepatol* 2020 [PMID: 32804853 DOI: 10.1097/MEG.0000000000001903]

76 **Ambrosino P**, Lupoli R, Di Minno A, Tarantino L, Spadarella G, Tarantino P, Nasto A, Celentano A, Di Minno MN. The risk of coronary artery disease and cerebrovascular disease in patients with hepatitis C: A systematic review and meta-analysis. *Int J Cardiol* 2016; **221**: 746-754 [PMID: 27428315 DOI: 10.1016/j.ijcard.2016.06.337]

77 **Petta S**, Maida M, Macaluso FS, Barbara M, Licata A, Craxì A, Cammà C. Hepatitis C Virus Infection Is Associated With Increased Cardiovascular Mortality: A Meta-Analysis of Observational Studies. *Gastroenterology* 2016; **150**: 145-155. quiz e15-6 [PMID: 26386298 DOI: 10.1053/j.gastro.2015.09.007]

78 **Vassalle C**, Masini S, Bianchi F, Zucchelli GC. Evidence for association between hepatitis C virus seropositivity and coronary artery disease. *Heart* 2004; **90**: 565-566 [PMID: 15084562 DOI: 10.1136/hrt.2003.018937]

79 **Sawayama Y**, Okada K, Maeda S, Ohnishi H, Furusyo N, Hayashi J. Both hepatitis C virus and Chlamydia pneumoniae infection are related to the progression of carotid atherosclerosis in patients undergoing lipid lowering therapy. *Fukuoka Igaku Zasshi* 2006; **97**: 245-255 [PMID: 17087362]

80 **Adinolfi LE**, Zampino R, Restivo L, Lonardo A, Guerrera B, Marrone A, Nascimbeni F, Florio A, Loria P. Chronic hepatitis C virus infection and atherosclerosis: clinical impact and mechanisms. *World J Gastroenterol* 2014; **20**: 3410-3417 [PMID: 24707124 DOI: 10.3748/wjg.v20.i13.3410]

81 **Boddi M**, Abbate R, Chellini B, Giusti B, Giannini C, Pratesi G, Rossi L, Pratesi C, Gensini GF, Paperetti L, Zignego AL. Hepatitis C virus RNA localization in human carotid plaques. *J Clin Virol* 2010; **47**: 72-75 [PMID: 19896417 DOI: 10.1016/j.jcv.2009.10.005]

82 **Mehta SH**, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL. Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. *Ann Intern Med* 2000; **133**: 592-599 [PMID: 11033586 DOI: 10.7326/0003-4819-133-8-200010170-00009]

83 **Butt AA**, Fultz SL, Kwoh CK, Kelley D, Skanderson M, Justice AC. Risk of diabetes in HIV infected veterans pre- and post-HAART and the role of HCV coinfection. *Hepatology* 2004; **40**: 115-119 [PMID: 15239093 DOI: 10.1002/hep.20289]

84 **Shaheen M**, Echeverry D, Oblad MG, Montoya MI, Teklehaimanot S, Akhtar AJ. Hepatitis C, metabolic syndrome, and inflammatory markers: results from the Third National Health and Nutrition Examination Survey [NHANES III]. *Diabetes Res Clin Pract* 2007; **75**: 320-326 [PMID: 16919355 DOI: 10.1016/j.diabres.2006.07.008]

85 **Arcari CM**, Nelson KE, Netski DM, Nieto FJ, Gaydos CA. No association between hepatitis C virus seropositivity and acute myocardial infarction. *Clin Infect Dis* 2006; **43**: e53-e56 [PMID: 16912934 DOI: 10.1086/507031]

86 **Völzke H**, Schwahn C, Wolff B, Mentel R, Robinson DM, Kleine V, Felix SB, John U. Hepatitis B and C virus infection and the risk of atherosclerosis in a general population. *Atherosclerosis* 2004; **174**: 99-103 [PMID: 15135257 DOI: 10.1016/j.atherosclerosis.2004.01.010]

87 **Butt AA**, Yan P, Shuaib A, Abou-Samra AB, Shaikh OS, Freiberg MS. Direct-Acting Antiviral Therapy for HCV Infection Is Associated With a Reduced Risk of Cardiovascular Disease Events. *Gastroenterology* 2019; **156**: 987-996. e8 [PMID: 30445009 DOI: 10.1053/j.gastro.2018.11.022]

88 **Hsu YC**, Ho HJ, Huang YT, Wang HH, Wu MS, Lin JT, Wu CY. Association between antiviral treatment and extrahepatic outcomes in patients with hepatitis C virus infection. *Gut* 2015; **64**: 495-503 [PMID: 25398770 DOI: 10.1136/gutjnl-2014-308163]

89 **Innes HA**, McDonald SA, Dillon JF, Allen S, Hayes PC, Goldberg D, Mills PR, Barclay ST, Wilks D, Valerio H, Fox R, Bhattacharyya D, Kennedy N, Morris J, Fraser A, Stanley AJ, Bramley P, Hutchinson SJ. Toward a more complete understanding of the association between a hepatitis C sustained viral response and cause-specific outcomes. *Hepatology* 2015; **62**: 355-364 [PMID: 25716707 DOI: 10.1002/hep.27766]

90 **Nahon P**, Bourcier V, Layese R, Audureau E, Cagnot C, Marcellin P, Guyader D, Fontaine H, Larrey D, De Lédinghen V, Ouzan D, Zoulim F, Roulot D, Tran A, Bronowicki JP, Zarski JP, Leroy V, Riachi G, Calès P, Péron JM, Alric L, Bourlière M, Mathurin P, Dharancy S, Blanc JF, Abergel A, Serfaty L, Mallat A, Grangé JD, Attali P, Bacq Y, Wartelle C, Dao T, Benhamou Y, Pilette C, Silvain C, Christidis C, Capron D, Bernard-Chabert B, Zucman D, Di Martino V, Thibaut V, Salmon D, Ziol M, Sutton A, Pol S, Roudot-Thoraval F; ANRS CO12 CirVir Group. Eradication of Hepatitis C Virus Infection in Patients With Cirrhosis Reduces Risk of Liver and Non-Liver Complications. *Gastroenterology* 2017; **152**: 142-156. e2 [PMID: 27641509 DOI: 10.1053/j.gastro.2016.09.009]

91 **Lapumnuaypol K**, Thongprayoon C, Wijarnpreecha K, Cheungpasitporn W. Impact of hepatitis C sustained viral response on cardiovascular diseases: a meta-analysis. *Hosp Pract (1995)* 2019; **47**: 105-110 [PMID: 31018721 DOI: 10.1080/21548331.2019.1612066]

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ORIGINAL ARTICLE

Basic Study

S100 calcium binding protein A6 and associated long noncoding ribonucleic acids as biomarkers in the diagnosis and staging of primary biliary cholangitis

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Abstract

BACKGROUND

Primary biliary cholangitis (PBC) is a chronic and slowly progressing cholestatic disease, which causes damage to the small intrahepatic bile duct by immuno-regulation, and may lead to cholestasis, liver fibrosis, cirrhosis and, eventually, liver failure.

AIM

To explore the potential diagnosis and staging value of plasma S100 calcium binding protein A6 (S100A6) messenger ribonucleic acid (mRNA), LINC00312, LINC00472, and LINC01257 in primary biliary cholangitis.

METHODS

A total of 145 PBC patients and 110 healthy controls (HCs) were enrolled. Among them, 80 PBC patients and 60 HCs were used as the training set, and 65 PBC patients and 50 HCs were used as the validation set. The relative expression levels of plasma S100A6 mRNA, long noncoding ribonucleic acids LINC00312, LINC00472 and LINC01257 were analyzed using quantitative reverse transcription-polymerase chain reaction. The bile duct ligation (BDL) mouse model was used to simulate PBC. Then double immunofluorescence was conducted to verify the overexpression of S100A6 protein in intrahepatic bile duct cells of BDL mice. Human intrahepatic biliary epithelial cells were treated with glycochenodeoxycholate to simulate the cholestatic environment of intrahepatic biliary epithelial cells in PBC.

RESULTS

The expression of S100A6 protein in intrahepatic bile duct cells was up-regulated in the BDL mouse model compared with sham mice. The relative expression

internationally accepted principles for the care and use of laboratory animals [China Medical University Application for Laboratory Animal Welfare and Ethical review (201702 Edition)].

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levels of plasma S100A6 mRNA, log10 LINC00472 and LINC01257 were up-regulated while LINC00312 was down-regulated in plasma of PBC patients compared with HCs (3.01 ± 1.04 *vs* 2.09 ± 0.87, $P < 0.0001$; 2.46 ± 1.03 *vs* 1.77 ± 0.84, $P < 0.0001$; 3.49 ± 1.64 *vs* 2.37 ± 0.96, $P < 0.0001$; 1.70 ± 0.33 *vs* 2.07 ± 0.53, $P < 0.0001$, respectively). The relative expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated and LINC00312 was down-regulated in human intrahepatic biliary epithelial cells treated with glycochenodeoxycholate compared with control (2.97 ± 0.43 *vs* 1.09 ± 0.08, $P = 0.0018$; 2.70 ± 0.26 *vs* 1.10 ± 0.10, $P = 0.0006$; 2.23 ± 0.21 *vs* 1.10 ± 0.10, $P = 0.0011$; 1.20 ± 0.04 *vs* 3.03 ± 0.15, $P < 0.0001$, respectively). The mean expression of S100A6 in the advanced stage (III and IV) of PBC was up-regulated compared to that in HCs and the early stage (II) (3.38 ± 0.71 *vs* 2.09 ± 0.87, $P < 0.0001$; 3.38 ± 0.71 *vs* 2.57 ± 1.21, $P = 0.0003$, respectively); and in the early stage (II), it was higher than that in HCs (2.57 ± 1.21 *vs* 2.09 ± 0.87, $P = 0.03$). The mean expression of LINC00312 in the advanced stage was lower than that in the early stage and HCs (1.39 ± 0.29 *vs* 1.56 ± 0.33, $P = 0.01$; 1.39 ± 0.29 *vs* 2.07 ± 0.53, $P < 0.0001$, respectively); in addition, the mean expression of LINC00312 in the early stage was lower than that in HCs (1.56 ± 0.33 *vs* 2.07 ± 0.53, $P < 0.0001$). The mean expression of log10 LINC00472 in the advanced stage was higher than those in the early stage and HCs (2.99 ± 0.87 *vs* 1.81 ± 0.83, $P < 0.0001$; 2.99 ± 0.87 *vs* 1.77 ± 0.84, $P < 0.0001$, respectively). The mean expression of LINC01257 in both the early stage and advanced stage were up-regulated compared with HCs (3.88 ± 1.55 *vs* 2.37 ± 0.96, $P < 0.0001$; 3.57 ± 1.79 *vs* 2.37 ± 0.96, $P < 0.0001$, respectively). The areas under the curves (AUC) for S100A6, LINC00312, log10 LINC00472 and LINC01257 in PBC diagnosis were 0.759, 0.7292, 0.6942 and 0.7158, respectively. Furthermore, the AUC for these four genes in PBC staging were 0.666, 0.661, 0.839 and 0.5549, respectively. The expression levels of S100A6 mRNA, log10 LINC00472, and LINC01257 in plasma of PBC patients were decreased (2.35 ± 1.02 *vs* 3.06 ± 1.04, $P = 0.0018$; 1.99 ± 0.83 *vs* 2.33 ± 0.96, $P = 0.036$; 2.84 ± 0.92 *vs* 3.69 ± 1.54, $P = 0.0006$), and the expression level of LINC00312 was increased (1.95 ± 0.35 *vs* 1.73 ± 0.32, $P = 0.0007$) after treatment compared with before treatment using the paired *t*-test. Relative expression of S100A6 mRNA was positively correlated with log10 LINC00472 ($r = 0.683$, $P < 0.0001$); serum level of collagen type IV was positively correlated with the relative expression of log10 LINC00472 ($r = 0.482$, $P < 0.0001$); relative expression of S100A6 mRNA was positively correlated with the serum level of collagen type IV ($r = 0.732$, $P < 0.0001$). The AUC for the four biomarkers obtained in the validation set were close to the training set.

CONCLUSION

These four genes may potentially act as novel biomarkers for the diagnosis of PBC. Moreover, LINC00472 acts as a potential biomarker for staging in PBC.

Key Words: S100 calcium binding protein A6; Long noncoding ribonucleic acids; Primary biliary cholangitis; Biomarker; Diagnosis; Staging

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Core Tip: Primary biliary cholangitis (PBC) is an autoimmune liver disease which is characterized by intrahepatic cholestasis. The expression of S100 calcium binding protein A6 (S100A6) was up-regulated in a bile duct ligation mouse model compared with sham mice. The relative expression levels of plasma S100A6 messenger ribonucleic acid, LINC00472 and LINC01257 were up-regulated and the relative expression of LINC00312 was down-regulated in PBC patients. S100A6 and the three long noncoding ribonucleic acids can be used as biomarkers for PBC diagnosis and staging using receiver operating characteristic curve analysis. The results were further verified *in vitro* using intrahepatic biliary epithelial cells.

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INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic and slowly progressing cholestatic disease, which causes damage to the small intrahepatic bile duct by immuno-regulation, and may lead to cholestasis, liver fibrosis, cirrhosis and, eventually, liver failure. The injury mechanism of intrahepatic biliary epithelial cells (iBECs) is the key to investigate the pathogenesis of PBC, but the accurate relationship between cholestasis and liver fibrosis is still indistinct. Currently, liver injury caused by cholestasis is mainly studied using liver cell lines or liver cancer cell lines treated with hydrophobic bile acids[1], while iBECs, the main target cells of PBC, have rarely been studied.

S100 calcium binding protein A6 (S100A6), also known as calcyclin, is a Ca^{2+} binding protein and is a member of the S100 family. Its distribution in the body is specific to cells and tissues, having a high expression in normal epithelial cells and fibroblasts, as well as in some tumor cells[2]. As an intracellular protein, S100A6 is involved in the regulation of various cellular functions, such as proliferation, apoptosis, cytoskeletal dynamics, and cell response to different stressors. It is believed that S100A6 may be involved in the ubiquitination of beta catenin and play an important role in controlling the cell cycle process[3]. S100A6 can interact with the calcyclin-binding protein/Siah-1-interacting protein, which is a component of the ubiquitin ligase complex[4].

Long non-coding ribonucleic acids (lncRNAs) are involved in the regulation of a variety of intracellular processes[5]. As a structural component, lncRNAs can form a nucleic acid protein complex with gene regulatory transcription factors[6]. LncRNAs can also bind to specific transcription factors and change their cellular localization, thus affecting the transcription of target genes. Abnormal expression of lncRNAs in plasma has been shown to accurately predict several human diseases[7,8].

As a general rule, PBC diagnosis depends on titers of antimitochondrial antibody (AMA), serum level of alkaline phosphatase (ALP) and liver biopsy[9-11]. However, it is difficult to achieve an early diagnosis in AMA-negative patients, or to differentiate from other autoimmune liver diseases; thus, an invasive liver biopsy is required to make a definitive diagnosis, and this not only increases the financial burden of patients, but also brings mental and physical trauma to patients, often delaying the best time for treatment. However, after definite diagnosis, some patients fail to respond to ursodeoxycholic acid treatment and often have a poor prognosis or even progress to liver failure. The majority of PBC cases are diagnosed mostly at an advanced stage, so diagnosis and staging biomarkers of PBC are urgently needed.

In this study, we explored the value of S100A6 and its associated lncRNAs as potential biomarkers for the diagnosis and staging of PBC.

MATERIALS AND METHODS

Study design

This study included three phases (Figure 1): (1) The discovery phase, in which candidate genes and lncRNAs were searched using bioinformatics methods, and were then verified by a mouse model and cell model; (2) The training phase, in which quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to evaluate the relative expression levels of target gene and lncRNAs in the plasma of PBC patients and healthy controls, as well as to estimate their diagnosis and staging value; and (3) the validation phase, in which the diagnosis and staging value of target genes and lncRNAs was verified in another independent PBC cohort.

Identification of differentially expressed genes from the gene expression omnibus dataset

The GSE29776 array dataset was analyzed on the gene expression omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/)[12]. The dataset contains 6 mouse liver tissue samples, including 3 bile duct ligation (BDL) mouse samples and 3 sham mouse samples. "GEO2R" in the webpage was used to analyze the array database.

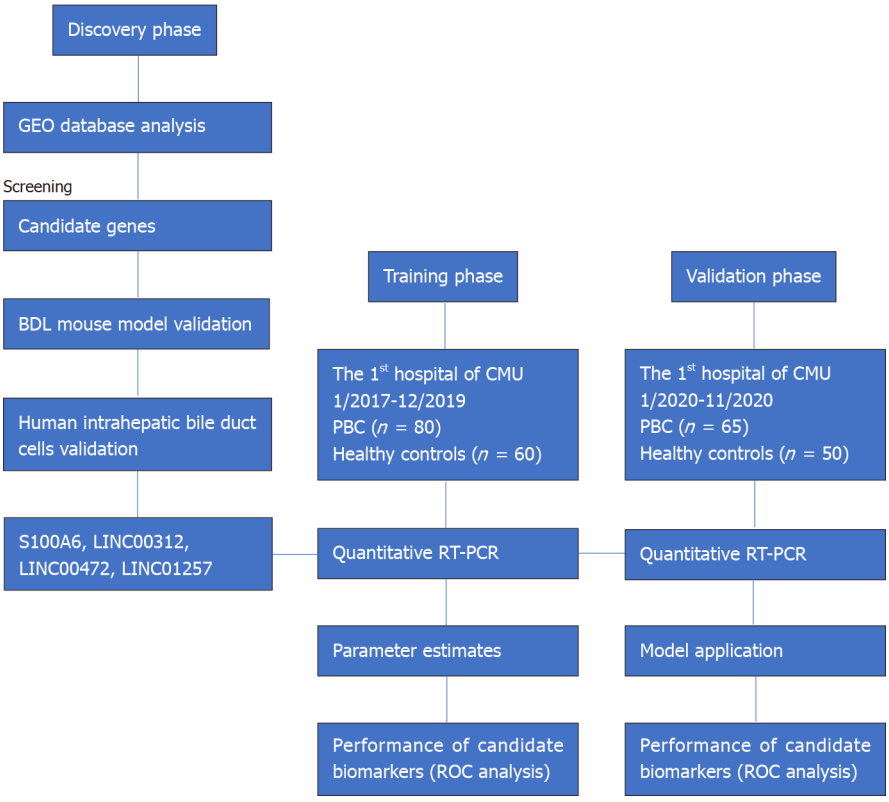


Figure 1 Study design. BDL: Bile duct ligation; GEO: Gene Expression Omnibus; PBC: Primary biliary cholangitis; ROC: Receiver operating characteristic curve; RT-PCR: Reverse transcriptase polymerase chain reaction.

LncRNAs selection

The PROMO usage database (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to predict the transcription factors of the S100A6 promoter[13]. There were multiple binding sites between the transcription factor estrogen receptor alpha (also known as ESR1) and the promoter of S100A6. Experiments with BDL mice and PBC patients suggested that the expression of estrogen receptor in bile duct epithelial cells was associated with cholestasis or bile duct epithelial cells in PBC[14]. We hypothesized that ESR1 could regulate the transcription of S100A6 as a transcription factor and thus play an important role in the injury of bile duct cells in PBC. The Gene-Cloud of Biotechnology Information database (https://www.gcbi.com.cn/gcanalyze/html/generadar/index) was used to screen lncRNAs associated with ESR1[15]. The binding force between lncRNAs and ESR1 was calculated by RNA-Protein Interaction Prediction (http://pridb.gdcb.iastate.edu/RPISeq/)[16]. As RF and SVM scores of LINC00312, LINC00472, and LINC01257 were all found to be close to 1.0, these three lncRNAs were selected as candidate lncRNAs in this study.

Animal studies, bile duct ligation model

Male C57BL/6J mice (aged 6-8 wk) were purchased from the Animal Experiment Department of China Medical University (Shenyang, Liaoning Province, China). All mice were weighed and randomly grouped with an average weight of 20-25 g into the BDL group and the sham group. To simulate cholestasis, 9 mice underwent BDL[17]. The animal protocol was designed to minimize pain or discomfort to the mice. The animals were acclimatized to laboratory conditions (24 °C, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. The BDL procedure was performed with the common bile duct doubly ligated under anesthesia *via* laparotomy[18]. The sham procedure was performed *via* a similar laparotomy without BDL. Animal experiments were approved by the Ethics Committee of the Animal Experiment Department of China Medical University.

A portion of the liver tissue was placed in a 4% p-formaldehyde solution and routinely processed for histological assessment, while the remaining tissue was snap frozen and stored at -80 °C.

Histological analysis

The mice were sacrificed by cervical dislocation and the liver was immediately removed by laparotomy. Part of the right lobe of the liver was fixed in 4% formaldehyde. The liver tissues were embedded in paraffin and sliced. Hematoxylin and eosin stained liver sections were observed under a light microscope at x 400 magnification to evaluate whether the cholestasis model was successfully established[19].

Double immunofluorescence

To identify whether the expression of S100A6 protein was up-regulated in bile duct epithelial cells in BDL mice, we performed double immunofluorescence[20] for S100A6 antibodies (Abcam, USA, Cat. No. ab181975) with cytokeratin 19 (CK19) antibodies (Abcam, USA, Cat. No. ab52625) which was specifically expressed in epithelial cells[21]. The primary antibody was replaced by rabbit or mouse IgG for negative controls. The working concentration of fluorescein isothiocyanate and tetraethyl rhodamine isothiocyanate was 1:50. Nuclei were counterstained with DAPI. The empirical procedure was performed according to the manufacturer's instructions. The sections were counterstained with DAPI and evaluated under a conventional fluorescence microscope.

Cells culture and treatments

Human intrahepatic biliary epithelial cells (HiBECs) were purchased from Guangzhou Jennio Biotech Company Limited (Guangzhou, Guangdong Province, China). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (GEMINI, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin in incubators at 37 °C with 5% CO_2. HiBECs were treated with 1000 mmol/L glycochenodeoxycholate (GCDC)[22] for 24 h to mimic cholestasis in PBC patients.

Patients

A total of 80 untreated PBC patients and 60 healthy controls as the training set were enrolled in order to differentially evaluate S100A6 and lncRNAs. In addition, another cohort consisting of 65 PBC patients and 50 healthy controls was used as the validation set. PBC patients were diagnosed by the Department of Gastroenterology or Rheumatology of The First Affiliated Hospital of China Medical University between January 2017 and November 2020. The diagnosis of PBC needed to meet two of the following three criteria[9-11]: (1) AMA titer > 1:40; (2) ALP level 1.5-times higher than the normal upper limit for more than 24 wk; and (3) liver biopsy revealing non-suppurative cholangitis and interlobular bile duct damage. Written informed consent was obtained from all patients who participated in the study. This study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University and was carried out in accordance with the Declaration of Helsinki.

Percutaneous ultrasound-guided puncture biopsy of the right liver was performed in all PBC patients, followed by histopathological examination and pathological stage identification[23]. Four stages were defined based on intrahepatic bile duct injury[24]: Stage I: Cholangitis stage, chronic inflammation in the interlobular and septal bile duct. Lymphocytes and plasma cells around the damaged bile ducts infiltrate or form granuloma, but the inflammation in the portal area does not involve the liver parenchyma and there is no cholestasis; stage II: Periportal inflammation stage, with a continuous reduction in the number of interlobular bile ducts, reactive hyperplasia of bile ducts around the portal area, inflammation involving adjacent liver parenchyma and destruction of liver cells, and common focal necrosis, cholestasis also occurs; stage III: Progressive fibrosis stage, the portal area is continuously enlarged by inflammation and fibrosis progression, the fibrous septa formed gradually widens, and cholestasis is aggravated; stage IV: Liver cirrhosis stage, fibrous septa divides the liver parenchyma into patchy nodules, regenerating nodules, and forming pseudo lobules.

Extraction of total RNA from plasma samples and cells

The relative expression levels of S100A6 and lncRNAs in plasma were measured in PBC patients, as well as human intrahepatic biliary epithelial cell lines. Total RNA was extracted from plasma and HiBECs by an RNA extraction kit (Bioteke, China), according to the manufacturer's instructions.

Reverse transcription and quantitative PCR for S100A6 and lncRNAs

Total RNA was amplified by reverse transcription using a reverse transcription kit (PrimeScript™ RT Master Mix, TaKaRa, China)[25]. All reactions were completed in a

Themocycler (Mastercycler nexus, Eppendorf, Germany). Then, quantitative PCR was performed using SYBR® Premix Ex TaqTM II kit (Takara, China) on the LightCycler 480 (Roche, Germany). GAPDH was used as an internal reference, and served as an internal control for plasma RNA quality. S100A6 and lncRNAs expression were calculated by the $2^{-\Delta\Delta Ct}$ method {$2^{[(\text{Mean ct of RNA–mean ct of GAPDH}) - (\text{mean ct of control–mean ct of GAPDH})]}$}[26]. The calculated result was the relative quantitative expression value of S100A6 and lncRNAs compared with the internal reference. Primers for reactions were designed by Primer Premier 6.0 (Canada) software (Table 1)[27].

Statistical analysis

Statistical Package for Social Science 23.0 software (IBM Solutions Statistical Package for the Social Sciences Incorporated, USA) and GraphPad Prism 8 (GraphPad Software, Incorporated, San Diego, CA, USA) were used for all statistical analyses. The normal distribution data were recorded (mean ± SD), and comparisons between the two groups were performed using the unpaired *t*-test. The paired *t*-test was used to compare the expression levels before and after treatment. Non-normal distribution data were analyzed using the non-parametric Mann-Whitney *U* test[28]. Categorical data were analyzed using the χ^2 test. The correlation between the plasma level of S100A6 mRNA and lncRNAs was analyzed using Pearson or Spearman correlation analysis. Receiver operating characteristic (ROC) curves were constructed and the areas under the curves (AUC) were used to evaluate the value of plasma S100A6 mRNA and lncRNAs as biomarkers for the diagnosis and staging of PBC[29]. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of the target gene

"GEO2R" was used to analyze the differentially expressed genes in liver tissues of BDL and sham mice of GSE29776. The top 10 up- and down-regulated genes of GSE29776 in the BDL and sham group are listed in Table 2. To identify potential biomarkers for PBC diagnosis and staging, we used qRT-PCR to validate the analysis of bioinformatics up-regulated genes in plasma of 30 PBC patients and 30 healthy controls. It was found that S100A6 showed the greatest change in the plasma of PBC patients ($t = 20.28$, $P < 0.0001$) (Figure 2). Therefore, S100A6 was selected as the target gene in this study.

Expression of S100A6 protein in the BDL mouse model

HE staining revealed histological changes in liver tissues, with the BDL group showing liver cell swelling, vacuolar degeneration, and coagulative necrosis. Inflammatory cell infiltration was observed in the portal area and around the bile duct, and fibrosis around the bile duct (Figure 3A-C). In the sham group, there was no or minimal inflammatory cell infiltration around the portal area and bile duct (Figure 3D-F).

Double immunofluorescence staining was used to label CK19 and S100A6 proteins. A fluorescence microscope was used for observation and Image J software was used for graph analysis. The results showed that S100A6 labeled with fluorescein isothiocyanate showed emerald green fluorescence and CK19 labeled with tetraethyl rhodamine isothiocyanate showed red fluorescence. Red and green fluorescent overlapping images showed that CK19 and S100A6 proteins were positively expressed in the iBECs of BDL mice (Figure 4A-C), while these two proteins were weakly expressed in the iBECs of mice in the sham group (Figure 4D-F).

The expression of S100A6 and lncRNAs analyzed in HiBECs

To investigate the mechanism of S100A6 and lncRNAs, the expression of S100A6 and lncRNAs was studied in HiBECs. Normal and HiBECs treated with GCDC were detected by qRT-PCR. The relative expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated and LINC00312 was down-regulated in HiBECs treated with GCDC compared with controls (2.97 ± 0.43 *vs* 1.09 ± 0.08, $P = 0.0018$; 2.70 ± 0.26 *vs* 1.10 ± 0.10, $P = 0.0006$; 2.23 ± 0.21 *vs* 1.10 ± 0.10, $P = 0.0011$; 1.20 ± 0.04 *vs* 3.03 ± 0.15, $P < 0.0001$, respectively) (Figure 5).

Demographics and clinical features of PBC patients compared with healthy controls

There were no differences in age and gender between the training set and validation

Table 1 Primer sequences used in this study

Target gene	Forward sequence (5'→3')	Reverse sequence (5'→3')
S100A6	AATGTGCGTTGTGTAAGC	CGGTCCAAGTCTTCCATC
LINC00312	GGAAGGAATACCACAGAAGT	TGAAGAACAGGACATTGACA
LINC00472	AGAGTTGCTGTAGAAGAAGG	AGGAGGAGAGTAGAAGAGAC
LINC01257	TGCTGCGAATGATGACTT	AGGACTTGAATCTGCTACTG
HMGB2	TTACGTTCCTCCCAAAGGTG	TCTTTGGCTGACTGCTCAGA
RC3H2	TTGCAAAGAAATGCGTTGAG	GATTGGCAGACAACTGCTGA
ADAMTS1	CCTCTGTCTGTGTGCAAGGA	GTGGCTCCAGTTGGAATTGT
SERPINE1	CTCTCTCTGCCCTCACCAAC	GTGGAGAGGCTCTTGGTCTG
PALD1	GCCGAAGTTGTTCCCATTTA	GCTGAAAGTCAGAGCCAACC
GSTA4	TCCGTGAGATGGGTTTTAGC	TGCCAAAGAGATTGTGCTTG
ACTA2	TTCAATGTCCCAGCCATGTA	GAAGGAATAGCCACGCTCAG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

ACTA2: Actin alpha 2, smooth muscle; ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GSTA4: Glutathione S-transferase alpha 4; HMGB2: High mobility group box 2; PALD1: Phosphatase domain containing paladin 1; RC3H2: Ring finger and CCCH-type domains 2; S100A6: S100 calcium binding protein A6; SERPINE1: Serpin family E member 1.

set (P = 0.504 and P = 1.0, respectively, Table 3). Moreover, there were no differences in age and gender between PBC patients and healthy controls (P = 0.58 and P = 1.0, respectively). Clinical serological data including alanine aminotransferase, aspartate aminotransferase, ALP, gamma-glutamyl transpeptidase, total bilirubin, direct bilirubin, total bile acid, hyaluronic acid, laminin, collagen type IV (C-IV) and procollagen III were all significantly higher in PBC patients than in healthy controls (P < 0.0001, Table 4).

Differential expression of S100A6 and lncRNAs in PBC patients compared with HCs

To compare the mean expression levels of S100A6 mRNA, LINC00312, and LINC01257 in PBC patients and healthy controls, the Kolmogorov–Smirnov test was used to check normality. The results showed that these variables had a normal distribution (P > 0.05), and the t-test was used for analysis, as the relative expression of LINC00472 showed a skewed distribution, and was normally distributed after logarithmic conversion based on 10. The results showed that the expression levels of S100A6 mRNA, log10 LINC00472 and LINC1257 in PBC patients were significantly up-regulated compared to the healthy controls (3.01 ± 1.04 *vs* 2.09 ± 0.87; 2.46 ± 1.03 *vs* 1.77 ± 0.84; 3.49 ± 1.64 *vs* 2.37 ± 0.96, P values were all less than 0.0001, Figure 6A, C and D). The mean expression level of LINC00312 was significantly lower in PBC plasma samples compared with HCs (1.70 ± 0.33 *vs* 2.07 ± 0.53, P < 0.0001, Figure 6B).

Distribution of S100A6 and lncRNAs expression levels in different stages of PBC

The unpaired t-test analysis of variance was performed to evaluate differences in the expression of S100A6 and lncRNAs among different PBC stages and healthy controls (Figure 7). The results showed that the mean expression of S100A6 in the advanced stage (III and IV) of PBC was up-regulated compared to that in HCs and the early stage (II) (3.38 ± 0.71 *vs* 2.09 ± 0.87, P < 0.0001; 3.38 ± 0.71 *vs* 2.57 ± 1.21, P = 0.0003, respectively); and in the early stage (II), it was higher than that in HCs (2.57 ± 1.21 *vs* 2.09 ± 0.87, P = 0.03) (Figure 7A). The mean expression of LINC00312 in the advanced stage was lower than that in the early stage and HCs (1.39 ± 0.29 *vs* 1.56 ± 0.33, P = 0.01; 1.39 ± 0.29 *vs* 2.07 ± 0.53, P < 0.0001, respectively) (Figure 7B); in addition, the mean expression of LINC00312 in the early stage was lower than that in HCs (1.56 ± 0.33 *vs* 2.07 ± 0.53, P < 0.0001) (Figure 7B). The mean expression of log10 LINC00472 in the advanced stage was higher than that in the early stage and HCs (2.99 ± 0.87 *vs* 1.81 ± 0.83, P < 0.0001; 2.99 ± 0.87 *vs* 1.77 ± 0.84, P < 0.0001, respectively) (Figure 7C). The mean expression of LINC01257 in both the early stage and advanced stage were up-regulated compared with HCs (3.88 ± 1.55 *vs* 2.37 ± 0.96, P < 0.0001; 3.57 ± 1.79 *vs* 2.37 ± 0.96, P < 0.0001, respectively) (Figure 7D).

Table 2 Top 10 dysregulated genes in bile duct ligation and sham mice

Gene name	Transcript	Lg fold change
Up-regulated		
Hmgb2	ENSMUSG00000054717	3.53
Rc3h2	ENSMUSG00000075376	3.33
Adamts1	ENSMUSG00000022893	3.15
Serpine1	ENSMUSG00000037411	3.08
S100a6	ENSMUSG00000001025	2.98
Pald1	ENSMUSG00000020092	2.67
Gsta4	ENSMUSG00000032348	2.50
D17H6S56E-5	NM_033075	2.46
Acta2	ENSMUSG00000035783	2.39
Ifi204	ENSMUSG00000073489	2.33
Down-regulated		
Mcm10	ENSMUSG00000026669	3.23
Upp2	ENSMUSG00000026839	2.85
2810043O03Rik	AK012901.1	2.59
Dnaaf5	ENSMUSG00000025857	2.41
Sva	ENSMUSG00000023289	2.40
Naca	ENSMUSG00000061315	2.35
Dhps	ENSMUSG00000060038	2.33
Cdh15	ENSMUSG00000031962	2.26
Gzmm	ENSMUSG00000054206	2.20
Alox12	ENSMUSG00000000320	2.15

2810043O03Rik: RIKEN complementary deoxyribonucleic acid 2810043O03 gene; Acta2: Actin alpha 2, smooth muscle; Adamts1: A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1; Alox12: Arachidonate 12-lipoxygenase; Cdh15: Cadherin 15; D17H6S56E-5: Deoxyribonucleic acid segment, Chr 17, human D6S56E 5; Dhps: Deoxyhypusine synthase; Dnaaf5: Dynein, axonemal assembly factor 5; Gsta4: Glutathione S-transferase alpha 4; Gzmm: Granzyme M (lymphocyte met-ase 1); Hmgb2: High mobility group box 2; Ifi204: Interferon activated gene 204; Mcm10: Minichromosome maintenance 10 replication initiation factor; Naca: Nascent polypeptide-associated complex alpha polypeptide; Pald1: Phosphatase domain containing paladin 1; Rc3h2: Ring finger and CCCH-type domains 2; S100A6: S100 calcium binding protein A6; Serpine1: Serpin family E member 1; Sva: Seminal vesicle antigen; Upp2: Uridine phosphorylase 2.

Diagnosis and staging value of plasma S100A6 and lncRNAs for PBC patients

ROC curves were used to evaluate the potential diagnostic value of each biomarker for PBC. The AUC for S100A6, LINC00312, log10 LINC00472 and LINC01257 in PBC diagnosis were 0.759, 0.7292, 0.6942 and 0.7158, respectively (Figure 8A-D). Furthermore, AUC for these four genes in PBC staging were 0.666, 0.661, 0.839 and 0.5549, respectively (Figure 8E-H).

Pearson or Spearman correlation analysis was performed to evaluate the correlation between relative expression of S100A6 mRNA and lncRNAs, as well as relative expression of S100A6 mRNA or lncRNAs and clinical serological data in PBC patients. Relative expression of S100A6 mRNA was positively correlated with log10 LINC00472 (r = 0.683, P < 0.0001); serum level of C-IV was positively correlated with relative expression of log10 LINC00472 (r = 0.482, P < 0.0001); relative expression of S100A6 mRNA was positively correlated with serum level of C-IV (r = 0.732, P < 0.0001) (Figure 9).

Comparison of expression levels of biomarkers before and after treatment

A total of 58 PBC patients were followed up after their treatment for one year. Paired t-test analysis was used to compare the expression levels of these four genes before and after treatment. The relative expression of S100A6 mRNA, log10 LINC00472, and

Table 3 Demographics and clinical characteristics in the training and validation datasets

Characteristics	Training	Validation	*P* value
No.	140	115	-
Age, mean ± SD, yr	56.0 ± 13.9	57.2 ± 13.2	0.504
Gender, *n* (%)			
Male	17 (12.1)	13 (11.3)	
Female	123 (87.9)	102 (88.7)	1.0
Pathological stage			
I and II	36 (45.0)	26 (40.0)	
III and IV	44 (55.0)	39 (60.0)	0.614

Normally distributed data are expressed as mean ± SD. Categorical variable values are described as *n* (%). SD: Standard deviation.

Table 4 Demographics and clinical characteristics of primary biliary cholangitis patients and healthy controls[1]

Characteristics	PBC (*n* = 145)	HCs (*n* = 110)	*P* value
Age, mean ± SD, yr	56.1 ± 13.4	55.3 ± 11.8	0.58
Gender, *n* (%)			
Male	17 (12.5)	13 (16.7)	
Female	128 (87.5)	97 (83.3)	1.00
ALT, U/L	78.6 ± 35.7	18.4 ± 6.5	< 0.001
AST, U/L	104.8 ± 43.5	20.2 ± 4.3	< 0.001
ALP, U/L	257.4 ± 79.9	64.7 ± 14.5	< 0.001
γGT, U/L	416.7 ± 209.2	26.3 ± 10.4	< 0.001
TBIL, μmol/L	66.8 ± 10.6	11.8 ± 4.0	< 0.001
DBIL, μmol/L	51.9 ± 11.4	6.4 ± 0.5	< 0.001
TBA, μmol/L	71.3 ± 11.6	2.8 ± 0.4	< 0.001
HA, ng/mL	146.9 (104.6-190.1)	67.0 (53.9-79.7)	< 0.001
LN, ng/mL	148.9 (76.7-182.8)	70.4 (58.7-82.9)	< 0.001
C-IV, ng/mL	154.8 (121.1-192.0)	60.1 (55.2-66.7)	< 0.001
PC-III, ng/mL	161.0 (135.1-184.5)	57.3 (49.9-63.5)	< 0.001
Pathological stage			
I and II	62 (42.8)	-	
III and IV	83 (57.2)	-	

[1]Normally distributed data are expressed as means ± SD, variables with a skewed distribution are presented as median (interquartile range). Categorical variable values are described as *n* (%). γGT: Gamma-glutamyl transpeptidase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; C-IV: Collagen type IV; DBIL: Direct bilirubin; HA: Hyaluronic acid; LN, Laminin; PC-III: Procollagen III; TBA: Total bile acid; TBIL: Total bilirubin; SD: Standard deviation; PBC: Primary biliary cholangitis; HCs: Healthy controls.

LINC01257 were significantly decreased after treatment (2.35 ± 1.02 *vs* 3.06 ± 1.04, P = 0.0018; 1.99 ± 0.83 *vs* 2.33 ± 0.96, P = 0.036; 2.84 ± 0.92 *vs* 3.69 ± 1.54, P = 0.0006, respectively); in addition, the relative expression of LINC00312 increased significantly after treatment compared with before treatment (1.95 ± 0.35 *vs* 1.73 ± 0.32, P = 0.0007) (Figure 10).

Differences between PBC patients with high and low levels of LINC00472

According to ROC curves analysis, the AUC of log10 LINC00472 was 0.839 (P < 0.0001)

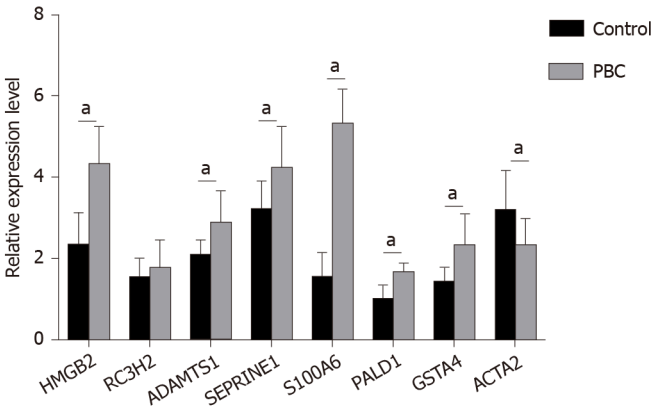


Figure 2 Validation of top 10 up-regulated genes in the plasma of primary biliary cholangitis patients and healthy controls by reverse transcriptase polymerase chain reaction. $^{a}P < 0.0001$. ACTA2: Actin alpha 2, smooth muscle; ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif 1; GSTA4: Glutathione S-transferase alpha 4; HMGB2: High mobility group box 2; PALD1: Phosphatase domain containing paladin 1; RC3H2: Ring finger and CCCH-type domains 2; S100A6: S100 calcium binding protein A6; SERPINE1: Serpin family E member 1; PBC: Primary biliary cholangitis.

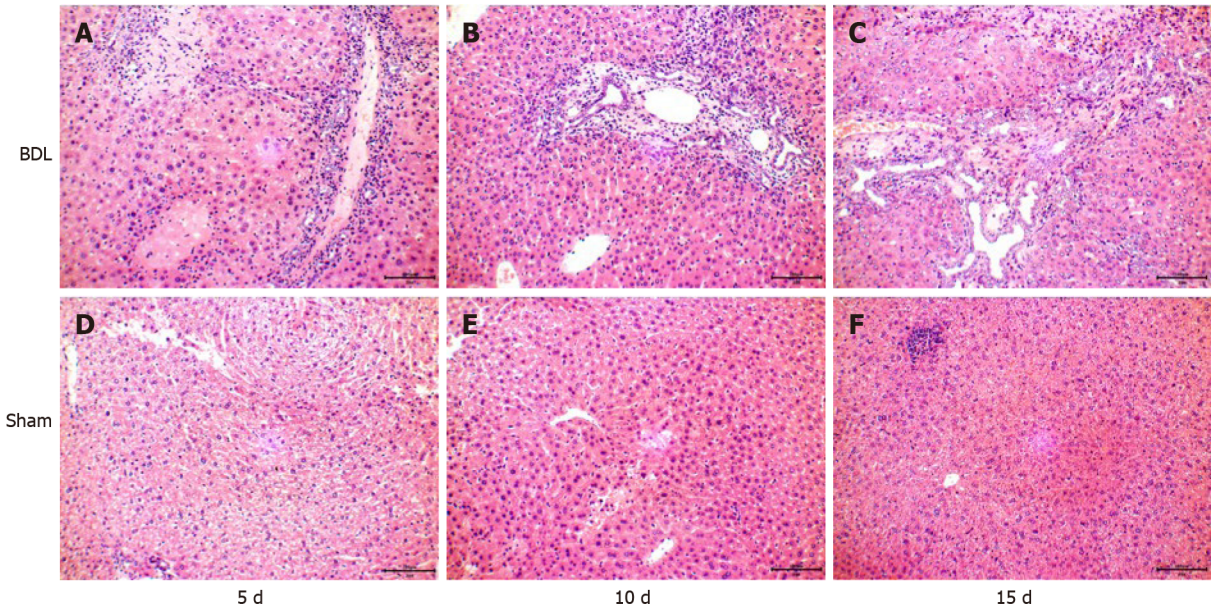


Figure 3 Liver tissues of mice in the bile duct ligation group and sham group were observed under an optical microscope (hematoxylin-eosin stain, × 400). A-C: 5, 10 and 15 d after surgery in the bile duct ligation group, respectively; D-F: 5, 10 and 15 d after surgery in the sham group, respectively. BDL: Bile duct ligation.

and the Youden index was 1.551. Accordingly, the patients in the PBC group were divided into L1 (log10 LINC00472 < 2.33) and L2 (log10 LINC00472 ≥ 2.33) subgroups. The baseline characteristics of PBC patients classified by the relative expression of the log10 LINC00472 cutoff value (2.33) is shown in Table 5. The relative expression of S100A6 mRNA and serum level of C-IV were lower in the L1 subgroup ($P < 0.0001$, Table 5); in addition, the relative expression of LINC01257 was higher in the L1 subgroup compared to the L2 subgroup ($P = 0.005$, Table 5).

Validation of diagnosis and staging value

The parameters estimated from the training data set were used to predict the probability of being diagnosed with PBC and staging of PBC for the independent validation data set. ROC curves were also constructed to predict the probability of diagnosis and staging. The AUC of S100A6 mRNA, LINC00312, log10 LINC00472 and LINC01257 in PBC diagnosis were 0.769, 0.772, 0.755 and 0.695, respectively (Figure 11A-D). Moreover, the AUC for log10 LINC00472 in PBC staging was 0.835 (Figure 11E).

Table 5 Characteristics of primary biliary cholangitis patients based on the expression of log10 LINC00472 cutoff value

	Relative Expression of log10 LINC00472		
	L1 (< 2.33) (*n* = 38)	L2 (≥ 2.33) (*n* = 42)	*P* value
Age, mean ± SD, years	60.3 ± 14.9	55.1 ± 14.0	0.109
Gender, *n* (%)			
Male	5 (13.2)	5 (11.9)	
Female	33 (86.8)	37 (88.1)	0.886
ALT, U/L	73.2 (52.7-100.1)	73.2 (46.2-100.7)	0.985
AST, U/L	109.2 ± 45.9	103.0 ± 44.6	0.543
ALP, U/L	264.0 ± 89.4	252.2 ± 78.3	0.532
γGT, U/L	420.2 ± 197.9	413.2 ± 237.1	0.887
TBA, μmol/L	73.2 ± 12.4	70.9 ± 13.3	0.438
TBiL, μmol/L	68.0 (63.0-73.0)	63.0 (58.0-73.0)	0.166
DBiL, μmol/L	50.6 ± 9.8	52.2 ± 11.4	0.505
LINC00312	1.51 ± 0.32	1.43 ± 0.31	0.261
S100A6	2.40 ± 1.05	3.57 ± 0.66	< 0.0001
LINC01257	4.25 ± 1.39	3.22 ± 1.78	0.005
HA, ng/mL	144.8 (101.6-208.8)	135.5 (95.4-195.4)	0.537
LN, ng/mL	126.1 (48.4-178.4)	156.1 (57.6-175.8)	0.78
C-IV, ng/mL	127.2 (100.9-170.4)	176.0 (154.7-232.0)	< 0.0001
PC-III, ng/mL	156.6 (125.8-190.1)	161.6 (128.0-184.5)	0.916

SD: Standard deviation; γGT: Gamma-glutamyl transpeptidase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; C-IV: Collagen type IV; DBIL: Direct bilirubin; HA: Hyaluronic acid; LN: Laminin; PC-III: Procollagen III; S100A6: S100 calcium binding protein A6; TBA: Total bile acid; TBIL: Total bilirubin.

DISCUSSION

PBC is a type of cholestatic liver disease which is a pathophysiological process caused by the obstruction of bile secretion and excretion. After analyzing the expression levels of the top 10 up-regulated genes of GSE29776 in the plasma of PBC patients, it was found that the difference in S100A6 mRNA expression levels between PBC patients and healthy controls was greatest (t = 20.28, P < 0.0001). Therefore, S100A6 was selected as the target gene. BDL is a common procedure for biliary obstruction widely used in rodent models of cholestasis and liver damage[30]. Immunofluorescence double labeling analysis was performed to identify the overexpression of S100A6 protein in the intrahepatic bile duct epithelial cells of BDL mice compared with sham mice, which verified the results predicted by bioinformatics analysis. In this study, the bile duct cells proliferated greatly in the liver tissue 10 d after the operation in the BDL group[31,32], and S100A6 protein was expressed in large quantities during the corresponding period. However, the number of bile duct cells in the sham group was relatively low, and the expression of S100A6 protein was also relatively low. Therefore, it can be seen that proliferation of bile duct cells was specifically enhanced when cholestatic liver injury occurred; thus, there was a difference in S100A6 between the two groups. S100A6 is expressed as a 89-amino acid protein in mice and rats, a 90-amino acid protein in humans and rabbits, and subtypes A (92 amino acids) and B (91 amino acids) in chickens, which may be produced by mRNA selective splicing[33]. In this study, the S100A6 antibodies used were universal in humans and mice, so the results of the BDL mouse model could indirectly reflect the up-regulation of S100A6 expression in human intrahepatic cholestasis.

In this study, S100A6 mRNA was overexpressed in the plasma of PBC patients compared with healthy controls. S100A6 expression is up-regulated in breast cancer, thyroid cancer, colorectal cancer, various types of skin tumors, acute myelogenous leukemia, epithelial tissues and other highly proliferating cell lines[34]. Apoptosis in

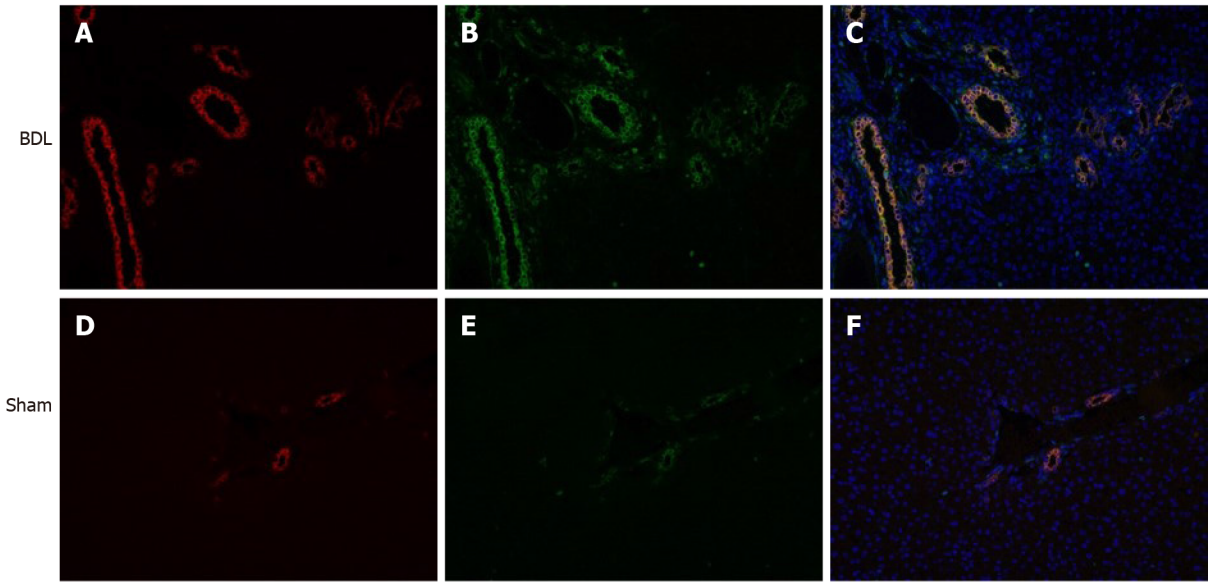


Figure 4 C57BL/6J mouse liver tissue double immunofluorescence (red light: Cytokeratin 19 protein, green light: S100 calcium binding protein A6 protein, × 200). A: Cytokeratin 19 (CK19) protein in bile duct ligation (BDL) mouse; B: S100 calcium binding protein A6 protein (S100A6) protein in BDL mouse; C: CK19 and S100A6 proteins merge in BDL mouse; D: CK19 protein in sham mouse; E: S100A6 protein in sham mouse; F: CK19 and S100A6 proteins merge in sham mouse. BDL: Bile duct ligation.

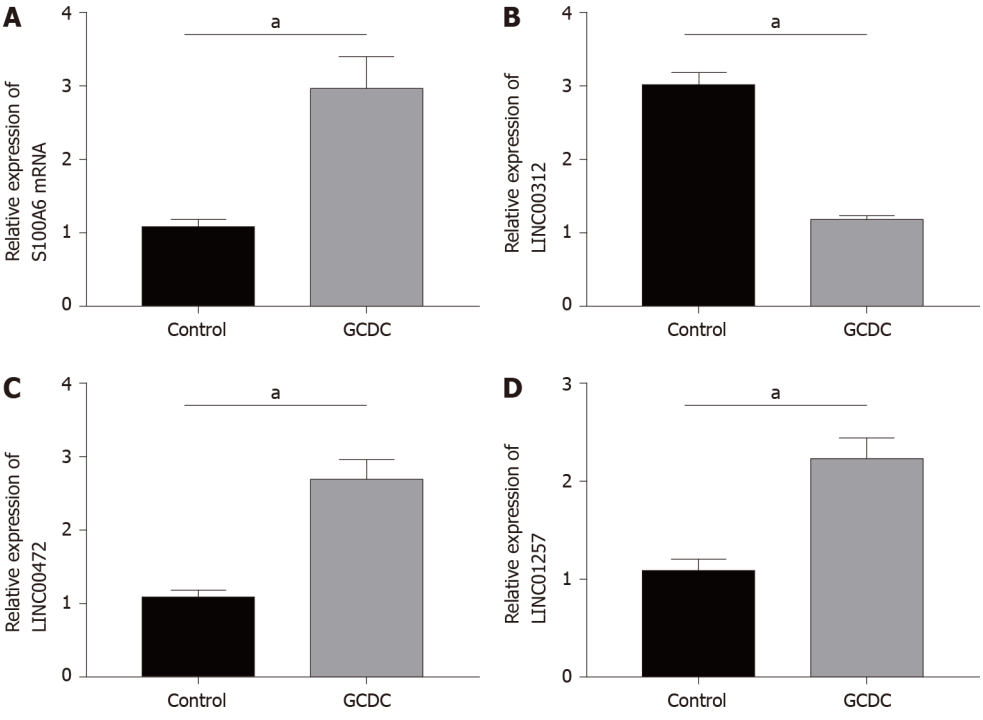


Figure 5 The expression of S100 calcium binding protein A6 protein messenger ribonucleic acid, LINC00312, LINC00472 and LINC01257 in human intrahepatic biliary epithelial cells (control and treated with glycochenodeoxycholate) analyzed by quantitative reverse transcription-polymerase chain reaction. A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: LINC00472; D: LINC01257. $^{a}P < 0.005$. GCDC: Glycochenodeoxycholate.

PBC is considered to be the cell effector injury mediated by T cells. Changes in apoptosis and apoptosis-related molecular expression of bile duct cells have been reported in bile duct lesions, but immune-mediated injury of bile duct epithelial cells has not been fully elucidated[35]. Joo *et al*[36] found that S100A6 may be involved in the process of apoptosis by regulating the transcriptional regulation of caspase-3. Therefore, it seems that S100A6 may play an important role in the pathogenesis of PBC.

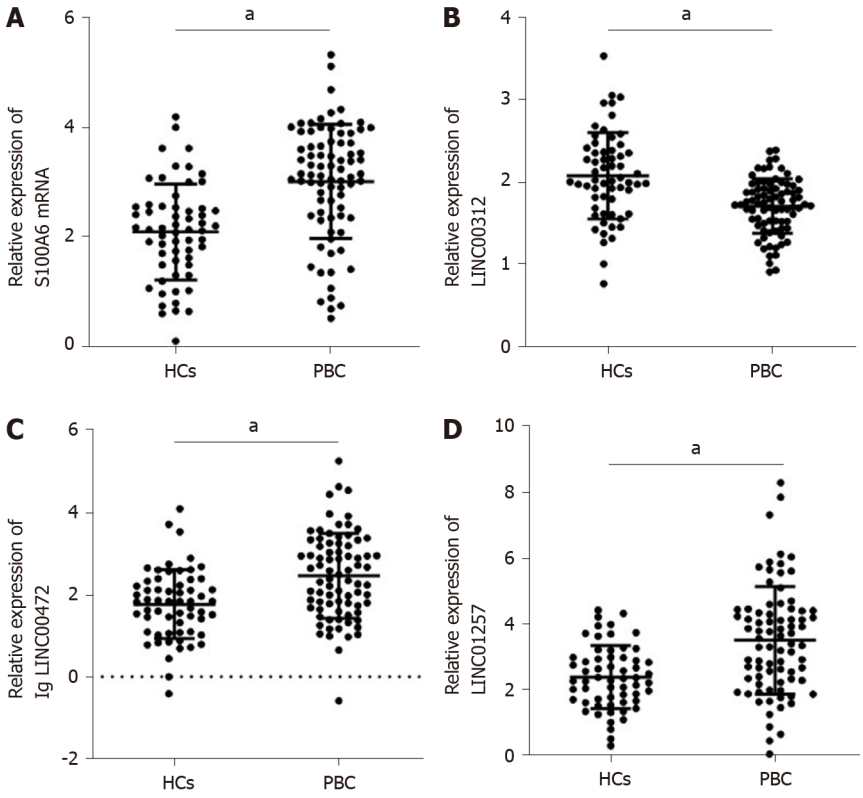


Figure 6 Differential expression of plasma s100 calcium binding protein A6 protein messenger ribonucleic acid and long non-coding ribonucleic acids in primary biliary cholangitis plasma samples compared with healthy controls. A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: log10 LINC00472; D: LINC01257. [a]*P* < 0.0001. HCs: Healthy controls; PBC: Primary biliary cholangitis.

The expression of lncRNAs is not only closely related to the occurrence and development of tumors[37], but also associated with autoimmune diseases[38]. In this study, the expression of lncRNAs selected by bioinformatics analysis was differentially expressed in the plasma of PBC patients compared with healthy controls. The levels of plasma LINC00312 was significantly down-regulated in PBC patients, while LINC00472 and LINC01257 were up-regulated in PBC patients, indicating that these lncRNAs might be valuable for PBC diagnosis. ROC curves were used to evaluate the diagnostic value of each marker. The differential expression in plasma between PBC patients and heathy controls indicated that S100A6 mRNA (AUC = 0.76, $P < 0.0001$), LINC00312 (AUC = 0.73, $P < 0.0001$), log10 LINC00472 (AUC = 0.69, $P < 0.0001$) and LINC01257 (AUC = 0.72, $P < 0.0001$) may be potential biomarkers for the diagnosis of PBC.

Furthermore, the ROC curves analysis also showed that plasma S100A6 mRNA (AUC = 0.67, $P = 0.01$), LINC00312 (AUC = 0.66, $P = 0.01$) and log10 LINC00472 (AUC = 0.84, $P < 0.0001$) could also be used to predict disease progression in PBC. In particular, LINC00472 had high diagnostic value for PBC staging (sensitivity was 77.27%, specificity was 77.78%). According to the cutoff value (2.33) of log10 LINC00472, the relative expression of S100A6 mRNA and serum level of C-IV in the high-level group were higher than those in the low-level group.

LINC00312, also known as NAG7, was found to inhibit proliferation and induce apoptosis in nasopharyngeal carcinoma (NPC) cells but also stimulate NPC cell invasion. LINC00312 was significantly down-regulated in NPC tissues compared with non-cancerous nasopharyngeal epithelium tissues. Positive expression of LINC00312 was negatively correlated with tumor size but positively correlated with lymph node metastasis[39]. High expression of LINC00472 was associated with less aggressive breast tumors and better prognosis. Patients with high expression of LINC00472 had a significantly reduced risk of recurrence and death compared to those with low expression. Patients with high expression of LINC00472 also responded better to adjuvant chemotherapy or hormone therapy than those with low expression[40]. Therefore, studies on S100A6, LINC00312 and LINC00472 have all been related to tumors. This study is the first to explore the relationship between these three genes and autoimmune diseases. In addition, we investigated the relationship between the expression of LINC01257 and diseases for the first time.

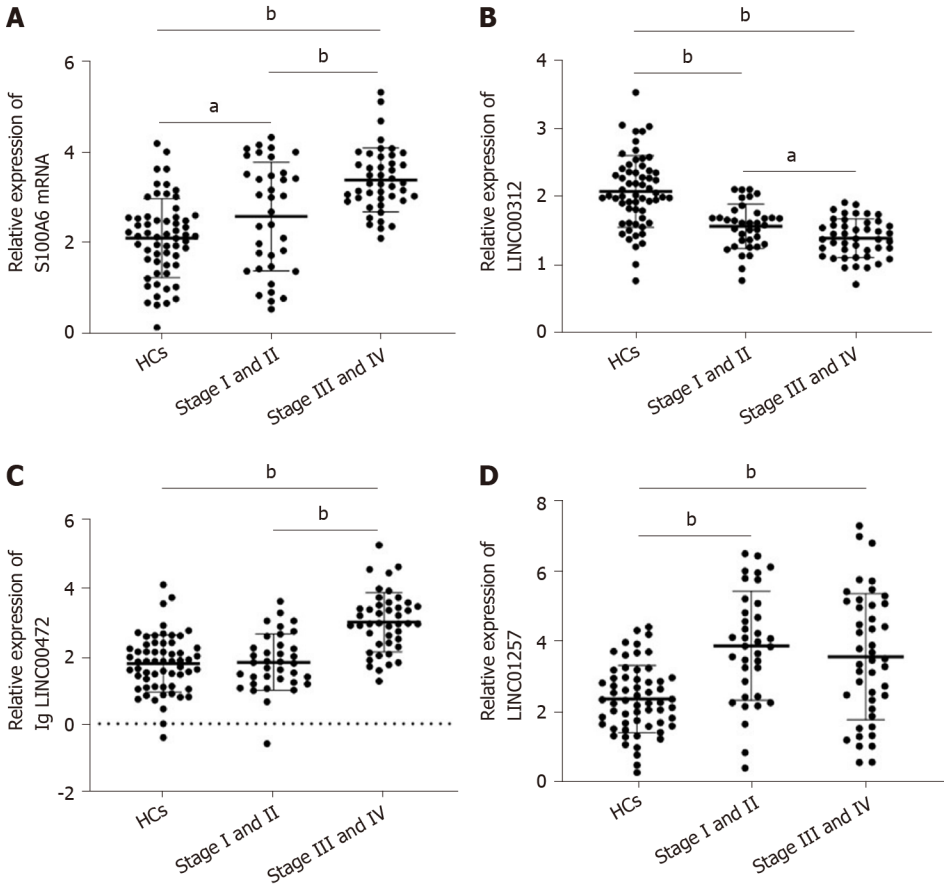


Figure 7 Scatter plot and distribution of expression levels of s100 calcium binding protein A6 protein messenger ribonucleic acid and long non-coding ribonucleic acids in different stages of primary biliary cholangitis compared with healthy controls. The unpaired *t*-test analysis of variance was performed to examine differences in S100 calcium binding protein A6 protein messenger ribonucleic acid and long non-coding ribonucleic acids expression levels between various groups. A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: log10 LINC00472; D: LINC01257. $^{a}P < 0.05$, $^{b}P < 0.0001$. HCs: Healthy controls.

The expression levels of plasma S100A6, LINC00312, LINC00472 and LINC01257 in PBC patients before and after treatment were analyzed by the paired *t*-test. It was found that the elevated biomarkers decreased after treatment, while the reduced biomarker increased. This provides further evidence that these four genes are biomarkers for PBC diagnosis.

The correlation analysis showed that relative expression of S100A6 mRNA was positively correlated with log10 LINC00472 ($r = 0.683$, $P < 0.0001$) and the serum level of C-IV ($r = 0.732$, $P < 0.0001$). C-IV serves as a histochemical marker of perisinusoidal basement membrane formation in liver disease[41]. It was further illustrated that S100A6 may be associated with PBC liver injury. The relative expression of log10 LINC00472 was positively correlated with the serum level of C-IV ($r = 0.482$, $P < 0.0001$), indicating that it was related to the disease severity of PBC. It was suggested that LINC00472 can be used as a marker of PBC staging. However, in our study, the four biomarkers did not correlate with the cholestasis indicator ALP, and we think this may be due to the following reasons: (1) The S100A6 protein was expressed in large quantities during the early period of cholestasis. This process may precede the increase in serum ALP level; (2) Proliferation of bile duct cells is characterized by irregular proliferation of intrahepatic bile ducts not only confined to portal areas, but also sprouting into periportal and parenchymal regions. This implies that the newly formed bile ducts are functionally ineffective[42,43]; and (3) In the late stage of liver fibrosis, considerable hepatocyte necrosis occurs.

Hepatocytes exposed to bile acids have been used in many studies on PBC. The most commonly used bile acid is GCDC, which is a type of toxic hydrophobic bile acid and can induce apoptosis of iBECs, form apoptotic bodies, and can lead to the pyruvate dehydrogenase complex E2 subunit as an autoimmune antigen to be exposed. A series of immune responses are then activated[44]. Hisamoto *et al*[45] studied the effects of hydrophobic bile acid on human BECs and autologous spleen mononuclear cells, especially the effects of GCDC on anion exchange protein

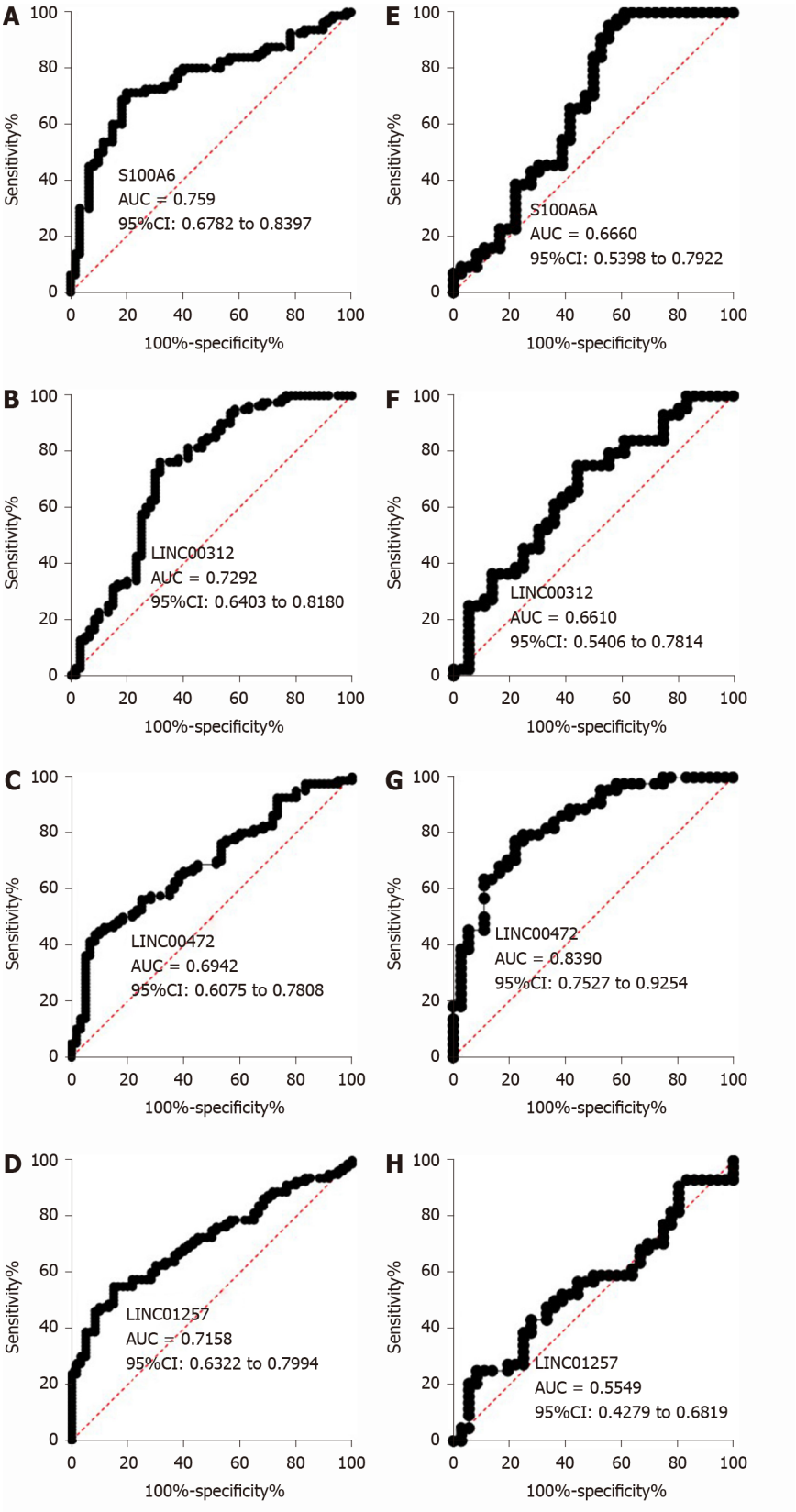


Figure 8 Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis and staging in the training set. A-D: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis in the training set; E-H: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis staging in the training set. AUC: Area under the curve; CI: Confidence interval.

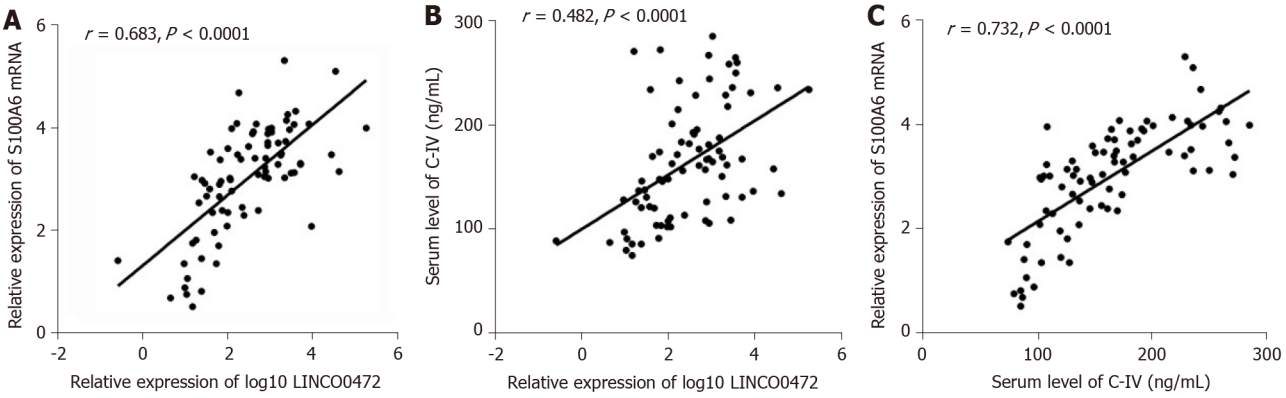


Figure 9 Correlation analysis of biomarkers and clinical serological indices. A: The positive correlation between relative expression of s100 calcium binding protein A6 protein messenger ribonucleic acid and log10 LINC00472, $r = 0.683$, $P < 0.0001$; B: The positive correlation between relative expression of log10 LINC00472 and serum level of collagen type IV, $r = 0.482$, $P < 0.0001$; C: The positive correlation between serum level of collagen type IV and relative expression of s100 calcium binding protein A6 protein messenger ribonucleic acid, $r = 0.732$, $P < 0.0001$.

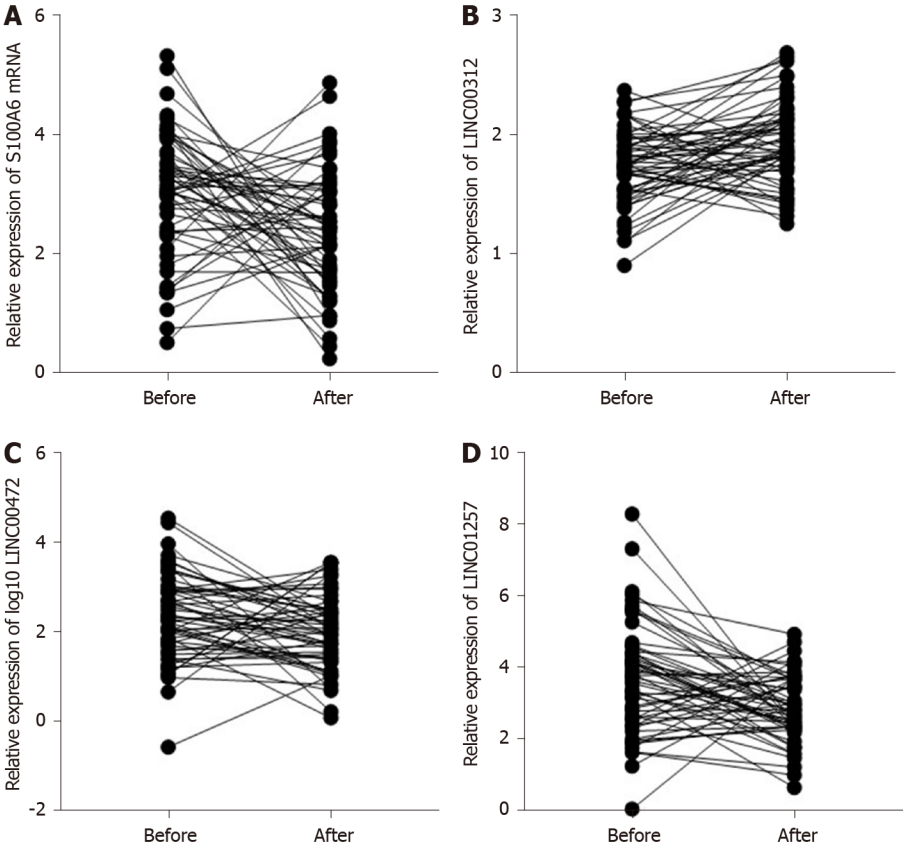


Figure 10 Comparison and analysis of s100 calcium binding protein A6 protein messenger ribonucleic acid, LINC00312, log10 LINC00472, LINC01257 expression levels in primary biliary cholangitis patients before and after treatment using the paired *t*-test. A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: log10 LINC00472; D: LINC01257.

expression of BECs and on the phenotype of BECs and local inflammatory response. It was proved that GCDC reduced the expression of anion exchange in BECs and accelerated the aging of BECs by inducing reactive oxygen species. Therefore, this study used GCDC to treat HiBECs to simulate a cholestatic environment and assess its damage to HiBECs. In this study, the expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated while LINC00312 was down-regulated in GCDC-treated HiBECs compared with controls, consistent with the expression in plasma of PBC patients. It was further proved that these four indicators are related to PBC diagnosis and staging.

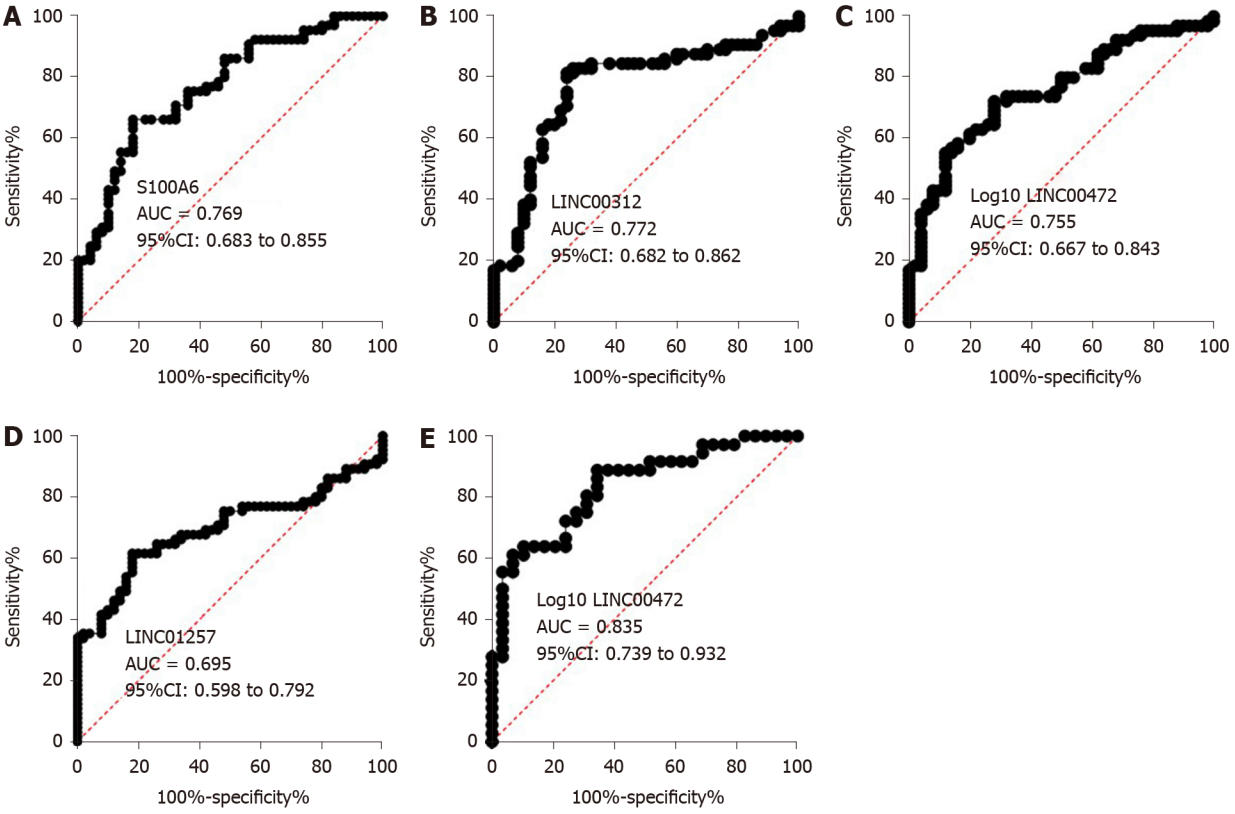


Figure 11 Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis and staging in the validation set. A-D: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis in the validation set; E: Receiver operating characteristic curves of LINC00472 for primary biliary cholangitis staging in the validation set. AUC: Area under the curve; CI: Confidence interval.

The value of the above four biomarkers should be validated in an additional cohort of PBC patients and their specificity needs to be examined in other patient populations[46]. We chose another PBC cohort as the validation set. The AUC of the four genes were close to those in the training set. Therefore, the value of these four biomarkers in the diagnosis and staging of PCB was validated. However, in China, the vast majority are Han Chinese; therefore, it is difficult to verify these findings in other ethnic groups.

CONCLUSION

In conclusion, the expression of S100A6 protein in BDL mice was up-regulated, the expression of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated, while LINC00312 was down-regulated both in the plasma of PBC patients and HiBECs treated with GCDC compared with controls. Although our study was confined to the expression analysis of S100A6 mRNA, LINC00312, LINC00472 and LINC01257, warranting further studies to investigate the mechanisms underlying the functional role of these four markers, nevertheless their potential as biomarkers for diagnosis and staging of PBC was elucidated by multiple evaluations in this study.

ARTICLE HIGHLIGHTS

Research background

Primary biliary cholangitis (PBC) is an autoimmune liver disease that mostly affects women. Fatigue and persistent pruritus are the most obvious symptoms. PBC may lead to cholestasis, liver fibrosis, cirrhosis and, eventually, liver failure. The injury mechanism of intrahepatic biliary epithelial cells is the key to investigating the pathogenesis of PBC, but the accurate relationship between cholestasis and liver

fibrosis is still indistinct.

Research motivation

To explore the target genes of intrahepatic biliary epithelial cell injury in PBC. To search for plasma biomarkers for early diagnosis and staging of PBC. To lay a foundation for further study on the pathogenesis of PBC.

Research objectives

To explore the potential diagnosis and staging value of plasma S100 calcium binding protein A6 (S100A6) messenger ribonucleic acid (mRNA), LINC00312, LINC00472, and LINC01257 in primary biliary cholangitis.

Research methods

The up-regulation of S100A6 was identified by double immunofluorescence in a bile duct ligation mouse model. We used quantitative reverse transcription-polymerase chain reaction to analyze the relative expression levels of S100A6 mRNA, long noncoding ribonucleic acids (lncRNAs) LINC00312, LINC00472 and LINC01257 both in patients with PBC and in human intrahepatic biliary epithelial cells treated with glycochenodeoxycholate.

Research results

The relative expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated while LINC00312 was down-regulated in both the plasma of patients with PBC and in human intrahepatic biliary epithelial cells treated with glycochenodeoxycholate.

Research conclusions

These four genes may potentially act as novel biomarkers for the diagnosis of PBC. Moreover, LINC00472 acts as a biomarker for staging in PBC.

Research perspectives

Although we have demonstrated that S100A6 and related lncRNAs may be biomarkers for the diagnosis and staging of PBC, their detailed value needs to be analyzed in a large sample. The specific mechanisms of S100A6 and lncRNAs require further investigation.

REFERENCES

1 **Woolbright BL**, McGill MR, Yan H, Jaeschke H. Bile Acid-Induced Toxicity in HepaRG Cells Recapitulates the Response in Primary Human Hepatocytes. *Basic Clin Pharmacol Toxicol* 2016; **118**: 160-167 [PMID: 26176423 DOI: 10.1111/bcpt.12449]

2 **Salama I**, Malone PS, Mihaimeed F, Jones JL. A review of the S100 proteins in cancer. *Eur J Surg Oncol* 2008; **34**: 357-364 [PMID: 17566693 DOI: 10.1016/j.ejso.2007.04.009]

3 **Matsuzawa SI**, Reed JC. Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. *Mol Cell* 2001; **7**: 915-926 [PMID: 11389839 DOI: 10.1016/s1097-2765(01)00242-8]

4 **Filipek A**, Kuźnicki J. Molecular cloning and expression of a mouse brain cDNA encoding a novel protein target of calcyclin. *J Neurochem* 1998; **70**: 1793-1798 [PMID: 9572262 DOI: 10.1046/j.1471-4159.1998.70051793.x]

5 **Veneziano D**, Di Bella S, Nigita G, Laganà A, Ferro A, Croce CM. Noncoding RNA: Current Deep Sequencing Data Analysis Approaches and Challenges. *Hum Mutat* 2016; **37**: 1283-1298 [PMID: 27516218 DOI: 10.1002/humu.23066]

6 **Geisler S**, Coller J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. *Nat Rev Mol Cell Biol* 2013; **14**: 699-712 [PMID: 24105322 DOI: 10.1038/nrm3679]

7 **Yang Y**, Cai Y, Wu G, Chen X, Liu Y, Wang X, Yu J, Li C, Chen X, Jose PA, Zhou L, Zeng C. Plasma long non-coding RNA, CoroMarker, a novel biomarker for diagnosis of coronary artery disease. *Clin Sci (Lond)* 2015; **129**: 675-685 [PMID: 26201019 DOI: 10.1042/CS20150121]

8 **Tang J**, Jiang R, Deng L, Zhang X, Wang K, Sun B. Circulation long non-coding RNAs act as biomarkers for predicting tumorigenesis and metastasis in hepatocellular carcinoma. *Oncotarget* 2015; **6**: 4505-4515 [PMID: 25714016 DOI: 10.18632/oncotarget.2934]

9 **Bowlus CL**, Gershwin ME. The diagnosis of primary biliary cirrhosis. *Autoimmun Rev* 2014; **13**: 441-444 [PMID: 24424173 DOI: 10.1016/j.autrev.2014.01.041]

10 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: management of cholestatic liver diseases. *J Hepatol* 2009; **51**: 237-267 [PMID: 19501929 DOI:

10.1016/j.jhep.2009.04.009]
11 **Zweers SJ**, de Vries EM, Lenicek M, Tolenaars D, de Waart DR, Koelfat KV, Groen AK, Olde Damink SW, Beuers U, Ponsioen C, Jansen PL, Schaap FG. Prolonged fibroblast growth factor 19 response in patients with primary sclerosing cholangitis after an oral chenodeoxycholic acid challenge. *Hepatol Int* 2017; **11**: 132-140 [PMID: 27696157 DOI: 10.1007/s12072-016-9769-7]
12 **Barrett T**, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M, Yefanov A, Lee H, Zhang N, Robertson CL, Serova N, Davis S, Soboleva A. NCBI GEO: archive for functional genomics data sets--update. *Nucleic Acids Res* 2013; **41**: D991-D995 [PMID: 23193258 DOI: 10.1093/nar/gks1193]
13 **Messeguer X**, Escudero R, Farré D, Núñez O, Martínez J, Albà MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* 2002; **18**: 333-334 [PMID: 11847087 DOI: 10.1093/bioinformatics/18.2.333]
14 **Alvaro D**, Alpini G, Onori P, Franchitto A, Glaser S, Le Sage G, Gigliozzi A, Vetuschi A, Morini S, Attili AF, Gaudio E. Effect of ovariectomy on the proliferative capacity of intrahepatic rat cholangiocytes. *Gastroenterology* 2002; **123**: 336-344 [PMID: 12105861 DOI: 10.1053/gast.2002.34169]
15 **Xiao J**, Liu A, Lu X, Chen X, Li W, He S, He B, Chen Q. Prognostic significance of TCF21 mRNA expression in patients with lung adenocarcinoma. *Sci Rep* 2017; **7**: 2027 [PMID: 28515486 DOI: 10.1038/s41598-017-02290-2]
16 **Wang L**, Yan X, Liu ML, Song KJ, Sun XF, Pan WW. Prediction of RNA-protein interactions by combining deep convolutional neural network with feature selection ensemble method. *J Theor Biol* 2019; **461**: 230-238 [PMID: 30321541 DOI: 10.1016/j.jtbi.2018.10.029]
17 **Grattagliano I**, Portincasa P, Palmieri VO, Palasciano G. Mutual changes of thioredoxin and nitrosothiols during biliary cirrhosis: results from humans and cholestatic rats. *Hepatology* 2007; **45**: 331-339 [PMID: 17256724 DOI: 10.1002/hep.21519]
18 **Soroka CJ**, Lee JM, Azzaroli F, Boyer JL. Cellular localization and up-regulation of multidrug resistance-associated protein 3 in hepatocytes and cholangiocytes during obstructive cholestasis in rat liver. *Hepatology* 2001; **33**: 783-791 [PMID: 11283840 DOI: 10.1053/jhep.2001.23501]
19 **Wen YA**, Liu D, Zhou QY, Huang SF, Luo P, Xiang Y, Sun S, Luo D, Dong YF, Zhang LP. Biliary intervention aggravates cholestatic liver injury, and induces hepatic inflammation, proliferation and fibrogenesis in BDL mice. *Exp Toxicol Pathol* 2011; **63**: 277-284 [PMID: 20149605 DOI: 10.1016/j.etp.2010.01.006]
20 **Bouter Y**, Meller B, Sahlmann CO, Wolf BJ, Langer L, Bankstahl JP, Wester HJ, Kropf S, Meller J, Bouter C. Immunohistochemical detection of chemokine receptor 4 expression in chronic osteomyelitis confirms specific uptake in 68Ga-Pentixafor-PET/CT. *Nuklearmedizin* 2018; **57**: 198-203 [PMID: 30267402 DOI: 10.3413/Nukmed-0971-18-04]
21 **Gurung A**, Yoshida EM, Scudamore CH, Hashim A, Erb SR, Webber DL. Primary hepatic neuroendocrine tumour requiring live donor liver transplantation: case report and concise review. *Ann Hepatol* 2012; **11**: 715-720 [PMID: 22947536]
22 **Woolbright BL**, Dorko K, Antoine DJ, Clarke JI, Gholami P, Li F, Kumer SC, Schmitt TM, Forster J, Fan F, Jenkins RE, Park BK, Hagenbuch B, Olyaee M, Jaeschke H. Bile acid-induced necrosis in primary human hepatocytes and in patients with obstructive cholestasis. *Toxicol Appl Pharmacol* 2015; **283**: 168-177 [PMID: 25636263 DOI: 10.1016/j.taap.2015.01.015]
23 **Czul F**, Levy C. Novel Therapies on Primary Biliary Cirrhosis. *Clin Liver Dis* 2016; **20**: 113-130 [PMID: 26593294 DOI: 10.1016/j.cld.2015.08.006]
24 **Trivedi PJ**, Hirschfield GM. Primary biliary cirrhosis: Renaming primary biliary cirrhosis-clarity or confusion? *Nat Rev Gastroenterol Hepatol* 2015; **12**: 678-679 [PMID: 26553417 DOI: 10.1038/nrgastro.2015.187]
25 **Li J**, Zhang M, An G, Ma Q. LncRNA TUG1 acts as a tumor suppressor in human glioma by promoting cell apoptosis. *Exp Biol Med (Maywood)* 2016; **241**: 644-649 [PMID: 26748401 DOI: 10.1177/1535370215622708]
26 **Livak KJ**, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-408 [PMID: 11846609 DOI: 10.1006/meth.2001.1262]
27 **Xu L**, Bao L, Gu S, Qin C. [Real-time PCR Detection Method for the Reston Subtype of the Ebola Virus]. *Bing Du Xue Bao* 2015; **31**: 276-281 [PMID: 26470534]
28 **Zimmerman DW**, Zumbo BD. The Relative Power of the Wilcoxon-Mann-Whitney Test and Student t Test Under Simple Bounded Transformations. *J Gen Psychol* 1990; **117**: 425-436 [PMID: 28142339 DOI: 10.1080/00221309.1990.9921148]
29 **Lorbeer R**, Ittermann T, Völzke H, Gläser S, Ewert R, Felix SB, Dörr M. Assessing cutoff values for increased exercise blood pressure to predict incident hypertension in a general population. *J Hypertens* 2015; **33**: 1386-1393 [PMID: 25807218 DOI: 10.1097/HJH.0000000000000568]
30 **Tag CG**, Sauer-Lehnen S, Weiskirchen S, Borkham-Kamphorst E, Tolba RH, Tacke F, Weiskirchen R. Bile duct ligation in mice: induction of inflammatory liver injury and fibrosis by obstructive cholestasis. *J Vis Exp* 2015 [PMID: 25741630 DOI: 10.3791/52438]
31 **Afroze SH**, Munshi MK, Martínez AK, Uddin M, Gergely M, Szynkarski C, Guerrier M, Nizamutdinov D, Dostal D, Glaser S. Activation of the renin-angiotensin system stimulates biliary hyperplasia during cholestasis induced by extrahepatic bile duct ligation. *Am J Physiol Gastrointest Liver Physiol* 2015; **308**: G691-G701 [PMID: 25678505 DOI: 10.1152/ajpgi.00116.2014]

32 **Graf A**, Meng F, Hargrove L, Kennedy L, Han Y, Francis T, Hodges K, Ueno Y, Nguyen Q, Greene JF, Francis H. Knockout of histidine decarboxylase decreases bile duct ligation-induced biliary hyperplasia *via* downregulation of the histidine decarboxylase/VEGF axis through PKA-ERK1/2 signaling. *Am J Physiol Gastrointest Liver Physiol* 2014; **307**: G813-G823 [PMID: 25169977 DOI: 10.1152/ajpgi.00188.2014]

33 **Allen BG**, Andrea JE, Sutherland C, Schönekess BO, Walsh MP. Molecular cloning of chicken calcyclin (S100A6) and identification of putative isoforms. *Biochem Cell Biol* 1997; **75**: 733-738 [PMID: 9599662]

34 **Ilg EC**, Schäfer BW, Heizmann CW. Expression pattern of S100 calcium-binding proteins in human tumors. *Int J Cancer* 1996; **68**: 325-332 [PMID: 8903474 DOI: 10.1002/(SICI)1097-0215(19961104)68:3<325::AID-IJC10>3.0.CO;2-7]

35 **Koga H**, Sakisaka S, Ohishi M, Sata M, Tanikawa K. Nuclear DNA fragmentation and expression of Bcl-2 in primary biliary cirrhosis. *Hepatology* 1997; **25**: 1077-1084 [PMID: 9141420 DOI: 10.1002/hep.510250505]

36 **Joo JH**, Yoon SY, Kim JH, Paik SG, Min SR, Lim JS, Choe IS, Choi I, Kim JW. S100A6 (calcyclin) enhances the sensitivity to apoptosis *via* the upregulation of caspase-3 activity in Hep3B cells. *J Cell Biochem* 2008; **103**: 1183-1197 [PMID: 17721932 DOI: 10.1002/jcb.21496]

37 **Hauptman N**, Glavač D. Long non-coding RNA in cancer. *Int J Mol Sci* 2013; **14**: 4655-4669 [PMID: 23443164 DOI: 10.3390/ijms14034655]

38 **Taghizadeh E**, Taheri F, Samadian MM, Soudyab M, Abi A, Gheibi Hayat SM. Role of long non-coding RNAs (LncRNAs) in multiple sclerosis: a brief review. *Neurol Sci* 2020; **41**: 2443-2451 [PMID: 32350675 DOI: 10.1007/s10072-020-04425-2]

39 **Zhang W**, Huang C, Gong Z, Zhao Y, Tang K, Li X, Fan S, Shi L, Li X, Zhang P, Zhou Y, Huang D, Liang F, Zhang X, Wu M, Cao L, Wang J, Li Y, Xiong W, Zeng Z, Li G. Expression of LINC00312, a long intergenic non-coding RNA, is negatively correlated with tumor size but positively correlated with lymph node metastasis in nasopharyngeal carcinoma. *J Mol Histol* 2013; **44**: 545-554 [PMID: 23529758 DOI: 10.1007/s10735-013-9503-x]

40 **Shen Y**, Katsaros D, Loo LW, Hernandez BY, Chong C, Canuto EM, Biglia N, Lu L, Risch H, Chu WM, Yu H. Prognostic and predictive values of long non-coding RNA LINC00472 in breast cancer. *Oncotarget* 2015; **6**: 8579-8592 [PMID: 25865225 DOI: 10.18632/oncotarget.3287]

41 **Mak KM**, Mei R. Basement Membrane Type IV Collagen and Laminin: An Overview of Their Biology and Value as Fibrosis Biomarkers of Liver Disease. *Anat Rec (Hoboken)* 2017; **300**: 1371-1390 [PMID: 28187500 DOI: 10.1002/ar.23567]

42 **Alvaro D**, Gigliozzi A, Attili AF. Regulation and deregulation of cholangiocyte proliferation. *J Hepatol* 2000; **33**: 333-340 [PMID: 10952254 DOI: 10.1016/s0168-8278(00)80377-3]

43 **Priester S**, Wise C, Glaser SS. Involvement of cholangiocyte proliferation in biliary fibrosis. *World J Gastrointest Pathophysiol* 2010; **1**: 30-37 [PMID: 21607140 DOI: 10.4291/wjgp.v1.i2.30]

44 **Lleo A**, Selmi C, Invernizzi P, Podda M, Coppel RL, Mackay IR, Gores GJ, Ansari AA, Van de Water J, Gershwin ME. Apotopes and the biliary specificity of primary biliary cirrhosis. *Hepatology* 2009; **49**: 871-879 [PMID: 19185000 DOI: 10.1002/hep.22736]

45 **Hisamoto S**, Shimoda S, Harada K, Iwasaka S, Onohara S, Chong Y, Nakamura M, Bekki Y, Yoshizumi T, Ikegami T, Maehara Y, He XS, Gershwin ME, Akashi K. Hydrophobic bile acids suppress expression of AE2 in biliary epithelial cells and induce bile duct inflammation in primary biliary cholangitis. *J Autoimmun* 2016; **75**: 150-160 [PMID: 27592379 DOI: 10.1016/j.jaut.2016.08.006]

46 **Wilson JL**, Altman RB. Biomarkers: Delivering on the expectation of molecularly driven, quantitative health. *Exp Biol Med (Maywood)* 2018; **243**: 313-322 [PMID: 29199461 DOI: 10.1177/1535370217744775]

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ORIGINAL ARTICLE

Basic Study

Long non-coding RNA TP73-AS1 promotes pancreatic cancer growth and metastasis through miRNA-128-3p/GOLM1 axis

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Author contributions: Qiu ZJ and Wang B conceived and supervised the study and wrote the manuscript; Wang B performed most of the experiments and data analyses; Sun X, Zhou LS, and Huang KJ performed Western blot experiments and data analysis; Zhou LS took part in the discussions; all the authors discussed the results, commented on the manuscript, and approved the final version of the article.

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Abstract

BACKGROUND

Previous studies have suggested that long non-coding RNAs (lncRNA) TP73-AS1 is significantly upregulated in several cancers. However, the biological role and clinical significance of TP73-AS1 in pancreatic cancer (PC) remain unclear.

AIM

To investigate the role of TP73-AS1 in the growth and metastasis of PC.

METHODS

The expression of lncRNA TP73-AS1, miR-128-3p, and *GOLM1* in PC tissues and cells was detected by quantitative real-time polymerase chain reaction. The bioinformatics prediction software ENCORI was used to predict the putative binding sites of miR-128-3p. The regulatory roles of TP73-AS1 and miR-128-3p in cell proliferation, migration, and invasion abilities were verified by Cell Counting Kit-8, wound-healing, and transwell assays, as well as flow cytometry and Western blot analysis. The interactions among TP73-AS1, miR-128-3p, and GOLM1 were explored by bioinformatics prediction, luciferase assay, and Western blot.

RESULTS

The expression of TP73-AS1 and miRNA-128-3p was dysregulated in PC tissues and cells. High TP73-AS1 expression was correlated with a poor prognosis. TP73-AS1 silencing inhibited PC cell proliferation, migration, and invasion *in vitro* as well as suppressed tumor growth *in vivo*. Mechanistically, TP73-AS1 was validated to promote PC progression through GOLM1 upregulation by competitively binding to miR-128-3p.

CONCLUSION

Our results demonstrated that TP73-AS1 promotes PC progression by regulating

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the miR-128-3p/GOLM1 axis, which might provide a potential treatment strategy for patients with PC.

Key Words: Pancreatic cancer; Long non-coding RNA; TP73-AS1; miR-128-3p; GOLM1

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Core Tip: In this study, the expression level of TP73-AS1 in pancreatic cancer (PC) was measured and its clinical significance was assessed. *In vitro* and *in vivo* experiments were performed to determine the roles of TP73-AS1 in the progression and development of PC. Moreover, the underlying molecular mechanisms were also illustrated, which could provide a novel therapeutic target for patients with PC.

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INTRODUCTION

Pancreatic cancer (PC) is the fourth most frequent cause of cancer-related deaths with an extremely poor prognosis, especially in patients with advanced-stage PC [1,2]. Although standard treatments have been improved in recent years, the effectiveness of these treatments was still limited and surgical resection was the only chance to obtain curative treatment[2,3]. Hence, it is necessary to seek for new treatment to optimize therapeutic approaches.

Long non-coding RNAs (lncRNAs) are small endogenous non-coding RNAs whose lengths are larger than 200 nucleotides. LncRNAs have the capacity to regulate various biological processes such as tumor initiation, growth, metastasis, chemoresistance, and radioresistance by directly binding to partially complimentary sequences in their target genes[4-7]. Moreover, emerging evidence has revealed that lncRNAs could play crucial roles in the progression of PC[8,9]. LncRNA-BX111 was upregulated in pancreatic cancer and high BX111 expression was correlated with advance tumor-node-metastasis (TNM) stage, lymphatic invasion, and distant metastasis, as well as poor clinical prognosis in patients with PC[10]. Further investigation revealed that BX111 contributed to metastasis and progression of PC by regulating expression of ZEB1 and its downstream proteins E-cadherin and MMP2[10]. PVT1 was identified as a regulator of gemcitabine sensitivity with a genome-wide and piggyBac transposon-based genetic screening platform[11]. Therefore, lncRNAs may be new biological markers for disease diagnosis and could be taken as new drug targets, which would provide a new strategy for PC.

Dysregulation of TP73-AS1 has been identified in several human cancer types, including glioma, hepatocellular carcinoma, and non-small cell lung cancer[12-14]. However, little is known about the expression pattern and biological roles of TP73-AS1 in PC. In this study, the expression level of TP73-AS1 in PC was measured and its clinical significance was assessed. *In vitro* and *in vivo* experiments were performed to determine the roles of TP73-AS1 in the progression and development of PC. Further investigation indicated that the 3′ untranslated region (UTR) of *GOLM1* harbors a functional response element for miR-128-3p. Besides, miR-128-3p-3p could abrogate TP73-AS1-mediated expression of GOLM1, which suggested that TP73-AS1 could act as a molecular sponge to decrease miR-128-3p expression, thereby resulting in partial abolition of the translational repression of its target gene *GOLM1* in PC cells. Therefore, we hope that the underlying molecular mechanisms of TP73-AS1 could provide a novel therapeutic target for patients with PC.

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MATERIALS AND METHODS

Clinical specimens

A total of 116 clinical PC tissues and corresponding normal tissues from surgical resection were collected at Shanghai General Hospital of Shanghai Jiao Tong University between April 2007 and July 2010. PC was diagnosed by pathological examinations. Patients were excluded if they received any treatments such as chemotherapy, radiotherapy, or molecular targeted therapy prior to surgery. All patients provided informed written consent prior to the use of these clinical materials for research purpose. This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Shanghai First People's Hospital (No. 2014-07DF), School of Medicine, Shanghai Jiaotong University (Shanghai, China). All human tissues were immediately frozen in liquid nitrogen until being used.

Cell lines and transfection

Human PC cell lines (SW1990, PANC-1, BXPC-3, AsPc-1, and Capan-1) and human pancreatic duct epithelial cell line (H6C7) were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, United States) supplemented with 10% fatal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Hyclone, South Logan, UT, United States) in a humidified incubator (5% CO_2) at 37 °C. Small interfering RNAs (siRNAs) targeting TP73-AS1 (si-TP73-AS1#1, si-TP73-AS1#2, and si-TP73-AS1#3) and negative control (si-control) were purchased from GenePharma (Shanghai, China). MiR-128-3p, miR-NC, anti-miR-128-3p, and anti-miR-NC were obtained from Thermofisher. Cell transfection was performed using FuGENE HD Transfection Reagent (Roche, United States) according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen) from PC tissues and cell lines. RNA was reversely transcribed into cDNA using the PrimeScript™RT reagent Kit with gDNA Eraser (TakaRa, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) on a Bio-Rad RT-PCR cycler (Bio-Rad, Hercules, United States). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and small RNA RNU6B (U6) were used as the internal controls for lncRNA/mRNA and miRNAs, respectively. Relative expression values of genes were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation, colony formation, and apoptosis assays

For cell proliferation assay, cells were plated into 96-well plates with four replicate wells per group and then incubated with 10 μL of CCK-8 reagent (Dojindo, Kumamoto, Japan). The absorbance was measured at 450 nm with a microplate reader (Bio-Tek, Winooski, United States) 2 h later. For colony formation assay, approximately 600 cells were plated into 6-well plates with three replicates. Cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet 14 d later. Cell apoptosis was detected using Annexin-V-fluorescein isothiocyanate apoptosis detection kit (BD, Franklin Lakes, United States) according to the manufacturer's instructions. The apoptosis rate of cells was measured on a BD FACSAria™ II flow cytometer (BD).

Transwell assay

Cell migration and invasion were evaluated using the Boyden chambers (Millipore; Merck KGaA, Germany) with an 8 mm pore size. Briefly, a total of 2×10^4 cells in 100 μL serum-free medium were transferred into the upper chamber, and the lower chamber was filled with medium containing 10% FBS. After 24 h incubation, cells were fixed with 10% formaldehyde for 15 min and stained with 0.5% crystal violet for 20 min at room temperate. The migrated cells were counted under an X71 inverted microscope in six randomly selected fields and captured using a microscope (Nikon). The invasion assay was performed in the same way as the migration assay did except that the inserts were pre-coated with Matrigel (BD).

Luciferase reporter assay

The putative miR-128-3p binding sites in TP73-AS1 and GOLM1 3′UTR were synthesized and inserted into pMIR-REPORT™ miRNA Expression Reporter Vector

(Thermofisher). Their corresponding mutants were generated using MutanBEST Kit (TaKaRa). AsPc-1 and Capan-1 cells were co-transfected with these reporter plasmids and pMIR-REPORT β-gal, miR-128-3p mimics, or miR-128-3p inhibitors using Lipofectamine 3000 (Invitrogen). Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega, WI, United States) 48 h after the transfection.

RNA immunoprecipitation assay

PANC-1 and ASPC-1 cells transfected with miR-NC or miR-128-3p were harvested with revised importance-performance analysis lysis buffer (Cell Signaling Technology, Danvers, MA, United States) containing a proteinase inhibitor cocktail (Roche, IN, United States). The lysates were incubated with magnetic beads conjugated with human anti-Ago2 antibody and normal rabbit immunoglobulin G. Then RNA was isolated from the mixture with TRIzol reagent for qRT-PCR analysis.

Western blot analysis

Western blot was performed as we previously described[15]. The primary antibodies used in this study are listed as following: Anti-GOLM1 (H00051280-PW1, Abnova, Taiwan), anti-GAPDH (#10494-1-AP, Proteintech, IL, United States), anti-E-cadherin (14-3249-82, CST, United States), anti-N-cadherin (MA1-91128, CST, United States), anti-Vimentin (PA5-27231, CST, United States), anti-Caspase-3 (700182, CST, United States), and anti-Bcl-2 (MA5-11757, CST, United States).

In vivo xenograft experiment

All animal experiments were performed in compliance to institutional guidelines approved by the Use Committee for Animal Care and this study was approved by the Ethics Committees of Shanghai First People's Hospital of Shanghai Jiao Tong University (approval No. 201804SF). Female BALB/c-nude mice (4–6 wk of age) were purchased from Shanghai SJA Laboratory Animal Company (Shanghai, China) and maintained under specific pathogen free conditions. Capan-1 cells (1×10^7; transfected with si-control or si-TP73-AS1#1) mingled with 100 μL serum-free medium were injected subcutaneously into to the flanks of the nude mice. All mice were sacrificed 4 wk after injection and then tumors were isolated and photographed. Tumor volumes were calculated using the formula length × width^2/2 and tumor weights were measured. For tail vein injection, 1×10^6 cells in serum-free medium were injected into 6 wk-old BALB/c-nude mice *via* the tail vein. Five weeks after injection, all mice were sacrificed and lung tissues were finally embedded with paraffin and subjected to hematoxylin and eosin (H&E) staining.

Statistical analysis

Data are shown as the mean ± SD. The differences between groups were analyzed by Student's *t*-test or Chi-square test. The cumulative overall survival was calculated using the Kaplan-Meier method, and the log-rank test was used to analyze differences in the survival times. Data were analyzed using GraphPad software 7.0. $P < 0.05$ was considered significant.

RESULTS

TP73-AS1 is upregulated in PC and associated with a poor prognosis

To explore the roles of TP73-AS1 in PC, we first detected the expression of TP73-AS1 in the human pancreatic duct epithelial cell line (H6C7) and five PC cell lines by qRT-PCR. Our data indicated that TP73-AS1 expression was higher in all PC cell lines than in H6C7, especially in AsPc-1 and Capan-1 cells (Figure 1A). Then the expression of TP73-AS1 in 116 pairs of PC tissues and adjacent non-cancerous tissues was measured, and the results revealed that TP73-AS1 expression was significantly increased in PC tissues compared to the corresponding non-cancerous tissues (Figure 1B and C). Furthermore, it was shown that in the tissue samples of stages I and II PC patients, the expression levels of TP73-AS1 were lower than those in stage III PC patients (Figure 1D). Moreover, the associations between TP73-AS1 expression and the clinicopathological characteristics in PC patients were analyzed. The results suggested that increased TP73-AS1 expression was significantly correlated with tumor size, vessel infiltration, and TNM stage. Besides, no correlation was found between TP73-AS1 expression and other clinical pathological features (Table 1). Kaplan-Meier

Table 1 Relationship between TP73-AS1 expression and clinicopathological features

Characteristic	(*n*)	TP73-AS1 expression		*P* value
	n = 116	Low expression	High expression	
Age				0.7101
< 60	65	32	33	
≥ 60	51	23	28	
Gender				0.7026
Male	74	34	40	
Female	42	21	21	
Tumor differentiation				0.5723
Poor	69	31	38	
Middle and well	47	24	23	
Tumor size				0.008
≤ 2 cm	48	31	17	
> 2 cm	68	26	42	
Tumor site				0.4555
Head	64	28	36	
Body	52	27	25	
Vessel infiltration				0.001
Negative	82	47	35	
Positive	34	8	26	
Lymph node metastasis				0.5526
No	78	35	43	
Yes	38	20	18	
TNM stage				0.0008
I-II	84	48	36	
III	32	7	25	

TNM: Tumor-node-metastasis.

survival results suggested that patients with higher TP73-AS1 expression had a shorter overall survival than those with lower TP73-AS1 expression (Figure 1E). These data indicated that TP73-AS1 might play a vital role in the progression of PC.

LncRNA TP73-AS1 is required for efficient PC cell proliferation, migration, and invasion

In order to assess the biological functions of TP73-AS1, we knocked down TP73-AS1 by transfecting specific siRNAs in AsPc-1 and Capan-1 cells, which have higher endogenous TP73-AS1 expression. The knockdown efficacy was confirmed by qRT-PCR analysis. The expression of TP73-AS1 was markedly decreased in AsPc-1 and Capan-1 cells after transfecting with siRNAs targeting TP73-AS1 (Figure 2A). CCK-8 (Figure 2B) and colony formation assay (Figure 2C) showed that knockdown of TP73-AS1 in PC cells markedly restrained cell proliferation. Furthermore, cell apoptosis was highly promoted by depletion of TP73-AS1 in AsPc-1 and Capan-1 cells (Figure 2D). In addition, in the transwell assay, TP73-AS1 silencing could effectively impede the invasive ability of PC cells (Figure 2E and F). These data revealed that TP73-AS1 acts as an oncogene and depletion of TP73-AS1 inhibits PC cell growth and invasion *in vitro*.

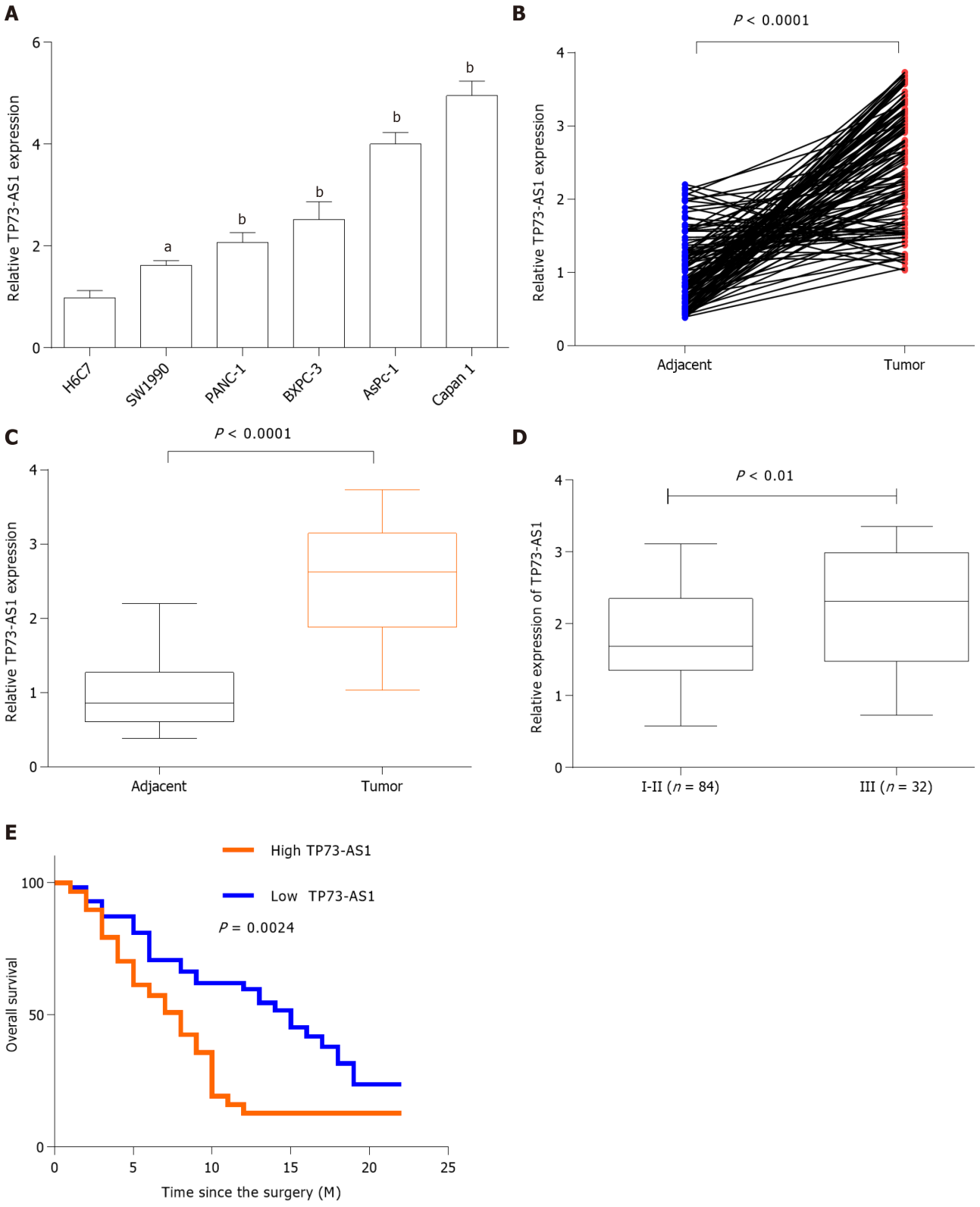
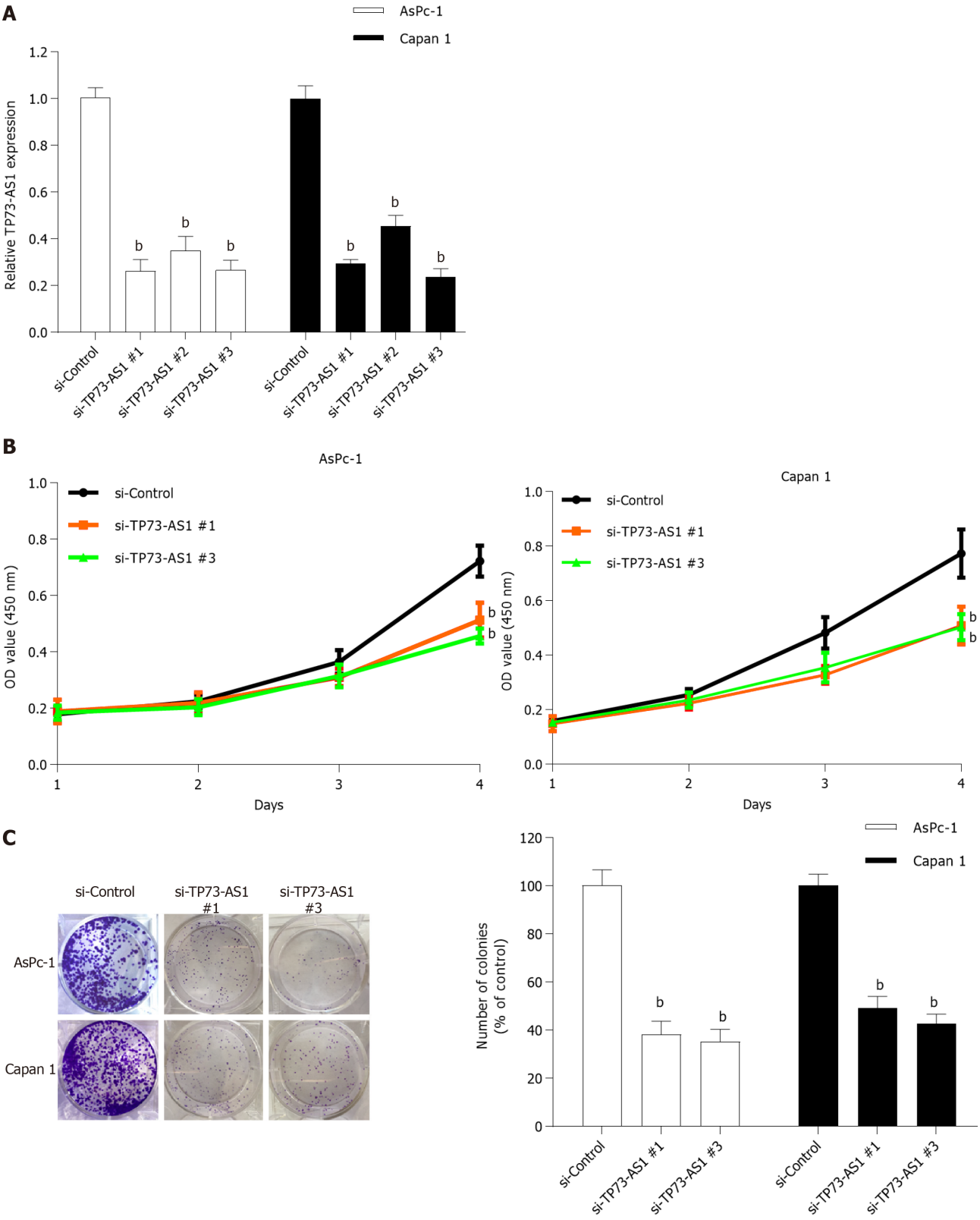


Figure 1 Aberrantly expressed TP73-AS1 in pancreatic cancer cells and tissues. A: TP73-AS1 expression in pancreatic cancer (PC) cell lines and an immortalized human pancreatic duct epithelial cell (H6C7) detected by quantitative real-time polymerase chain reaction; B: The relative expression of TP73-AS1 in 116 paired PC tissues and adjacent non-tumor tissues; C: TP73-AS1 is highly expressed in collected PC tissues; D: The different expression of TP73-AS1 between tumor-node-metastasis (TNM) stage I-II; and TNM stage III; E: Kaplan-Meier analysis of the overall survival in patients with PC based on the levels of TP73-AS1 expression. $^{a}P < 0.05$; $^{b}P < 0.01$.

LncRNA TP73-AS1 functions as a competing endogenous RNA and sponges miR-128-3p in PC cells

Accumulating evidence suggests that lncRNAs could bind to miRNAs and function as a molecular sponge in the tumorigenesis of various cancers[4]. To elucidate the


A
AsPc-1
Capan 1
Relative TP73-AS1 expression
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #2
si-TP73-AS1 #3
B
AsPc-1
Capan 1
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #3
OD value (450 nm)
Days
C
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #3
AsPc-1
Capan 1
Number of colonies (% of control)

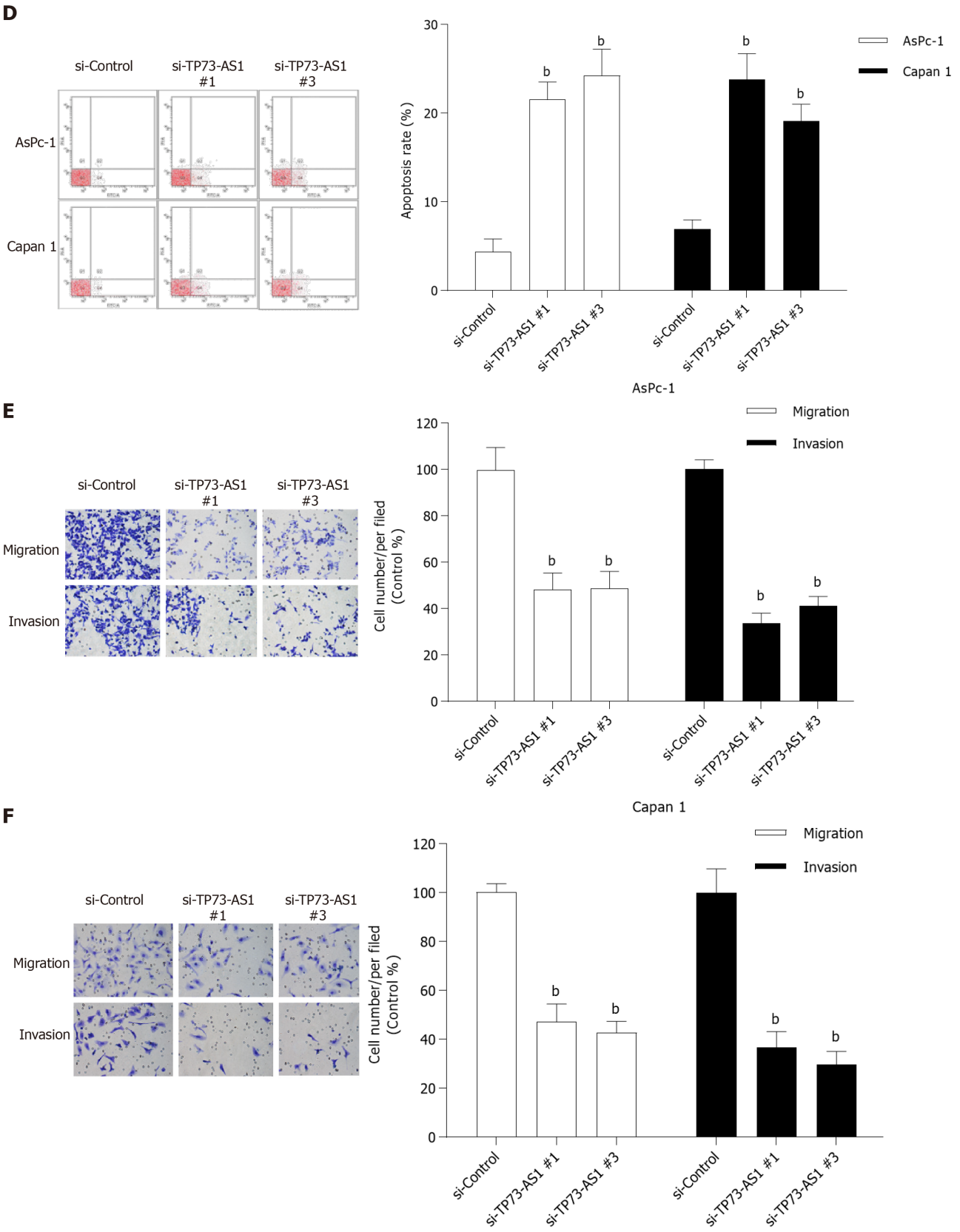


Figure 2 Knockdown of TP73-AS1 suppresses cell proliferation and invasion *in vitro*. A: Quantitative real-time polymerase chain reaction analysis of TP73-AS1 expression in AsPc-1 and Capan-1 cells transfected with si-control and small interfering RNAs targeting TP73-AS1; B: CCK-8 proliferation assay in pancreatic cancer cells transfected with si-control, si-TP73-AS1#1, or si-TP73-AS1#3; C: Colony formation assay in AsPc-1 and Capan-1 cells transfected with si-control, si-TP73-AS1#1, or si-TP73-AS1#3; D: Role of TP73-AS1 on AsPc-1 and Capan-1 cells apoptosis checked by flow cytometry assay; E and F: Effect of TP73-AS1 silencing on the migration and invasion ability of AsPc-1 (E) and Capan-1 (F) cell determined by transwell assay. $^{a}P < 0.05$; $^{b}P < 0.01$.

underlying mechanism of TP73-AS1 involved in PC progression, the potential target miRNAs of TP73-AS1 were forecasted with bioinformatics analysis software (http://starbase.sysu.edu.cn). Among these potential targets, miR-128-3p was chosen for further study because it had been validated as a tumor suppressor in PC (Figure 3A)[16]. Dual-luciferase reporter assay was used to validate the relationship between TP73-AS1 and miR-128-3p. The activity of the wild-type luciferase reporter gene was significantly reduced following transfection with miR-128-3p mimics, whereas the activity of the reporter gene containing the mutant sequence showed no significant change, which indicated that TP73-AS1 could bind to the specific sites of miR-128-3p (Figure 3B). Moreover, anti-Ago2 RNA immunoprecipitation in AsPc-1 and Capan-1 cells transiently overexpressing miR-128-3p could significantly increase the amount of TP73-AS1 (Figure 3C), which could further validate their binding potential. The expression of miR-128-3p was significantly increased in PC cells transfected with si-TP73-AS1#1 and si-TP73-AS1#3 (Figure 3D). Then, we measured miR-128-3p expression level and the relationship between TP73-AS1 and miR-128-3p expression in PC tissues. Interestingly, qRT-PCR assay showed that the miR-128-3p level was remarkably reduced in PC tissues (Figure 3E) and the endogenous miR-128-3p level was negatively correlated with TP73-AS1 in PC tissues (Figure 3F). These results suggested that TP73-AS1 might function as a competing endogenous RNA (ceRNA) for miR-128-3p.

MiR-128-3p-3p inhibits pancreatic cell proliferation, migration, and invasion

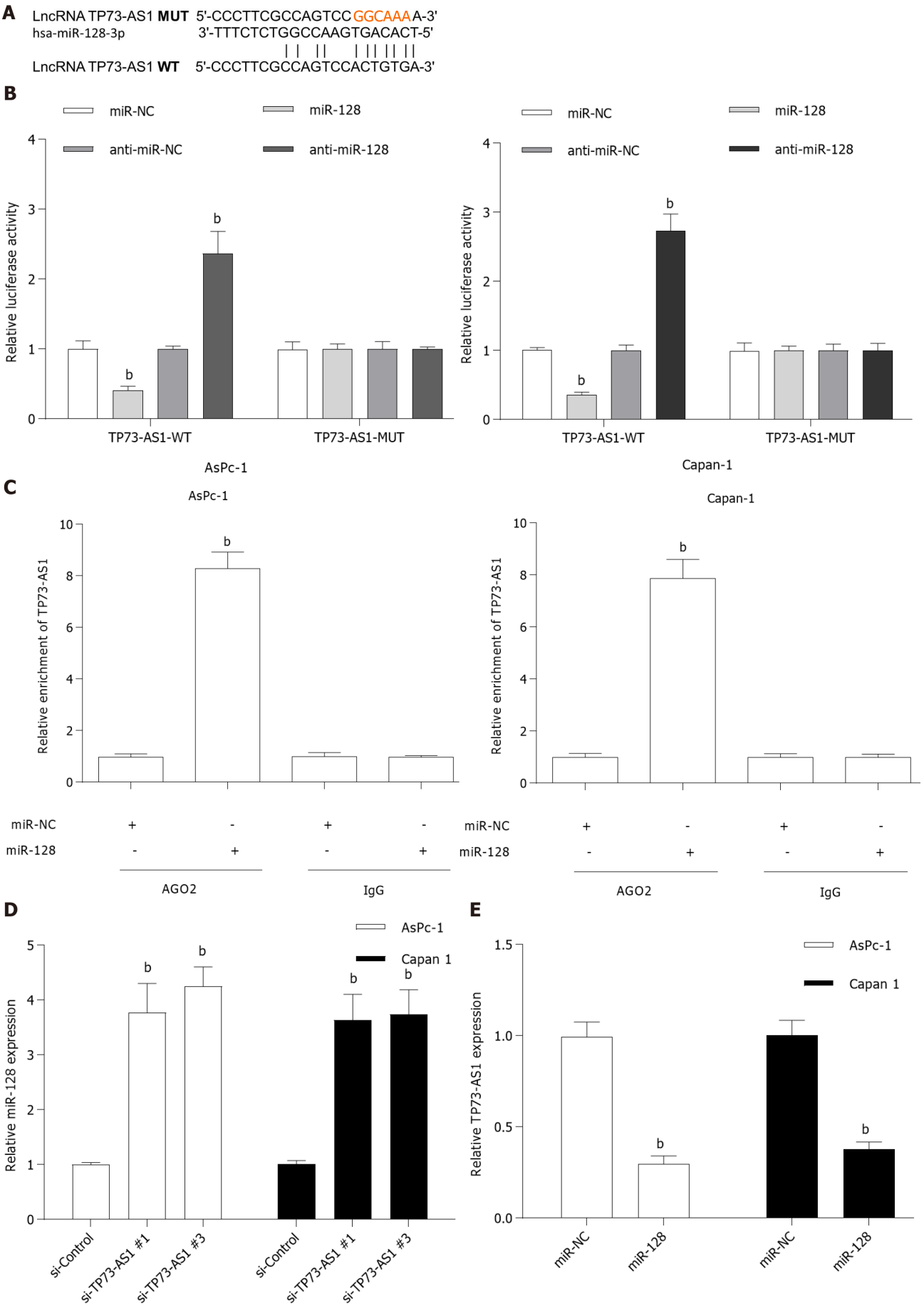
MiR-128-3p was reported to be a tumor suppressor in several cancers, including PC[16,17]. But its effects in the progression of PC are largely unknown. To explore the roles of miR-128-3p in PC cell growth and mobility, miR-128-3p mimics were transfected in AsPc-1 and Capan-1 cells (Figure 4A). CCK-8 (Figure 4B) and colony formation assays (Figure 4C) revealed that miR-128-3p had significant negative regulation effects on the ability of cell proliferation in AsPc-1 and Capan-1 cells. Flow cytometry assay indicated that overexpression of miR-128-3p increased the number of apoptotic cells both in AsPc-1 and Capan-1 cells (Figure 4D). Meanwhile, transwell assays demonstrated that miR-128-3p overexpression significantly restrained PC cell migration and invasion (Figure 4E and F). Considering the downregulation of miR-128-3p in PC tissues, our results manifested that miR-128-3p could act as a tumor suppressor by regulating PC cell proliferation and invasion.

Inhibition of miR-128-3p partly reverses regulatory effects induced by depletion of TP73-AS1

To determine if TP73-AS1 knockdown could exert anti-proliferation and anti-metastasis function by mediating miR-128-3p, anti-miR-128-3p was transfected into AsPc-1 and Capan-1 cells after TP73-AS1 silencing. Functional experiments demonstrated that the TP73-AS1-mediated pro-proliferation (Figure 5A) and anti-apoptosis (Figure 5B) effect was dramatically abrogated by anti-miR-128-3p transfection in TP73-AS1 silencing PC cells. In addition, the inhibitory effects of TP73-AS1 silencing on cell metastasis were rescued by anti-miR-128-3p transfection (Figure 5C and D). Moreover, epithelial-mesenchymal transition (EMT)-related proteins were detected by Western blot. Consistent with the functional assays above, the results showed that TP73-AS1 could regulate EMT-related proteins by regulating miR-128-3p (Figure 5E). All these data indicated that TP73-AS1 is involved in PC progression, at least partly through miR-128-3p.

LncRNA TP73-AS1 regulates GOLM1 expression by competing for miR-128-3p-3p

Since TP73-AS1 was demonstrated to bind to miR-128-3p, we assessed whether TP73-AS1 could indirectly affect the target gene of miR-128-3p by serving as a ceRNA. Based on online bioinformatics analysis, GOLM1 3'UTR was found to possess a putative recognition site for miR-128-3p (Figure 6A). The luciferase reporter assay was carried out and the results showed that the luciferase activity of plasmid carrying GOLM1 3'UTR-WT was significantly decreased by transfecting miR-128-3p mimics both in AsPc-1 and Capan-1 cells (Figure 6B). However, these effects were abolished when the binding sequences were mutated. Transfecting miR-128-3p mimics led to a significant decrease of GOLM1 mRNA and protein expression in AsPc-1 and Capan-1 cells (Figure 6C). To further explore the correlation between TP73-AS1 and GOLM1, the mRNA and protein expression of GOLM1 was detected after TP73-AS1 silencing. As expected, the mRNA and protein expression of GOLM1 was remarkably decreased after silencing TP73-AS1 both in AsPc-1 and Capan-1 cells (Figure 6D). Moreover, the level of GOLM1 mRNA was significantly down-regulated in PC tissues compared to


A
LncRNA TP73-AS1 MUT 5'-CCCTTCGCCAGTCC GGCAAA A-3'
hsa-miR-128-3p 3'-TTTCTCTGGCCAAGTGACACT-5'
LncRNA TP73-AS1 WT 5'-CCCTTCGCCAGTCCACTGTGA-3'
B
miR-NC
miR-128
anti-miR-NC
anti-miR-128
Relative luciferase activity
TP73-AS1-WT
TP73-AS1-MUT
AsPc-1
Capan-1
C
Relative enrichment of TP73-AS1
miR-NC
miR-128
AGO2
IgG
D
Relative miR-128 expression
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #3
E
Relative TP73-AS1 expression

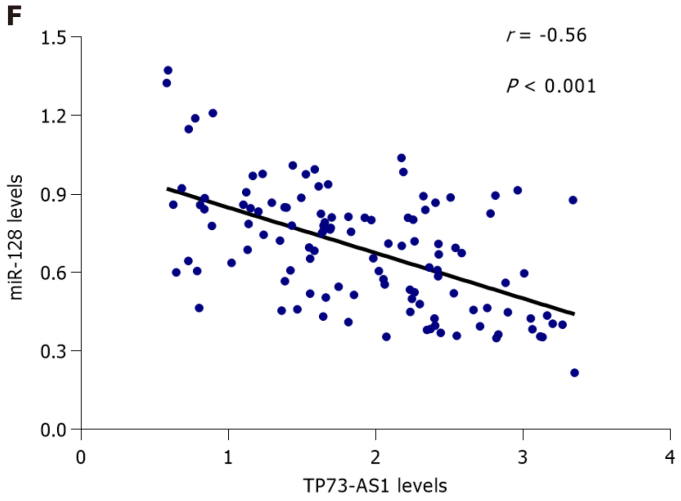


Figure 3 TP73-AS1 physically interacts with miR-128-3p-3p. A: miR-128-3p and its predicted binding sites in the TP73-AS1 sequence; B: Luciferase activity of pancreatic cancer (PC) cells co-transfected with miRNA mimics and luciferase reporter vectors containing TP73-AS1-WT or TP73-AS1-MUT; C: RNA immunoprecipitation assay of the enrichment of TP73-AS1 in AsPc-1 and Capan-1 transfected with miR-NC or miR-128-3p. Immunoglobulin G was used as negative control; D: Expression levels of miR-128-3p in AsPc-1 and Capan-1 cells transfected with si-control or small interfering RNAs targeting TP73-AS1; E: Expression levels of miR-128-3p in PC tissues and adjacent non-tumor tissues; F: The expression of TP73-AS1 and miR-128-3p exhibits a negative correlation in PC tissues. $^{b}P < 0.01$. IgG: Immunoglobulin G.

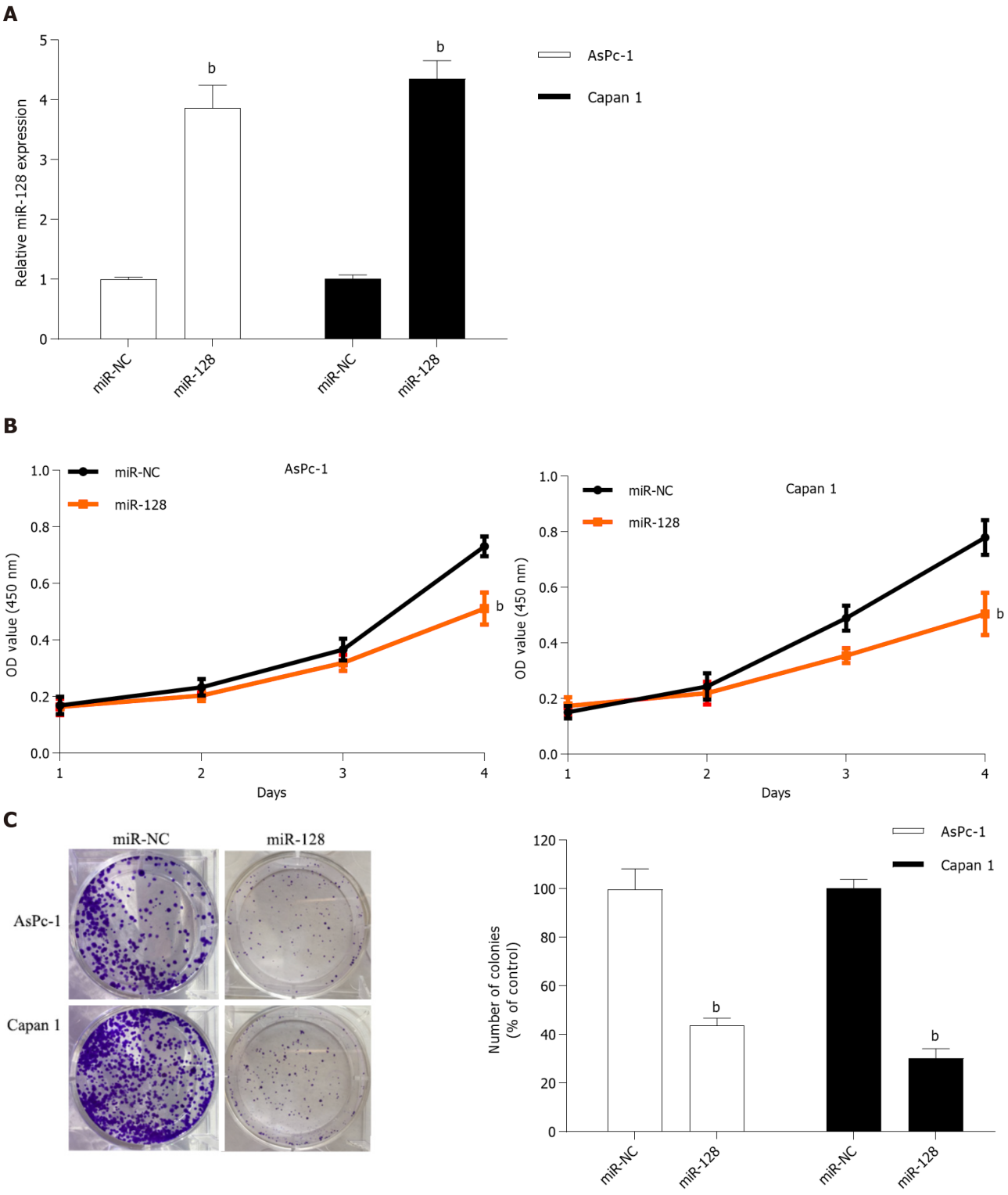
the corresponding non-cancerous tissues (Figure 6E). Interestingly, an inverse correlation was identified between miR-128-3p and GOLM1 mRNA levels in PC tissues (Figure 6F). In contrast, the positive relationship between TP73-AS1 and GOLM1 mRNA levels was observed in PC tissues (Figure 6G). The protein expression of GOLM1 was increased after anti-miR-128-3p transfection in TP73-AS1 silencing PC cells (Figure 6H), which suggested that TP73-AS1 could regulate the expression of GOLM1 by acting as a sponge for miR-128-3p *in vitro*.

TP73-AS1 silencing inhibits tumor growth and metastasis of PC cells

To further elucidate the biological roles of TP73-AS1 in PC tumorigenesis *in vivo*, Capan-1 cells transfected with si-TP73-AS1#1 or si-Control were implanted into nude mice *via* subcutaneous injection. Four weeks later, the subcutaneous tumors were collected. Tumor growth curve and tumor weight from the si-TP73-AS1#1 group showed lower size and lighter tumor weight (Figure 7A-C). Moreover, qRT-PCR analysis suggested that the expression of TP73-AS1 was decreased (Figure 7D) and the expression of miR-128-3p (Figure 7E) was increased in the si-TP73-AS1#1 group. In addition, Ki-67 immunostaining indicated that the subcutaneous tumors formed by TP73-AS1 silencing Capan-1 cells showed fewer Ki-67 positive cells compared to the control group (Figure 7F). Together, the *in vitro* and *in vivo* results suggested that TP73-AS1 might function as an oncogene in the progression of PC. To investigate the metastatic potential of TP73-AS1 *in vivo*, Capan-1 cells transfected with si-TP73-AS1#1 or si-Control were injected into the mice *via* the tail vein. As shown in Figure 7G, silencing TP73-AS1 remarkably decreased the number and size of lung metastatic lesions as detected by H&E staining. Moreover, we detected apoptotic markers in the tumors from the two groups and the results showed that apoptosis-related genes were significantly altered (Figure 7H).

DISCUSSION

Increasing numbers of studies have shown that lncRNAs are involved in both normal development and pathological processes of human diseases by chromatin modification, genomic imprinting, RNA decay, and sponge-like miRNAs[5,18,19]. Dysregulation of lncRNAs might influence cell proliferation, metastasis, angiogenesis, and drug resistance[18,20,21]. It has been previously reported that increased expression of TP73-AS1 is associated with a poorer prognosis and shorter survival in patients with hepatocellular carcinoma[13]. High TP73-AS1 expression was also observed and associated with poor overall survival of patients with osteosarcoma[22]. TP73-AS1 was up-regulated in both colorectal cancer tissues and colorectal cancer cells


A
Relative miR-128 expression
AsPc-1
Capan 1
b
miR-NC
miR-128
B
AsPc-1
Capan 1
miR-NC
miR-128
OD value (450 nm)
Days
C
miR-NC
miR-128
AsPc-1
Capan 1
Number of colonies (% of control)

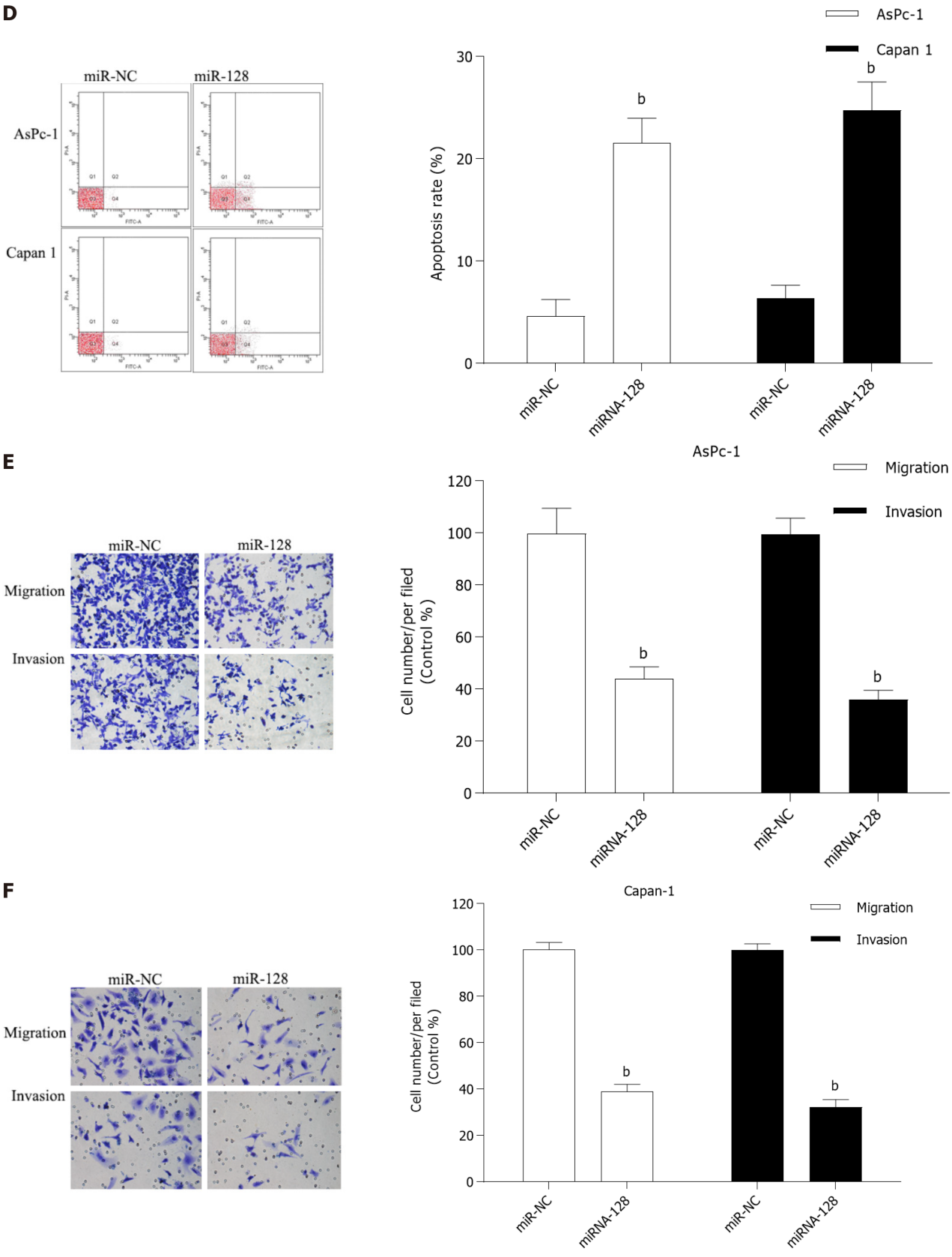


Figure 4 MiR-128-3p inhibits the proliferation, migration, and invasion of pancreatic cancer cells. A: The efficiencies of overexpression for miR-128-3p determined by quantitative real-time polymerase chain reaction; B and C: CCK-8 assay (B) and colony formation assay (C) for detecting cell proliferative ability after overexpression of miR-128-3p; D: Effect of miR-128-3p on pancreatic cancer cell apoptosis determined by flow cytometry; E and F: Transwell assay with or without matrigel for assessing the effect of miR-128-3p on cell migration and invasion in AsPc-1 (E) and Capan-1 (F) cells. $^{b}P < 0.01$.

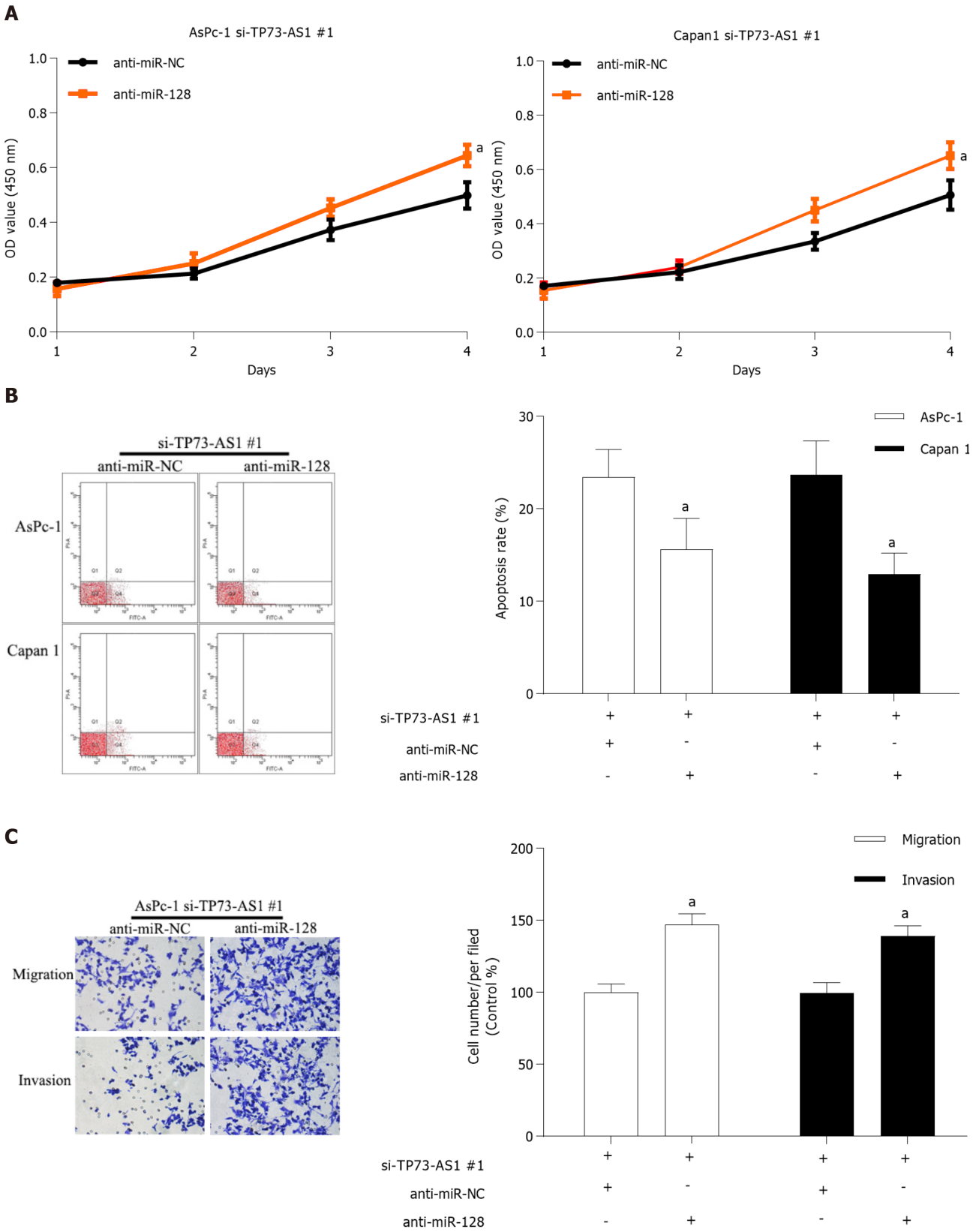
and high TP73-AS1 expression was associated with metastasis and advanced clinical stages in patients with colorectal cancer[23]. Above studies suggested that TP73-AS1 might act as an oncogene in tumor progression, which encouraged us to explore the expression and biological function of TP73-AS1 in PC. In agreement with these studies, we found that TP73-AS1 was significantly increased and associated with tumor size, vessel infiltration, and poor prognosis in PC patients. Furthermore, our results showed that knockdown of TP73-AS1 suppressed the proliferation and invasion of PC cells *in vitro* and the tumor growth *in vivo*.

Emerging studies demonstrated that lncRNAs could function as ceRNAs to regulate gene expression through competitively binding to miRNAs[4,5]. To further investigate the mechanism of the TP73-AS1 in PC, bioinformatics analysis predicted that TP73-AS1 is a target of miR-128-3p. Numerous studies have indicated that miR-128-3p could act as a tumor suppressive role in many tumors, including glioma, breast cancer, and non-small cell lung cancer[24-26]. In our present study, we found that miR-128-3p could significantly suppress PC cell growth and invasion. Luciferase reporter assay confirmed the relationship between TP73-AS1 and miR-128-3p. Mechanistical study showed that TP73-AS1 could mediate PC cell proliferation, migration, and invasion by sponging miR-128-3p and a negative correlation between TP73-AS1 and miR-128-3p expression was observed in PC tissues. Further investigation indicated that the 3'UTR of *GOLM1* harbors a functional response element for miR-128-3p. GOLM1, a type II transmembrane protein, has been reported to be induced by virus infection[27,28]. Recent studies have shown that GOLM1 commonly expressed in epithelial cells of normal tissues was significantly upregulated in tumor tissues, which suggested a possible oncogenic role of GOLM1 in tumor progression[29,30]. Moreover, clinicopathological features showed that GOLM1 was correlated with Edmondson grade, vascular invasion, TNM stage, overall survival, as well as Vimentin expression[31]. GOLM1 was also reported to promote prostate cancer cell growth, migration, and invasion, and inhibited cell apoptosis *via* the PI3K/AKT/mTOR signaling axis[32]. The role of GOLM1 was unclear in PC and our data showed that the mRNA expression of *GOLM1* was increased in PC tissues. Moreover, ectopic expression of miR-128-3p significantly inhibited the expression of GOLM1 at both the mRNA and protein level. Most interestingly, miR-128-3p-3p could abrogate TP73-AS1-mediated expression of GOLM1, which suggested that TP73-AS1 could act as a molecular sponge to decrease miR-128-3p expression, thereby resulting in partial abolition of the translational repression of its target gene *GOLM1* in PC cells.

KRAS gene, the most common genetic driver in PC, is mutated in about 93% of PC s[33,34]. The KRAS protein is a small GTPase, which is responsible for interacting with cell membrane growth factor receptors and controlling the switch of multiple signaling pathways and cellular processes. Oncogenic KRAS mutations have been found in 95% of pancreatic ductal adenocarcinoma tissues[35,36]. Decades of research have discovered and clarified the complex picture of KRAS-regulated biological processes, including cell metabolism, tumor cell signaling, the tumor microenvironment, micropinocytosis, apoptosis, and redox homeostasis[37,38]. In our research, ASPC-1 and Capan-1 cells were the two PC cell lines that we selected, both of which contained mutations in the *KRAS* gene. As our results show, the regulatory roles of TP73-AS1 in cell proliferation, migration, and invasion ability were verified by Cell Counting Kit-8, wound-healing, and transwell assays in ASPC-1 and Capan-1 cells. Due to the vital role that KRAS could play in PC, we are also curious about the role of TP73-AS1 in KRAS wild cells. Therefore, in our further research, we would select BXPC-3 cell line, which contains wild *KRAS* gene, for *in vitro* and *in vivo* functional assays of TP73-AS1 to detect whether *KRAS* gene could modulate the function of TP73-AS1 in PC.

CONCLUSION

In summary, our data suggested that TP73-AS1 could function as an oncogenic lncRNA in PC progression. Moreover, TP73-AS1 could promote tumor growth and invasion by acting as a ceRNA to promote GOLM1 expression by sponging miR-128-3p in PC.


A
AsPc-1 si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
OD value (450 nm)
Days
a
Capan1 si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
OD value (450 nm)
Days
a
B
si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
AsPc-1
Capan 1
AsPc-1
Capan 1
Apoptosis rate (%)
a
a
si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
C
AsPc-1 si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
Migration
Invasion
Migration
Invasion
Cell number/per filed (Control %)
a
a
si-TP73-AS1 #1
anti-miR-NC
anti-miR-128

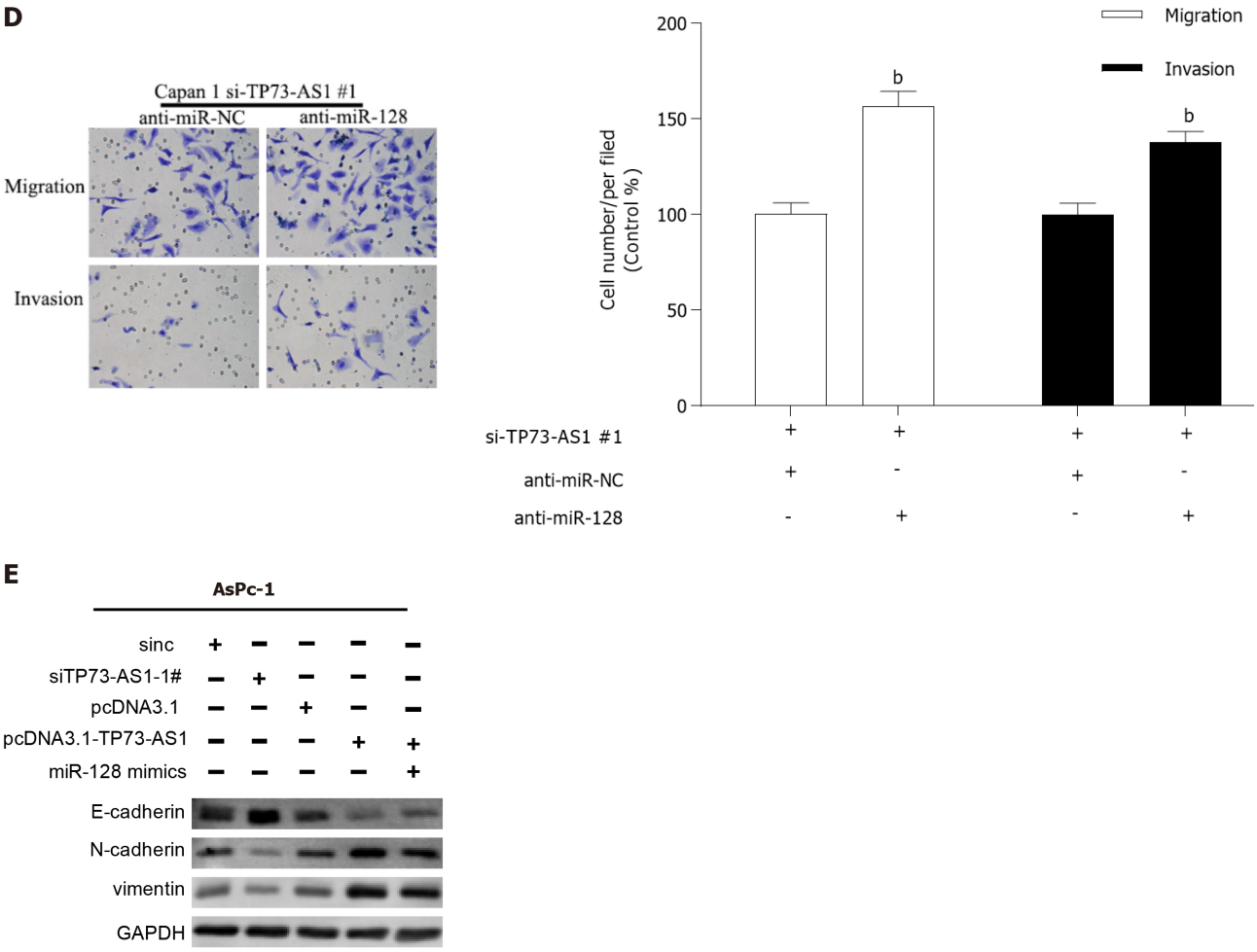
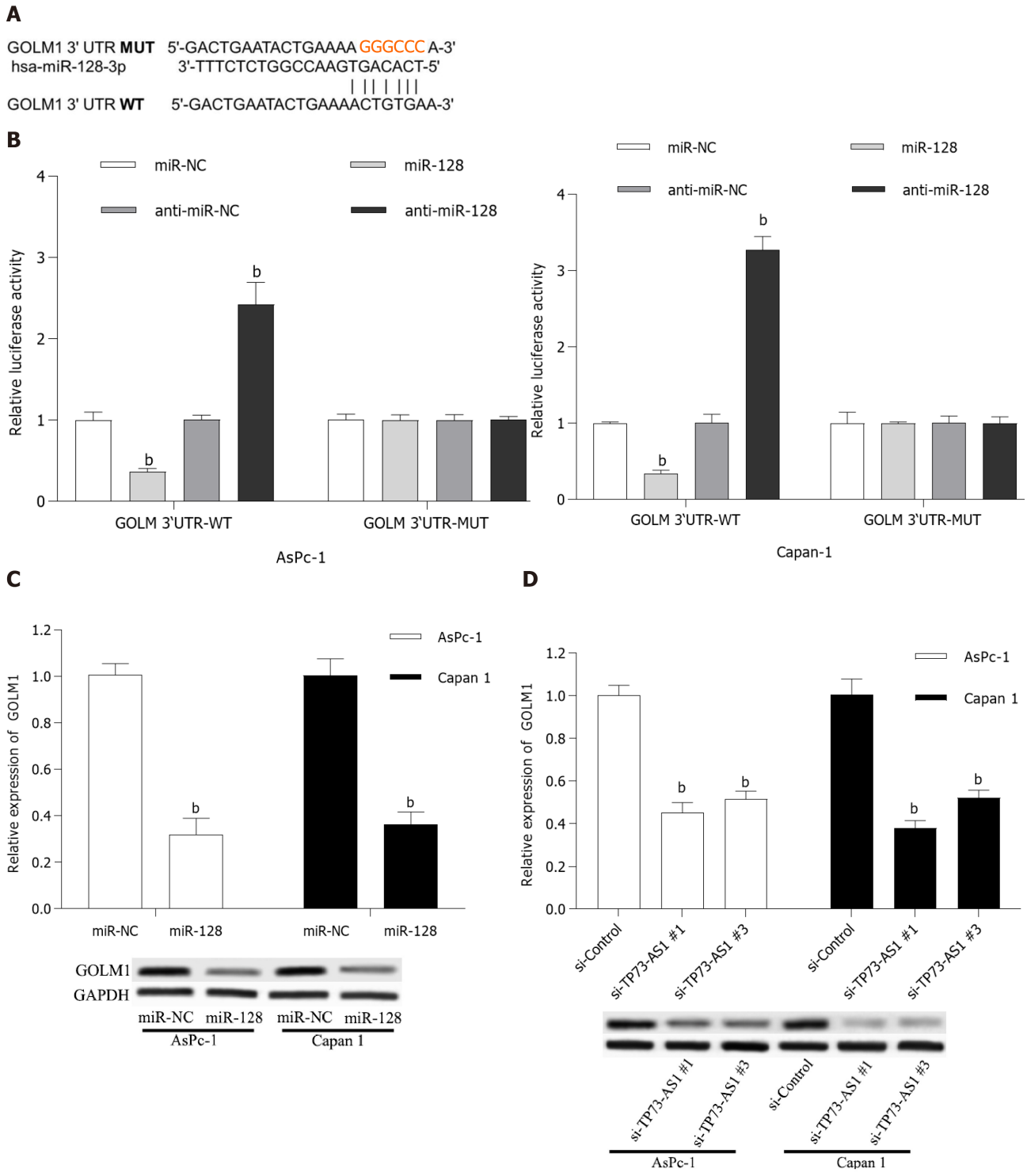


Figure 5 MiR-128-3p is responsible for TP73-AS1-mediated malignant behavior. A: Knockdown of miR-128-3p eliminates the inhibition on cell proliferation by TP73-AS1 depletion; B: Depletion of miR-128-3p abolishes cell apoptosis induced by TP73-AS1 silencing; C and D: Representative images of migrated and invaded cells treated with si-TP73-AS1#1 and anti-miR-NC or anti-miR-128-3p in AsPc-1 (C) and Capan-1 (D) cells; E: Epithelial-mesenchymal transition-related proteins detected by Western blot. $^{a}P < 0.05$; $^{b}P < 0.01$. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.


A
GOLM1 3' UTR MUT 5'-GACTGAATACTGAAAA GGGCCC A-3'
hsa-miR-128-3p 3'-TTTCTCTGGCCAAGTGACACT-5'
GOLM1 3' UTR WT 5'-GACTGAATACTGAAAACTGTGAA-3'
B
miR-NC
miR-128
anti-miR-NC
anti-miR-128
Relative luciferase activity
GOLM 3'UTR-WT
GOLM 3'UTR-MUT
AsPc-1
Capan-1
C
AsPc-1
Capan 1
Relative expression of GOLM1
GOLM1
GAPDH
D
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #3

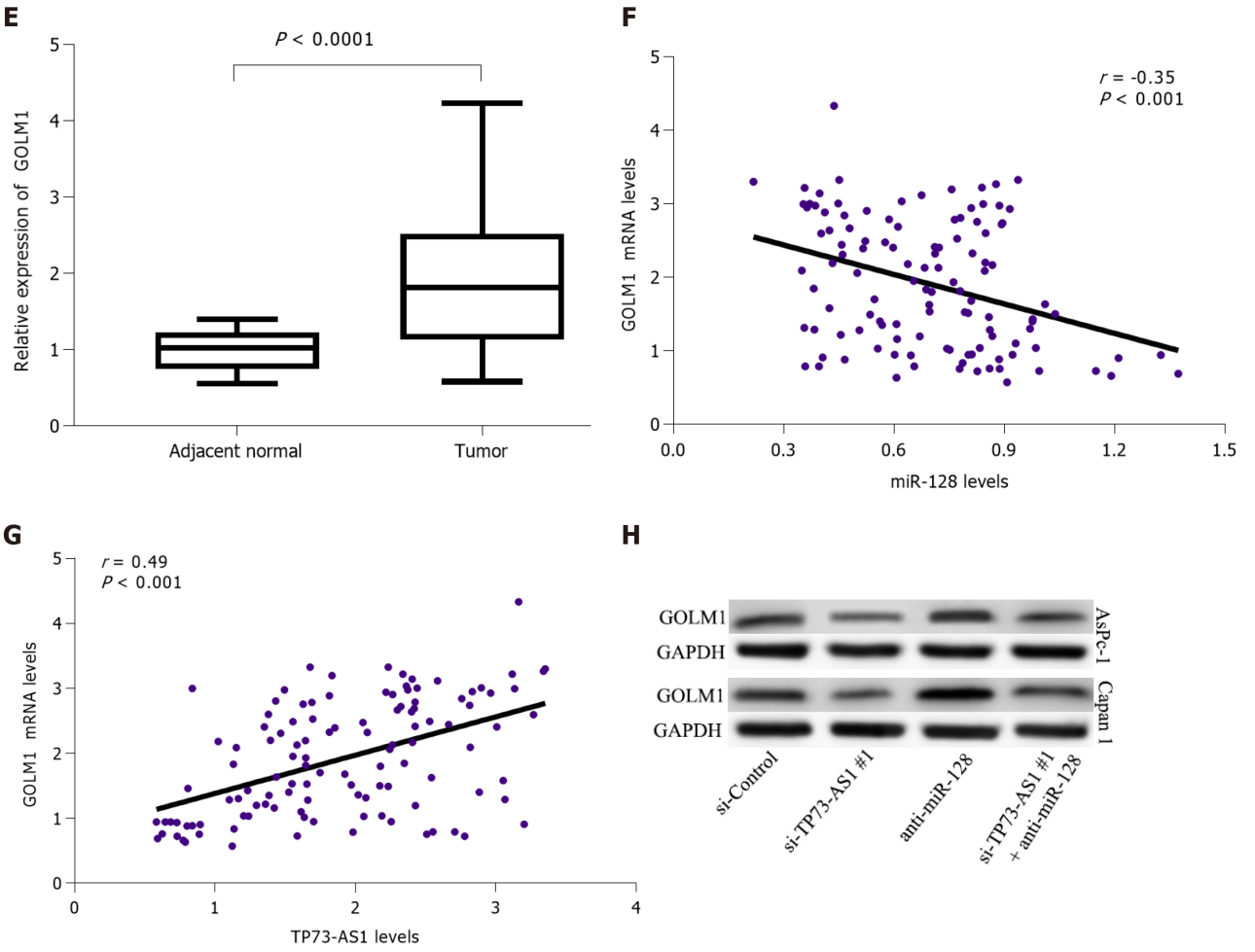
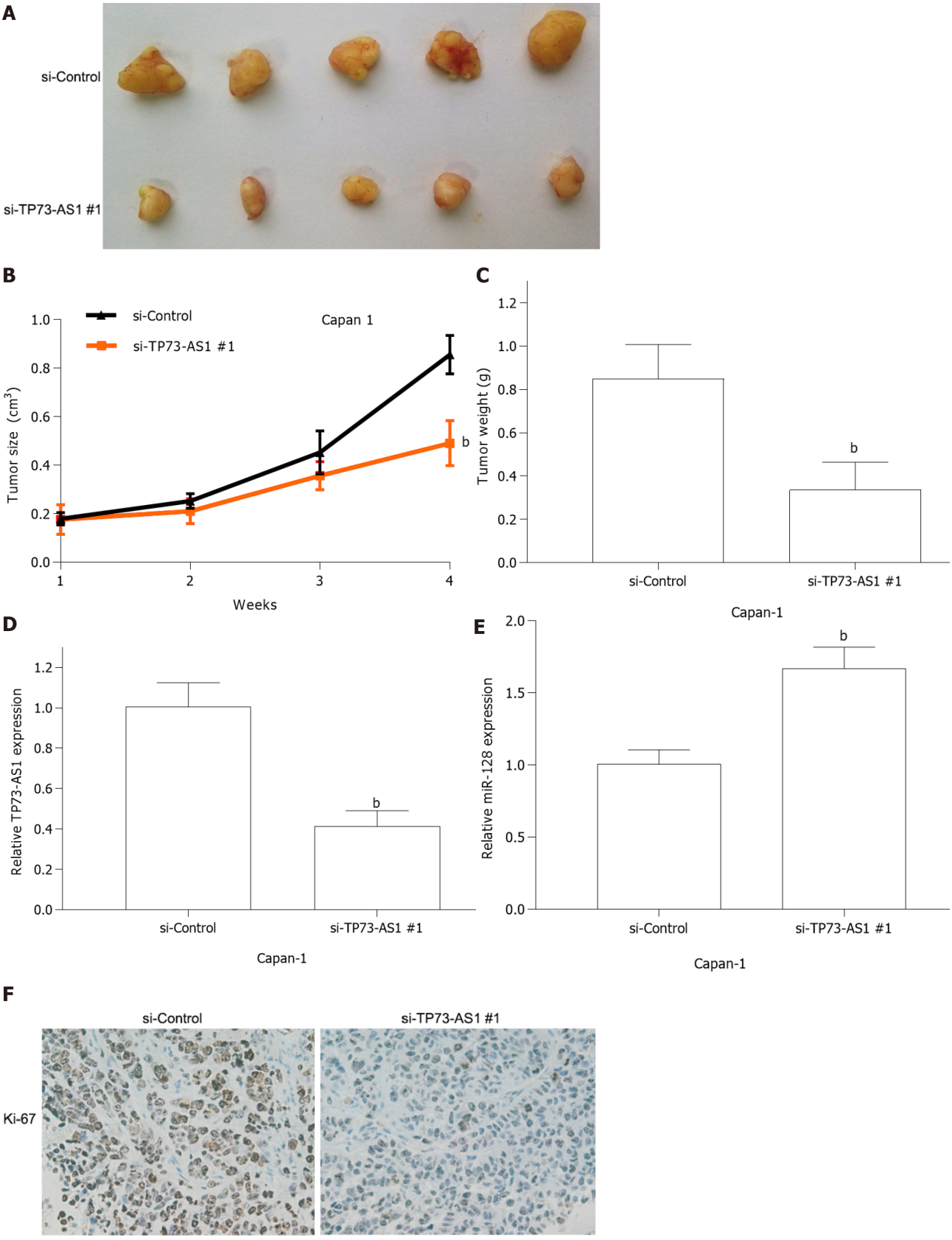


Figure 6 TP73-AS1 increases GOLM1 expression by decreasing miR-128-3p. A: Schematic diagram presenting the putative miR-128-3p recognition site in the GOLM1 sequence; B: Luciferase activity in pancreatic cancer (PC) cells transfected with miR-128-3p and reporter plasmids containing WT or MUT GOLM1 3'UTR; C: The GOLM1 mRNA (up) and protein (down) levels in AsPc-1 and Capan-1 cells with miR-128-3p transfection; D: The GOLM1 mRNA (up) and protein (down) levels in AsPc-1 and Capan-1 cells after TP73-AS1 silencing; E: The expression levels of *GOLM1* mRNA in 116 pairs of PC and adjacent non-tumor tissues assessed by quantitative real-time polymerase chain reaction; F: The relationship between miR-128-3p and GOLM1 mRNA levels assessed by Pearson correlation analysis; G: The relationship between TP73-AS1 and GOLM1 mRNA levels assessed by Pearson correlation analysis; H: The protein expression levels of GOLM1 detected by Western blot assay as indicated. $^{b}P < 0.01$. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.


A
si-Control
si-TP73-AS1 #1
B
si-Control
si-TP73-AS1 #1
Capan 1
Tumor size (cm3)
Weeks
b
C
Tumor weight (g)
b
si-Control
si-TP73-AS1 #1
Capan-1
D
Relative TP73-AS1 expression
b
si-Control
si-TP73-AS1 #1
Capan-1
E
Relative miR-128 expression
b
si-Control
si-TP73-AS1 #1
Capan-1
F
si-Control
si-TP73-AS1 #1
Ki-67

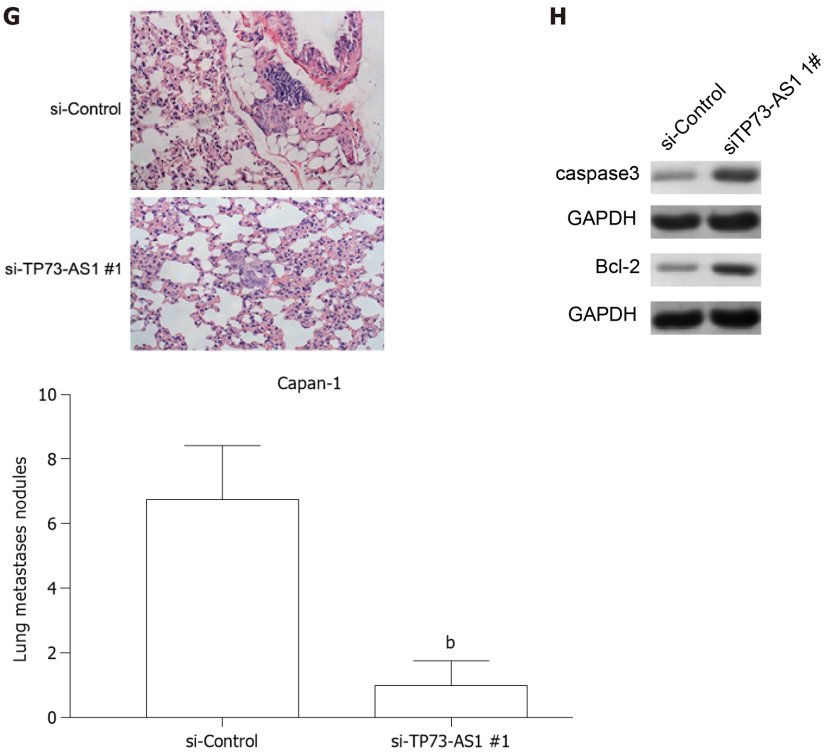


Figure 7 TP73-AS1 knockdown inhibits tumor growth and metastasis in nude mice. A: Images of resected tumors from nude mice from the indicated groups. *n* = 5 mice per group; B: Tumor growth curves established by measuring tumor volume after injection at the indicated time points; C: Weights of resected tumors from nude mice from the indicated groups; D and E: Quantitative real-time polymerase chain reaction to detect the expression of TP73-AS1 (D) and miR-128-3p (E) in xenograft tumor tissues; F: Ki67 expression in resected tumor tissues evaluated by immunohistochemistry analysis; G: Representative images and quantification of hematoxylin and eosin staining of lungs isolated from mice; H: Apoptosis markers detected by Western blot. $^{b}P < 0.01$. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cancer (PC) is the fourth most frequent cause of cancer-related deaths in the world. Emerging evidence has revealed that long non-coding RNAs (lncRNAs) could play crucial roles in the progression of PC. However, the biological role and clinical significance of TP73-AS1 in PC remain unclear.

Research motivation

Treatments for PC are still limited, and surgical resection could be the only chance to obtain curative treatment. We hope to provide a novel therapeutic target for patients with PC.

Research objectives

The present study aimed to investigate the role of TP73-AS1 in the growth and metastasis of PC.

Research methods

Quantitative reverse transcription-polymerase chain reaction was used to detect the expression of lncRNA TP73-AS1, miR-128-3p, and *GOLM1* in PC tissues and cells. The regulatory roles of TP73-AS1 in cell proliferation, migration, and invasion ability were verified by Cell Counting Kit-8, wound-healing, and transwell assays. The bioinformatics prediction software ENCORI was used to predict the putative binding sites of miR-128-3p. The interactions among TP73-AS1, miR-128-3p, and GOLM1 were explored by bioinformatics prediction, luciferase assay, and Western blot.

Research results

Our data suggested that TP73-AS1 and miRNA-128-3p were dysregulated in PC tissues and cells. TP73-AS1 silencing inhibited PC cell proliferation, migration, and invasion *in vitro* as well as suppressed tumor growth *in vivo*. Moreover, TP73-AS1

could promote tumor growth and invasion by acting as a competing endogenous RNA to promote GOLM1 expression by sponging miR-128-3p in PC.

Research conclusions

TP73-AS1 could promote PC cell proliferation and metastasis by modulating the miR-128-3p/GOLM1 axis.

Research perspectives

TP73-AS1 could promote PC progression, which might provide a potential treatment strategy for patients with PC.

REFERENCES

1 **Siegel R**, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014; **64**: 9-29 [PMID: 24399786 DOI: 10.3322/caac.21208]

2 **Kamisawa T**, Wood LD, Itoi T, Takaori K. Pancreatic cancer. *Lancet* 2016; **388**: 73-85 [PMID: 26830752 DOI: 10.1016/S0140-6736(16)00141-0]

3 **Tsai S**, Evans DB. Therapeutic Advances in Localized Pancreatic Cancer. *JAMA Surg* 2016; **151**: 862-868 [PMID: 27276001 DOI: 10.1001/jamasurg.2016.1113]

4 **Hansen TB**, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013; **495**: 384-388 [PMID: 23446346 DOI: 10.1038/nature11993]

5 **Wang KC**, Chang HY. Molecular mechanisms of long noncoding RNAs. *Mol Cell* 2011; **43**: 904-914 [PMID: 21925379 DOI: 10.1016/j.molcel.2011.08.018]

6 **Ebahimzadeh K**, Shoorei H, Mousavinejad SA, Anamag FT, Dinger ME, Taheri M, Ghafouri-Fard S. Emerging role of non-coding RNAs in response of cancer cells to radiotherapy. *Pathol Res Pract* 2021; **218**: 153327 [PMID: 33422780 DOI: 10.1016/j.prp.2020.153327]

7 **Wang C**, Chen Y, Chen K, Zhang L. Long Noncoding RNA LINC01134 Promotes Hepatocellular Carcinoma Metastasis *via* Activating AKT1S1 and NF-κB Signaling. *Front Cell Dev Biol* 2020; **8**: 429 [PMID: 32656205 DOI: 10.3389/fcell.2020.00429]

8 **Duguang L**, Jin H, Xiaowei Q, Peng X, Xiaodong W, Zhennan L, Jianjun Q, Jie Y. The involvement of lncRNAs in the development and progression of pancreatic cancer. *Cancer Biol Ther* 2017; **18**: 927-936 [PMID: 29053398 DOI: 10.1080/15384047.2017.1385682]

9 **Chen X**, Wang J, Xie F, Mou T, Zhong P, Hua H, Liu P, Yang Q. Long noncoding RNA LINC01559 promotes pancreatic cancer progression by acting as a competing endogenous RNA of miR-1343-3p to upregulate RAF1 expression. *Aging (Albany NY)* 2020; **12**: 14452-14466 [PMID: 32678071 DOI: 10.18632/aging.103487]

10 **Deng SJ**, Chen HY, Ye Z, Deng SC, Zhu S, Zeng Z, He C, Liu ML, Huang K, Zhong JX, Xu FY, Li Q, Liu Y, Wang CY, Zhao G. Hypoxia-induced LncRNA-BX111 promotes metastasis and progression of pancreatic cancer through regulating ZEB1 transcription. *Oncogene* 2018; **37**: 5811-5828 [PMID: 29970904 DOI: 10.1038/s41388-018-0382-1]

11 **You L**, Chang D, Du HZ, Zhao YP. Genome-wide screen identifies PVT1 as a regulator of Gemcitabine sensitivity in human pancreatic cancer cells. *Biochem Biophys Res Commun* 2011; **407**: 1-6 [PMID: 21316338 DOI: 10.1016/j.bbrc.2011.02.027]

12 **Zhang R**, Jin H, Lou F. The Long Non-Coding RNA TP73-AS1 Interacted With miR-142 to Modulate Brain Glioma Growth Through HMGB1/RAGE Pathway. *J Cell Biochem* 2018; **119**: 3007-3016 [PMID: 28379612 DOI: 10.1002/jcb.26021]

13 **Li S**, Huang Y, Fu Y, Tang D, Kang R, Zhou R, Fan XG. The long non-coding RNA TP73-AS1 modulates HCC cell proliferation through miR-200a-dependent HMGB1/RAGE regulation. *J Exp Clin Cancer Res* 2017; **36**: 51 [PMID: 28403886 DOI: 10.1186/s13046-017-0519-z]

14 **Zhang L**, Fang F, He X. Long noncoding RNA TP73-AS1 promotes non-small cell lung cancer progression by competitively sponging miR-449a/EZH2. *Biomed Pharmacother* 2018; **104**: 705-711 [PMID: 29803931 DOI: 10.1016/j.biopha.2018.05.089]

15 **Deng B**, Wang B, Fang J, Zhu X, Cao Z, Lin Q, Zhou L, Sun X. MiRNA-203 suppresses cell proliferation, migration and invasion in colorectal cancer *via* targeting of EIF5A2. *Sci Rep* 2016; **6**: 28301 [PMID: 27376958 DOI: 10.1038/srep28301]

16 **Jiang J**, Feng X, Zhou W, Wu Y, Yang Y. MiR-128 reverses the gefitinib resistance of the lung cancer stem cells by inhibiting the c-met/PI3K/AKT pathway. *Oncotarget* 2016; **7**: 73188-73199 [PMID: 27690301 DOI: 10.18632/oncotarget.12283]

17 **Liu T**, Zhang X, Du L, Wang Y, Liu X, Tian H, Wang L, Li P, Zhao Y, Duan W, Xie Y, Sun Z, Wang C. Exosome-transmitted miR-128-3p increase chemosensitivity of oxaliplatin-resistant colorectal cancer. *Mol Cancer* 2019; **18**: 43 [PMID: 30890168 DOI: 10.1186/s12943-019-0981-7]

18 **Batista PJ**, Chang HY. Long noncoding RNAs: cellular address codes in development and disease. *Cell* 2013; **152**: 1298-1307 [PMID: 23498938 DOI: 10.1016/j.cell.2013.02.012]

19 **Li N**, Yang G, Luo L, Ling L, Wang X, Shi L, Lan J, Jia X, Zhang Q, Long Z, Liu J, Hu W, He Z, Liu H, Liu W, Zheng G. lncRNA *THAP9-AS1* Promotes Pancreatic Ductal Adenocarcinoma Growth and

Leads to a Poor Clinical Outcome *via* Sponging miR-484 and Interacting with YAP. *Clin Cancer Res* 2020; **26**: 1736-1748 [PMID: 31831555 DOI: 10.1158/1078-0432.CCR-19-0674]

20 **Pan S**, Shen M, Zhou M, Shi X, He R, Yin T, Wang M, Guo X, Qin R. Long noncoding RNA LINC01111 suppresses pancreatic cancer aggressiveness by regulating DUSP1 expression *via* microRNA-3924. *Cell Death Dis* 2019; **10**: 883 [PMID: 31767833 DOI: 10.1038/s41419-019-2123-y]

21 **Kong X**, Liu CX, Wang GD, Yang H, Yao XM, Hua Q, Li XY, Zhang HM, Ma MZ, Su Q, Lv K. LncRNA LEGLTBC Functions as a ceRNA to Antagonize the Effects of miR-34a on the Downregulation of SIRT1 in Glucolipotoxicity-Induced INS-1 Beta Cell Oxidative Stress and Apoptosis. *Oxid Med Cell Longev* 2019; **2019**: 4010764 [PMID: 31737170 DOI: 10.1155/2019/4010764]

22 **Yang G**, Song R, Wang L, Wu X. Knockdown of long non-coding RNA TP73-AS1 inhibits osteosarcoma cell proliferation and invasion through sponging miR-142. *Biomed Pharmacother* 2018; **103**: 1238-1245 [PMID: 29864904 DOI: 10.1016/j.biopha.2018.04.146]

23 **Cai Y**, Yan P, Zhang G, Yang W, Wang H, Cheng X. Long non-coding RNA TP73-AS1 sponges miR-194 to promote colorectal cancer cell proliferation, migration and invasion *via* up-regulating TGFα. *Cancer Biomark* 2018; **23**: 145-156 [PMID: 30010111 DOI: 10.3233/CBM-181503]

24 **Papagiannakopoulos T**, Friedmann-Morvinski D, Neveu P, Dugas JC, Gill RM, Huillard E, Liu C, Zong H, Rowitch DH, Barres BA, Verma IM, Kosik KS. Pro-neural miR-128 is a glioma tumor suppressor that targets mitogenic kinases. *Oncogene* 2012; **31**: 1884-1895 [PMID: 21874051 DOI: 10.1038/onc.2011.380]

25 **Zhu Y**, Yu F, Jiao Y, Feng J, Tang W, Yao H, Gong C, Chen J, Su F, Zhang Y, Song E. Reduced miR-128 in breast tumor-initiating cells induces chemotherapeutic resistance *via* Bmi-1 and ABCC5. *Clin Cancer Res* 2011; **17**: 7105-7115 [PMID: 21953503 DOI: 10.1158/1078-0432.CCR-11-0071]

26 **Cai J**, Fang L, Huang Y, Li R, Xu X, Hu Z, Zhang L, Yang Y, Zhu X, Zhang H, Wu J, Li J, Zeng M, Song E, He Y, Li M. Simultaneous overactivation of Wnt/β-catenin and TGFβ signalling by miR-128-3p confers chemoresistance-associated metastasis in NSCLC. *Nat Commun* 2017; **8**: 15870 [PMID: 28627514 DOI: 10.1038/ncomms15870]

27 **Wei H**, Hao X, Li B, Li X, Hou J, Qiao Y, Zhang R. GP73 is a potential marker for evaluating AIDS progression and antiretroviral therapy efficacy. *Mol Biol Rep* 2013; **40**: 6397-6405 [PMID: 24068434 DOI: 10.1007/s11033-013-2754-5]

28 **Kladney RD**, Cui X, Bulla GA, Brunt EM, Fimmel CJ. Expression of GP73, a resident Golgi membrane protein, in viral and nonviral liver disease. *Hepatology* 2002; **35**: 1431-1440 [PMID: 12029628 DOI: 10.1053/jhep.2002.32525]

29 **Mao Y**, Yang H, Xu H, Lu X, Sang X, Du S, Zhao H, Chen W, Xu Y, Chi T, Yang Z, Cai J, Li H, Chen J, Zhong S, Mohanti SR, Lopez-Soler R, Millis JM, Huang J, Zhang H. Golgi protein 73 (GOLPH2) is a valuable serum marker for hepatocellular carcinoma. *Gut* 2010; **59**: 1687-1693 [PMID: 20876776 DOI: 10.1136/gut.2010.214916]

30 **Ye QH**, Zhu WW, Zhang JB, Qin Y, Lu M, Lin GL, Guo L, Zhang B, Lin ZH, Roessler S, Forgues M, Jia HL, Lu L, Zhang XF, Lian BF, Xie L, Dong QZ, Tang ZY, Wang XW, Qin LX. GOLM1 Modulates EGFR/RTK Cell-Surface Recycling to Drive Hepatocellular Carcinoma Metastasis. *Cancer Cell* 2016; **30**: 444-458 [PMID: 27569582 DOI: 10.1016/j.ccell.2016.07.017]

31 **Bao YX**, Cao Q, Yang Y, Mao R, Xiao L, Zhang H, Zhao HR, Wen H. Expression and prognostic significance of golgiglycoprotein73 (GP73) with epithelial-mesenchymal transition (EMT) related molecules in hepatocellular carcinoma (HCC). *Diagn Pathol* 2013; **8**: 197 [PMID: 24313979 DOI: 10.1186/1746-1596-8-197]

32 **Yan G**, Ru Y, Wu K, Yan F, Wang Q, Wang J, Pan T, Zhang M, Han H, Li X, Zou L. GOLM1 promotes prostate cancer progression through activating PI3K-AKT-mTOR signaling. *Prostate* 2018; **78**: 166-177 [PMID: 29181846 DOI: 10.1002/pros.23461]

33 **Haigis KM**. KRAS Alleles: The Devil Is in the Detail. *Trends Cancer* 2017; **3**: 686-697 [PMID: 28958387 DOI: 10.1016/j.trecan.2017.08.006]

34 **di Magliano MP**, Logsdon CD. Roles for KRAS in pancreatic tumor development and progression. *Gastroenterology* 2013; **144**: 1220-1229 [PMID: 23622131 DOI: 10.1053/j.gastro.2013.01.071]

35 **Jones S**, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008; **321**: 1801-1806 [PMID: 18772397 DOI: 10.1126/science.1164368]

36 **Ryan DP**, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med* 2014; **371**: 2140-2141 [PMID: 25427123 DOI: 10.1056/NEJMc1412266]

37 **Tape CJ**, Ling S, Dimitriadi M, McMahon KM, Worboys JD, Leong HS, Norrie IC, Miller CJ, Poulogiannis G, Lauffenburger DA, Jørgensen C. Oncogenic KRAS Regulates Tumor Cell Signaling *via* Stromal Reciprocation. *Cell* 2016; **165**: 1818 [PMID: 27315484 DOI: 10.1016/j.cell.2016.05.079]

38 **Liang C**, Qin Y, Zhang B, Ji S, Shi S, Xu W, Liu J, Xiang J, Liang D, Hu Q, Ni Q, Xu J, Yu X. Metabolic plasticity in heterogeneous pancreatic ductal adenocarcinoma. *Biochim Biophys Acta* 2016; **1866**: 177-188 [PMID: 27600832 DOI: 10.1016/j.bbcan.2016.09.001]

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ORIGINAL ARTICLE

Retrospective Study

Prediction of microvascular invasion in solitary hepatocellular carcinoma ≤ 5 cm based on computed tomography radiomics

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Author contributions: Mao XH and Li YC contributed equally to this work; Liu P designed the research study; Zhang T and Tan XZ performed the research; Gu QB contributed new reagents and analytic tools; Liu P, Zhang T, and He YQ analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

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Informed consent statement: All patients provided written informed consent.

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Abstract

BACKGROUND

Liver cancer is one of the most common malignant tumors, and ranks as the fourth leading cause of cancer death worldwide. Microvascular invasion (MVI) is considered one of the most important factors for recurrence and poor prognosis of liver cancer. Thus, accurately identifying MVI before surgery is of great importance in making treatment strategies and predicting the prognosis of patients with hepatocellular carcinoma (HCC). Radiomics as an emerging field, aims to utilize artificial intelligence software to develop methods that may contribute to cancer diagnosis, treatment improvement and evaluation, and better prediction.

AIM

To investigate the predictive value of computed tomography radiomics for MVI in solitary HCC ≤ 5 cm.

METHODS

A total of 185 HCC patients, including 122 MVI negative and 63 MVI positive patients, were retrospectively analyzed. All patients were randomly assigned to

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the training group (n = 124) and validation group (n = 61). A total of 1351 radiomic features were extracted based on three-dimensional images. The diagnostic performance of the radiomics model was verified in the validation group, and the Delong test was applied to compare the radiomics and MVI-related imaging features (two-trait predictor of venous invasion and radiogenomic invasion).

RESULTS

A total of ten radiomics features were finally obtained after screening 1531 features. According to the weighting coefficient that corresponded to the features, the radiomics score (RS) calculation formula was obtained, and the RS score of each patient was calculated. The radiomics model exhibited a better correction and identification ability in the training and validation groups [area under the curve: 0.72 (95% confidence interval: 0.58-0.86) and 0.74 (95% confidence interval: 0.66-0.83), respectively]. Its prediction performance was significantly higher than that of the image features ($P < 0.05$).

CONCLUSION

Computed tomography radiomics has certain predictive value for MVI in solitary HCC ≤ 5 cm, and the predictive ability is higher than that of image features.

Key Words: Hepatocellular carcinoma; Microvascular invasion; Radiomics; Image features; Computed tomography

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Core Tip: Microvascular invasion (MVI) is considered one of the most important factors for recurrence and poor prognosis of liver cancer. Thus, accurately identifying MVI before surgery is of great importance in making treatment strategies and predicting the prognosis of patients with hepatocellular carcinoma (HCC). This study showed that radiomics as an emerging method at present had a good diagnostic efficiency and exhibited better accuracy in predicting MVI than image features, indicating that radiomics is a more suitable method in predicting MVI in solitary HCC ≤ 5 cm.

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DOI: https://dx.doi.org/10.3748/wjg.v27.i17.2015

INTRODUCTION

Liver cancer is one of the most common malignant tumors, and ranks as the fourth leading cause of cancer death worldwide[1]. Furthermore, more than half of liver cancers occur in China, where there is a high incidence of hepatitis B[2]. Moreover, the recurrence rate after hepatectomy is 70%[3], and microvascular invasion (MVI) is considered one of the most important factors for recurrence and poor prognosis of liver cancer[4]. MVI has a relatively high incidence in hepatocellular carcinoma (HCC), which ranges from 12.4% to 57.1%, and may occur even in solitary HCC ≤ 2 cm[5,6]. Thus, accurately identifying MVI before surgery is of great importance in making treatment strategies and predicting the prognosis of patients with HCC[7]. However, MVI can only be confirmed by histopathology *via* surgical resection at present. Therefore, the accurate prediction of MVI before surgery is desperately needed. Radiomics as an emerging field, which aims to utilize artificial intelligence software to develop methods that may contribute to cancer diagnosis, treatment improvement and evaluation, and better prediction[8]. At present, few studies have focused on the prediction of MVI in the early stage of HCC (which refers to solitary tumor with a size of ≤ 5 cm, without intrahepatic venous invasion[9]). The present study aimed to

investigate the predictive value of computed tomography (CT) radiomics for MVI in solitary HCC ≤ 5 cm.

MATERIALS AND METHODS

Patients

Patients were retrospectively collected from January 1, 2014 to November 15, 2018 (Hunan provincial People's Hospital). The inclusion criteria were: (1) Pathologically diagnosed hepatocellular carcinoma with MVI; (2) Solitary tumor with the maximum diameter of ≤ 5 cm; and (3) Enhanced CT scanning was performed before surgery. The exclusion criteria were: (1) Complicated with other malignant tumors, and multiple primary or recurrent liver cancer; (2) History of preoperative treatment; (3) CT revealed a vascular tumor thrombus or macrovascular invasion; or (4) The tumor boundary was difficult to determine. The flowchart for the screening of patients is presented in Figure 1.

Examination methods

The Philips (Brilliance iCT 256) and Neusoft (NeuViz 64EN) scanners were used, with a tube voltage of 100-200 kV, tube current of 171-313 mAs, scanning layer thickness of 5 mm, layer spacing of 5 mm, and matrix of 1024 × 1024. The contrast medium (iopromide injection, 300 mgI/mL) was injected using a high-pressure syringe through the anterior cubital vein at a rate of 3.5 mL/s and at a dose of 1.2 mL/kg. Dynamic contrast-enhanced imaging data acquisition was performed at fixed time points: For the arterial phase, acquisition occurred at approximately 25-33 s after administration; for the portal vein phase, it was 57-63 s, and for the delayed phase, 117-123 s.

Observation of imaging features

The two-trait predictor of venous invasion (TTPVI) was defined as having two independent imaging characteristics at the same time, and the development of an internal tumor artery without the signs of low density at the tumor margin[10]. Radiogenomic invasion (RVI) comprised of three independent image features: Intratumoral artery, low-density ring, and tumor-liver difference[11]. If there was an intratumoral artery, but there was no low-density ring or tumor-liver difference, the tumor was considered to have RVI. The imaging features (TTPVI and RVI) were evaluated double-blindly by two radiologists (with three years and seven years of experience in abdominal radiology, respectively). If these radiologists had inconsistent evaluation results, a third senior radiologist (with 13 years of experience in abdominal radiology) would make the further confirmation. The detailed description is presented in Figure 2.

Drawing, image feature extraction, selection, and construction of region of interest

Region of interest selection: The CT images of the patients in the arterial phase were exported in DICOM format. Without knowing the pathological results, the radiologist with three years of experience in abdominal radiology used the 3D-Slice software (www.slice.org) to delineate the region of interest (ROI) in each layer that contained the tumor, and finally formed the three-dimensional segmented image.

Extraction of radiomic features: After segmenting the images, the plug-in radiologics in the 3D-Slice software was used to analyze the original image data in the ROI, and 1351 candidate texture parameters were extracted, including the histogram features, morphological features, original features, and texture features.

Selection of features correlated to the MVI status and construction of the radiomics tags: First, the extracted intraclass features were evaluated by intraclass correlation coefficient (ICC), and features with ICC < 0.75 were removed. Then, the least absolute shrinkage and selection operator (LASSO) algorithm was used for dimension reduction and feature construction, and the least feature variables were selected by 10-fold cross-validation with the minimal value. The selected features were modeled by Logistic regression, in order to generate the formula of the radiomics score (rad-score, RS) and calculate the RS score of each patient.

Statistical analysis

The R language (version 3.4.0, https://www.r-project.org) was used for the statistical analyses, and $P < 0.05$ was considered statistically significant. The chi-square test for

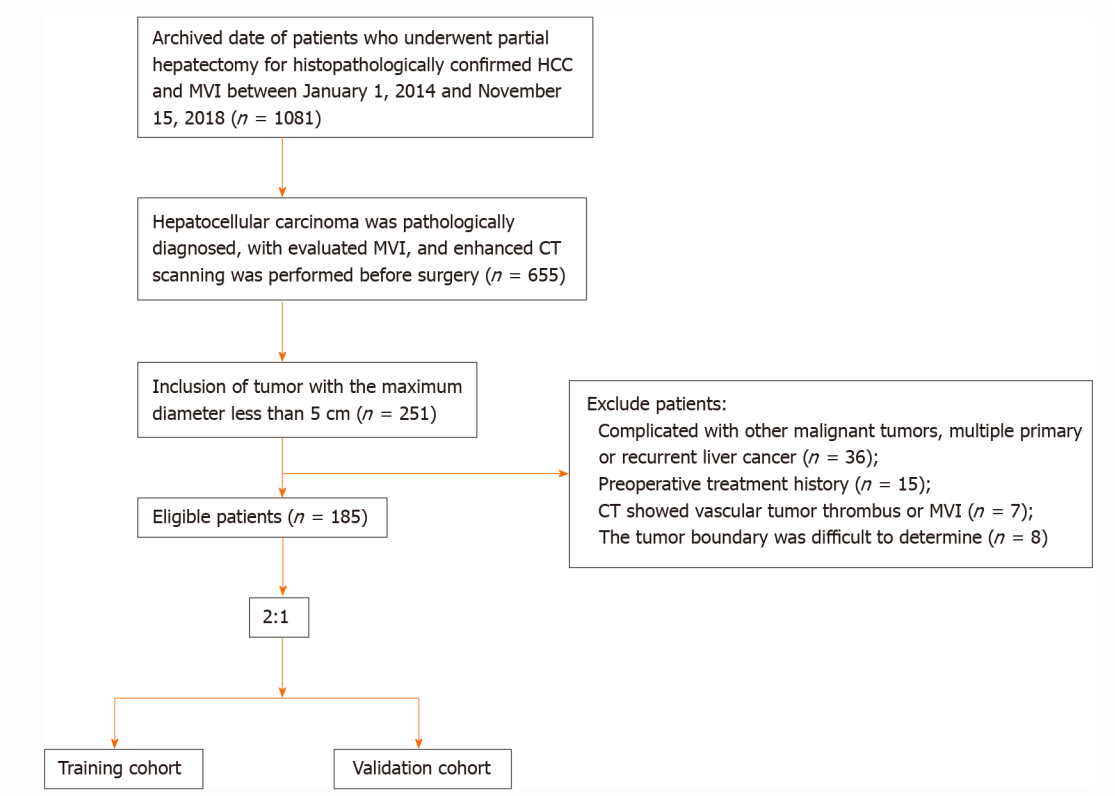


Figure 1 Screening process for patients with liver cancer. HCC: Hepatocellular carcinoma; CT: Computed tomography; MVI: Microvascular invasion.

two independent samples was used for categorical variables, and Mann-Whitney *U*-test for two independent samples was used for continuous variables, in order to analyze the difference between the training group and verification group. The receiver operator characteristic curve and the area under the curve (AUC) were used to evaluate the prediction efficiency of the radiomic and image features, and the Delong test was used to determine whether there was a statistical difference between the two methods.

RESULTS

Patient clinicopathologic features

Finally, the study consisted of 185 patients. Tumor size ranged from 10 mm to 50 mm. The clinicopathological and CT features in the training group and verification group are presented in Table 1. There was no significant difference in scores for MVI, other clinical data, imaging features (TTPVI and RVI), or RS between the two groups ($P > 0.05$), indicating the reasonable arrangement of the training group and verification group.

Construction of radiomics tags

After removal by high correlation, 185 of the 1531 features remained. Then, ten features were selected by dimension reduction using the LASSO algorithm, as shown in Figure 3.

According to the weighting coefficient corresponding to the feature (Figure 3), the radiomics formula was obtained and used to calculate the histological score of each lesion in the training group and verification group. The formula is as follows: Rad-score = -0.66692761 - 0.02491645 × originalshapeFlatness + 0.10564798 × log.sigma.1.0.mm.3DglcmContrast + 0.08789965 × log.sigma.2.0.mm.3DgldmDependenceVariance- 0.06847308 × log.sigma.2.0.mm.3DglszmSizeZoneNonUniformityNormalized - 0.01878760 × log.sigma.2.0.mm.3DglszmSizeZoneNonUniformity + 0.14275995 × log.sigma.3.0.mm.3Dfirstorder90Percentile + 0.04978394 × log.sigma.4.0.mm.3DglszmSmallAreaEmphasis - 0.02095096 × log.sigma.4.0.mm.3DngtdmBusyness + 0.06796763 × wavelet.HLLglcmSumSquares + 0.0767876 × 1wavelet.HLLglszmLargeAreaLow

Table 1 Clinical and imaging features of patients in the training group and verification group

Feature	Training group (*n* = 124)	Verification group (*n* = 61)	*Z* value/χ^2 value	*P* value
Age (yr), median (quartile)	54 (47; 63)	52 (46; 62)	-0.900	0.368
Gender/cases				
Male	102	53	0.349	0.555
Female	22	8		
Hepatitis B	104	56	1.575	0.210
Liver cirrhosis	84	43	0.044	0.833
AFP (ng/mL)				
≤ 20	73	30	1.188	0.276
> 20	51	31		
MVI				
Negative	82	40	0.000	1.000
Positive	42	21		
Tumor size (mm), median (quartile)	36 (28; 44)	34 (27.5; 41)	-0.746	0.456
TTPVI				
Negative	56	25	0.145	0.703
Positive	68	36		
RVI				
Negative	93	40	1.362	0.243
Positive	31	21		
Rad-score, median (quartile)	-0.669 (-0.831; -0.546)	-0.640 (-0.780; -0.494)	-0.917	0.359

AFP: α-fetoprotein; MVI: Microvascular invasion; TTPVI: Two-trait predictor of venous invasion; RVI: Radiogenomic invasion; Rad-score: Radiomics score.

GrayLevelEmphasis.

Predictive performance of radiomics tags

The median RS [quartile interval] of MVI positive patients [training group: -0.574 (-0.695, -0.412); verification group: -0.495 (-0.644, -0.429)] was significantly higher than that of MVI negative patients [training group: -0.710 (-0.899, -0.610); verification group: -0.709 (-0.805, -0.589)] in both the training and verification groups, and the difference was statistically significant ($P < 0.05$). The radiomics tags exhibited better diagnostic efficacy in both the training and verification groups, as shown in Table 2.

Comparison of predictive performance between radiomics tags and MVI-related image features

As shown in Figure 4 and Table 2, the diagnostic efficacy of the radiomics score was higher than that of the image features in the training group and verification group, and the difference was statistically significant ($P < 0.05$).

DISCUSSION

The present study revealed that radiomics, as an emerging method at present, exhibited good diagnostic efficiency and better accuracy in predicting MVI, when compared to image features, indicating that radiomics is a more suitable method for predicting MVI in solitary HCC ≤ 5 cm.

A number of studies have shown that tumor size and imaging features can predict MVI, in which TTPVI and RVI are good predictors with an ideal sensitivity and specificity[11]. However, the diagnostic performance of TTPVI and RVI in the present study was significantly lower than that in previous studies, which might be attributed

Table 2 Comparison of predictive performance between radiomics tags and image features

	Training group		Verification group	
	AUC (95%CI)	*P* value	AUC (95%CI)	*P* value
Rad-score	0.724 (0.584-0.863)		0.745 (0.655-0.834)	
TTPVI	0.590 (0.500-0.679)		0.522 (0.393-0.651)	
RVI	0.545 (0.462-0.628)		0.528 (0.401-0.655)	
Rad *vs* TTPVI		0.018		0.043
Rad *vs* RVI		0.002		0.048

AUC: Area under the curve; CI: Confidence interval; TTPVI: Two-trait predictor of venous invasion; RVI: Radiogenomic invasion; Rad-score: Radiomics score.

to the difference in tumor diameter of the study samples. Furthermore, previous studies did not define the tumor size, while the present study merely included patients with a tumor diameter of ≤ 5 cm. In addition, the imaging predictors, such as internal arteries and low-density shadow, were not commonly observed in cases with small tumors, which significantly reduced the positive rate of the MVI-related image features in the present study.

With the recent increase in development of radiomics, numerous studies have indicated that radiomics can reveal the pathological grade, prognosis, MVI, and treatment response of liver cancer. The nomogram of MVI in HCC based on CT radiomics established by Peng *et al*[12] exhibited good decision-making efficiency (AUC = 0.84), which was slightly higher than the results of the present study, but the model did not involve the tumor diameter. In another study[13], the diagnostic efficacy of predicting MVI in a tumor diameter of ≤ 5 cm based on radiomics (the AUC for the verification and verification group was 0.637 and 0.583, respectively) was slightly lower than that of the present study. Compared to that study[13], the present study adopted more stringent inclusion and exclusion criteria. Moreover, the present study only included solitary liver cancer with a diameter of ≤ 5 cm. Partial hepatectomy is the first choice for patients with solitary liver cancer ≤ 5 cm and good liver function[9]. However, if the lesion is not fully resected, the residual MVI near the surgical margin may be an important cause of recurrence in patients with HCC[14], and some studies have demonstrated that extended resection can reduce the early recurrence rate of patients with liver cancer complicated with MVI[15]. Therefore, the present study has certain reference value for surgery choice in patients with liver cancer. In addition, the present study excluded patients with a visible thrombus or the invasion of large blood vessels, rupture and bleeding of liver cancer, and intangible tumor boundary due to other reasons, because the ROI of these patients was difficult to delineate. Hence, measurement errors were hard to avoid. Furthermore, the present study employed the three-dimensional ROI of tumors to the extract radiomics features, which can better reflect the whole outline of the tumor, and allow for the extraction of more tumor information, when compared to two-dimensional ROI. Some studies have revealed that the feature extraction of the maximum cross-sectional area cannot represent the whole tumor[16]. Hence, the present study has obtained more objective prediction results.

The present study had some limitations. First, the present retrospective and single-center study may have selection bias. Second, the present study only used arterial phase images. Multi-phase images may be utilized to obtain more tumor information and improve the diagnostic efficiency. Therefore, multi-center studies with large samples and multi-phase images would become our future research content.

CONCLUSION

In conclusion, CT radiomics has certain predictive value for MVI in solitary HCC ≤ 5 cm. Compared to imaging features, the predictive ability of radiomics tags is significantly higher. The radiomics model of MVI would facilitate clinicians in choosing the appropriate treatment.

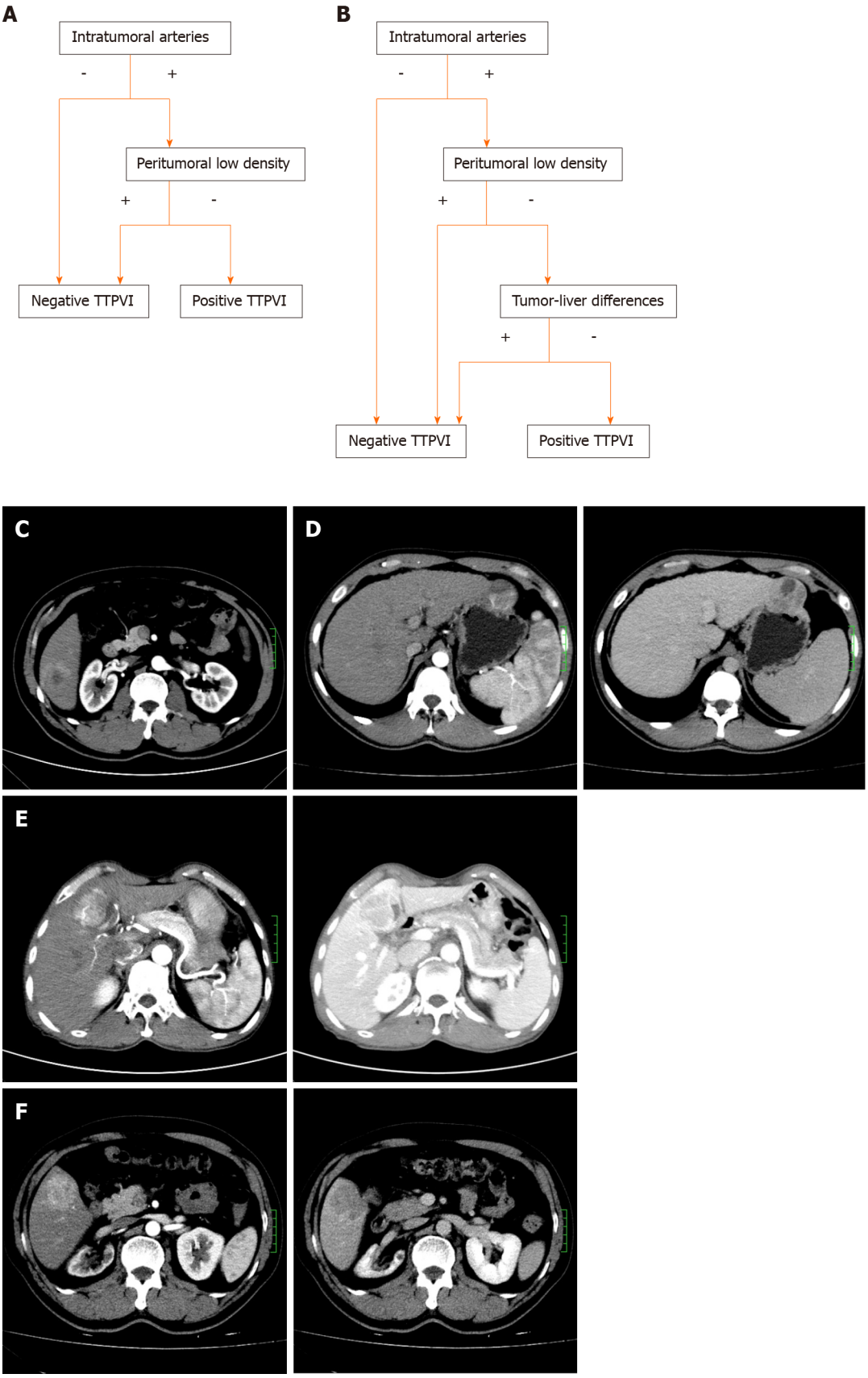


Figure 2 Specific performance of two-trait predictor of venous invasion and radiogenomic invasion. A and B: The discriminant process of two-trait predictor of venous invasion (TTPVI) (A) and radiogenomic invasion (RVI) (B); C: Negative intratumoral arteries: Negative TTPVI and RVI; D: Positive intratumoral arteries and peritumoral low density: Negative TTPVI and PVI; E: Positive intratumoral arteries, negative peritumoral low-density shadow, and positive tumor-liver differences: Positive TTPVI and negative RVI; F: Positive intratumoral arteries, negative peritumoral low density, and negative tumor-liver differences: Positive TTPVI and RVI. TTPVI: Two-trait predictor of venous invasion; RVI: Radiogenomic invasion.

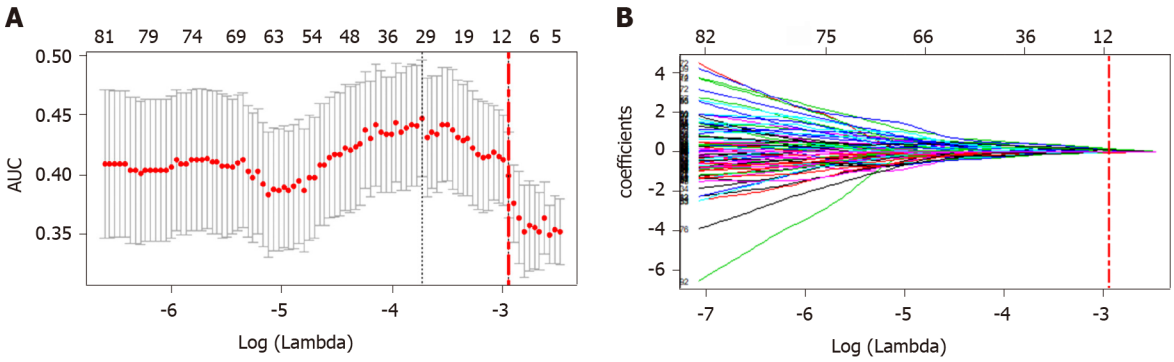


Figure 3 Selection of radiomic features using the least absolute shrinkage and selection operator-logistic regression model. A: Coefficient profile of 158 radiomics features against the area under the curve; B: Cross-validation curve. Red dotted vertical lines are drawn at the optimal log (Lambda) by using 10-fold cross-validation and the 1-SE criteria. Ten nonzero coefficients are chosen. AUC: Area under the curve.

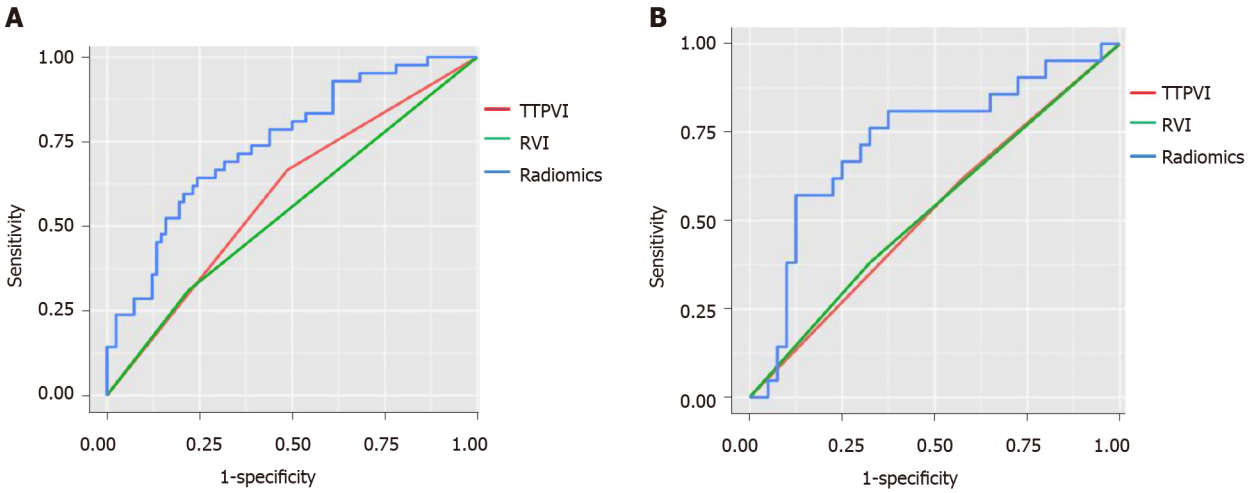


Figure 4 The two-trait predictor of venous invasion (green curve), radiogenomic invasion (red curve), and receiver operator characteristic of radiomics tag (blue curve) from the two groups. A: Training group; B: Verification group. TTPVI: Two-trait predictor of venous invasion; RVI: Radiogenomic invasion.

ARTICLE HIGHLIGHTS

Research background

Liver cancer is one of the most common malignant tumors, and ranks as the fourth leading cause of cancer death worldwide. Microvascular invasion (MVI) is considered one of the most important factors for recurrence and poor prognosis of liver cancer. Radiomics as an emerging field, aims to utilize artificial intelligence software to develop methods that may contribute to cancer diagnosis, treatment improvement, and evaluation and better prediction.

Research motivation

At present, few studies have focused on the prediction of MVI in the early stage of hepatocellular carcinoma (HCC) (which refers to solitary tumor with a size of ≤ 5 cm, without MVI). Our study aimed to investigate the predictive value of computed tomography (CT) radiomics for MVI in solitary HCC ≤ 5 cm.

Research objectives

This study aimed to investigate the predictive value of radiomics for MVI in solitary HCC ≤ 5 cm.

Research methods

A total of 185 HCC patients, including 122 MVI negative and 63 MVI positive patients, were retrospectively analyzed. All patients were randomly assigned to the training

group (n = 124) and validation group (n = 61), at a ratio of 2:1. A total of 1351 radiomic features were extracted based on three-dimensional images. In the training group, the least absolute shrinkage and selection operator feature selection algorithm was used to reduce the dimensions, and the most relevant radiomic features of MVI were selected to calculate the image score (Rad-score, RS) of each patient. The diagnostic performance of the radiomics model was verified in the validation group, and the Delong test was applied to compare the radiomics and MVI-related imaging features (two-trait predictor of venous invasion and radiogenomic invasion).

Research results

A total of ten radiomics features were finally obtained after screening 1531 features. According to the weighting coefficient that corresponded to the features, the RS calculation formula was obtained, and the RS score of each patient was calculated. The radiomics model exhibited a better correction and identification ability in the training and validation groups [area under the curve: 0.72 (95% confidence interval: 0.58-0.86) and 0.74 (95% confidence interval: 0.66-0.83), respectively]. Its prediction performance was significantly higher than that of the image features (P < 0.05).

Research conclusions

CT radiomics has certain predictive value for MVI in solitary HCC ≤ 5 cm, and the predictive ability is higher than that of image features.

Research perspectives

The accurate prediction of MVI before surgery is desperately needed. Radiomics as an emerging field, aims to utilize artificial intelligence software to develop methods that may contribute to cancer diagnosis, treatment improvement and evaluation, and better prediction. At present, few studies have focused on the prediction of MVI in the early stage of HCC (which refers to solitary tumor with a size of ≤ 5 cm, without MVI). The present study aimed to investigate the predictive value of CT radiomics for MVI in solitary HCC ≤ 5 cm.

REFERENCES

1 Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2020; **70**: 313 [PMID: 32767693 DOI: 10.3322/caac.21609]

2 **Chen W**, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; **66**: 115-132 [PMID: 26808342 DOI: 10.3322/caac.21338]

3 **Bruix J**, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. *Gut* 2014; **63**: 844-855 [PMID: 24531850 DOI: 10.1136/gutjnl-2013-306627]

4 **Kim KA**, Kim MJ, Jeon HM, Kim KS, Choi JS, Ahn SH, Cha SJ, Chung YE. Prediction of microvascular invasion of hepatocellular carcinoma: usefulness of peritumoral hypointensity seen on gadoxetate disodium-enhanced hepatobiliary phase images. *J Magn Reson Imaging* 2012; **35**: 629-634 [PMID: 22069244 DOI: 10.1002/jmri.22876]

5 **Rodríguez-Perálvarez M**, Luong TV, Andreana L, Meyer T, Dhillon AP, Burroughs AK. A systematic review of microvascular invasion in hepatocellular carcinoma: diagnostic and prognostic variability. *Ann Surg Oncol* 2013; **20**: 325-339 [PMID: 23149850 DOI: 10.1245/s10434-012-2513-1]

6 **Wang H**, Wu MC, Cong WM. Microvascular invasion predicts a poor prognosis of solitary hepatocellular carcinoma up to 2 cm based on propensity score matching analysis. *Hepatol Res* 2019; **49**: 344-354 [PMID: 30117236 DOI: 10.1111/hepr.13241]

7 **Pons F**, Varela M, Llovet JM. Staging systems in hepatocellular carcinoma. *HPB (Oxford)* 2005; **7**: 35-41 [PMID: 18333159 DOI: 10.1080/13651820410024058]

8 **Lambin P**, Rios-Velazquez E, Leijenaar R, Carvalho S, van Stiphout RG, Granton P, Zegers CM, Gillies R, Boellard R, Dekker A, Aerts HJ. Radiomics: extracting more information from medical images using advanced feature analysis. *Eur J Cancer* 2012; **48**: 441-446 [PMID: 22257792 DOI: 10.1016/j.ejca.2011.11.036]

9 **Yau T**, Tang VY, Yao TJ, Fan ST, Lo CM, Poon RT. Development of Hong Kong Liver Cancer staging system with treatment stratification for patients with hepatocellular carcinoma. *Gastroenterology* 2014; **146**: 1691-700. e3 [PMID: 24583061 DOI: 10.1053/j.gastro.2014.02.032]

10 **Renzulli M**, Brocchi S, Cucchetti A, Mazzotti F, Mosconi C, Sportoletti C, Brandi G, Pinna AD, Golfieri R. Can Current Preoperative Imaging Be Used to Detect Microvascular Invasion of Hepatocellular Carcinoma? *Radiology* 2016; **279**: 432-442 [PMID: 26653683 DOI: 10.1148/radiol.2015150998]

11 **Banerjee S**, Wang DS, Kim HJ, Sirlin CB, Chan MG, Korn RL, Rutman AM, Siripongsakun S, Lu D, Imanbayev G, Kuo MD. A computed tomography radiogenomic biomarker predicts microvascular

invasion and clinical outcomes in hepatocellular carcinoma. *Hepatology* 2015; **62**: 792-800 [PMID: 25930992 DOI: 10.1002/hep.27877]

12 **Peng J**, Zhang J, Zhang Q, Xu Y, Zhou J, Liu L. A radiomics nomogram for preoperative prediction of microvascular invasion risk in hepatitis B virus-related hepatocellular carcinoma. *Diagn Interv Radiol* 2018; **24**: 121-127 [PMID: 29770763 DOI: 10.5152/dir.2018.17467]

13 **He M**, Zhang P, Ma X, He B, Fang C, Jia F. Radiomic Feature-Based Predictive Model for Microvascular Invasion in Patients With Hepatocellular Carcinoma. *Front Oncol* 2020; **10**: 574228 [PMID: 33251138 DOI: 10.3389/fonc.2020.574228]

14 **Zhao H**, Hua Y, Lu Z, Gu S, Zhu L, Ji Y, Qiu Y, Dai T, Jin H. Prognostic value and preoperative predictors of microvascular invasion in solitary hepatocellular carcinoma ≤ 5 cm without macrovascular invasion. *Oncotarget* 2017; **8**: 61203-61214 [PMID: 28977857 DOI: 10.18632/oncotarget.18049]

15 **Wang H**, Yu H, Qian YW, Cao ZY, Wu MC, Cong WM. Impact of Surgical Margin on the Prognosis of Early Hepatocellular Carcinoma (≤5 cm): A Propensity Score Matching Analysis. *Front Med (Lausanne)* 2020; **7**: 139 [PMID: 32478080 DOI: 10.3389/fmed.2020.00139]

16 **Ng F**, Kozarski R, Ganeshan B, Goh V. Assessment of tumor heterogeneity by CT texture analysis: can the largest cross-sectional area be used as an alternative to whole tumor analysis? *Eur J Radiol* 2013; **82**: 342-348 [PMID: 23194641 DOI: 10.1016/j.ejrad.2012.10.023]

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ORIGINAL ARTICLE

Retrospective Study

Hepatocellular carcinoma progression in hepatitis B virus-related cirrhosis patients receiving nucleoside (acid) analogs therapy: A retrospective cross-sectional study

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Author contributions: Yang DH designed the research study; Yang DH and Wang WP analyzed the data and wrote the manuscript; Zhang Q, Pan HY, Huang YC and Zhang JJ collected the data; All authors have read and approved the final manuscript.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Zhejiang Provincial People's Hospital (2020QT155, Hangzhou, Zhejiang Province, China).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

BACKGROUND

Antiviral therapy cannot completely block the progression of hepatitis B to hepatocellular carcinoma (HCC). Furthermore, there are few predictors of early HCC progression and limited strategies to prevent progression in patients with HBV-related cirrhosis who receive nucleos(t)ide analog (NA) therapy.

AIM

The study aim was to clarify risk factors and the diagnostic value of alpha-fetoprotein (AFP) for HCC progression in NA-treated hepatitis B virus (HBV)-related cirrhosis patients.

METHODS

In this retrospective cross-sectional study, we analyzed the clinical data of 266 patients with HBV-related cirrhosis who received NA treatment between February 2014 and April 2020 at Zhejiang Provincial People's Hospital. The patients were divided into two groups, 145 who did not progress to HCC (No-HCC group), and 121 who progressed to HCC during NA treatment (HCC group). The logistic regression analysis was used to analyze the risk factors of HCC progression. The diagnostic value of AFP for HCC was evaluated by receiver operating characteristic (ROC) curve analysis.

RESULTS

Univariate analysis showed that age ≥ 60 years (P = 0.001), hepatitis B and alcoholic etiology (P = 0.007), smoking history (P < 0.001), family history of HBV-

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Data sharing statement: No additional data are available.

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related HCC (P = 0.002), lamivudine resistance (P = 0.011), HBV DNA negative (P = 0.023), aspartate aminotransferase > 80 U/L (P = 0.002), gamma-glutamyl transpeptidase > 120 U/L (P = 0.001), alkaline phosphatase > 250 U/L (P = 0.001), fasting blood glucose (FBG) ≥ 6.16 (mmol/L) (P = 0.001) and Child-Pugh class C (P = 0.005) were correlated with HCC progression. In multivariate analysis, age ≥ 60 years [hazard ratio (HR) = 3.089, 95% confidence interval (CI): 1.437-6.631, P = 0.004], smoking history (HR = 4.001, 95%CI: 1.836-8.716, P < 0.01), family history of HBV-related HCC (HR = 6.763, 95%CI: 1.253-36.499, P < 0.05), lamivudine resistance (HR = 2.949, 95%CI: 1.207-7.208, P = 0.018), HBV DNA negative (HR = 0.026, 95%CI: 0.007-0.139, P < 0.01), FBG ≥ 6.16 mmol/L (HR = 7.219, 95%CI: 3.716-14.024, P < 0.01) were independent risk factors of HCC progression. ROC of AFP for diagnosis of HCC was 0.746 (95%CI: 0.674-0.818). A cutoff value of AFP of 9.00 ug/L had a sensitivity of 0.609, and specificity of 0.818 for diagnosing HCC.

CONCLUSION

Age ≥ 60 years, smoking history, family history of HCC, lamivudine resistance, HBV DNA negative, FBG ≥ 6.16 mmol/L were risk factors of HCC progression. Serum AFP had limited diagnostic value for HCC.

Key Words: Hepatitis B virus; Hepatocellular carcinoma; Cirrhosis; Risk factors; Nucleos(t)ide analogs; Progression

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Core Tip: This retrospective cross-sectional study analyzed risk factors of hepatocellular carcinoma (HCC) progression in hepatitis B virus-related cirrhosis patients receiving nucleoside acid analog therapy for at least 6 mo. We discuss the diagnostic value of serum alpha-fetoprotein level in these patients. The results of the present study increase our understanding of HCC pathogenesis and help to provide HCC prevention and control strategies.

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INTRODUCTION

Liver cancer has the third-highest cancer mortality rate worldwide[1]. Hepatocellular carcinoma (HCC) is one of the most common subtypes of liver cancer and is the sixth most prevalent cancer type. In most countries, the HCC mortality rates have increased in recent decades[2]. HCC is the fourth most common malignant tumor type in China, accounting for over 55% of the total number of HCC cases[3]. Infection with the hepatitis B virus (HBV) greatly increases the incidence of HCC because HBV causes chronic hepatitis B (CHB), liver cirrhosis, and ultimately HCC[4,5]. The estimated risk of developing HCC was observed to be 25 to 37-fold higher in hepatitis B surface antigen (HBsAg) carriers compared with noninfected patients HBV infection is one of the most important contributors to the pathogenesis of HCC[6-8]. Over the past 30 years, antiviral drugs, especially nucleos(t)ide analogs (NAs), have been widely used in clinical practice and have substantial long-term effects on the inhibition of HBV replication, namely, delaying and reducing the occurrence and development of hepatitis B-related events. Many studies have shown that antiviral therapy can considerably decrease the incidence of HCC, even for patients in whom CHB has progressed to cirrhosis[9,10]. However, antiviral therapy does not completely block the progression of CHB to liver cancer[11,12]. In the current study, we analyzed the risk factors of HCC progression in patients with HBV-related cirrhosis who received

NAs therapy for at least 6 mo. The diagnostic value of the serum alpha-fetoprotein (AFP) level was evaluated in those patients by receiver operating characteristic (ROC) curve analysis. The study results increase our understanding of HCC pathogenesis and help to provide HCC prevention and control strategies.

MATERIALS AND METHODS

Patients and design

This cross-sectional study retrospectively enrolled 266 patients with HBV-related cirrhosis who were treated with NA antiviral therapy at Zhejiang Provincial People's Hospital between February 2014 and April 2020. The 266 patients were divided into two groups, 145with cirrhosis who did not progress to HCC during the observation period (No-HCC group), and 121 with cirrhosis who progressed to HCC (HCC group). The inclusion criteria were: (1) Age ≥ 18 years; (2) Treatment with lamivudine (LAM), adefovir (ADV), telbivudine, entecavir (ENT), or tenofovir (TDF) nucleoside or NAs for at least 6 mo; (3) Diagnosis of cirrhosis established by either histology (progressive fibrosis, nodule formation, and loss of hepatic architecture) or clinical data (symptoms and signs of cirrhosis, abnormal liver function, and ultrasonic identification). Demographic, clinical, laboratory, imaging, and pathology data were collected during the patient's hospital stay. The severity of cirrhosis was classified by the Child-Pugh criteria. Patients with HBV-related cirrhosis were diagnosed in accord with the guidelines for the prevention and treatment of chronic hepatitis B formulated by the Hepatology Branch and the Infectious Diseases Branch of the Chinese Medical Association[13,14]; and (4) HCC and hepatitis diagnoses confirmed by clinical and serological characteristics, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), hepatic arteriography with digital subtraction angiography (DSA) and pathological examination consistent with the hepatitis and primary liver cancer clinical diagnosis criteria[15,16]. The exclusion criteria were: (1) Hepatitis C virus, hepatitis D virus, or human immunodeficiency virus coinfection; (2) Autoimmune hepatitis and drug hepatitis; and (3) Hepatocarcinoma prior to antiviral treatment or within 6 mo after antiviral treatment. The study received no support from any pharmaceutical company and was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital (2020QT155), Hangzhou, Zhejiang Province, China.

Data collection and study design

The data collected from the electronic medical record system were age, sex, history of drinking and smoking, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), total bilirubin, fasting blood glucose (FBG), AFP, prothrombin time (PT), presence of ascites, hepatic encephalopathy, Child-Pugh score and classification, and family history of HBV, hepatitis B, and HCC. Serological markers of hepatitis B, serum content of HBV DNA, and history of NA treatment were reported by the patients or their families. All patients in the cohort were followed-up for 3 years or until death.

Serum liver function was tested by routine automated techniques using an Olympus AU5400 automated analyzer (Olympus, Tokyo, Japan). HBsAg, hepatitis B e-antigen (HBeAg), and hepatitis B e-antibody were assessed at baseline by chemiluminescence immunoassay (Abbott ARCHITECT i2000 SR analyzer; Abbott Diagnostics, Chicago, IL, United States). The serum HBV DNA load was assessed by RT-PCR using a LightCycler PCR system (Roche LightCycler480 II fluorescence quantitative PCR) in strict accordance with the instructions provided with the reagent kit (Shenzheng PG Biotech Co. Ltd, China). The detection limit was approximately 100 viral genome IU/mL. Genotypic resistance to LAM and ADV were determined at baseline by direct sequencing of the PCR amplification products. The serum AFP tumor marker was assayed by electrochemiluminescence. The study protocol conformed to the 1975 Declaration of Helsinki ethical guidelines for clinical studies.

Statistical analysis

Descriptive data were expressed as means ± SD or n (%). Continuous variables were compared using Student's t-test. Skewness distribution data were reported as the median with the range and were analyzed using the Mann–Whitney U test. Categorical variables were analyzed using Fisher's exact test or Pearson's χ^2 test. A logistic regression model was used to analyze single factors, and multivariate analysis with stepwise regression was used to identify statistically significant variables in the

single-factor analysis. The diagnostic performance of the serum AFP level was evaluated using ROC curves. The cutoff value, which was the maximum area under the ROC curve (AUROC), and accuracy were calculated with 95% confidence intervals (CIs). A *P* value of less than 0.05 was determined to indicate statistical significance. The SPSS statistical package (version 22.0; SPSS, Chicago, IL, United States) was used for the statistical analysis.

RESULTS

Clinical characteristics of HBV-related cirrhosis patients receiving NA therapy

The clinical features of the No-HCC and the HCC group were compared. There were no significant differences between the two groups in the sex ratio, duration of NA therapy, HBsAg level, HBeAg positivity, jaundice index, Child-Pugh class B ratio, and PT level ($P > 0.05$). The HCC group included significantly more patients older than 60 years of age and patients with increased levels of ALT, AST, GGT, ALP, and FBG, and decreased levels of ALB than the No-HCC group. The HCC group also contained more patients with mixed etiology (alcohol + HBV), history of smoking, family history of HBV-related HCC, LAM resistance, Child-Pugh class C status, and an AFP > 20 μg/L and fewer HBV DNA-negative and Child-Pugh class A patients than the No-HCC group (Table 1).

Risk factors for HCC progression in HBV-related cirrhosis patients receiving NA therapy

We analyzed factors associated with HCC progression in HBV-related cirrhosis patients who received NA therapy. Univariate analysis found age ≥ 60 years ($P = 0.002$), HBV + alcohol mixed etiology ($P = 0.007$), smoking history ($P < 0.001$), family history of HBV-related HCC ($P = 0.002$), LAM resistance ($P = 0.046$), HBV DNA negativity ($P = 0.023$), AST > 80 U/L ($P = 0.002$), GGT > 120 U/L ($P = 0.001$), ALP > 250 U/L ($P = 0.001$), FBG ≥ 6.16 mmol/L ($P = 0.001$), and Child-Pugh class C ($P = 0.005$) to be significantly related to HCC (Table 2). Multivariate analysis showed that age ≥ 60 years [hazard ratio (HR) = 3.089, 95%CI: 1.437-6.631, $P = 0.004$], smoking history (HR = 4.001 95%CI: 1.836-8.716, $P < 0.01$), family history of HBV-related HCC (HR = 6.763, 95%CI: 1.253-36.499, $P < 0.05$), LAM resistance (HR = 2.949, 95%CI: 1.207-7.208, $P = 0.018$), HBV DNA negative (HR = 0.026, 95%CI: 0.007-0.139, $P < 0.01$), and FBG ≥ 6.16 mmol/L (HR = 7.219, 95%CI: 3.716-14.024, $P < 0.01$) independently predicted HCC progression in patients with HBV-related cirrhosis who received NAs therapy (Table 3).

Serum AFP levels in the No-HCC and HCC groups

In the HCC group, there were 56 patients with serum AFP levels > 20 μg/L, but that was seen in only 17 patients in the No-HCC group ($P < 0.001$). In the HCC group, 65 patients had AFP levels < 20 μg/L. The AFP levels of 37 patients in the HCC group were > 400 μg/L, but only two patients in the No-HCC group levels > 400 μg/L ($P < 0.001$, Table 4). The 17 patients in the No-HCC group with AFP levels > 20 μg/L were followed for 1 year. Their AFP level was determined every month and showed a dynamic decline, returning to normal within 1 year. All patients underwent enhanced MRI of imaging of the liver. We believe that hepatitis B activity, rather than HCC, led to the AFP abnormality. The AFP level distributions in the two groups are shown in Figures 1 and 2.

Serum AFP has limited ability to diagnose HBV-related HCC

We investigated the value of using serum AFP to diagnose HCC in patients who had HBV-related cirrhosis and were receiving NAs therapy. The AUROC of serum AFP for the diagnosis of HCC was 0.746 (95%CI: 0.674-0.818). The sensitivity of serum AFP in diagnosing HCC in those patients was 0.609, and the specificity was 0.818. The positive predictive value of HCC was 22.51%, the negative predictive value of HCC was 46.07%, the cutoff was 9.00, and the Youden index was 0.427 (Figure 3).

DISCUSSION

HBV infection remains a major risk factor for the development of cirrhosis and HCC[17]. Patients with chronic HBV are at risk of developing liver-related complica-

Table 1 Comparison of the clinical characteristics of patients with and without hepatocellular carcinoma

Characteristic	no-HCC group (*n* = 145)	HCC group (*n* = 121)	*P* value
Age ≥ 60 yr, *n* (%)	41 (28.3)	64 (52.9)	< 0.001
Male, *n* (%)	99 (68.3)	91 (75.2)	0.133
Etiology of liver cirrhosis, *n* (%)			
HBV	101 (60.7)	76 (62.8)	0.363
HBV + alcohol	31 (21.4)	45 (37.2)	0.006
HBV + HEV	1 (0.6)	0 (0.0)	-
HBV + schistosome	1 (0.6)	0 (0.0)	-
Smoking history, *n* (%)	21 (14.5)	51 (42.1)	< 0.001
Family history of HBV-related HCC, *n* (%)	10 (6.9)	23 (19.0)	0.004
Medication history			
Duration of NA treatment, yr, median (P_{25}, P_{75})	3.9 (2.1, 5.8)	5.4 (2.3, 6.9)	0.067
LAM resistance, *n* (%)	18 (12.4)	27 (22.3)	0.021
HBsAg level, IU/L, median (P_{25}, P_{75})	255.0 (56.0, 678.0)	269.0 (67.0, 656.0)	0.456
HBeAg positive, *n* (%)	37 (25.5)	21 (17.4)	0.136
HBV DNA negative, *n* (%)	67 (46.2)	39 (32.2)	0.033
ALB, U/L	36.29 ± 7.98	33.34 ± 6.62	0.002
ALT, U/L, median (P_{25}, P_{75})	27.00 (18.00, 37.00)	32.00 (21.27, 62.00)	0.006
AST, U/L, median (P_{25}, P_{75})	33.00 (23.00, 47.00)	44.50 (31.65, 96.85)	< 0.001
GGT, U/L, median (P_{25}, P_{75})	33.00 (20.00, 46.00)	61.50 (32.75, 160.75)	< 0.001
ALP, U/L, median (P_{25}, P_{75})	99.00 (73.00, 126.00)	134.50 (92.00, 198.85)	< 0.001
TB, μmol/L, median (P_{25}, P_{75})	20.41 (13.81, 44.60)	24.46 (16.60, 42.80)	0.192
FBS, mmol/L, median (P_{25}, P_{75})	5.17 ± 0.68	6.99 ± 1.31	0.025
Ascites, *n* (%)	33 (20.0)	43 (34.7)	0.022
Child-Pugh class, *n* (%)			
A	81 (55.9)	55 (45.5)	0.001
B	38 (26.2)	26 (21.5)	0.561
C	26 (17.9)	40 (33.1)	0.002
AFP ≥ 20 μg/L, *n* (%)	14 (9.7)	56 (46.3)	< 0.001
PT s, median (P_{25}, P_{75})	13.25 (11.80, 14.20)	13.51 (12.41, 15.02)	0.475

AFP: Alpha-fetoprotein; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FBS: Fasting blood sugar; GGT: Glutamyl transpeptidase; HBeAb: Hepatitis B e-antibody; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: Lamivudine; PT: Prothrombin time; SD: Standard deviation; TB: Total bilirubin.

tions, namely, cirrhosis and HCC. In China, 77% of cirrhosis cases and 84% of HCC cases are caused by HBV infection[18]. Antiviral therapy can reduce but not eliminate the risk of developing HCC[19-21]. The annual incidence of HCC ranges from 0.01% to 5.4% in patients with CHB who are treated with ENT or TDF[19,22]. NAs antiviral treatments can markedly inhibit viral replication and improve liver necrosis, inflammation, and fibrosis. However, NAs cannot eliminate covalently closed template DNA (cccDNA) produced during hepatitis B viral replication or clear the integrated HBV genome; therefore, NAs cannot completely block hepatitis B cirrhosis from developing into HCC[12,21]. The persistence of cccDNA and an integrated HBV genome is the basis for hepatitis B cirrhosis progressing to primary liver cancer[23,24]. In this study, we investigated the clinical characteristics of HCC progression in patients with HBV-related cirrhosis who received antiviral therapy with NAs.

Table 2 Univariate logistic regression analysis of hepatitis B-related cirrhosis progressing to hepatocellular carcinoma in patients treated with nucleos(t)ide analogs

Characteristic	No-HCC group (*n* = 145)	HCC group (*n* = 121)	Univariate adjusted HR (95%CI)	*P* value
Age, yr				
≥ 60	40 (27.6)	65 (52.9)	2.664 (1.606-4.418)	0.001
< 60	105 (72.4)	56 (46.3)		
HBV + alcohol				
Yes	31 (21.4)	45 (37.2)	2.384 (1.271-4.473)	0.007
No				
Smoking history				
Yes	21 (14.5)	51 (42.1)	4.073 (2.281-7.273)	< 0.001
No				
Family history of HBV-related HCC				
Yes	10 (6.9)	23 (19.0)	3.546 (1.573-7.998)	0.002
No				
LAM resistance				
Yes	18 (12.4)	27 (22.3)	2.284 (1.214-4.297)	0.011
No				
HBV DNA negative				
Yes	67 (46.2)	39 (32.2)	0.559 (0.339-0.922)	0.023
No				
ALB (g/L)				
< 35	67(46.2)	62(51.2)	1.223 (0.754-1.984)	0.414
≥ 35	78 (53.8)	59 (48.8)		
ALT (U/L)				
50-100	13 (9.0)	15 (12.4)	1.324 (0.612-2.866)	0.476
> 100	10 (7.0)	14 (11.6)	1.138(0.482-2.688)	0.768
AST (U/L)				
40-80	32 (22.1)	24 (19.8)	0.919 (−0.713-0.514)	0.783
> 80	16 (10.3)	31 (29.8)	2.899 (0.436-1.767)	0.002
GGT (U/L)				
60-120	15 (10.3)	20 (16.5)	1.853 (0.892-3.847)	0.098
> 120	8 (5.1)	28 (23.1)	5.663 (1.075-2.573)	0.001
ALP (U/L)				
125-250	36(24.8)	45 (38.8)	1.609 (−0.062-1.028)	0.073
> 250	6 (1.4)	21 (17.4)	4.865 (0.667-2.993)	0.001
FBG (mmol/L)				
≥ 6.16	19 (13.1)	37 (30.6)	3.3179 (0.587-1.902)	0.001
< 6.16				
Ascites class				
Yes	33 (22.8)	43 (35.5)	0.834 (−0.412-0.060)	0.142
No				

Child-Pugh class				
A	81 (58.6)	55 (37.9)	0.658 (−0.938-0.064)	0.091
B	38 (26.2)	26 (21.5)	0.671 (−0.981-0.112)	0.165
C	26 (17.9)	40 (33.1)	2.260 (0.247-1.427)	0.005

Data are *n* (%) or mean ± SD as shown. AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; FBG: Fasting blood glucose; GGT: Glutamyl transpeptidase; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HR: Hazard ratio; LAM: Lamivudine. .

Table 3 Multivariate analysis of factors associated with hepatitis B-related cirrhosis progression to hepatocellular carcinoma in nucleos(t)ide analog-treated patients

Risk factor	β	SE	Wald	*P* value	OR (95%CI)
Age ≥ 60 yr	1.127	0.390	8.347	0.004	3.089 (1.437-6.631)
Smoking history	1.387	0.397	12.180	< 0.01	4.001 (1.836-8.716)
Family history of HBV-related HCC	1.911	0.860	4.938	< 0.05	6.763 (1.253-36.499)
LAM resistance	1.082	0.456	5.638	0.018	2.949 (1.207-7.208)
HBV DNA negative	-3.479	0.816	19.427	< 0.01	0.026 (0.007-0.139)
FBG ≥ 6.16 mmol/L	1.977	0.339	34.030	< 0.01	7.219 (3.716-14.024)

CI: Confidence interval; FBG: Fasting blood glucose; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: Lamivudine OR: Odds ratio;; SE: Standard error.

Table 4 Comparison of the alpha-fetoprotein level distributions in patients with and without hepatocellular carcinoma, *n* (%)

AFP (μg/L)	No-HCC group (*n* = 145)	HCC group (*n* = 121)	*P* value
< 20	128 (88.27)	65 (53.72)	< 0.001
20-100	12 (8.27)	14 (11.57)	0.51
100-200	1 (0.69)	2 (2.17)	0.231
200-400	2 (1.38)	3 (2.48)	1
≥ 400	2 (1.38)	37 (30.58)	< 0.001

AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

Medical and healthcare progress, improved living standards, and decreased population fertility have led to population aging in China. Data from 22 tumor registration centers in China have shown that the average age of liver cancer onset increased from 58.80 to 62.35 years for men and from 64.02 to 68.99 years for women between 2000 and 2014[25]. Our study found that age ≥ 60 years was an independent risk factor for the progression of hepatitis B-related cirrhosis to HCC while receiving NAs antiviral therapy. An aging population and the burden caused by HCC mortality could be a future challenge for China. Previous studies have confirmed that poor lifestyle habits are related to a high incidence of liver cancer; in particular, a history of smoking and drinking increases the risk of HCC[26,27]. Tobacco smoke contains various carcinogens, 11 of which are classified as International Agency for Research on Cancer Group 1 human carcinogens. Epidemiologic evidence from a recent meta-analysis showed a positive association between current tobacco smoking and liver cancer risk (risk ratio: 1.55, 95%CI: 1.46-1.65), suggesting a causal role of smoking in liver cancer development[28]. Liu *et al*[29] found that tobacco smoking and HBV infection positively interact in the development of liver cancer. Our results revealed smoking to be an independent risk factor of HCC progression (95%CI: 1.836-8.716, $P < 0.01$) even if the patients were receiving antiviral treatment. Studies have frequently reported that a family history of liver cancer increases HCC risk independent of

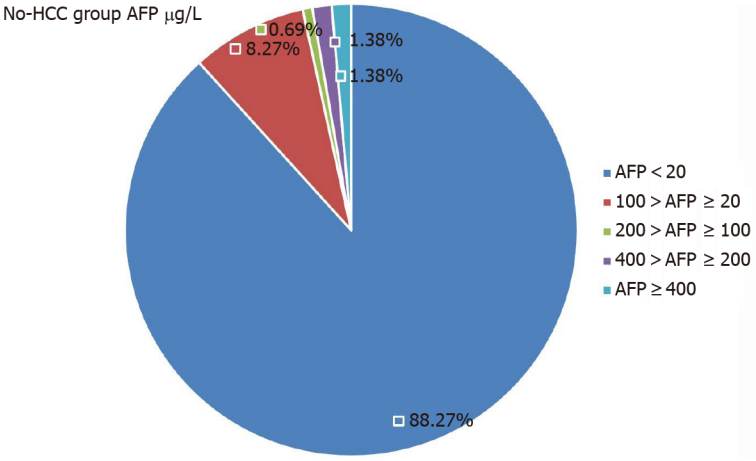


Figure 1 Distribution of alpha-fetoprotein levels in patients without hepatocellular carcinoma. AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

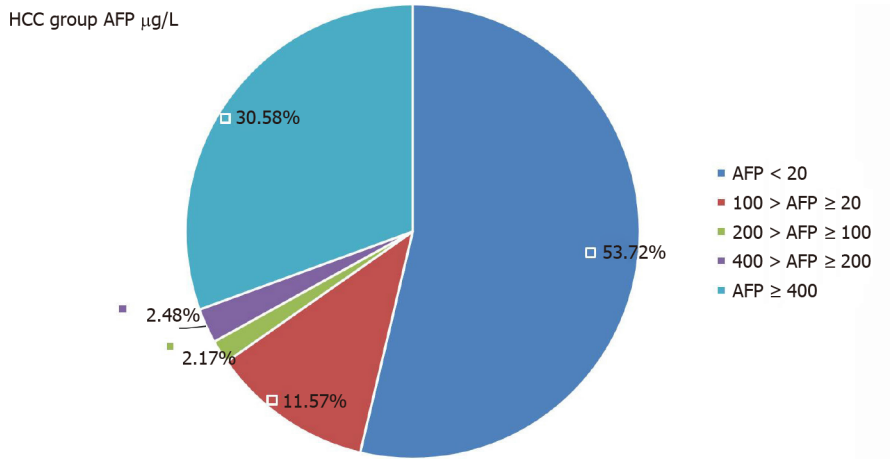


Figure 2 Distribution of alpha-fetoprotein level in patients with hepatocellular carcinoma. AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

hepatitis. The combination of a family history of liver cancer and hepatitis B serum markers is associated with a greater than 70-fold increase in HCC risk[30]. Super-additive and super-multiplicative interactions may exist between a family history of liver cancer and HBV infection that increase the risk of the development of liver cancer[31]. Our study found that a family history of HBV-related HCC is a risk factor of progression in cirrhotic patients receiving antiviral therapy (95%CI: 1.253-36.499, $P < 0.05$). Our results suggest that patients with HBV-related cirrhosis who smoke should quit smoking and cultivate a healthy lifestyle to reduce the risk of developing HCC[32,33]. Furthermore, close monitoring should be carried out if a patient's first-degree relative develops HBV-related HCC during antiviral treatment.

LAM has been used as an antiviral treatment for hepatitis B for the past 20 years in China. Some Chinese patients with hepatitis B-related cirrhosis have previously undergone primary LAM monotherapy. Studies have shown that long-term LAM antiviral therapy can delay disease progression, reduce liver function decompensation, and reduce the incidence of HCC[34,35]. The inflammation seen in liver histology can improve in patients with hepatitis B-related cirrhosis when they are treated with LAM, but with a prolonged treatment time, the incidence of drug-resistant viral mutation increases[34,36]. The clinical benefit of LAM is limited by the emergence of resistant mutant strains and viral breakthrough. Although some LAM-resistant CHB patients have received ADV combination therapy or sequential ENT or TDF monotherapy, some of these patients did not experience a beneficial treatment effect and still had continuous replication of HBV in the liver[37,38]. In patients with LAM resistance, those with cirrhosis had a higher HCC risk than non-cirrhotic patients. Rescue treatment with ADV in patients who developed viral breakthrough did not appear to reduce the risk of HCC compared with untreated patients without remission[11]. In

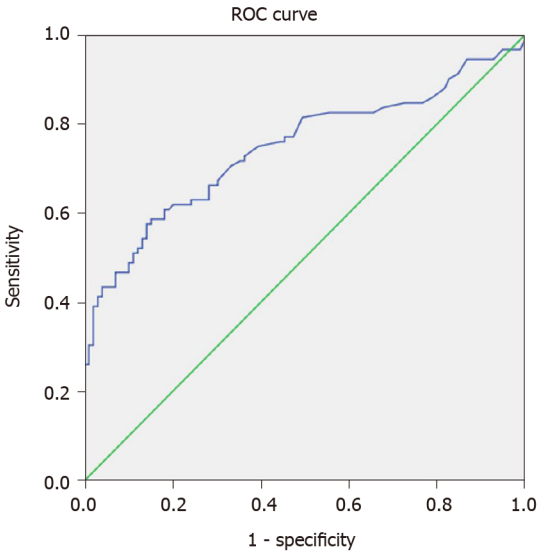


Figure 3 Receiver operating characteristic curve analysis of alpha-fetoprotein to diagnose hepatocellular carcinoma in patients with hepatitis B virus-related cirrhosis receiving nucleos(t)ide analog therapy. ROC: Receiver operating characteristic.

our study of patients treated with antiviral therapy for more than 6 mo, the number of HBV DNA-negative patients in the HCC group was lower than that in the No-HCC group. Many studies have found a correlation between serum HBV DNA levels and the occurrence of HCC in patients with hepatitis B. Kaneko *et al*[39] reported that detectable HBV DNA was significantly associated with a higher risk of HCC development compared with continuously undetectable HBV DNA. Chen *et al*[40] found that the incidence of HCC increased with serum HBV DNA level at study entry in a dose-response relationship ranging from 108/100,000 person-years for an HBV DNA level of < 300 copies/mL to 1152/100,000 person-years for an HBV DNA level of 1 million copies/mL or more. The corresponding cumulative incidence rates of HCC were 1.3% and 14.9%.

Even if the serum HBV DNA level in patients with hepatitis B-related cirrhosis is kept at a low level (< 2000 IU/mL), the risk of HCC is still high[41]. As shown in our study, HBV DNA negativity (HR = 0.026, 95%CI: 0.007-0.139, $P < 0.01$) independently predicted HCC progression in patients with HBV-related cirrhosis who received NAs therapy. Thus, to help avoid HCC progression, hepatitis B patients should continue to maintain an HBV DNA-negative status. Therefore, drugs with a high genetic barrier to resistance are suggested as first-line antiviral drugs for HBV therapy, and are recommended by current guidelines. Drugs such as ETV, TDF, and TDF alafenamide fumarate should be selected to generate a sustained antiviral treatment response and to reduce the occurrence of HBV resistance and the incidence of HCC[42,43].

As an important metabolic organ, the liver plays a key role in maintaining glucose homeostasis. Studies have found a positive relationship between liver cancer and diabetes mellitus[44,45]. Cell and animal experiments have shown that type 2 diabetes and male sex are associated with HCC development. Gao *et al*[46] demonstrated that heterozygous deletion of the *Ncoa5* gene caused spontaneous development of HCC exclusively in male mice, and NCOA5 deficiency increased susceptibility to both glucose intolerance and HCC. In a prospective cohort study, adjusted multivariable analysis showed that participants with 4.82 mmol/L ≤ FBG ≤ 5.49 mmol/L had a 47% increased risk of HCC, and those with an FBG > 5.49 mmol/L had a 69% increased risk[47]. In our study, the FBG level in the HCC group was higher than that in the No-HCC group (Table 1), and FBG ≥ 6.16 mmol/L was an independent risk factor for the HCC progression in patients with hepatitis B-related cirrhosis receiving NA antiviral treatment. Controlling blood sugar concentrations might be a way to decrease the risk of HCC in the Chinese population.

AFP is a glycoprotein that exists in a variety of different glycotypes. AFP has been used in the screening, diagnosis, efficacy evaluation, and prognosis evaluation of HCC. AFP elevation is commonly seen in active hepatitis, pregnancy, liver cancer, and embryonic tumors[48,49]. Previous studies reported that there was a significant correlation between serum AFP levels and the tumor size in liver cancer, and that the sensitivity and specificity of AFP depended on the selected serum level threshold[20,50]. Liu *et al*[51] found that approximately one-third of patients with

HCC had normal serum AFP levels and that the level was related to the volume of liver cancer lesions, vascular invasion, and differentiation. In our study, we found 56 patients with a serum AFP > 20 μg/L, and 19 with serum AFP levels between 20 and 400 μg/L and with HCC confirmed by imaging and histopathological examination of liver masses. However, an AFP level of < 20 μg/L did not exclude the possibility of HCC. This study showed that 65 patients with a serum AFP of < 20 μg/L had space-occupying lesions that were confirmed as HCC by MRI enhancement, histopathology, and liver DSA examination. That indicates that more sensitive diagnostic markers of HCC must be developed. In addition, more sensitive serum tumor markers such as milk fat globule-EGF factor 8, osteopontin, miRNA classifier, glypican-3[52-54], and others should be actively investigated or combined to identify HCC at an early stage[55]. However, their clinical sensitivity and specificity must first be confirmed.

This study had some limitations. First, it was not prospective. The impact of antiviral treatment time and the amount of smoking and drinking on the development of HCC require confirmation in prospective studies with larger sample sizes. Second, longer follow-up and surveillance of HBV-related cirrhosis patients receiving NA therapy is necessary to observe whether they progress to HCC in their lifetime, even though the 145 cirrhotic patients with NA therapy did not progress to HCC during the observation period. Third, additional molecular markers should be assessed for their ability to provide an early diagnosis of HCC in patients with HBV-related cirrhosis. Some data indicate that the currently used potent NAs can reduce but not eliminate the risk of HCC. The inability to eliminate HCC risk might persist because of risk factors that are not amenable to change by antiviral therapy or because of events that may have taken place before treatment initiation.

CONCLUSION

In conclusion, age ≥ 60 years, a history of smoking, family history of HBV-related HCC, LAM resistance, HBV DNA negativity, and FBG ≥ 6.16 mmol/L were risk factors for HCC progression in patients with HBV-related cirrhosis who received NAs therapy. Patients with HBV-related cirrhosis should be treated with NA antiviral therapy that has a high genetic barrier to resistance[7] in order to improve the long-term response to antiviral therapy, to maintain an HBV DNA-negative status, and to prevent subsequent hepatitis activity. The early identification of HCC in patients with HBV-related cirrhosis remains difficult. Patients who receive antiviral therapy with NAs, especially those older than 60 years of age, should avoid smoking, control their blood sugar at a reasonable level, and undergo routine imaging examination of liver biochemistry and serum AFP evaluation. If space-occupying lesions are identified, the patient should undergo liver CT or MRI enhancement, or even liver DSA examination to identify HCC as promptly as possible, even if the AFP level is within the normal range.

ARTICLE HIGHLIGHTS

Research background

Antiviral therapy cannot completely block the progression of hepatitis B to hepatocellular carcinoma (HCC). Furthermore, there are few early predictors of HCC progression and early identification is difficult in patients with HBV-related cirrhosis who receive nucleos(t)ide analog (NA) therapy. The study is helpful to provide HCC prevention and control strategies by analyzing the risk factors of HCC progression and the diagnostic value of AFP for HCC in those people.

Research motivation

The study objective was to identify factors that affect the occurrence of HCC and how to identify early HCC in patients with hepatitis B virus (HBV)-related cirrhosis who receive NA therapy. The results can improve the understanding of the development of HCC in those patients so as to improve the early detection and prevention of HCC.

Research objectives

The study objectives were to clarify risk factors and the diagnostic value of alpha-fetoprotein (AFP) for HCC progression in patients with HBV-related cirrhosis treated with NAs and to provide new strategies for prevention and control of HCC in those

patients.

Research methods

Logistic regression analysis was used to analyze the risk factors of HCC progression. The diagnostic value of AFP for HCC was evaluated by receiver operating characteristic curve analysis.

Research results

The study showed that age ≥ 60 years, smoking history, family history of HCC, lamivudine resistance, HBV DNA negativity, fasting blood sugar ≥ 6.16 mmol/L were independent risk factors of HCC progression. Serum AFP had limited diagnostic value for HCC. The results provide a meaningful strategy for early prediction and identification for HCC in those patients.

Research conclusions

A retrospective cross-sectional study was conducted to analyze risk factors of HCC progression in HBV-related cirrhosis patients receiving NA therapy. Metabolic effects of fasting blood sugar levels on the progress of HCC were seen during the receipt of NA therapy. The diagnostic value of the serum AFP level was evaluated in those patients.

Research perspectives

The study results will change the strategies used to prevent HCC in patients with HBV-related cirrhosis an receive NA therapy.

REFERENCES

1 **Sayiner M**, Golabi P, Younossi ZM. Disease Burden of Hepatocellular Carcinoma: A Global Perspective. *Dig Dis Sci* 2019; **64**: 910-917 [PMID: 30835028 DOI: 10.1007/s10620-019-05537-2]

2 **Bertuccio P**, Turati F, Carioli G, Rodriguez T, La Vecchia C, Malvezzi M, Negri E. Global trends and predictions in hepatocellular carcinoma mortality. *J Hepatol* 2017; **67**: 302-309 [PMID: 28336466 DOI: 10.1016/j.jhep.2017.03.011]

3 **Forner A**, Llovet IM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; **379**: 1245-1255 [PMID: 22353262 DOI: 10.1016/S0140-6736(11)61347-0]

4 **McClune AC**, Tong MJ. Chronic hepatitis B and hepatocellular carcinoma. *Clin Liver Dis* 2010; **14**: 461-476 [PMID: 20638025 DOI: 10.1016/j.cld.2010.05.009]

5 **Ou DP**, Yang LY, Huang GW, Tao YM, Ding X, Chang ZG. Clinical analysis of the risk factors for recurrence of HCC and its relationship with HBV. *World J Gastroenterol* 2005; **11**: 2061-2066 [PMID: 15810069 DOI: 10.3748/wjg.v11.i14.2061]

6 **Chu CM**, Liaw YF. Hepatitis B virus-related cirrhosis: natural history and treatment. *Semin Liver Dis* 2006; **26**: 142-152 [PMID: 16673292 DOI: 10.1055/s-2006-939752]

7 **Chen QY**, Dong BQ, Yang JY, Wei SC, Fang KX, Wang XY, Fang ZL. [A prospective study of the relationship between serum hepatitis B virus DNA and the risk of primary liver cancer]. *Zhonghua Gan Zang Bing Za Zhi* 2009; **17**: 930-934 [PMID: 20038336]

8 **Chen YC**, Chu CM, Yeh CT, Liaw YF. Natural course following the onset of cirrhosis in patients with chronic hepatitis B: a long-term follow-up study. *Hepatol Int* 2007; **1**: 267-273 [PMID: 19669348 DOI: 10.1007/s12072-007-5001-0]

9 **Lu T**, Seto WK, Zhu RX, Lai CL, Yuen MF. Prevention of hepatocellular carcinoma in chronic viral hepatitis B and C infection. *World J Gastroenterol* 2013; **19**: 8887-8894 [PMID: 24379612 DOI: 10.3748/wjg.v19.i47.8887]

10 **Wu CY**, Lin JT, Ho HJ, Su CW, Lee TY, Wang SY, Wu C, Wu JC. Association of nucleos(t)ide analogue therapy with reduced risk of hepatocellular carcinoma in patients with chronic hepatitis B: a nationwide cohort study. *Gastroenterology* 2014; **147**: 143-151. e5 [PMID: 24704525 DOI: 10.1053/j.gastro.2014.03.048]

11 **Papatheodoridis GV**, Lampertico P, Manolakopoulos S, Lok A. Incidence of hepatocellular carcinoma in chronic hepatitis B patients receiving nucleos(t)ide therapy: a systematic review. *J Hepatol* 2010; **53**: 348-356 [PMID: 20483498 DOI: 10.1016/j.jhep.2010.02.035]

12 **Papatheodoridis GV**, Manolakopoulos S, Touloumi G, Vourli G, Raptopoulou-Gigi M, Vafiadis-Zoumbouli I, Vasiliadis T, Mimidis K, Gogos C, Ketikoglou I, Manesis EK; HEPNET. Greece Cohort Study Group. Virological suppression does not prevent the development of hepatocellular carcinoma in HBeAg-negative chronic hepatitis B patients with cirrhosis receiving oral antiviral(s) starting with lamivudine monotherapy: results of the nationwide HEPNET. Greece cohort study. *Gut* 2011; **60**: 1109-1116 [PMID: 21270118 DOI: 10.1136/gut.2010.221846]

13 **Hepatology Branch of Chinese Medical Association**. Infectious diseases branch of Chinese Medical Association. Guidelines for the prevention and treatment of chronic hepatitis B (2015 Edition).

Zhonghua Shiyan He linchuang Ganranbing Zazhi 2015; **9**: 570-589 [DOI: 10.3877/cma.j.issn.1674-1358.2015.05.001]

14 **Hepatology Branch of Chinese Medical Association**. Infectious diseases branch of Chinese Medical Association. Guidelines for the prevention and treatment of chronic hepatitis B (2019 version). *Zhonghua Shiyan He linchuang Ganranbing Zazhi* 2019; **37**: 711-736 [DOI: 10.3760/cma.j.issn.1000-6680.2019.12.003]

15 **Ministry of Health of the People's Republic of China**. Standardization for diagnosis and treatment for hepatocellular carcinoma. *Zhonghua Zhongliu Linchunag* 2011; **16**: 929-946

16 **Health and Bureau of Medical Administration**, National Health and Family Planning Commission of the People's Republic of China. Standardization of diagnosis and treatment for hepatocellular carcinoma (2017 edition). *Zhonghua Waike Xiaohua Zazhi* 2017; **16**: 635-647 [DOI: 10.3760/cma.j.issn.1673-9752.2017.07.001]

17 **Chen JD**, Yang HI, Iloeje UH, You SL, Lu SN, Wang LY, Su J, Sun CA, Liaw YF, Chen CJ; Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer in HBV (REVEAL-HBV) Study Group. Carriers of inactive hepatitis B virus are still at risk for hepatocellular carcinoma and liver-related death. *Gastroenterology* 2010; **138**: 1747-1754 [PMID: 20114048 DOI: 10.1053/j.gastro.2010.01.042]

18 **Wang X**, Lin SX, Tao J, Wei XQ, Liu YT, Chen YM, Wu B. Study of liver cirrhosis over ten consecutive years in Southern China. *World J Gastroenterol* 2014; **20**: 13546-13555 [PMID: 25309085 DOI: 10.3748/wjg.v20.i37.13546]

19 **Arends P**, Sonneveld MJ, Zoutendijk R, Carey I, Brown A, Fasano M, Mutimer D, Deterding K, Reijnders JG, Oo Y, Petersen J, van Bömmel F, de Knegt RJ, Santantonio T, Berg T, Welzel TM, Wedemeyer H, Buti M, Pradat P, Zoulim F, Hansen B, Janssen HL; VIRGIL Surveillance Study Group. Entecavir treatment does not eliminate the risk of hepatocellular carcinoma in chronic hepatitis B: limited role for risk scores in Caucasians. *Gut* 2015; **64**: 1289-1295 [PMID: 25011935 DOI: 10.1136/gutjnl-2014-307023]

20 **Vlachogiannakos J**, Papatheodoridis G. Hepatocellular carcinoma in chronic hepatitis B patients under antiviral therapy. *World J Gastroenterol* 2013; **19**: 8822-8830 [PMID: 24379605 DOI: 10.3748/wjg.v19.i47.8822]

21 **Loomba R**, Liu J, Yang HI, Lee MH, Lu SN, Wang LY, Iloeje UH, You SL, Brenner D, Chen CJ; REVEAL–HBV Study Group. Synergistic effects of family history of hepatocellular carcinoma and hepatitis B virus infection on risk for incident hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2013; **11**: 1636-45. e1-3 [PMID: 23669307 DOI: 10.1016/j.cgh.2013.04.043]

22 **Papatheodoridis GV**, Chan HL, Hansen BE, Janssen HL, Lampertico P. Risk of hepatocellular carcinoma in chronic hepatitis B: assessment and modification with current antiviral therapy. *J Hepatol* 2015; **62**: 956-967 [PMID: 25595883 DOI: 10.1016/j.jhep.2015.01.002]

23 **Yan H**, Yang Y, Zhang L, Tang G, Wang Y, Xue G, Zhou W, Sun S. Characterization of the genotype and integration patterns of hepatitis B virus in early- and late-onset hepatocellular carcinoma. *Hepatology* 2015; **61**: 1821-1831 [PMID: 25627239 DOI: 10.1002/hep.27722]

24 **Zeng HM**, Cao MM, Zheng RS, Zhang SC, Cai JQ, Qu CF, Bi XY, Zou XN, Chen WQ, He J. Trend analysis of mean age of diagnosis for liver cancer in cancer registry are as in China, 2000-2014. *Zhonghua Yufan Yixue Zazhi* 2018; **52**: 573-578

25 **Levrero M**, Zucman-Rossi J. Mechanisms of HBV-induced hepatocellular carcinoma. *J Hepatol* 2016; **64**: S84-S101 [PMID: 27084040 DOI: 10.1016/j.jhep.2016.02.021]

26 **Pascual S**, Herrera I, Irurzun J. New advances in hepatocellular carcinoma. *World J Hepatol* 2016; **8**: 421-438 [PMID: 27028578 DOI: 10.4254/wjh.v8.i9.421]

27 **Baecker A**, Liu X, La Vecchia C, Zhang ZF. Worldwide incidence of hepatocellular carcinoma cases attributable to major risk factors. *Eur J Cancer Prev* 2018; **27**: 205-212 [PMID: 29489473 DOI: 10.1097/CEJ.0000000000000428]

28 **Abdel-Rahman O**, Helbling D, Schöb O, Eltobgy M, Mohamed H, Schmidt J, Giryes A, Mehrabi A, Iype S, John H, Tekbas A, Zidan A, Oweira H. Cigarette smoking as a risk factor for the development of and mortality from hepatocellular carcinoma: An updated systematic review of 81 epidemiological studies. *J Evid Based Med* 2017; **10**: 245-254 [PMID: 28891275 DOI: 10.1111/jebm.12270]

29 **Liu X**, Baecker A, Wu M, Zhou JY, Yang J, Han RQ, Wang PH, Jin ZY, Liu AM, Gu X, Zhang XF, Wang XS, Su M, Hu X, Sun Z, Li G, Mu L, He N, Li L, Zhao JK, Zhang ZF. Interaction between tobacco smoking and hepatitis B virus infection on the risk of liver cancer in a Chinese population. *Int J Cancer* 2018; **142**: 1560-1567 [PMID: 29193051 DOI: 10.1002/ijc.31181]

30 **Turati F**, Edefonti V, Talamini R, Ferraroni M, Malvezzi M, Bravi F, Franceschi S, Montella M, Polesel J, Zucchetto A, La Vecchia C, Negri E, Decarli A. Family history of liver cancer and hepatocellular carcinoma. *Hepatology* 2012; **55**: 1416-1425 [PMID: 22095619 DOI: 10.1002/hep.24794]

31 **Liu X**, Baecker A, Wu M, Zhou JY, Yang J, Han RQ, Wang PH, Jin ZY, Liu AM, Gu X, Zhang XF, Wang XS, Su M, Hu X, Sun Z, Li G, Fu A, Jung SY, Mu L, He N, Li L, Zhao JK, Zhang ZF. Family history of liver cancer may modify the association between HBV infection and liver cancer in a Chinese population. *Liver Int* 2019; **39**: 1490-1503 [PMID: 31228882 DOI: 10.1111/liv.14182]

32 **Singal AK**, Bataller R, Ahn J, Kamath PS, Shah VH. ACG Clinical Guideline: Alcoholic Liver Disease. *Am J Gastroenterol* 2018; **113**: 175-194 [PMID: 29336434 DOI: 10.1038/ajg.2017.469]

33 **Massarweh NN**, El-Serag HB. Epidemiology of Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. *Cancer Control* 2017; **24**: 1073274817729245 [PMID: 28975830 DOI:

10.1177/1073274817729245]
34 **Liaw YF**, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J; Cirrhosis Asian Lamivudine Multicentre Study Group. Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351**: 1521-1531 [PMID: 15470215 DOI: 10.1056/NEJMoa033364]
35 **Su MH**, Lu AL, Li SH, Zhong SH, Wang BJ, Wu XL, Mo YY, Liang P, Liu ZH, Xie R, He LX, Fu WD, Jiang JN. Long-term lamivudine for chronic hepatitis B and cirrhosis: A real-life cohort study. *World J Gastroenterol* 2015; **21**: 13087-13094 [PMID: 26673249 DOI: 10.3748/wjg.v21.i46.13087]
36 **Yao GB**, Zhu M, Cui ZY, Wang BE, Yao JL, Zeng MD. A 7-year study of lamivudine therapy for hepatitis B virus e antigen-positive chronic hepatitis B patients in China. *J Dig Dis* 2009; **10**: 131-137 [PMID: 19426396 DOI: 10.1111/j.1751-2980.2009.00375.x]
37 **Chen CH**, Lin CL, Hu TH, Hung CH, Tseng PL, Wang JH, Chang JY, Lu SN, Chien RN, Lee CM. Entecavir vs. lamivudine in chronic hepatitis B patients with severe acute exacerbation and hepatic decompensation. *J Hepatol* 2014; **60**: 1127-1134 [PMID: 24583247 DOI: 10.1016/j.jhep.2014.02.013]
38 **Wang Y**, Liu S, Chen YU, Zheng S, Zhou LI, Lu F, Duan Z. Lamivudine-resistant rtL180M and rtM204I/V are persistently dominant during combination rescue therapy with entecavir and adefovir for hepatitis B. *Exp Ther Med* 2016; **11**: 2293-2299 [PMID: 27313669 DOI: 10.3892/etm.2016.3230]
39 **Kaneko S**, Kurosaki M, Joko K, Marusawa H, Kondo M, Kojima Y, Uchida Y, Kimura H, Tsuji K, Yagisawa H, Kusakabe A, Kobashi H, Akahane T, Tamaki N, Kirino S, Abe T, Yoshida H, Matsushita T, Hasebe C, Izumi N. Detectable HBV DNA during nucleos(t)ide analogues stratifies predictive hepatocellular carcinoma risk score. *Sci Rep* 2020; **10**: 13021 [PMID: 32747646 DOI: 10.1038/s41598-020-69522-w]
40 **Chen CJ**, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH; REVEAL-HBV Study Group. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65-73 [PMID: 16391218 DOI: 10.1001/jama.295.1.65]
41 **Kim JH**, Sinn DH, Kang W, Gwak GY, Paik YH, Choi MS, Lee JH, Koh KC, Paik SW. Low-level viremia and the increased risk of hepatocellular carcinoma in patients receiving entecavir treatment. *Hepatology* 2017; **66**: 335-343 [PMID: 28012257 DOI: 10.1002/hep.28916]
42 **European Association for the Study of the Liver**. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]
43 **Ghany MG**. Current treatment guidelines of chronic hepatitis B: The role of nucleos(t)ide analogues and peginterferon. *Best Pract Res Clin Gastroenterol* 2017; **31**: 299-309 [PMID: 28774412 DOI: 10.1016/j.bpg.2017.04.012]
44 **El-Serag HB**, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* 2004; **126**: 460-468 [PMID: 14762783 DOI: 10.1053/j.gastro.2003.10.065]
45 **Shang RZ**, Qu SB, Wang DS. Reprogramming of glucose metabolism in hepatocellular carcinoma: Progress and prospects. *World J Gastroenterol* 2016; **22**: 9933-9943 [PMID: 28018100 DOI: 10.3748/wjg.v22.i45.9933]
46 **Gao S**, Li A, Liu F, Chen F, Williams M, Zhang C, Kelley Z, Wu CL, Luo R, Xiao H. NCOA5 haploinsufficiency results in glucose intolerance and subsequent hepatocellular carcinoma. *Cancer Cell* 2013; **24**: 725-737 [PMID: 24332041 DOI: 10.1016/j.ccr.2013.11.005]
47 **Liu T**, Wang W, Cui H, Sun M, Wang Y, Liu X, Cao L, Liu H, Liu S. Elevated fasting serum glucose levels increase the risk of hepatocellular carcinoma: A prospective cohort study. *Medicine (Baltimore)* 2019; **98**: e16369 [PMID: 31348238 DOI: 10.1097/MD.0000000000016369]
48 **Meng W**, Bai B, Bai Z, Li Y, Yue P, Li X, Qiao L. The immunosuppression role of alpha-fetoprotein in human hepatocellular carcinoma. *Discov Med* 2016; **21**: 489-494 [PMID: 27448785]
49 **Zhou L**, Liu C, Meng FD, Qu K, Tian F, Tai MH, Wei JC, Wang RT. Long-term prognosis in hepatocellular carcinoma patients after hepatectomy. *Asian Pac J Cancer Prev* 2012; **13**: 483-486 [PMID: 22524811 DOI: 10.7314/apjcp.2012.13.2.483]
50 **Abbasi A**, Bhutto AR, Butt N, Munir SM. Corelation of serum alpha fetoprotein and tumor size in hepatocellular carcinoma. *J Pak Med Assoc* 2012; **62**: 33-36 [PMID: 22352098]
51 **Liu S**, Wang M, Zheng C, Zhong Q, Shi Y, Han X. Diagnostic value of serum glypican-3 alone and in combination with AFP as an aid in the diagnosis of liver cancer. *Clin Biochem* 2020; **79**: 54-60 [PMID: 32087138 DOI: 10.1016/j.clinbiochem.2020.02.009]
52 **Shimagaki T**, Yoshio S, Kawai H, Sakamoto Y, Doi H, Matsuda M, Mori T, Osawa Y, Fukai M, Yoshida T, Ma Y, Akita T, Tanaka J, Taketomi A, Hanayama R, Yoshizumi T, Mori M, Kanto T. Serum milk fat globule-EGF factor 8 (MFG-E8) as a diagnostic and prognostic biomarker in patients with hepatocellular carcinoma. *Sci Rep* 2019; **9**: 15788 [PMID: 31673081 DOI: 10.1038/s41598-019-52356-6]
53 **Zhu M**, Zheng J, Wu F, Kang B, Liang J, Heskia F, Zhang X, Shan Y. OPN is a promising serological biomarker for hepatocellular carcinoma diagnosis. *J Med Virol* 2020 [PMID: 32043608 DOI: 10.1002/jmv.25704]
54 **Lin XJ**, Chong Y, Guo ZW, Xie C, Yang XJ, Zhang Q, Li SP, Xiong Y, Yuan Y, Min J, Jia WH, Jie Y, Chen MS, Chen MX, Fang JH, Zeng C, Zhang Y, Guo RP, Wu Y, Lin G, Zheng L, Zhuang SM. A serum microRNA classifier for early detection of hepatocellular carcinoma: a multicentre, retrospective, longitudinal biomarker identification study with a nested case-control study. *Lancet*

Oncol 2015; **16**: 804-815 [PMID: 26088272 DOI: 10.1016/S1470-2045(15)00048-0]
55 **Forner A**, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018; **391**: 1301-1314 [PMID: 29307467 DOI: 10.1016/S0140-6736(18)30010-2]

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ORIGINAL ARTICLE

Observational Study

Hypophosphatemia after high-dose intravenous iron treatment in patients with inflammatory bowel disease: Mechanisms and possible clinical impact

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Abstract

BACKGROUND

High-dose intravenous iron is an effective treatment option for iron deficiency (ID) or ID anaemia (IDA) in inflammatory bowel disease (IBD). However, treatment with ferric carboxymaltose (FCM) has been associated with the development of hypophosphatemia.

AIM

To investigate mechanisms behind the development of hypophosphatemia after intravenous iron treatment, and disclose symptoms and clinical manifestations related to hypophosphatemia short-term.

METHODS

A prospective observational study of adult IBD patients with ID or IDA was conducted between February 1, 2017 and July 1, 2018 at two separate university hospitals in the southeast region of Norway. Patients received one dose of 1000

Regionale Komiteer for Medisinsk og Helsefaglig Forskningsetikk (REK) in Helse Sør-Øst, Norway/Regional Ethics Committee from the South-East Health Region of Norway.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Detlie TE has served as a speaker, consultant or advisory board member for AbbVie, Ferring, Pfizer, Pharmacosmos, Tillotts, and Vifor Pharma. He has received unrestricted research grants from AbbVie, and Pharmacosmos. Jahnsen ME has received lecture fees from Pharmacosmos. Zoller H has served as a speaker, consultant or advisory board member for AbbVie, BMS, Gilead, MSD, Merck, Merz, Novartis, Pharmacosmos, and Vifor Pharma. Moum B has served as a speaker, consultant or advisory board member for AbbVie, Ferring, Janssen, Roche, and Takeda. Jahnsen J has served as a speaker, consultant or advisory board member for AbbVie, Astro Pharma, Boehringer Ingelheim, BMS, Celltrion, Ferring, Hikma, Janssen, Meda, MSD, Napp Pharma, Novartis, Orion Pharma, Pfizer, Pharmacosmos, Roche, Takeda, Tillotts, and Sandoz. The other authors have no disclosures.

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mg of either FCM or ferric derisomaltose (FDI) and were followed for an observation period of at least 7 wk. Blood and urine samples were collected for relevant analyses at baseline, week 2 and at week 6. Clinical symptoms were assessed at the same timepoints using a respiratory function test, a visual analogue scale, and a health-related quality of life questionnaire.

RESULTS

A total of 106 patients was available for analysis in this study. The FCM treatment group consisted of 52 patients and hypophosphatemia was present in 72.5% of the patients at week 2, and in 21.6% at week 6. In comparison, the FDI treatment group consisted of 54 patients and 11.3% of the patients had hypophosphatemia at week 2, and 3.7% at week 6. The difference in incidence was highly significant at both week 2 and 6 ($P < 0.001$ and $P < 0.013$, respectively). We observed a significantly higher mean concentration of intact fibroblast growth factor 23 ($P < 0.001$), a significant rise in mean urine fractional excretion of phosphate ($P = 0.004$), a significant decrease of 1,25-dihydroxyvitamin D ($P < 0.001$) and of ionised calcium levels ($P < 0.012$) in the FCM-treated patients compared with patients who received FDI. No clinical symptoms could with certainty be related to hypophosphatemia, since neither the respiratory function test, SF-36 (36-item short form health survey) or the visual analogue scale scores resulted in significant differences between patients who developed hypophosphatemia or not.

CONCLUSION

Fibroblast growth factor 23 has a key role in FCM induced hypophosphatemia, probably by inducing loss of phosphate in the urine. Short-term clinical impact of hypophosphatemia was not demonstrated.

Key Words: Iron deficiency; Hypophosphatemia; Inflammatory bowel disease; Ferric carboxymaltose; Ferric derisomaltose

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Core Tip: High-dose intravenous iron is an effective treatment for iron deficiency anaemia (IDA) in inflammatory bowel disease (IBD). However, ferric carboxymaltose (FCM) is associated with development of hypophosphatemia. This study of adult IBD patients with IDA investigated the mechanisms and clinical manifestations related to hypophosphatemia after treatment of either FCM or ferric derisomaltose (FDI). The incidence of hypophosphatemia was significantly higher after FCM than FDI, and fibroblast growth factor 23 had a key role, inducing loss of phosphate in the urine along with a significant lowering of 1,25-dihydroxyvitamin D and ionised calcium levels. Short-term clinical impact was not demonstrated.

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INTRODUCTION

Iron replacement therapy is often needed in patients with inflammatory bowel disease (IBD) because iron deficiency (ID) and ID anaemia (IDA) occur frequently in this patient group[1-3]. A large proportion of IBD patients experience intolerance to oral iron[4]. Additionally, it is asserted that oral iron can lead to an exacerbation of inflammation in the bowel mucosa due to a local effect on the enterocytes[5-7]. Therefore, administration of high-dose iron as an intravenous infusion is an effective, suitable

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and convenient treatment option in IBD. Ferric carboxymaltose (FCM; Ferinject®; Vifor Pharma) and ferric derisomaltose (FDI), previously known as iron isomaltoside (Monofer®; Pharmacosmos A/S), are the most widely used preparations in Europe when high-dose intravenous iron is indicated.

In a recent publication, we described a high incidence of hypophosphatemia in IBD patients who had received treatment with FCM[8]. The mechanism behind the development of hypophosphatemia has been described by Wolf *et al*[9], but probably is not yet fully understood, and has not been investigated in patients with IBD. Fibroblast growth factor 23 (FGF23) is a small peptide hormone, synthesized by osteocytes, which regulates phosphate and vitamin D homeostasis[9]. FGF23 consists of a biologically-active component (full-length, intact FGF23) and inactive C-terminal fragments (C-terminal FGF23). FCM causes an increase in intact FGF23, which triggers the pathophysiological cascade of renal phosphate wasting, suppressed levels of 1,25-dihydroxyvitamin D, and secondary hyperparathyroidism[9]. In contrast, FDI does not appear to induce increased intact FGF23 levels, and is associated with a low incidence of hypophosphatemia[9].

Moderate to severe hypophosphatemia over time, as well as acute severe hypophosphatemia, can lead to serious complications, *e.g.*, respiratory failure, haemolysis, left ventricular failure, and rhabdomyolysis[10-13]. Development of osteomalacia with pseudo-fractures has been found in patients with sustained hypophosphatemia [14-17]. However, there are uncertainties with regard to both the frequency of symptoms and the clinical impact of hypophosphatemia.

Reduced quality of life (QoL) is common and well-documented in IBD patients due to chronic inflammation in the gut and the occurrence of extra-intestinal manifestations[18,19]. Therefore, addressing additional symptoms and implications of hypophosphatemia in this patient group is a challenge, and no specific questionnaire related to hypophosphatemia is available.

In this short-term study, we aimed to investigate the mechanisms causing the development of hypophosphatemia in IBD patients, with ID or IDA, who received one high-dose (1000 mg) infusion of iron. Moreover, we aimed to document symptoms and clinical manifestations related to hypophosphatemia.

MATERIALS AND METHODS

Study design and patient population

This prospective observational study was conducted between February 1, 2017 and July 1, 2018. The study design and patient recruitment have previously been described in detail (Detlie *et al*[8]). In brief, adult IBD patients (> 18 years) diagnosed with ID or IDA (according to European Crohn's and Colitis Organisation guidelines)[2] were recruited at two separate study sites in the southeast region of Norway and treated with either FCM or FDI.

Eligible patients were prescribed 1000 mg of high-dose intravenous iron, FCM (50 mg/mL) or iron derisomaltose (100 mg/mL), administered as a single dose. Patients who had received high-dose intravenous iron treatment or a packed red blood cell transfusion within 3 mo of study entry, or for whom high-dose intravenous iron treatment was contraindicated, were not included in the study.

Enrolment continued until at least 50 consecutive patients with complete adherence to the study protocol were recruited at each site (a total of more than 100 patients) (Supplementary Figure 1). The enrolment period was followed by a prospective observation period, which lasted ≤ 7 wk for each patient and included three study visits.

Study inclusion was performed at baseline, at which time intravenous iron treatment was administered. Patients attended the clinic at week 2 (10-15 d) and at week 6 (5-7 wk) following intravenous iron treatment. Each patient could receive only one infusion within an approximate 2-mo period after consenting to study participation.

Study assessments and data collection

Blood analysis at each study visit included ionised calcium, creatinine, phosphate, parathyroid hormone (PTH) and vitamin D (25-hydroxyvitamin D).

Blood samples were also frozen and sent to Medizinische Universität Innsbruck, Universitätsklinik für Innere Medizin I, for analysis of 1,25-dihydroxyvitamin D, intact and C-terminal FGF23. The Kainos FGF-23 ELISA Kit was used for the FGF23 analysis. The assay for intact FGF23 measures only full-length peptide, whereas the assay for C-

terminal FGF23 measures full-length peptide and the C-terminal fragments thereby representing total FGF23.

Spot urine samples were collected at each study visit and analysed for urine phosphate and urine creatinine. A calculation of the fractional excretion of phosphate rate ($FEPO_4$) was then performed using the formula, $FEPO_4$ = (urine phosphate × plasma creatinine × 100)/(plasma phosphate × urine creatinine). Oslo University Hospital Ullevål used the Roche analysis method (Roche/Hitachi Cobas® C systems PHOS2 and CREP2) while Akershus University Hospital used the Vitros analysis (VITROS® MicroSlide Assay 5.1 FS Diluent Pack 3). The slight sensitivity difference between the two analytical methods was minimized by recalculating $FEPO_4$ using the above-mentioned formula.

Symptoms that might be related to hypophosphatemia were assessed at each of the three study visits using the MicroRPM™ (CareFusion) test to determine respiratory muscle function by measuring maximum inspiratory and maximum expiratory pressure, a health-related QoL questionnaire (36-item short form health survey, SF-36), and a visual analogue scale (VAS).

For the MicroRPM™ respiratory function test, patients were asked to inhale and exhale as hard as possible. The test was repeated three times at every visit, and the best result of the three attempts was registered.

The SF-36 is a generic, self-administered questionnaire containing 36 items[20]. The items are divided into eight multi-item scales that reflect general health, physical functioning, role limitations due to physical problems, bodily pain, vitality, mental health, social functioning, and role limitations due to emotional problems. Each scale is transformed into a 1-100 scale, where a lower score represents more disability. The processing of raw SF-36 data into results was executed according to the SF-36 scoring algorithms[21].

The VAS is a 10 cm line on which the patient is asked to place a vertical mark to indicate the level of intensity of a symptom that best fits his or her experience. Scores range from 0-100 (mm) where a higher score represents greater symptom intensity. The VAS was used to assess general weakness, fatigue, joint pain, joint stiffness, muscle pain, bone and skeletal pain, and difficulties performing daily activities.

All demographic information was collected from patients' medical records and was entered into an electronic case report form.

The study was completed when all enrolled patients had received intravenous iron administration, had attended all three study visits, and had fulfilled the requirements of the study protocol.

Study outcomes

Serum phosphate, PTH, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, ionised calcium, creatinine, intact and C-terminal FGF23, and $FEPO_4$ (urine phosphate and urine creatinine) were measured in order to assess possible mechanisms behind the development of hypophosphatemia after intravenous iron treatment. Results from the FCM treatment group were compared with results from the FDI treatment group.

Hypophosphatemia was defined as a serum phosphate level < 0.8 mmol/L (< 2.5 mg/dL). The clinical impact of hypophosphatemia was evaluated at week 2 and week 6 using the respiratory muscle function test, SF-36, and the VAS score. In relation to the assessment of clinical impact, the hypophosphatemia group was defined as patients experiencing hypophosphatemia at both week 2 and week 6. Results for patients with hypophosphatemia were compared with results for patients without hypophosphatemia, independent of treatment group.

Statistical analysis

This study was designed to achieve 80% power to detect a difference in the primary outcome, which was the incidence of hypophosphatemia (previously described by Detlie *et al*[8]). Hence, the MicroRPM™ respiratory test, SF-36, and VAS scores were not used to justify sample size.

Data are presented descriptively, as mean with SD or 95% confidence intervals for continuous variables, and as the number of exposed patients (with proportions) for categorical variables. Hypothesis tests for differences in change between treatment groups, change from baseline, and groups with or without hypophosphatemia, were conducted using paired *t*-tests. All analyses were performed in R. A P value of < 0.05 was considered significant.

Ethical considerations

The study protocol was approved by the relevant local regulatory and ethical

committees and adhered to the applicable laws on data protection. A study registration application was sent to the EudraCT system with the application No. 2016-003476-41, but the application was deemed unnecessary since there were no indications of a medical intervention study.

All patients gave informed consent before inclusion into the study, and the study was performed in accordance with the principles for post-authorisation safety studies, according to Good Clinical Practice guidelines.

All biological material obtained from patients was destroyed after analysis, as were the frozen blood samples sent to the Medical University of Innsbruck.

Study nurses were blinded to the results of laboratory findings but, for safety reasons, the primary investigator at each study centre was not blinded.

RESULTS

Of the 130 patients screened for this study, 106 patients (52 patients at Oslo University Hospital Ullevål and 54 patients at Akershus University Hospital) were included in the analyses. Demographic and clinical characteristics of the patients have previously been described[8].

Data for serum phosphate, $FEPO_4$, intact and C-terminal FGF23, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, ionised calcium, and PTH at baseline and at each study visit are shown in Table 1. A sub-analysis of the same data, stratified according to hypophosphatemia status (with/without) at week 2 and at week 6, is shown in Table 2.

Serum phosphate and urinary excretion of phosphate

As previously described, following treatment with FCM, hypophosphatemia was present in 72.5% (37/51) of patients at week 2, and in 21.6% (11/51) of patients at week 6. In comparison, in the FDI treatment group, 11.3% (6/53) of patients had hypophosphatemia at week 2, and 3.7% (2/54) at week 6. There were no new incidences of hypophosphatemia at week 6. The difference in incidence was highly significant at both week 2 and 6 ($P < 0.001$ and $P < 0.013$, respectively)[8]. These findings are consistent with the mean urine $FEPO_4$ that was significantly ($P = 0.004$) higher at week 2 in the FCM treatment group compared with the FDI treatment group, and still elevated (though declining) at week 6 in the FCM group (Table 1 and Figure 1A). In the sub-analysis, the FDI-treated patients with hypophosphatemia ($n = 6$) had numerically increased $FEPO_4$ (Table 2).

Patients in both treatment groups without hypophosphatemia at week 2 also experienced an increase in $FEPO_4$ at week 2 compared to baseline values, but urinary phosphate excretion declined again at week 6 in these patients.

FGF23

There was a significant ($P < 0.001$) increase in intact FGF23 from baseline to week 2 after infusion of FCM, compared with the FDI treatment group (Table 1 and Figure 1B). At week 6, intact FGF23 values in the FCM treatment group had returned close to baseline. In comparison, after FDI treatment no such increases were found (Figure 1B). At baseline, the serum concentration of C-terminal FGF23 was higher in the FDI treatment group than in the FCM treatment group (Table 1), and declined after FDI infusion (Figure 1C). This high value at baseline was not seen in the FCM treatment group (Table 1), which is probably compatible with the less severe ID/IDA seen in the FCM group[8].

In the sub-analysis, for the FCM-treated patients with hypophosphatemia, intact FGF23 was significantly increased compared with FCM-treated patients without hypophosphatemia at week 2 and at week 6 (Table 2). In the FCM-treated patients who had normal phosphate, intact FGF23 was not increased at week 2 or week 6.

For FDI-treated patients, the sub-analysis showed that there was no significant difference in mean intact FGF23 Levels between patients with/without hypophosphatemia at week 2 or at week 6 (Table 2). At week 2, only one FDI-treated patient with hypophosphatemia had significantly increased intact FGF23; the other five patients with hypophosphatemia had minimal change in their intact FGF23 values.

Vitamin D

There were no significant differences between the treatment groups in the concentration of 25-hydroxyvitamin D throughout the study period (Figure 1D). However, the sub-analysis showed that 25-hydroxyvitamin D concentrations were lower at week

Table 1 Descriptive data for laboratory parameters at baseline, at week 2 and at week 6

Analysis	High-dose intravenous iron	Baseline, mean ± SD	Week 2, mean ± SD	Difference from baseline at week 2	Difference between FCM and FDI at week 2 (95%CI)	*P* value	Week 6, mean ± SD	Difference from baseline at week 6	Difference between FCM and FDI at week 6 (95%CI)	*P* value
Serum phosphate, mmol/L (ref. value 0.8-1.65)	FCM	1.07 ± 0.2	0.65 ± 0.2	-0.417	-0.344 (-0.427 to -0.260)	< 0.001	1.00 ± 0.3	-0.072	-0.070 (-0.144 to 0.004)	0.064
	FDI	1.15 ± 0.2	1.07 ± 0.2	-0.073			1.14 ± 0.2	-0.002		
Intact FGF23, pg/mL (ref. value 11.50-48.90)	FCM	43.42 ± 14.2	91.61 ± 63.8	49.205	45.312 (25.982 to 64.697)	< 0.001	44.79 ± 23.1	1.718	1.559 (-6.407 to 9.525)	0.698
	FDI	43.88 ± 14.5	47.77 ± 22.1	3.892			44.04 ± 16.6	0.159		
C-terminal FGF23, pmol/L (ref. value 0.30-3.00)	FCM	2.46 ± 3.2	1.68 ± 1.3	-0.756	6.783 (-1.319 to 14.885)	0.099	0.94 ± 1.2	-1.507	5.124 (-1.310 to 11.558)	0.116
	FDI	8.87 ± 30.6	1.33 ± 1.4	-7.539			2.24 ± 7.6	-6.632		
$FEPO_4$, % (ref. value N/A)	FCM	9.95 ± 5.8	18.70 ± 10.8	9.946	5.375 (1.801 to 8.95)	0.004	13.68 ± 11.3	4.210	3.326 (-0.309 to 6.96)	0.072
	FDI	12.55 ± 5.9	17.03 ± 8.6	4.570			13.37 ± 6.0	0.884		
PTH, pmol/L (ref. value 1.5-7.0)	FCM	5.46 ± 2.6	7.02 ± 3.4	1.608	0.442 (-0.561 to 1.445)	0.384	5.97 ± 3.3	0.767	0.590 (-0.358 to 1.539)	0.220
	FDI	5.51 ± 2.6	6.72 ± 3.4	1.166			5.69 ± 2.3	0.176		
Ionised calcium, mmol/L (ref. value 1.16-1.32)	FCM	1.21 ± 0.0	1.20 ± 0.0	-0.015	-0.020 (-0.035 to -0.004)	0.012	1.21 ± 0.0	0.000	-0.018 (-0.036 to -0.001)	0.044
	FDI	1.23 ± 0.0	1.23 ± 0.1	0.005			1.25 ± 0.0	0.018		
25-hydroxyvitamin D, nmol/L (ref. value 50-125)	FCM	58.35 ± 24.4	57.13 ± 23.1	-1.212	-2.133 (-6.238 to 1.972)	0.305	57.48 ± 20.8	-0.865	-0.160 (-7.078 to 6.759)	0.964
	FDI	63.51 ± 21.9	64.63 ± 20.0	0.922			62.75 ± 21.1	-0.706		
1,25-dihydroxyvitamin D, ng/L (ref. value 20-79)	FCM	51.10 ± 19.2	28.77 ± 17.9	-21.074	-16.463 (-24.487 to -8.438)	< 0.001	53.78 ± 20.2	3.218	5.357 (-3.164 to 13.878)	0.215
	FDI	52.85 ± 20.4	48.24 ± 17.7	-4.611			50.71 ± 19.8	-2.139		

Normal phosphate levels: > 0.8 mmol/L = > 2.48 mg/dL; Mild hypophosphatemia 0.79-0.6 mmol/L = 2.44-1.86 mg/dL; Moderate hypophosphatemia 0.59-0.32 mmol/L = 1.83-0.99 mg/dL; Severe hypophosphatemia < 0.32 mmol/L = < 0.99 mg/dL. CI: Confidence interval; FCM: Ferric carboxymaltose; FDI: Ferric derisomaltose; FEPO4: Fractional excretion of phosphate; FGF23: Fibroblast growth factor 23; PTH: Parathyroid hormone; N/A: Not applicable.

6 in the two FDI-treated patients with hypophosphatemia when compared with baseline concentrations within the same group (Tables 1 and 2). At week 2, 1,25-dihydroxyvitamin D concentrations were significantly lower in patients who received FCM compared with patients who received FDI (Table 1). In the FCM-treatment group, the mean concentration of 1,25-dihydroxyvitamin D returned to baseline at week 6 (Table 1 and Figure 1E). However, the sub-analysis revealed that, for the FCM-treated patients with hypophosphatemia, low 1,25-dihydroxyvitamin D levels persisted at week 6 (Table 2). In the subgroups of patients without hypophosphatemia, 1,25-dihydroxyvitamin D levels were relatively unchanged.

In our cohort, we identified 36 patients (34.0%) with vitamin D deficiency (25-hydroxyvitamin D < 50 nmol/L) at baseline; 10 of these patients had severe vitamin D deficiency (25-hydroxyvitamin D < 30 nmol/L). The distribution of these patients was equal in the two treatment groups, as well as equally distributed across disease states – ulcerative colitis and Crohn's disease. Moreover, we found no association between low levels of vitamin D and development of hypophosphatemia.

Table 2 Laboratory parameters for patients stratified by hypophosphatemia status (with/without) at week 2 and at week 6

Analysis	High-dose intravenous iron	Week 2 serum phosphate ≥ 0.8 mmol/L, mean ± SD	Week 2 serum phosphate < 0.8 mmol/L, mean ± SD	Difference	*P* value	Week 6 serum phosphate ≥ 0.8 mmol/L, mean ± SD	Week 6 serum phosphate < 0.8 mmol/L, mean ± SD	Difference	*P* value
Intact FGF23, pg/mL (ref. value 11.50-48.90)	FCM	33.71 ± 10.56 (*n* = 14/51)	114.33 ± 62.22 (*n* = 37/51)	80.62	< 0.001	39.30 ± 16.79) (*n* = 40/51)	66.86 ± 30.05 (*n* = 11/51)	27.56	0.013
	FDI	45.82 ± 16.90 (*n* = 47/53)	57.16 ± 46.35 (*n* = 6/53)	11.43	0.577	43.85 ± 16.83 (*n* = 52/54)	48.93 ± 10.13 (*n* = 2/54)	5.08	0.604
C-terminal FGF23, pmol/L (ref. value 0.30-3.00)	FCM	1.03 ± 0.99 (*n* = 14/51)	1.93 ± 1.33 (*n* = 37/51)	0.9	0.014	0.76 ± 0.63 (*n* = 40/51)	1.64 ± 2.14 (*n* = 11/51)	0.88	0.206
	FDI	1.36 ± 1.47 (*n* = 47/53)	1.08 ± 0.72 (*n* = 6/53)	-0.28	0.465	2.27 ± 7.79 (*n* = 52/54)	1.31 ± 0.33 (*n* = 2/54)	-0.97	0.384
$FEPO_4$, % (ref. value N/A)	FCM	13.35 ± 5.94 (*n* = 12/46)	20.59 ± 11.60 (*n* = 34/46)	7.24	0.009	9.62 ± 6.56 (*n* = 36/47)	26.99 ± 13.38 (*n* = 11/47)	17.37	0.001
	FDI	15.97 ± 7.85 (*n* = 46/52)	25.11 ± 10.22 (*n* = 6/52)	9.14	0.080	13.00 ± 5.79 (*n* = 52/54)	22.85 ± 4.41 (*n* = 2/54)	9.85	0.176
PTH, pmol/L (ref. value 1.5-7.0)	FCM	5.46 ± 2.83 (*n* = 14/48)	7.46 ± 3.23 (*n* = 34/48)	2	0.042	5.22 ± 2.39 (*n* = 39/47)	9.93 ± 4.43) (*n* = 8/47)	4.71	0.020
	FDI	6.28 ± 2.97 (*n* = 47/53)	10.13 ± 4.70 (*n* = 6/53)	3.85	0.102	5.66 ± 2.32 (*n* = 52/54)	6.40 ± 1.70 (*n* = 2/54)	0.74	0.649
Ionised calcium, mmol/L (ref. value 1.16-1.32)	FCM	1.21 ± 0.32 (*n* = 13/50)	1.19 ± 0.05 (*n* = 37/50)	-0.02	0.336	1.21 ± 0.05 (*n* = 40/51)	1.22 ± 0.04 (*n* = 11/51)	0.01	0.496
	FDI	1.24 ± 0.45 (*n* = 46/52)	1.21 ± 0.09 (*n* = 6/52)	-0.03	0.422	1.25 ± 0.05 (*n* = 51/53)	1.23 ± 0.02 (*n* = 2/53)	-0.02	0.314
25-hydroxyvitamin D, nmol/L (ref. value 50-125)	FCM	63.00 ± 30.94 (*n* = 14/51)	54.54 ± 19.69 (*n* = 37/51)	-8.46	0.354	60.05 ± 21.68 (*n* = 40/51)	45.45 ± 10.00 (*n* = 11/51)	-14.6	0.002
	FDI	64.16 ± 20.30 (*n* = 45/51)	64.83 ± 18.93 (*n* = 6/51)	0.67	0.937	63.68 ± 20.97 (*n* = 50/52)	39.50 ± 2.12 (*n* = 2/52)	-24.18	< 0.001
1,25-dihydroxyvitamin D, ng/L (ref. value 20-79)	FCM	46.34 ± 19.10 (*n* = 14/50)	21.46 ± 11.63 (*n* = 36/50)	-24.88	< 0.001	56.76 ± 20.27 (*n* = 40/50)	40.89 ± 15.95 (*n* = 10/50)	-15.87	0.016
	FDI	49.05 ± 18.52 (*n* = 47/53)	45.72 ± 7.15 (*n* = 6/53)	-3.33	0.414	50.88 ± 19.92 (*n* = 52/54)	46.45 ± 21.85 (*n* = 2/54)	-4.43	0.822

Normal phosphate levels: > 0.8 mmol/L = > 2.48 mg/dL; Mild hypophosphatemia 0.79-0.6 mmol/L = 2.44-1.86 mg/dL; Moderate hypophosphatemia 0.59-0.32 mmol/L = 1.83-0.99 mg/dL; Severe hypophosphatemia < 0.32 mmol/L = < 0.99 mg/dL. FCM: Ferric carboxymaltose; FDI: Ferric derisomaltose; FEPO4: Fractional excretion of phosphate; FGF23: Fibroblast growth factor 23; PTH: Parathyroid hormone; N/A: Not applicable.

Calcium and PTH

Ionised calcium values dropped significantly from baseline to week 2 in the FCM treatment group compared with the FDI treatment group ($P < 0.012$) but stayed within normal range. The mean values in the FCM group had increased by week 6, but the between-group difference was still significant ($P < 0.044$). Calcium values remained stable throughout the study in the FDI treatment group (Figure 1F), and in the subgroup of FCM-treated patients who did not develop hypophosphatemia. The sub-analysis showed that there was a numerically lower level of ionised calcium in the FDI-treated patients with hypophosphatemia than in the FDI-treated patients without hypophosphatemia (Table 2).

PTH values were elevated (> 7 pmol/L) in 28 patients (26.4%) at baseline; the distribution was similar between treatment groups. PTH concentrations were similar between treatment groups at baseline, and no significant between-group differences were observed in mean PTH concentrations at week 2, and at week 6 (Table 1). PTH values increased in both treatment groups at week 2 and decreased again at week 6 (Figure 1G). The sub-analysis indicated that the increase in PTH in both treatment groups was mainly driven by the patients who developed hypophosphatemia, with significant differences at week 2 and week 6 for the FCM-treated patients with hypophosphatemia compared to FCM-treated patients without hypophosphatemia (Table 2).

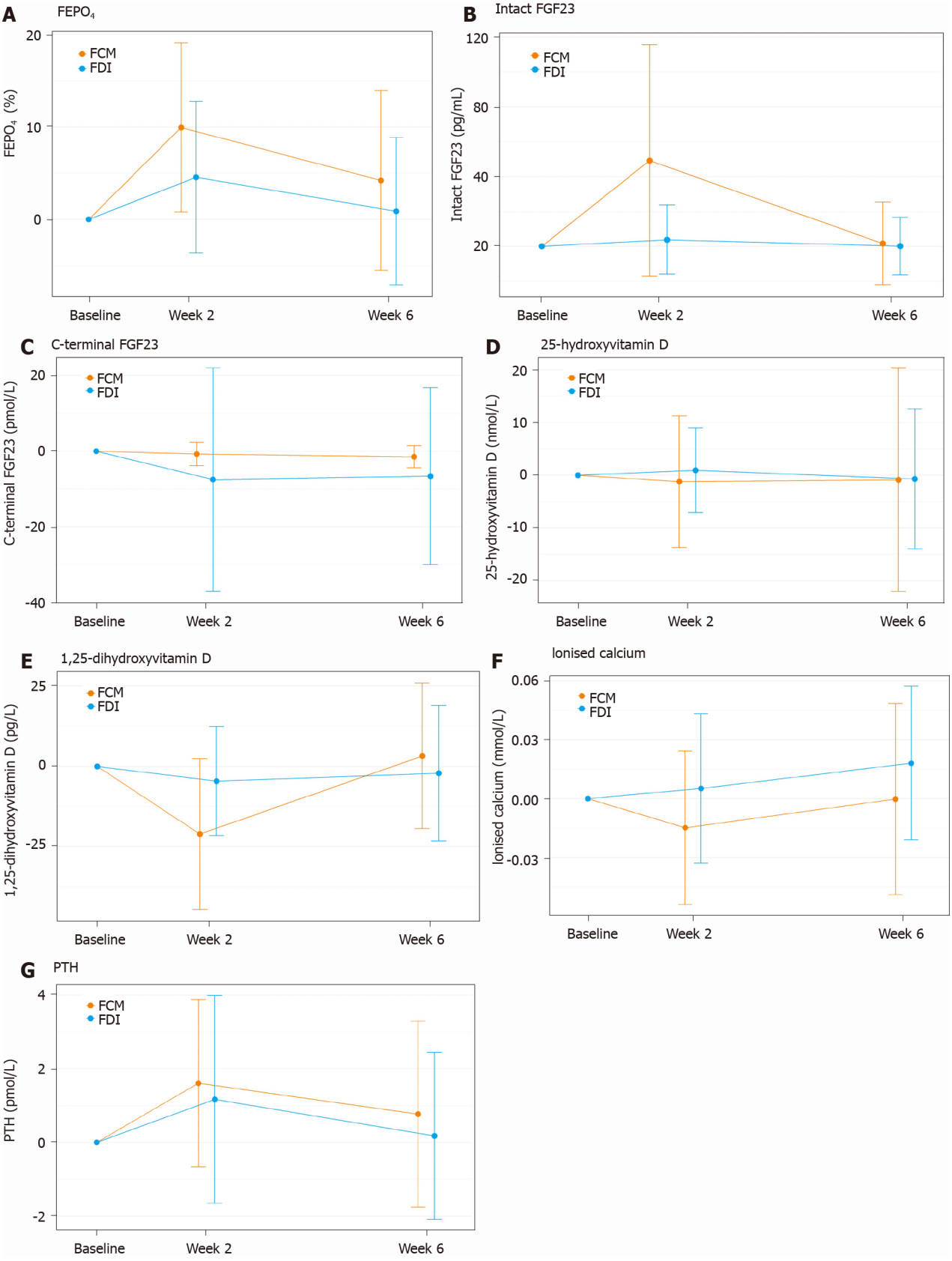


Figure 1 mean ± SD change from baseline in laboratory parameters in inflammatory bowel disease patients with iron deficiency/iron deficiency anaemia treated with a single 1000 mg intravenous dose of ferric carboxymaltose or ferric derisomaltose. A: Fractional excretion of phosphate; B: Intact fibroblast growth factor 23; C: C-terminal fibroblast growth factor 23; D: 25-Hydroxyvitamin D; E: 1,25-Hydroxyvitamin D; F: Ionised calcium; G: Parathyroid hormone. FCM: Ferric carboxymaltose; FDI: Ferric derisomaltose; $FEPO_4$: Fractional excretion of phosphate; FGF23: Fibroblast growth factor 23; PTH: Parathyroid hormone.

Respiratory muscle function tests

In the comparison of patients who developed hypophosphatemia *vs* those who did not develop hypophosphatemia, independent of treatment group, no significant differences were observed in the respiratory muscle function test results. The differences between patients with hypophosphatemia and those with normal phosphate values were minimal and the standard deviation was wide in both groups (Figure 2).

SF-36

The results of the SF-36 QoL assessment are presented in Table 3. Overall, there were no significant differences between patient groups with or without hypophosphatemia at baseline and at any time point during the study. The mean scores at baseline in both treatment groups were generally low.

VAS scores

There were no significant differences in VAS scores between the groups of patients with/without hypophosphatemia at week 2 and at week 6 (Table 4). Overall, VAS scores were elevated at baseline. However, the group of patients who developed hypophosphatemia had lower VAS scores at baseline for the items joint pain, muscle pain, and bone and skeletal pain, compared to the group of patients who did not develop hypophosphatemia; between-group differences were not significant for these items. There was, however, a significant between-group difference ($P < 0.001$) at baseline for the VAS joint stiffness item score, with lower values in the group of patients who developed hypophosphatemia.

DISCUSSION

Our study indicates that FGF23 plays an important role in the development of hypophosphatemia in IBD patients treated with FCM. In these patients, a high level of intact FGF23, an increased excretion of phosphate in the urine, a decrease of 1,25-dihydroxyvitamin D and of serum calcium levels, and a slight elevation of PTH, was demonstrated.

Previous clinical trials of FCM have shown similar results[9,22]. However, for the most part, these studies have been conducted in healthy and, predominantly, female populations. The role of FGF23 has also been described in earlier publications[23-26]. Regulation of phosphate concentrations in the body seems to be strongly influenced by intact FGF23, which reduces phosphate reabsorption in the proximal tubules in the kidneys and inhibits production of 1,25-dihydroxyvitamin D, probably by inhibiting the activity of the enzyme 25-hydroxyvitamin D-1a-hydroxylase and increased expression of 24-hydroxylase[24,26]. Our findings suggest that FCM could have a direct impact on cleavage of FGF23, resulting in a high level of intact FGF23 and consequent phosphate wasting. This might also explain why baseline phosphate level does not predict the development of mild or severe hypophosphatemia, due to the inappropriate excretion of available phosphate in the urine, following FCM treatment[8]. We also observed a decrease in 1,25-dihydroxyvitamin D (the active vitamin D metabolite), a decrease in ionised calcium, and development of secondary hyperparathyroidism. This might explain why some patients treated with FCM still had hypophosphatemia six weeks after treatment, when the intact FGF23 values had normalized (Table 2) since elevated PTH promotes excretion of phosphate in the urine[9,27,28].

The majority of patients in the FCM treatment group developed hypophosphatemia at week 2. The remaining patients did not develop hypophosphatemia and had unchanged levels of intact FGF23. So, there is a clear association between the development of high levels of intact FGF23 and hypophosphatemia. Therefore, it can only be speculated that there might be some individual factors related to the handling of FCM that cause the majority of patients treated with FCM to develop hypophosphatemia, whereas others do not. Neither is it known if any individual patient would develop hypophosphatemia on subsequent administrations of FCM, or if the effect of FCM treatment on phosphate wasting is indiscriminate. Perhaps some patients are protected against the influence of FCM on the enzyme responsible for FGF23 protein cleavage. From our results, we postulate that the mechanism of FCM-induced hypophosphatemia is not related to IBD; instead, it appears to be independently connected to the drug itself.

Table 3 Descriptive 36-item short form health survey scores for patient groups with/without hypophosphatemia independent of treatment group

SF-36 scale item	Normal phosphate population, baseline, mean ± SD	Hypophosphatemia population, baseline, mean ± SD	Difference[1] at baseline, mean (95%CI)	Normal phosphate population, change at week 2, mean ± SD	Hypophosphatemia population, change at week 2, mean ± SD	Difference[1] at week 2, mean (95%CI)	Normal phosphate population, change at week 6, mean ± SD	Hypophosphatemia population, change at week 6, mean ± SD	Difference[1] at week 6, mean (95%CI)
General health	50.91 ± 20.1	48.50 ± 26.1	2.4 (-14.5 to 19.4)	-0.45 ± 12.9	0.75 ± 12.2	-1.2 (-9.2 to 6.8)	3.06 ± 16.3	3.25 ± 15.1	-0.2 (-10.2 to 9.8)
Physical functioning	78.72 ± 22.4	86.11 ± 21.1	-7.4 (-21.3 to 6.6)	2.63 ± 10.9	-0.28 ± 6.3	2.9 (-1.6 to 7.4)	3.97 ± 14.5	-0.28 ± 4.7	4.2 (0.2 to 8.3)
Role limitations due to physical problems	47.61 ± 42.8	66.67 ± 40.4	-19.1 (-45.7 to 7.6)	-4.08 ± 28.1	-6.25 ± 18.8	2.2 (-10.8 to 15.1)	11.32 ± 39.2	2.08 ± 16.7	9.2 (-3.7 to 22.1)
Bodily pain	65.65 ± 25.1	67.17 ± 27.6	-1.5 (-19.6 to 16.5)	0.68 ± 18.9	4.42 ± 12.2	-3.7 (-12.2 to 4.7)	3.40 ± 19.8	10.83 ± 19.1	-7.4 (-20.0 to 5.2)
Vitality	37.98 ± 22.0	40.00 ± 22.4	-2.0 (-16.7 to 12.7)	4.05 ± 13.1	7.50 ± 15.9	-3.4 (-13.8 to 6.9)	10.89 ± 18.0	12.92 ± 16.0	-2.0 (-12.7 to 8.6)
Mental health	71.72 ± 18.9	70.33 ± 16.9	1.4 (-9.9 to 12.6)	3.37 ± 9.0	4.33 ± 5.0	-1.0 (-4.5 to 2.6)	4.89 ± 13.2	1.67 ± 11.1	3.2 (-4.2 to 10.7)
Social functioning	68.35 ± 26.3	68.75 ± 30.4	-0.4 (-20.2 to 19.4)	3.86 ± 16.7	7.29 ± 13.5	-3.4 (-12.5 to 5.6)	8.70 ± 20.7	7.29 ± 15.5	1.4 (-9.1 to 11.9)
Role limitations due to emotional problems	67.91 ± 41.5	72.22 ± 42.2	-4.3 (-32.1 to 23.4)	-2.66 ± 34.8	-8.33 ± 37.9	5.7 (-19.1 to 30.5)	6.52 ± 38.0	-2.78 ± 26.4	9.3 (-8.8 to 27.4)

Hypophosphatemia defined as serum phosphate < 0.8 mmol/L (< 2.5 mg/dL).

[1]Differences are normal phosphate group minus hypophosphatemia group. CI: Confidence interval; SF-36: 36-Item short form health survey.

A few patients who received treatment with FDI also developed hypophosphatemia but, unlike those receiving FCM, these patients did not on average have significantly elevated intact FGF23 Levels when assessed at week 2, which would suggest a different underlying mechanism. A transient increase in intact FGF23 during the first 2 wk in patients experiencing hypophosphatemia cannot be ruled out, as data were not collected during this time period. A numerical increase in PTH was observed at week 2 along with decreased ionised calcium, and decreased 25-hydroxyvitamin D at week 6. It is not clear whether these observations are the result of a transient increase of intact FGF23 during the first 2 wk, or solely a physiological response to a rapid correction of ID, or simply an artefact due to the low numbers of FDI patients who developed hypophosphatemia. The general physiological response of mineral metabolism markers to rapid ID correction is not fully elucidated and is an area of further research.

Table 4 Descriptive visual analogue scale score for patient groups with/without hypophosphatemia independent of treatment group

VAS item	Normal phosphate group, baseline, mean ± SD	Hypophosphatemia group, baseline, mean ± SD	Difference[1] at baseline, mean (95%CI)	*P* value	Normal phosphate group, change at week 2, mean ± SD	Hypophosphatemia group, change at week 2, mean ± SD	Difference[1] at week 2, mean (95%CI)	*P* value	Normal phosphate group, change at week 6, mean ± SD	Hypophosphatemia group, change at week 6, mean ± SD	Difference[1] at week 6, mean (95%CI)	*P* value
General weakness	43.54 ± 28.7	31.17 ± 30.5	12.4 (-7.6 to 32.4)	0.205	-4.98 ± 14.9	-1.50 ± 17.2	-3.5 (-14.7 to 7.7)	0.515	-10.64 ± 21.9)	-6.33 ± 25.7	-4.3 (-21.1 to 12.5)	0.589
Fatigue	37.53 ± 30.0	29.58 ± 30.1	7.9 (-11.8 to 27.7)	0.403	-3.31 ± 19.4	-1.33 ± 17.9	-2.0 (-13.9 to 9.9)	0.728	-8.93 ± 22.0	-5.17 ± 26.7	-3.8 (-21.2 to 13.6)	0.648
Joint pain	17.30 ± 23.3	8.25 ± 14.8	9.1 (-1.2 to 19.3)	0.081	0.32 ± 14.3	-3.09 ± 6.9	3.4 (-1.9 to 8.7)	0.197	2.17 ± 18.0	0.17 ± 7.4	2.0 (-3.8 to 7.8)	0..489
Joint stiffness	13.31 ± 22.3	1.42 ± 3.4	11.9 (6.9 to 16.8)	< 0.001	-0.57 ± 18.1	-0.64 (2.1)	0.1 (-3.9 to 4.0)	0.972	0.99 ± 17.7	1.75 ± 8.5	-0.8 (-7.1 to 5.6)	0.807
Muscle pain	15.35 ± 23.1	5.83 ± 14.5	9.5 (-0.5 to 19.6)	0.062	-1.71 ± 14.6	2.45 (8.9)	-4.2 (-10.7 to 2.3)	0.194	-0.50 ± 20.6	-0.17 ± 11.0	-0.3 (-8.3 to 7.6)	0.932
Bone and skeletal pain	7.24 ± 19.4	1.92 ± 6.6	5.3 (-0.3 to 10.9)	0.061	1.85 ± 20.2	2.64 (8.7)	-0.8 (-7.7 to 6.1)	0.817	3.42 ± 18.7	-1.67 ± 6.8	5.1 (-0.5 to 10.7)	0.076
Difficulties performing daily activities	34.38 ± 28.5	29.67 ± 29.7	4.7 (-14.8 to 24.2)	0.611	-3.80 ± 14.9	-2.17 (23.5)	-1.6 (-16.8 to 13.5)	0.819	-9.45 ± 20.0	-11.00 ± 24.8	1.6 (-14.6 to 17.7)	0.839

Hypophosphatemia defined as serum phosphate < 0.8 mmol/L (< 2.5 mg/dL).

[1]Differences are normal phosphate group minus hypophosphatemia group. CI: Confidence interval; VAS: Visual analogue scale.

An important observation is that 34% of the study population was vitamin D deficient at baseline with 25-hydroxyvitamin D values < 50 nmol/L and, perhaps more interestingly, 24% of the patients had PTH values compatible with secondary hyperparathyroidism. These findings were equally distributed between the two treatment groups. This disturbance in vitamin D metabolism is unlikely to be a consequence of previous iron infusions since no patients received high-dose intravenous iron treatment during the 6 mo prior to inclusion in this study. The high prevalence of vitamin D deficiency at baseline is in agreement with previous studies of patients with IBD[29]. However, it is important to note that, in our study, many of the samples were taken during the winter months when sun exposure is reduced in Norway, and individuals could therefore be expected to be somewhat vitamin D deficient during this time. Nevertheless, this finding is important since both hypophosphatemia and vitamin D deficiency can contribute to the development of metabolic bone disease, including osteomalacia.

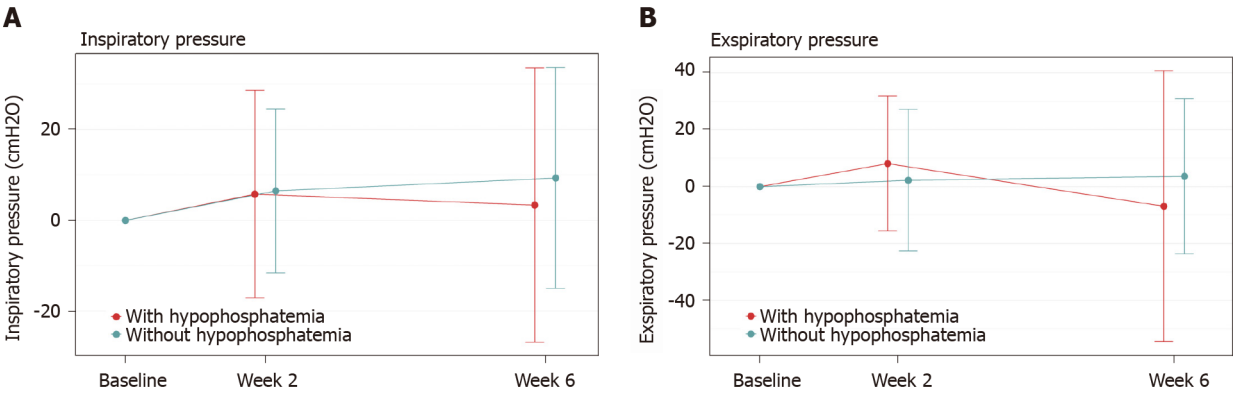


Figure 2 mean ± SD changes from baseline in respiratory pressure in inflammatory bowel disease patients with iron deficiency/iron deficiency anaemia treated with a single 1000 mg intravenous dose of ferric carboxymaltose or ferric derisomaltose. A: Inspiratory pressure; B: Expiratory pressure.

Guidelines regarding hypophosphatemia diagnosis, treatment, and follow-up are available, but the possible risk or incidence rate of developing hypophosphatemia with symptoms or complications are rarely mentioned[30]. A risk of developing respiratory failure, rhabdomyolysis, and left ventricular failure due to severe hypophosphatemia has been reported in case series[10]. More recent data also predict an increased risk of developing osteomalacia, especially in long-standing hypophosphatemia[14,15]. What is less well known is the number of patients developing more subtle, but identifiable, symptoms related to hypophosphatemia that are experienced as troublesome and might influence QoL.

With respect to the clinical impact of hypophosphatemia, measuring forced inspiratory and expiratory respiratory pressure can be used as a proxy to assess the physical effect of hypophosphatemia on skeletal and proximal muscles. There are no specific questionnaires available to evaluate the clinical impact of hypophosphatemia. The SF-36 is, however, one of the most commonly applied QoL questionnaires used world-wide in health surveys. Additionally, the VAS score can be used as a general assessment of impact of symptoms, such as fatigue, general weakness, bone and skeletal pain, and joint and muscle conditions. In our study, these three methods were applied to assess clinical impact in patients who developed hypophosphatemia compared to those who did not develop hypophosphatemia. All three methods failed to demonstrate significant differences in clinical impact following one administration of high-dose intravenous iron in this short-term study.

We hypothesize several reasons that might explain these results. In addition to the fact that a type II error cannot be excluded, it can be speculated that the positive effect of the correction of ID or IDA plays a more important role than any short-term negative clinical impact of hypophosphatemia and, hence, the effects of hypophosphatemia would be difficult to discern in our study. Another challenge is that IBD, ID, IDA and hypophosphatemia are associated with similar symptoms and, possibly, similar impacts on daily life. Indeed, assessing the specific impact of hypophosphatemia with questionnaires would, therefore, prove difficult. Since the SF-36 and the VAS questionnaires are not disease- or population-specific, there may be uncertainty surrounding the reliability of the results. Additionally, the patient cohort had more than one dynamic medical condition, with overlapping symptoms, and patients were observed in a longitudinal manner. Certainly, it would be almost impossible to determine which disease state or co-morbidity is reflecting improvement or worsening of clinical status.

The already affected baseline recordings in SF-36 and the VAS score should not go unnoticed. These findings mirror previous studies of IBD populations[31], and reflect the reduced QoL and the intensity of symptoms that these patients experience in general. Finally, the fact that we did not detect clinical consequences in patients who developed hypophosphatemia suggests that, in order to detect overt symptoms and complications, the population size needs to be larger than our sample, as one might expect such complications to be relatively rare. Hence, this needs to be taken into account when considering the expectation of finding significant changes in the clinical outcomes in this study.

CONCLUSION

In summary, our study has implicated the small peptide hormone FGF23 in the development of hypophosphatemia in IBD patients treated with FCM. An increase in intact FGF23 occurs, which probably results in phosphate wasting in the urine. Assessment of symptoms did not exclude, nor did they demonstrate, any short-term clinical impact of hypophosphatemia in IBD patients treated for ID or IDA with high-dose intravenous iron.

ARTICLE HIGHLIGHTS

Research background

High-dose intravenous iron is an effective and frequently used treatment option for iron deficiency (ID) or ID anaemia (IDA) in inflammatory bowel disease (IBD). However, treatment with ferric carboxymaltose (FCM) has been associated with the development of hypophosphatemia.

Research motivation

We aimed to investigate the occurrence of hypophosphatemia after treatment with either FCM and ferric derisomaltose (FDI) for ID or IDA in patients with IBD.

Research objectives

In this part of the study, we aimed to disclose underlying mechanism behind the development of hypophosphatemia after treatment with high dose intravenous iron and whether hypophosphatemia had a clinical impact on these patients.

Research methods

A prospective observational study of adult IBD patients with ID or IDA was conducted between February 1, 2017 and July 1, 2018 at two separate university hospitals in the southeast region of Norway. Patients were recruited consecutively and received one dose of 1000 mg of either FCM or FDI, and were followed for an observation period of at least 7 wk at three timepoints; baseline, week 2 and week 6. Blood and urine samples were collected for relevant analyses at all three visits in addition to assessment of clinical symptoms using a respiratory function test, a visual analogue scale, and a health-related quality of life questionnaire.

Research results

Our study results demonstrate an association between FCM treatment and the development of hypophosphatemia by increasing the level of intact Fibroblast Growth Factor 23 (iFGF23) and phosphate wasting in the urine. Moreover, we observed a significant decline in active Vitamin D and ionised calcium. No clinical impact was detected by applying Short Form-36 questionnaire, visual analogue scale score and real-time position management breathing test in an observation period of 6 wk.

Research conclusions

FCM treatment is associated with the development of hypophosphatemia in patients with IBD. This is due to increased formation of iFGF23 which in turn probably results in an increase of urinary phosphate output. No clinical impact was detected nor excluded. Assumably our study is underpowered together with a too short observation period to provide solid information with regard to clinical impact of hypophosphatemia.

Research perspectives

Based on our results we encourage clinicians to be aware of the risk of developing hypophosphatemia after treatment with FCM. Larger studies with a longer observation period to detect possible clinical impact of hypophosphatemia is desirable.

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REFERENCES

1 **Kulnigg S**, Gasche C. Systematic review: managing anaemia in Crohn's disease. *Aliment Pharmacol Ther* 2006; **24**: 1507-1523 [PMID: 17206940 DOI: 10.1111/j.1365-2036.2006.03146.x]

2 **Dignass AU**, Gasche C, Bettenworth D, Birgegård G, Danese S, Gisbert JP, Gomollon F, Iqbal T, Katsanos K, Koutroubakis I, Magro F, Savoye G, Stein J, Vavricka S; European Crohn's and Colitis Organisation [ECCO]. European consensus on the diagnosis and management of iron deficiency and anaemia in inflammatory bowel diseases. *J Crohns Colitis* 2015; **9**: 211-222 [PMID: 25518052 DOI: 10.1093/ecco-jcc/jju009]

3 **Eriksson C**, Henriksson I, Brus O, Zhulina Y, Nyhlin N, Tysk C, Montgomery S, Halfvarson J. Incidence, prevalence and clinical outcome of anaemia in inflammatory bowel disease: a population-based cohort study. *Aliment Pharmacol Ther* 2018; **48**: 638-645 [PMID: 30069892 DOI: 10.1111/apt.14920]

4 **Rampton DS**, Goodhand JR, Joshi NM, Karim AB, Koodun Y, Barakat FM, Macken L, Ward DG, Iqbal TH, Epstein J, Fell JM, Sanderson IR. Oral Iron Treatment Response and Predictors in Anaemic Adolescents and Adults with IBD: A Prospective Controlled Open-Label Trial. *J Crohns Colitis* 2017; **11**: 706-715 [PMID: 27932449 DOI: 10.1093/ecco-jcc/jjw208]

5 **Lobo V**, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev* 2010; **4**: 118-126 [PMID: 22228951 DOI: 10.4103/0973-7847.70902]

6 **Mahalhal A**, Williams JM, Johnson S, Ellaby N, Duckworth CA, Burkitt MD, Liu X, Hold GL, Campbell BJ, Pritchard DM, Probert CS. Oral iron exacerbates colitis and influences the intestinal microbiome. *PLoS One* 2018; **13**: e0202460 [PMID: 30308045 DOI: 10.1371/journal.pone.0202460]

7 **Carrier J**, Aghdassi E, Platt I, Cullen J, Allard JP. Effect of oral iron supplementation on oxidative stress and colonic inflammation in rats with induced colitis. *Aliment Pharmacol Ther* 2001; **15**: 1989-1999 [PMID: 11736731 DOI: 10.1046/j.1365-2036.2001.01113.x]

8 **Detlie TE**, Lindstrøm JC, Jahnsen ME, Finnes E, Zoller H, Moum B, Jahnsen J. Incidence of hypophosphatemia in patients with inflammatory bowel disease treated with ferric carboxymaltose or iron isomaltoside. *Aliment Pharmacol Ther* 2019; **50**: 397-406 [PMID: 31264261 DOI: 10.1111/apt.15386]

9 **Wolf M**, Rubin J, Achebe M, Econs MJ, Peacock M, Imel EA, Thomsen LL, Carpenter TO, Weber T, Brandenburg V, Zoller H. Effects of Iron Isomaltoside *vs* Ferric Carboxymaltose on Hypophosphatemia in Iron-Deficiency Anemia: Two Randomized Clinical Trials. *JAMA* 2020; **323**: 432-443 [PMID: 32016310 DOI: 10.1001/jama.2019.22450]

10 **Subramanian R**, Khardori R. Severe hypophosphatemia. Pathophysiologic implications, clinical presentations, and treatment. *Medicine (Baltimore)* 2000; **79**: 1-8 [PMID: 10670405 DOI: 10.1097/00005792-200001000-00001]

11 **Amanzadeh J**, Reilly RF Jr. Hypophosphatemia: an evidence-based approach to its clinical consequences and management. *Nat Clin Pract Nephrol* 2006; **2**: 136-148 [PMID: 16932412 DOI: 10.1038/ncpneph0124]

12 **Padelli M**, Leven C, Sakka M, Plée-Gautier E, Carré JL. [Causes, consequences and treatment of hypophosphatemia: A systematic review]. *Presse Med* 2017; **46**: 987-999 [PMID: 29089216 DOI: 10.1016/j.lpm.2017.09.002]

13 **Geerse DA**, Bindels AJ, Kuiper MA, Roos AN, Spronk PE, Schultz MJ. Treatment of hypophosphatemia in the intensive care unit: a review. *Crit Care* 2010; **14**: R147 [PMID: 20682049 DOI: 10.1186/cc9215]

14 **Schaefer B**, Glodny B, Zoller H. Blood and Bone Loser. *Gastroenterology* 2017; **152**: e5-e6 [PMID: 28384445 DOI: 10.1053/j.gastro.2016.09.050]

15 **Klein K**, Asaad S, Econs M, Rubin JE. Severe FGF23-based hypophosphataemic osteomalacia due to ferric carboxymaltose administration. *BMJ Case Rep* 2018; **2018** [PMID: 29298794 DOI: 10.1136/bcr-2017-222851]

16 **Urbina T**, Belkhir R, Rossi G, Carbonnel F, Pavy S, Collins M, Mariette X, Seror R. Iron Supplementation-Induced Phosphaturic Osteomalacia: FGF23 is the Culprit. *J Bone Miner Res* 2018; **33**: 540-542 [PMID: 29281120 DOI: 10.1002/jbmr.3369]

17 **Reyes M**, Diamond T. Hypophosphataemic Rickets Due to Parenteral Ferrous Carboxymaltose in a Young Man with Crohn Disease and Iron Deficiency: A Case Report and Review of Literature. *J Clin Case Rep* 2017; **7**: 931 [DOI: 10.4172/2165-7920.1000931]

18 **Moradkhani A**, Beckman LJ, Tabibian JH. Health-related quality of life in inflammatory bowel disease: psychosocial, clinical, socioeconomic, and demographic predictors. *J Crohns Colitis* 2013; **7**: 467-473 [PMID: 22884758 DOI: 10.1016/j.crohns.2012.07.012]

19 **Vavricka SR**, Schoepfer A, Scharl M, Lakatos PL, Navarini A, Rogler G. Extraintestinal Manifestations of Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2015; **21**: 1982-1992 [PMID: 26154136 DOI: 10.1097/MIB.0000000000000392]

20 **Ware JE Jr**, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. *Med Care* 1992; **30**: 473-483 [PMID: 1593914]

21 **Ware JE**, Snow KK, Kosinski M, Gandek B. Health survey manual and interpretation guide. Boston: The Health Institute, New England Medical Center, 1993

22 **Wolf M**, Chertow GM, Macdougall IC, Kaper R, Krop J, Strauss W. Randomized trial of intravenous iron-induced hypophosphatemia. *JCI Insight* 2018; **3** [PMID: 30518682 DOI: 10.1172/jci.insight.124486]

23 **Fukumoto S**. Phosphate metabolism and vitamin D. *Bonekey Rep* 2014; **3**: 497 [PMID: 24605214 DOI: 10.1038/bonekey.2013.231]

24 **Hasegawa H**, Nagano N, Urakawa I, Yamazaki Y, Iijima K, Fujita T, Yamashita T, Fukumoto S, Shimada T. Direct evidence for a causative role of FGF23 in the abnormal renal phosphate handling and vitamin D metabolism in rats with early-stage chronic kidney disease. *Kidney Int* 2010; **78**: 975-980 [PMID: 20844473 DOI: 10.1038/ki.2010.313]

25 **Shimada T**, Hasegawa H, Yamazaki Y, Muto T, Hino R, Takeuchi Y, Fujita T, Nakahara K, Fukumoto S, Yamashita T. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J Bone Miner Res* 2004; **19**: 429-435 [PMID: 15040831 DOI: 10.1359/JBMR.0301264]

26 **Prié D**, Friedlander G. Reciprocal control of 1,25-dihydroxyvitamin D and FGF23 formation involving the FGF23/Klotho system. *Clin J Am Soc Nephrol* 2010; **5**: 1717-1722 [PMID: 20798257 DOI: 10.2215/CJN.02680310]

27 **Quarles LD**. Endocrine functions of bone in mineral metabolism regulation. *J Clin Invest* 2008; **118**: 3820-3828 [PMID: 19033649 DOI: 10.1172/JCI36479]

28 **Potts JT**. Parathyroid hormone: past and present. *J Endocrinol* 2005; **187**: 311-325 [PMID: 16423810 DOI: 10.1677/joe.1.06057]

29 **Fletcher J**, Cooper SC, Ghosh S, Hewison M. The Role of Vitamin D in Inflammatory Bowel Disease: Mechanism to Management. *Nutrients* 2019; **11** [PMID: 31067701 DOI: 10.3390/nu11051019]

30 **Nottingham University Hospitals**. Guideline for the Treatment of Hypophosphatemia in Adults. [cited 15 September 2020]. In: Nottingham University Hospitals [Internet]. Available from: https://studyres.com/doc/14104630/hypophosphataemia-in-adults---nottingham-university-hospi

31 **Jelsness-Jørgensen LP**, Bernklev T, Henriksen M, Torp R, Moum BA. Chronic fatigue is associated with impaired health-related quality of life in inflammatory bowel disease. *Aliment Pharmacol Ther* 2011; **33**: 106-114 [PMID: 21083587 DOI: 10.1111/j.1365-2036.2010.04498.x]

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EVIDENCE REVIEW

Role of microbial dysbiosis in the pathogenesis of esophageal mucosal disease: A paradigm shift from acid to bacteria?

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Abstract

Genomic sequencing, bioinformatics, and initial speciation (*e.g.*, relative abundance) of the commensal microbiome have revolutionized the way we think about the "human" body in health and disease. The interactions between the gut bacteria and the immune system of the host play a key role in the pathogenesis of gastrointestinal diseases, including those impacting the esophagus. Although relatively stable, there are a number of factors that may disrupt the delicate balance between the luminal esophageal microbiome (EM) and the host. These changes are thought to be a product of age, diet, antibiotic and other medication use, oral hygiene, smoking, and/or expression of antibiotic products (bacteriocins) by other flora. These effects may lead to persistent dysbiosis which in turn increases the risk of local inflammation, systemic inflammation, and ultimately disease progression. Research has suggested that the etiology of gastroesophageal reflux disease-related esophagitis includes a cytokine-mediated inflammatory component and is, therefore, not merely the result of esophageal mucosal exposure to corrosives (*i.e.*, acid). Emerging evidence also suggests that the EM plays a major role in the pathogenesis of disease by inciting an immunogenic response which ultimately propagates the inflammatory cascade. Here, we discuss the potential role for manipulating the EM as a therapeutic option for treating the root cause of various esophageal disease rather than just providing symptomatic relief (*i.e.*, acid suppression).

Key Words: Microbiome; Gastroesophageal reflux disease; Probiotics; Prebiotics; Bacteriocins; Dysbiosis; Barrett's esophagus; Esophageal cancer; Esophagitis; Eosinophilic esophagitis

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Core Tip: The interactions between the gut bacteria and the immune system of the host play a key role in the pathogenesis of gastrointestinal diseases, including those impacting the esophagus. This evidence-based review brings forward the emerging data on the microbial changes related to esophageal disease. Better understanding of these data will lead to mitigation strategies for intervention and innovation.

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INTRODUCTION

Investigation of the gut microbiome has progressively changed the understanding of esophageal disease. During the past two decades, 16S rRNA gene sequencing was used to characterize and compare the esophageal microbiomes (EMs) of healthy individuals with those in patients with esophageal diseases including gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), esophageal adenocarcinoma (EAC), eosinophilic esophagitis (EoE), and esophageal motility disorders[1,2]. Analyzing the compositional differences between healthy and dysbiotic microbiomes in the esophagus has provided further insight into the pathogenesis of GERD and related sequelae along with other associated pathology (Table 1). In particular, it is noted that the diseased esophagus, relative to healthy controls, is colonized by a bacterial population that is unusually rich in gram-negative species. Furthermore, the aberrant bacteria conform, to a great extent, with pro-inflammatory oral pathogens. This insight into the EM can help guide further investigation into new therapeutic tools that target these mechanisms.

NORMAL GASTROESOPHAGEAL MICROFLORA

Characterizing the 'normal' vs 'abnormal' esophageal luminal flora

Metagenomic sequencing of the human population revealed that the gastroesophageal (GE) microbiome is predominated roughly in order of prevalence by six major phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Saccharibacteria[3]. Typically, Bacteroidetes and Firmicutes often predominate-primarily in response to abundance of either *Bacteroides* or *Clostridium spp.*[4]. Here, phylum-level classification is an oversimplification that does not account for the diversity that exists in a relatively simple microbiome like that found in the distal esophagus. Considerable variation of both the identity and relative abundance of specific bacteria exists, especially when the taxa are characterized with greater resolution by elucidation of taxa to species or strain-level. In 2009, one group identified two distinct types of GE microbiomes[5]. Comparing the results from individuals with GERD to healthy controls[5], they characterized the control population, which is predominated by gram-positive organisms, *Streptococcus spp.*, as a type I microbiome. The type II microbiome was largely associated with pathological states, such as GERD and BE, and demonstrated a relative increase in abundance of gram-negative anaerobes[5].

Further work delineated the taxa and observed that three distinct clusters of esophageal microbiotas were predominant in biopsies of human esophageal tissue. Each is characterized by their relative abundance of *Streptococcus* and *Prevotella spp.*[6]. Cluster 1 is intermediate, with an approximately equal proportion of both genera with an increased presence of *Haemophilus* and *Rothia spp*. Cluster 2 consists predominantly of *Streptococcus spp*. Cluster 3 is primarily represented by *Prevotella spp.*[6].

In addition to inter-individual variation, the composition of luminal microbiota also varies in the esophagus from the mouth to the stomach both in health and disease. Specifically, the commensal flora of the proximal, mid-, and distal esophagus varies both in makeup, and relative abundance. In a study of 12 patients under routine

Table 1 Changes in local flora that occur with particular esophageal disease states

Disease states	Changes in microbiome
GERD	Non-erosive reflux disease: A shift towards Proteobacteria (*Neisseria oralis*, *Moraxella spp.*) and Bacteroidetes (*Bacteroides uniformis*, *Capnocytophaga spp.*, and *Prevotella pallens*); A shift away from Fusobacteria (*Leptotrichia*) and Actinobacteria (*Rothia spp.*); Increased abundance of *Dorea spp.* Reflux esophagitis: Decreased Firmicutes (*Mogibacterium spp.*, *Streptococcus infantis*, *Solobacterium moorei*) and increased Fusobacteria (*Leptotrichia spp.*) and Proteobacteria (*Marivita spp.*, *Nisaea spp.*, *Mesorhizobium spp.*)
Barrett's esophagus	Increased Fusobacteria, and Proteobacteria (*Neisseria spp.*, and *Campylobacter spp.*); Decreased alpha diversity as well as *Bacteroidetes* and *Prevotella*
Esophageal adenocarcinoma	Increased abundance of Proteobacteria and decreased Firmicutes; Relatively unchanged *Streptococci* abundance
Eosinophilic esophagitis	Increased Proteobacteria (*Neisseria* and *Haemophilus*) and *Corynebacterium*; Decrease in *Clostridia spp.*

GERD: Gastroesophageal reflux disease.

surveillance for BE, the proximal esophagus was more similar to the oropharynx in that it had higher concentrations of gram-positive organisms than the distal esophagus[7]. *Streptococcus spp.* were found throughout the entirety of the esophagus, increasing in relative abundance from the proximal to mid-esophagus and markedly decreasing thereafter in the distal esophagus[7]. Gram-negative organisms included *Prevotella* and *Delftia spp.*, which overall were more concentrated in the distal esophagus[7]. This is not surprising, because the lipopolysaccharide (LPS) "shell" around gram-negative organisms hardens them to variation in pH, bile salt concentration, proteases, and to some extent, temperature[8-15]. This is why most enteric pathogens are gram negative—they can survive the selection and potentially adverse effects related to proximity to gastric contents. It would be thereby expected that the microbiome becomes much less diverse and increasingly enriched in gram-negative species. Diversity is greatest in the region nearest to the source (oronasal cavity) and with mildest conditions, *e.g.*, saliva/mucous, luminal physiological pH, and moderate physiological temperatures[16,17]. It would be expected to see a gradient toward facultative and obligate anaerobes (probably from the subgingival space) in the esophageal lumen distally towards the stomach. Notably, many bacteria also have increased abundance because of the protective nature of sporulation (*e.g.*, *Clostridia spp.*) within the harsh surrounding environment[18,19]. Accordingly, the underlying GE pathology appears to be associated with alterations in the composition of this gram positive/gram negative continuum and balance[7].

The biomic differences for esophageal disease is notable. Patients with BE had overall higher levels of *Streptococcus spp.* in tissue biopsies throughout the entirety of the tract compared to those without the disease. While this appears to be in contrast with the gram-positive/gram-negative imbalance discussed previously, this may be an indirect effect of persistent local irritation caused by immunogenic gram-negative species that facilitates bacterial proliferation and the infiltration of underlying tissue with gram-positive bacteria—of which streptococci are a major part. Whether this is a cause of BE metaplasia or a consequence is unknown. Furthermore, the sharper decrease in overall abundance of *Streptococcus spp.* from mid- to distal-esophagus is greater in individuals with BE compared to those without metaplasia, which suggests that the overall effect on relative composition of *Streptococcus spp.* is negative despite an increased tissue prevalence[7].

These findings suggest that an increase in relative abundance of gram-negative flora in the distal esophagus leads to a local inflammatory response which negatively impacts the barrier function. This ultimately leads to tissue proliferation of all flora, including commensal *Streptococcus spp.* The proximal-distal variation in specific flora changes from healthy to diseased states, in conjunction with the previous GE microbiome subtyping, illustrate the pathological potential of dysbiosis. The emerging data may offer insight into new treatment paradigms focused on microbial alteration which could supplement, and even possibly replace, the need for acid suppression in these disease states.

Factors affecting the composition of the microbiota

The GE microbiome is shaped by the oral cavity, oropharynx, and stomach due to migration of oral bacteria to the esophagus and reflux of gastric microbiota[20].

Recognizably, this varies considerably from person to person, even in the apparently healthy population. In addition to anatomic location, factors that have been noted to alter the EM composition include age, diet, proton pump inhibitor (PPI) use, oral hygiene, and smoking. Studies of these factors have helped provide a framework for understanding the GE microbiota.

Contrary to the philosophy that, for example, the colon is a discrete microbiome that stands alone, we view the whole of the gastrointestinal tract as a contiguous system separated by "gates" imposed by selection pressure driven by factors related to function (pH, osmolarity, proteases, indigenous flora, *etc.*). That is, a series of discrete "neighborhoods" connected by means of a tube and the assumption that the oronasal cavity if the sole source of inoculum. Prior to weaning, infants do not have established "gates," and an evolving colonization with what will ultimately become the adult microbiome takes place until the age of approximately three[21]. Thereafter, barring unnatural perturbation, the gastrointestinal neighborhoods are established, stable, and at equilibrium with the host. Unfortunately, humans are pioneers of the unnatural, and a number of behaviors, many now considered essential, systematically undermine the balance. Recognizably, there may be sequential changes associated with age, medication exposures, diet, hygiene, sleep efficiency, and environmental exposures.

Age: Age has an effect on the GE microbiome, although the full significance has not yet been determined[22-25]. During early life, the human colonic microbiome varies greatly. Analysis of the microbial composition of the human colonic microbiome in patients ranging from newborns to 80 years old, and across three distinct geographic locations (United States, Venezuela, and Malawi) has found that the phylogenetic composition fluctuates dramatically during the first 3 years of life before stabilizing into a more stable adult-like composition, regardless of geographic location[26]. Conceivably, a similar dynamic microbial shift exists for the esophagus given the same multifactorial environmental factors in early life, based on mode of delivery (vaginal birth or cesarean section),the type of dietary feeding (breast or formula feeding), as well as the timing of adult food introduction[27,28].

With aging, humans seem to have a less dramatic, but still notable shift in the GE microbiome. Evaluation of the EM of adults of ages 30 years to 60 years, using 16S rRNA-, 18S rRNA-amplicon sequencing, and shotgun sequencing, has found age to be a significant factor driving microbiome composition. Notably, they indicated a positive correlation with age and the relative abundance of Firmicutes such as some *Streptococcus spp.*, including *Streptococcus parasanguinis* with increasing age[6]. Furthermore, increasing age was inversely correlated with prevalence of Bacteroidetes including *Prevotella melaninogenica*[6]. To better place this in the context of our current understanding of the GE microbiome and the previously demonstrated community clusters (*Streptococcus* predominant, *Prevotella* predominant, and intermediate predominant), this study showed that regardless of disease state, with increased age, there is a more robust microbiome composition and a higher number of gram-positive (*Streptococcus parasanguinis*) species and a lower number of gram-negative (*Prevotella melaninogenica*) species[6]. Thus, age may contribute to the different esophageal microbial community types. Despite this, gram-negative proliferation is associated with progression of esophageal disease at all ages[6]. It may be that age may affect and predict a 'baseline' microbiome that is incrementally altered by microbial imbalance.

Notably, there is a degenerative effect of aging on esophageal motor function which may play a role in the differences seen in the GE microbiome of the elderly population as esophageal function naturally deteriorates after the age of 40[29]. The presence of GERD has a significant impact on esophageal contraction wave amplitude but not on peristalsis[30]. Accordingly and hypothetically, the mechanistic and functional changes of the esophagus influence the microbiota as a direct or indirect consequence of the aging process.

Diet: Dietary factors influence the colonic microbiome both as an infant (breast *vs* formula feeding) and as an adult (affecting the colonic microbiome with short-term macronutrient changes)[31-33]. With specific focus on the GE microbiome, dietary intake has been associated with the development of esophageal diseases such as BE, EAC, and esophageal squamous cell carcinoma (ESCC)[34,35]. In particular, consumption of leafy and cruciferous vegetables, as well as raw fruits is associated with decreased risk of BE and EAC, while red meat intake is associated with increased risk[35].

In early life, breastfeeding, formula feeding and the introduction of solid foods, play a large role in development of the gastrointestinal microbiome[36]. While more specific investigation is needed to evaluate the specific effects on the EM, it is likely

that similarities exist in the progression due to the same factors. Human breast milk is predominantly composed of the microbes *Corynebacterium, Ralstonia, Staphylococcus, Streptococcus, Serratia, Pseudomonas, Propionibacterium, Sphingomonas, and Bradyrhizobiaceae* in addition to milk oligosaccharides[37-39].

The impact of breastfeeding on the infant gastrointestinal microbiome was highlighted in two studies that found formula-fed infants to have a lower proportion of *Bifidobacterium* and *Lactobacillus spp.* and a higher proportion of Clostridiales and Proteobacteria when compared with breast-fed infants[40]. Furthermore, formula fed infants have lower microbial diversity after the first year of life when compared to breast-fed infants[41]. Several other epidemiologic studies have suggested breast-feeding to have a protective role against asthma, autism, and type 1 diabetes, while also showing a lower association of inflammatory and autoimmune diseases[37,42]. As stated earlier, the phylogenetic composition fluctuates dramatically during the first 3 years of life before evolving into a more mature and stable adult-like configuration[26]. This shift is likely to allow infants to be better equipped to handle processing of a more robust diet.

In adults, the GE microbiome and the relationship to diet is still under investigation. Our focus here will be on diet and its relationship to esophageal disease as a foundation for possible future studies into the GE microbiome role. There are several difficulties, particularly with confounding and study-design issues, when correlating dietary factors in adults with esophageal disease[34]. Thus, most of the existing literature on diet and BE or EAC is based on case-control studies in which minor to moderate inverse associations have been reported with a diet low in fruits and vegetables (green, leafy, and cruciferous vegetables). It has been theorized that fruits and vegetables, which are high in antioxidants, phytosterols, and other substances, may inhibit carcinogenesis by free-radical reduction or by blocking the formation of N-nitroso compounds in the alimentary and respiratory tract[43,44]. Other case-control studies have shown an association with a diet high in red and processed meats and an increased risk of esophageal cancers, likely due to processed meats being a major source of nitrites and nitrosamines[35,45]. Given the potential for multiple interactions between specific macronutrients, other studies have turned to looking at diet-regimens for easier study design. They found that the Mediterranean diet is inversely associated with both BE and EAC, whereas the "Western diet", high in meat consumption and low in fruits and vegetables, appears to increase the risk of these diseases[46,47]. Although this relationship between dietary intake of fruits, vegetables, as well as red and processed meats, has been more recently implicated, further evaluation is needed in regard to the interaction of these diets and the GE microbiome composition.

A study specifically looking at the relationship of diet with the GE microbiome evaluated patients with high overall fiber *vs* high fat intake and found that dietary fiber, but not fat intake was associated with a distinct EM[48]. In particular, increasing fiber intake was significantly associated with increasing relative abundance of Firmicutes, including *Streptococcus spp*., and decreasing relative abundance of gram-negative bacteria overall[48]. Low fiber intake was associated with increased relative abundance of several gram-negative flora, including *Prevotella, Neisseria,* and *Eikenella spp*.[48]. These findings offer a potential dietary therapeutic option for prevention or slowing the progression of esophageal disease by decreasing exposure to a higher abundance of gram-negative influence, and thereby reducing the induction of a gram-negative-LPS induced inflammatory cascade.

PPIs: PPIs are the therapeutic first-line treatment for many esophageal disorders such as GERD, erosive esophagitis, and BE. The main mechanistic action of PPIs is to lower acid production at the level of the stomach by inhibiting the hydrogen-potassium ATPase pump, a transmembrane protein responsible for releasing hydrochloric acid into the stomach lumen. PPIs inhibit acid secretion by binding within this domain, promoting a higher gastric pH, and thus increasing the pH of the refluxate[49].

The use of PPIs has been demonstrated to alter both GE and colonic microbiomes, although the full extent is yet unknown. The clearest defined role is reduction of gastric acid, thereby allowing survival of orally ingested organisms to populate the more distal esophagus. This pH-related microflora change may allow propagation of bacterial species that would otherwise not flourish under more acidic conditions. For example, a significant increase in oral microbiome species such as *Rothia dentocariosa, Rothia mucilaginosa, Scardovia spp*., and *Actinomyces spp.* in the gut microbiome has been noted following PPI use[50-52].

In the distal esophagus, the effect of PPIs may be more likely to be due to microbial related inflammatory changes, whereas previously attributed to direct acid contact mucosal injury. A study of patients with non-erosive reflux disease (NERD), erosive

GERD, and BE compared PPI use *vs* no use within each respective group and found no change in α diversity or β diversity between PPI and non-PPI users of each group was reported, but composition of specific bacteria taxa at the phylum level was noted[53]. In particular, PPI use was associated with an increase in Firmicutes and Proteobacteria in BE, and a decrease in Bacteroidetes in NERD and reflux esophagitis (RE)[53]. In another study, biopsies taken before and after 8 wk of PPI treatment (lansoprazole 30 mg twice daily) revealed a significant decrease in the gram-negative *Comamonadaceae spp.* and increased gram-positive Clostridia (*Clostridiaceae* and *Lachnospiraceae spp.*) and Actinomycetales (*Micrococcaceae* and *Actinomycetaceae spp.*)[54].

These studies offer evidence that PPI use may have effects beyond that of acid suppression. This supports a possible mechanistic role for PPIs altering the GE microbiome, favoring gram-positive bacteria that prefer environments with higher pH. This effect would reduce induction of the Toll-like-receptor (TLR)/inflammatory cascade by gram-negative LPS producing bacteria.

Although their association with GERD is unknown, acid-producing bacteria are found in the esophagus and oral cavity. The use of PPIs may directly target the proton pumps (P-type ATPase enzymes) of these bacteria (notably *Streptococcus pneumoniae* and *Helicobacter pylori*)[55]. Further studies are warranted to determine if these bacteria are a causal factor of GERD by directly producing acid, which are in turn inhibited by PPIs. In addition, PPI use may indirectly change the natural bacterial flora in non-gastric tissues that express H^+/K^+-ATPases by shutting down proton pumps[55,56].

PPIs may also reduce inflammation apart from direct acid suppression. In esophageal squamous epithelial cells, omeprazole has been shown to inhibit interleukin (IL)-8 expression by blocking the nuclear translocation of a nuclear factor-kappa beta (NF-kB) subunit and the binding of AP-1 subunits to the IL-8 promoter[56]. IL-8 is an inflammatory mediator that has been implicated in the GERD, BE, and EAC pathways. Increased expression of LPS from gram-negative bacteria, and subsequent activation of the TLR-4-NF-κB pathway are associated with expression of downstream mediators such as IL-8 and cyclooxygenase (COX)-2[57]. The levels of both are directly correlated with transition from metaplasia to dysplasia in BE[57]. Thus, if PPI therapy has an effect on IL-8 expression by blocking NF-kb and AP-1, there may be a role for therapeutic use outside of direct acid suppression.

Oral hygiene: Oral hygiene is thought to play a vital role in the GE microbiome. Bacteria found in the oral cavity can migrate distally *via* deglutition[20]. The role of oral microbiota in colonizing the esophagus, and becoming part of the commensal GE microbiota, remains uncertain.

Maintained oral hygiene is associated with a higher proportion of gram-positive cocci and rods, mostly comprised of *Streptococcus spp.*, which contrasts with those with poor oral hygiene showing shifts to a higher proportion of anaerobic gram-negative bacteria such as *Prevotella spp.*[58]. The oral microbiome shift to a more gram-negative dominant flora may have distal effects of LPS-inducing TLRs and activation of an inflammatory cascade in the esophagus as described previously. It is also unclear whether antibiotic mouthwashes damage an otherwise healthy microbiome. Further research is needed to better define the relationship between oral hygiene and the GE microbiome.

One recent population-based, case-control study reported that poor oral health was associated with an increased risk of ESCC[59]. More specifically, they found that tooth loss was associated with a moderately significant increased risk of esophageal cancer and that brushing once per day or less was associated with an 80% increased risk of developing ESCC in this population. They propose that tooth brushing influences the balance of microorganisms by directly removing plaque, food residue, and carcinogenic products of tobacco and alcohol. Accordingly, this affects the levels of inflammation and/or production of the carcinogenic by-products of nitrosamines and acetaldehyde. While this is currently theoretical, given the proximity of the oral cavity to the distal esophagus, it is reasonable that oral hygiene would have downstream effects on the GE microbiome and esophageal disease as well as perhaps on intestinal microbial mediated disease as well.

Smoking: Up to 20% of United States adults use a tobacco product, and tobacco's effect on the GE microbiome is uncertain[60]. Esophageal balloon procured cytology found that current smoking was associated with an increase in both α and β diversity of the esophagus[61]. It is suspected that the increase is due to smoking-related immunosuppression, permitting novel bacteria to colonize the upper gastrointestinal tract[61]. The study also found two anaerobic bacteria, *Dialister invisus* and *Megasphaera micronuciformis*, are more commonly detected in current smokers[61].

Increased α and β diversity after smoking exposure may also be a result of biofilm formation[62]. There is some evidence that cigarette smoking induces staphylococcal biofilm formation in an oxidant-dependent manner by increasing fibronectin binding protein-A. This leads to increased binding of staphylococci to fibronectin and increased adherence to human cells[62].

Smoking exposure can affect a wide range of human physiologic processes by inducing a proinflammatory state, increasing cytokines such as tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-8 all while decreasing anti-inflammatory cytokines such as IL-10[63]. Further investigation into the EM and its relationship to smoking and the development of disease are needed.

Bacteriocins

Bacteriocins are ribosomal derived peptides produced by microorganisms colonizing the gastrointestinal tract that are thought to inhibit competitive flora, thereby out-competing other pathogens. Additionally, in humans, bacteriocins are thought to maintain barrier function, partake in immune modulation, have direct antimicrobial activity, and also exhibit anti-neoplastic activity[64]. Bacteriocins' diverse functional role is reflected in their wide-ranging bactericidal properties, amino acid sequence and peptide structures, bacteriocin operon type, and different molecular weights and charges[65,66].

In a normal, healthy individual, the gut-blood barrier is responsible for maintaining homeostasis between the bloodstream and the gastrointestinal tract, which ultimately regulates water and nutrient absorption[67]. Along with a growing interest of the GE microbiome and its relationship with the intestinal barrier, recent *in vitro* studies have reported that the size and properties of bacteriocins allow them to cross this gut-blood barrier[64,68]. The size and charge contribute to bacteriocin movement across membranes and barriers and account for the large role in different physiologic mechanisms[64].

The bacteriocins in the gastrointestinal tract also have specific, potent antimicrobial properties[69,70]. This antibacterial property makes it essential in maintaining and affecting the composition of the local microbiome. The anti-neoplastic role of bacteriocins is still an area warranting further investigation. *In vitro* studies evaluating the effect of nisin have shown that bacteriocin may trigger apoptosis in head, neck, and squamous cell carcinoma, effectively reducing the size of tumor xenografts[71,72].

Defensins

Defensins are small host-derived polypeptide molecules of host-origin that play a role in innate immunity with both direct and indirect bactericidal effects. They serve a similar purpose to bacteriocins, but are eukaryote derived[73]. Human defensins are typically classified into α-defensins, which are mainly neutrophil-derived, and β-defensins, which are mainly epithelial cell-derived. Defensin expression is typically inducible through cellular exposure to bacterial products such as LPS or cytokines, which include TNF and IL-1[73]. Binding of defensins can lead to direct bacterial cell membrane disruption. The positive charge of some defensins is attracted to the negative components of bacterial capsules, leading to oligomerization and pore formation[74]. Alternately, defensin binding can stimulate recruitment of the adaptive immune response[73].

Defensins have been demonstrated to play a role in normal gut-microbe interactions. Loss of human β-defensin (hBD) 1 and hBD3 is associated with progression of EoE, and this may be a result of unmitigated microbe-immune interactions[75]. Uncontrolled defensin production may also negatively impact gut health. The production of hBD5 by metaplastic Paneth cells, can lead to a loss of E-cadherin, and thus inhibit cell adhesion[76]. This loss of structural integrity has been associated with progression of BE[76].

Similar to bacteriocins, modification of defensin expression or structure may provide a therapeutic venue for alteration of the commensal microbiome. Chimeric variants of defensins have been explored as possible antimicrobial agents, but this potential treatment modality is still in early stages of exploration, and further work is needed to evaluate their therapeutic safety and efficacy[77].

MECHANISMS OF DYSBIOSIS

Initial paradigms for dysbiosis

Dysbiosis is a term that encompasses a change in the composition of commensal microbiome relative to that found in healthy individuals[78]. When distinguishing the biome of a normal healthy esophagus to dysbiotic disease states such as GERD, EE, BE, or EAC, it is evident that dysbiosis may precede inflammation. Thus, the composition of the microbiota may play a large role in the downstream events[79]. Gram-negative bacterial products activate TLRs that are present on the esophageal endothelial cells causing an inflammatory cascade which leads to downstream LES relaxation[79]. *Prevotella*, which are found in greater abundance in the dysbiotic distal esophagus, have been shown to be a key producer of LPS that contributes to TLR activation[6]. The activation results in chemokine-induced production of nitric oxide and COX-2, which may promote LES relaxation and decrease gastric emptying[80].

DYSBIOSIS IN DISEASE STATES

GERD

GERD is an inflammatory disease state that is most commonly caused by inappropriate transient relaxation or secondly, by a chronically decreased tonicity of the lower esophageal sphincter[81]. It is further classified into two large phenotypes: RE and NERD, as determined by endoscopy. Additionally, there is a category of uninvestigated GERD wherein treatment is initiated without direction from an endoscopic evaluation[82].

Inflammatory pathogenesis of GERD: Classically, GERD has been described as the result of reflux of gastric acid and/or duodenal bile salts, causing direct chemical mediated mucosal injury and inflammation[83]. The majority of patients that present with clinical symptoms of GERD have no endoscopic evidence of reflux, suggesting alternative pathophysiologic pathways. An animal study demonstrated that RE does not develop as a chemical injury starting at the epithelial surface but rather, begins with a submucosal infiltration by lymphocytes that later progresses upward to the epithelial surface[84]. Subsequent work analyzed human esophageal squamous cell lines exposed to acidified bile salts and found evidence that reflux did not directly damage the mucosal esophagus, but instead stimulated epithelial cells and led to subepithelial cytokine-mediated and *retrograde* directed mucosal damage of the tissue[85].

Acid exposure is believed to contribute to mucosal expression of inflammatory markers, including IL-8, as well as several other chemokines, that promote local migration of leukocytes, mainly neutrophils[85]. This is done through activation of transient receptor potential cation channel subfamily V member 1 on epithelial cells and neurons, which results in calcitonin gene-related peptide (CGRP) and substance P expression[86]. Both CGRP and neutrophil activation initiate a cascade of cytokine expression, leading to local submucosal inflammation as well as hydrogen peroxide production as well as further immune cell, including lymphocytic proliferation into the mucosa (Figure 1). Peroxide-mediated smooth muscle relaxation of the lower esophageal sphincter further contributes to reflux[87].

Role of the microbiome in GERD: Gram-negative bacterial products, mainly LPS, bind to TLR-4, which stimulates IL-18 production, and initiates a cascade of IL and TNF production. This leads to downstream effects including lower esophageal sphincter relaxation as well as decreased gastric motility[1].

It has been theorized that the bacterial biofilm may be involved in the pathogenesis of GERD[88]. Biofilm is an organized community of microbes that produce protective factors and adhesion molecules which enhance the survival of the local microbial community. Biofilm has been implicated in the pathogenesis of a variety of disease states elsewhere in the gastrointestinal tract, notably in the oral cavity and colon, while the extent of contribution to esophageal disease is still unclear[89-91].

Compositional variation in the localized microbiome may help explain the mechanistic and clinical difference between the two phenotypes of GERD. A recent study found distinct microbiota in patients with NERD when compared with controls and RE subjects. The NERD microbiota composition shifted towards Proteobacteria (*Neisseria oralis* and *Moraxella spp.*) and Bacteroidetes (*Bacteroides uniformis, Capnocytophaga spp.*, and *Prevotella pallens*), and away from Fusobacteria (*Leptotrichia*) and

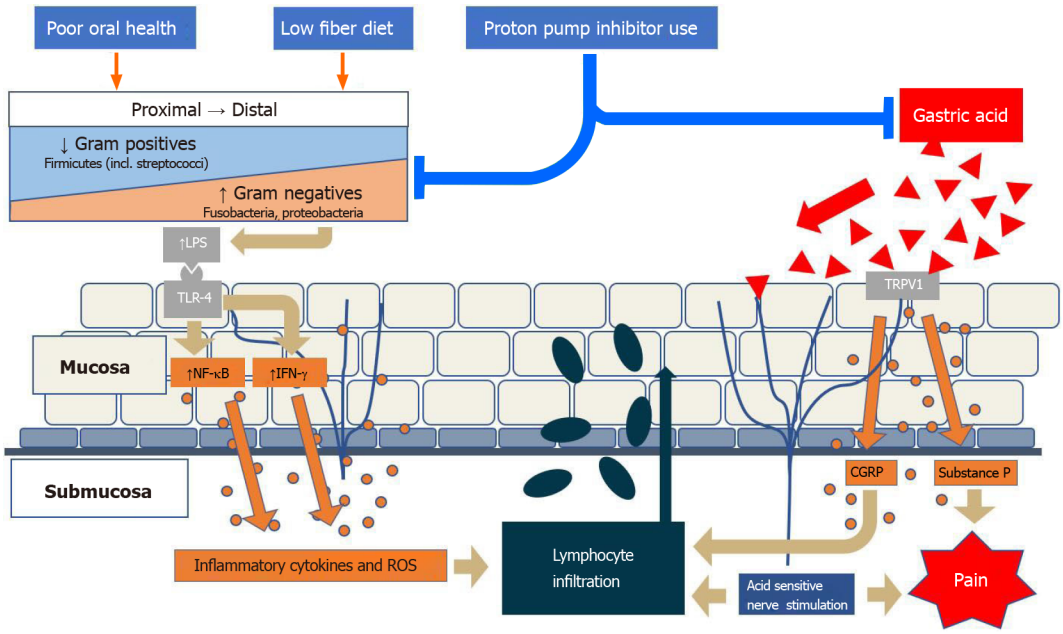


Figure 1 Environmental factors alter the local esophageal microbiome, which normally has a gram-positive to gram-negative gradient, towards increased proportion of gram-negatives. Activation of Toll-like-receptor-4 by gram-negative lipopolysaccharide leads towards an inflammatory cascade that results in lymphocyte infiltration. In addition, gastric acid activates transient receptor potential cation channel subfamily V member 1 on local nerve fibers and results in calcitonin gene-related peptide and substance P expression, which contributes to the local inflammatory response as well as pain. LPS: Lipopolysaccharide; TLR: Toll-like-receptor; NF-κB: nuclear factor-kappa beta; IFN: Interferon; ROS: Reactive oxygen species; TRPV1: Transient receptor potential cation channel subfamily V member 1; CGRP: Calcitonin gene-related peptide.

Actinobacteria (*Rothia*)[53]. Several Firmicutes genera were reduced in NERD (*Peptococcus* and *Moryella*). An increased abundance of *Dorea spp.*, however, resulted in an overall higher Firmicutes composition compared with controls[53]. The same study speculated that the increase in sulfate-reducing *Proteobacteria spp.* and *Bacteroidetes spp.* along with hydrogen producing *Dorea spp.* is associated with a mechanistic role in visceral hypersensitivity present in NERD[53]. Alternatively, patients with RE have a decrease in Firmicutes (*Mogibacterium spp.*, *Streptococcus infantis*, *Solobacterium moorei*) and increase in gram-negative Fusobacteria (*Leptotrichia spp.*) and Proteobacteria (*Marivita, Neisseria,* and *Mesorhizobium spp.*)[53].

The compositional changes in esophageal flora can also occur as a response to environmental factors. High-fat diet has been heavily associated with localized mucosal inflammatory changes in murine models[92]. It is theorized that alterations in the luminal microenvironment seen with high-fat diet leads to increased gut-epithelial interactions and drives progression of the inflammatory response. In addition, these dietary changes have been demonstrated to contribute to colonic increased colonic Firmicutes/Bacteroidetes ratio, which is associated with obesity and an inflammatory response characterized by expression of chemokine IL-8[92]. Other dietary factors, including consumption of insoluble carbohydrates, can contribute to reflux through effect on gastric and esophageal tone. Colonic bacterial metabolism of carbohydrates into short-chain fatty acids (SCFAs) is associated with increase in peptide YY and oxyntomodulin, which inhibit gastric motility and LES function[93].

BE

BE is a disease state characterized by stratified squamous to columnar metaplasia of the distal esophageal epithelium, and is typically associated with longstanding GERD[94,95]. The incidence of BE has increased since the mid-20th century, which is thought to be related to population-level changes in EM composition following the introduction of antibiotics[96]. The activation of the LPS-TLR4-NF-κB pathway through dysbiotic changes may contribute to inflammation and malignant transformation[3].

Inflammatory pathogenesis of BE: The inflammatory-mediated model of reflux-related inflammation seen in GERD is also present in BE[97]. There are, however, several specific dysbiotic changes that contribute to inflammatory mediated metaplasia as well. Murine models of BE demonstrated localized cytokine expression,

especially in the distal esophagus, which can lead to an inflammatory response in gastric stem cells, ultimately promoting columnar epithelial formation[98]. Additionally, the same pro-inflammatory response associated with LPS and TLR-4 binding may be more notable in the distal esophagus with increased gram negative presence, and specifically contributes to metaplastic transformation[80].

Pro-inflammatory cytokine IL-1b, has been shown to be highly expressed in BE. The precursor for IL-1b necessitates proteolytic cleavage by caspase-1[98]. Caspase-1 also allows apoptosis of cells which can elicit further inflammation[99]. The functional role of caspase-1 is activated by inflammasomes which contain pattern-recognition receptors (PRRs). The PRR captures particular pathogen-associated molecular patterns (PAMPs) from microbes and damage-associated molecular patterns (DAMPs) from injured cells[100]. Once the PRR recognizes a certain PAMP or DAMP, a large complex of inflammasomes activates caspase-1 which initiates the inflammatory response pathway. One study demonstrated that LPS in Barrett's epithelial cells was shown to prime and activate NOD-like receptor protein 3 inflammasomes and caused a cascade of pro-inflammatory responses and induced apoptosis[101]. These data suggest that BE is perpetuated through a chronic inflammatory response from localized cytokine release which is initiated at the molecular level of the microbiome and related mucosal cell responses.

Role of the microbiome in BE: Patients with BE have been found to have both similarities and distinct features in EM profile when compared to those with GERD[102]. Using the original clustering model, Type I to Type II microbiome transition likely contributes to local inflammation[103,104]. More recently, it has been demonstrated that specific flora plays a contributing role in its pathogenesis. Fusobacteria, *Neisseria spp.*, and *Campylobacter spp.* have been linked to BE when compared to controls[105]. Additionally, comparison of metaplastic tissue to adjacent normal areas demonstrates decreased α diversity and decreased prevalence of Bacteroidetes including *Prevotella spp.*[106]. Distinct compositional differences in specific phyla occur along the NERD, BE, and EAC pathway. While Proteobacteria are massively over-represented in NERD (Bacteroidetes to a lesser extent), microbiota in in BE and RE demonstrate increase in Fusobacteria and Proteobacteria. The transition to the EAC microbiome demonstrates an increase in Firmicutes, which are decreased in NERD and BE[53]. This increase may mirror the increase in Firmicutes/Bacteroidetes ratio that is seen in obesity and colorectal adenocarcinoma, as impaired fermentation by *Bacteroidetes spp.* of fiber to anti-inflammatory SCFAs may contribute to the local inflammatory cascade.

EAC

Inflammatory pathogenesis: The pathogenesis of EAC has been attributed to a combination of genetic predisposition as well as environmental factors[107]. The majority of cases are associated with environmental triggers such as smoking, obesity, and GERD/BE[108]. The incidence of EAC in BE has been clearly established, albeit more recent data suggests lower rates, more in the range of 0.1-0.2%[109].

Role of the microbiome in EAC: All stages in the GERD-BE-EAC pathway have commonalities in composition of local flora. The change from type I to type II EM is generally associated with the initiation of reflux-associated inflammatory processes that are present in GERD and BE, and persist in EAC. The subsequent activation of the LPS-TLR4 cascade leads to NF-kB activation, which further increases COX-2 production[3]. COX-2 elevation is associated with the progression of BE to high-grade dysplasia[80]. More recent data, however, has identified specific floral changes in relative abundance that are associated with the progression from BE high-grade dysplasia and EAC. Patients with dysplastic disease have increased abundance of Proteobacteria and decreased Firmicutes[110]. Notably, *Streptococcus spp.* abundance is reportedly unchanged between non-dysplastic BE and high-grade dysplasia/EAC, suggesting that variation in *Streptococcus spp.* may not play a role in carcinogenesis[110].

The composition of the EM in EAC is closely linked to that of the oral cavity, with aboral movement of flora being theorized as one mechanism for compositional changes[111]. Specifically, oral flora such as *Treponema denticola*, *Streptococcus mitis*, and *Streptococcus anginosus* are associated with esophageal carcinogenesis[112]. While the overall abundance of *Streptococci* appears unchanged in between non-dysplastic BE and EAC, it is possible that individual species may have varied abundance.

In addition to pathogenic linkage between the oral and esophageal flora, compositional changes that are associated with decreased adenocarcinoma risk are also shared. Notably, the abundance of *Bifidobacteria spp.*, *Bacteroides spp.*, *Fusobacteria spp.*, *Veillonella spp.*, *Lactobacillus spp.*, and *Staphylococcus spp.* is associated with adenocarcinoma risk[88,106]. *Leptotrichia spp.* have been demonstrated to be a specific genus linked to EAC. In addition, reduction in some species, including *Neisseria* and *Streptococcus pneumoniae* decreased risk of progression to cancer[113,114].

ESCC

Inflammatory pathogenesis: ESCC is more common than EAC in regions such as Asia and South America[115]. Classically, ESCC has been associated with environmental exposures, including tobacco, alcohol, and hot-drink consumption, as well as genetic predisposition[116]. Other risk factors, such as poor oral health, have been linked to esophageal squamous dysplasia, a precursor for ESCC[117]. Exposure to various environmental triggers may directly stimulate epithelial expression of inflammatory markers, or may indirectly lead to proinflammatory state through changes in the microbiome[118].

Role of the microbiome in ESCC: Evaluation of microbiomes of patient's with ESCC demonstrates specific changes when compared to healthy controls, notably increased in proportion of *Actinomyces spp.* and *Atopobium spp.*, and decrease in *Fusobacterium spp.*, and *Porphyromonas spp.*[118]. Generally, there is also a decrease in bacterial diversity, and increase in interpersonal compositional variation, suggestive that the dysbiotic state is not stable[118,119]. As with EAC, there is a close association with oral cavity disease/dysbiosis and ESCC. Decrease diversity of oral flora is associated with ESCC, as aboral movement of microbes likely disrupts the normal esophageal microbial composition and contributes to dysbiosis[119].

Specific environmental agents may directly contribute to dysbiosis through nutrient availability. Tobacco smoking is associated with many oral microbiome changes that have some overlap with dysbiosis in ESCC. Particularly, increases in flora within phylum Actinobacteria and genera *Atopobium* and *Prevotella*, and decreases in *Fusobacteria spp.* have been reported[120]. Alcohol consumption may negatively affect epithelial barrier function that normally modulates epithelium-microbe interaction[121]. It may additionally contribute to local dysbiosis through metabolism by local flora into toxic metabolites such as acetaldehyde. Alcohol consumption is associated with colonic microbial changes that are reversible with probiotics, suggesting plasticity of the microbiome[122]. A combination of dysbiosis and epithelial dysfunction may contribute to endotoxemia and systemic inflammatory response[123], which may play a role in carcinogenesis.

Microbiome changes may also play a prognostic role in ESCC. Increases in phyla Firmicutes, Bacteroidetes, and Spirochaetes, and decrease in Proteobacteria are associated with lymph node spread, with *Streptococcus spp.* and *Prevotella spp.* being specifically indicated[124].

EoE

Inflammatory pathogenesis: EoE is a chronic immune mediated disease of the esophagus characterized by marked eosinophilic infiltration in response to T helper type 2 (Th2) cells[125]. Factors such as genetics, environment, allergens, and microbiome have been identified as triggers of the chronic disease[126]. Several genes have been identified as contributors to EoE, which include thymic stromal lymphopoietin (TSLP), calpain 14 (CAPN14), EMSY, LRRC32, STAT6, and ANKRD27[126]. Among the identified genes, TSLP appears to be the major contributor as it is activated by epithelial cells and induces Th2 differentiation[127]. Different allergens also induce an inflammatory cascade which increases inflammatory markers such as IL-5 and IL-13, which introduce a potential inflammatory path of EoE[126]. In addition to the genetics and environmental exposure, the microbiome of the esophagus has recently emerged as a potentially key mediator in the pathogenesis of EoE.

Role of the microbiome: Analysis of the microbiome in EoE has reported an increase of proteobacteria, specifically *Neisseria spp.* and *Corynebacterium spp.*, in children with active EoE[128]. This study, in conjunction with the evidence of altered gut microbiome with infantile antibiotic use and Cesarean section delivery, further supports that dysbiosis in the human microbiome has a role in EoE[129,130]. Analysis by an esophageal string test to evaluate the microbiome of children and adults with active EoE found a greater abundance of *Haemophilus spp.* in active EoE[131]. Notably,

there was also a decrease in specific taxa of Clostridia that had been observed in patients with active EoE[132]. In antibiotic treated mice, the addition of Clostridia-containing microbiota prevents sensitization to a specific food allergen. Induction of IL-22 by RAR-related orphan receptor innate lymphoid cells and T cells in the intestinal lamina propria are proposed as the mechanism of blocking sensitization to a food allergen[133]. The recent evidence suggests that the EM may be involved in the pathogenesis of EoE, and may represent new treatment approaches.

IMPLICATIONS FOR THERAPY

As previously discussed with modification of other environmental exposures, additional therapeutic options such as prebiotics, probiotics, and antibiotics have been investigated to reduce dysbiosis by directly or indirectly improving the gram-positive to gram-negative ratio for esophageal diseases. Although there is increasing evidence of altering dysbiosis utilizing these therapies in other gastrointestinal diseases, further research is needed in specific esophageal diseases.

Prebiotics

Supplements which increase the concentration of beneficial flora have been investigated. *Lactobacillus spp.*, which are found in the esophagus, metabolize maltosyl-isomaltooligosaccharides (MIMO), thereby enhancing the populations of favorable gram-positive organisms. Daily ingestion of MIMO has been reported to improve or eliminate symptoms in chronic GERD patients[134]. An expanded comprehensive evaluation of this promising approach is needed. A tolerability study is underway (NCT04491734), and a comprehensive multicenter randomized placebo-controlled study will begin in early 2021. Sugarcane flour has also been evaluated as a prebiotic with some benefit seen on one study[135]. The proposed mechanism for benefit is slower fermentation and thus greater luminal availability compared to traditional fiber-containing products[135].

Probiotics

Probiotics add bacterial strains as dietary supplementation, attempting to improve the gut flora composition to a preferential state. Probiotics containing *Lactobacilli spp.* and *Bifidobacteria spp.* have been evaluated and demonstrated relief of GERD symptoms[136-140]. However, long term effects and histological benefit have not been studied, and more recent guidelines have suggested no evidence based recommendation for routine use[141]. Further investigation is needed to evaluate potential efficacy of probiotics in esophageal diseases.

Antibiotics

Antibiotics are a potential therapeutic option for direct modification of the EM, and are widely used in the treatment of gastrointestinal infectious diseases. Antibiotics with focused intraluminal bioavailability have been used in treating small-intestinal bacterial overgrowth, hepatic encephalopathy, and *Clostridium difficile* infections[142-144]. There is, however, no evidence of the effectiveness of antibiotic use for esophageal dysbiosis in GERD, BE, or EAC.

Bacteriocins

Bacteriocins facilitate competition between local and foreign microbes, direct microbial properties, have potential for crossing the gut-blood barrier, and have antineoplastic properties[64]. Bacteriocin-based drug therapy, whether in the form of targeted drug therapy *via* encapsulation or attachment of bacteriocins to macromolecules, metals, or polymer-based nanoparticles, remains an area of ongoing investigation[145].

CONCLUSION

The EM plays a major role in the pathogenesis of esophageal disease. Various factors can affect the composition of the commensal flora, which may lead to dysbiosis and ultimately result in esophageal diseases. The dysbiosis likely contributes to a pro-inflammatory, cytokine-mediated state that starts in the submucosa. The pathogenic consequence, affecting the esophageal mucosa, has previously been attributed to

caustic acid mucosal injury, but now appears to be multifactorial, with the EM playing a major role. Specific flora has been more recently identified that may play a pathogenic role in this process. Several potential methods for therapeutic alteration of microbiome exist, including prebiotics, probiotics, antibiotics, and bacteriocin based therapies. The prebiotic treatment data is particularly promising as directed EM modification for effective disease treatment.

REFERENCES

1 **D'Souza SM**, Cundra LB, Yoo BS, Parekh PJ, Johnson DA. Microbiome and Gastroesophageal Disease: Pathogenesis and Implications for Therapy. *Ann Clin Gastroenterol Hepatol* 2020; **4**: 20-33 [DOI: 10.29328/journal.acgh.1001018]

2 **Corning B**, Copland AP, Frye JW. The Esophageal Microbiome in Health and Disease. *Curr Gastroenterol Rep* 2018; **20**: 39 [PMID: 30069679 DOI: 10.1007/s11894-018-0642-9]

3 **Lv J**, Guo L, Liu JJ, Zhao HP, Zhang J, Wang JH. Alteration of the esophageal microbiota in Barrett's esophagus and esophageal adenocarcinoma. *World J Gastroenterol* 2019; **25**: 2149-2161 [PMID: 31143067 DOI: 10.3748/wjg.v25.i18.2149]

4 **Rinninella E**, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, Mele MC. What is the Healthy Gut Microbiota Composition? *Microorganisms* 2019; **7** [PMID: 30634578 DOI: 10.3390/microorganisms7010014]

5 **Yang L**, Lu X, Nossa CW, Francois F, Peek RM, Pei Z. Inflammation and intestinal metaplasia of the distal esophagus are associated with alterations in the microbiome. *Gastroenterology* 2009; **137**: 588-597 [PMID: 19394334 DOI: 10.1053/j.gastro.2009.04.046]

6 **Deshpande NP**, Riordan SM, Castaño-Rodríguez N, Wilkins MR, Kaakoush NO. Signatures within the esophageal microbiome are associated with host genetics, age, and disease. *Microbiome* 2018; **6**: 227 [PMID: 30558669 DOI: 10.1186/s40168-018-0611-4]

7 **Okereke I**, Hamilton C, Reep G, Krill T, Booth A, Ghouri Y, Jala V, Andersen C, Pyles R. Microflora composition in the gastrointestinal tract in patients with Barrett's esophagus. *J Thorac Dis* 2019; **11**: S1581-S1587 [PMID: 31489224 DOI: 10.21037/jtd.2019.06.15]

8 **Li Y**, Powell DA, Shaffer SA, Rasko DA, Pelletier MR, Leszyk JD, Scott AJ, Masoudi A, Goodlett DR, Wang X, Raetz CR, Ernst RK. LPS remodeling is an evolved survival strategy for bacteria. *Proc Natl Acad Sci USA* 2012; **109**: 8716-8721 [PMID: 22586119 DOI: 10.1073/pnas.1202908109]

9 **Audia JP**, Webb CC, Foster JW. Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. *Int J Med Microbiol* 2001; **291**: 97-106 [PMID: 11437344 DOI: 10.1078/1438-4221-00106]

10 **Lund P**, Tramonti A, De Biase D. Coping with low pH: molecular strategies in neutralophilic bacteria. *FEMS Microbiol Rev* 2014; **38**: 1091-1125 [PMID: 24898062 DOI: 10.1111/1574-6976.12076]

11 **Begley M**, Gahan CG, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev* 2005; **29**: 625-651 [PMID: 16102595 DOI: 10.1016/j.femsre.2004.09.003]

12 **Brown AD**, Turner HP. Membrane stability and salt tolerance in gram-negative bacteria. *Nature* 1963; **199**: 301-302 [PMID: 14076708 DOI: 10.1038/199301a0]

13 **CHRISTIAN JH**, WALTHO JA. The sodium and potassium content of non-halophilic bacteria in relation to salt tolerance. *J Gen Microbiol* 1961; **25**: 97-102 [PMID: 13693390 DOI: 10.1099/00221287-25-1-97]

14 **Joo HS**, Fu CI, Otto M. Bacterial strategies of resistance to antimicrobial peptides. *Philos Trans R Soc Lond B Biol Sci* 2016; **371** [PMID: 27160595 DOI: 10.1098/rstb.2015.0292]

15 **Breijyeh Z**, Jubeh B, Karaman R. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. *Molecules* 2020; **25** [PMID: 32187986 DOI: 10.3390/molecules25061340]

16 **Dong L**, Yin J, Zhao J, Ma SR, Wang HR, Wang M, Chen W, Wei WQ. Microbial Similarity and Preference for Specific Sites in Healthy Oral Cavity and Esophagus. *Front Microbiol* 2018; **9**: 1603 [PMID: 30065718 DOI: 10.3389/fmicb.2018.01603]

17 **Schwab F**, Gastmeier P, Meyer E. The warmer the weather, the more gram-negative bacteria - impact of temperature on clinical isolates in intensive care units. *PLoS One* 2014; **9**: e91105 [PMID: 24599500 DOI: 10.1371/journal.pone.0091105]

18 **Tetz G**, Tetz V. Introducing the sporobiota and sporobiome. *Gut Pathog* 2017; **9**: 38 [PMID: 28680484 DOI: 10.1186/s13099-017-0187-8]

19 **Nicholson WL**, Munakata N, Horneck G, Melosh HJ, Setlow P. Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* 2000; **64**: 548-572 [PMID: 10974126 DOI: 10.1128/mmbr.64.3.548-572.2000]

20 **Bassis CM**, Erb-Downward JR, Dickson RP, Freeman CM, Schmidt TM, Young VB, Beck JM, Curtis JL, Huffnagle GB. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *mBio* 2015; **6**: e00037 [PMID: 25736890 DOI: 10.1128/mBio.00037-15]

21 **Tanaka M**, Nakayama J. Development of the gut microbiota in infancy and its impact on health in later life. *Allergol Int* 2017; **66**: 515-522 [PMID: 28826938 DOI: 10.1016/j.alit.2017.07.010]

22 **Claesson MJ**, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G, Stanton C, van Sinderen D, O'Connor M, Harnedy N, O'Connor K, Henry C, O'Mahony D, Fitzgerald AP, Shanahan F, Twomey C, Hill C, Ross RP, O'Toole PW. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc Natl Acad Sci USA* 2011; **108** Suppl 1: 4586-4591 [PMID: 20571116 DOI: 10.1073/pnas.1000097107]

23 **Feres M**, Teles F, Teles R, Figueiredo LC, Faveri M. The subgingival periodontal microbiota of the aging mouth. *Periodontol 2000* 2016; **72**: 30-53 [PMID: 27501490 DOI: 10.1111/prd.12136]

24 **Odamaki T**, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao JZ, Abe F, Osawa R. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol* 2016; **16**: 90 [PMID: 27220822 DOI: 10.1186/s12866-016-0708-5]

25 **Conlon MA**, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. *Nutrients* 2014; **7**: 17-44 [PMID: 25545101 DOI: 10.3390/nu7010017]

26 **Yatsunenko T**, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI. Human gut microbiome viewed across age and geography. *Nature* 2012; **486**: 222-227 [PMID: 22699611 DOI: 10.1038/nature11053]

27 **Dominguez-Bello MG**, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci USA* 2010; **107**: 11971-11975 [PMID: 20566857 DOI: 10.1073/pnas.1002601107]

28 **Holgerson PL**, Vestman NR, Claesson R, Ohman C, Domellöf M, Tanner AC, Hernell O, Johansson I. Oral microbial profile discriminates breast-fed from formula-fed infants. *J Pediatr Gastroenterol Nutr* 2013; **56**: 127-136 [PMID: 22955450 DOI: 10.1097/MPG.0b013e31826f2bc6]

29 **Gregersen H**, Pedersen J, Drewes AM. Deterioration of muscle function in the human esophagus with age. *Dig Dis Sci* 2008; **53**: 3065-3070 [PMID: 18461452 DOI: 10.1007/s10620-008-0278-y]

30 **Gutschow CA**, Leers JM, Schröder W, Prenzel KL, Fuchs H, Bollschweiler E, Bludau M, Hölscher AH. Effect of aging on esophageal motility in patients with and without GERD. *Ger Med Sci* 2011; **9**: Doc22 [PMID: 21863136 DOI: 10.3205/000145]

31 **Yoshioka H**, Iseki K, Fujita K. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics* 1983; **72**: 317-321 [PMID: 6412205]

32 **Le Huërou-Luron I**, Blat S, Boudry G. Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr Res Rev* 2010; **23**: 23-36 [PMID: 20450531 DOI: 10.1017/S0954422410000065]

33 **David LA**, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014; **505**: 559-563 [PMID: 24336217 DOI: 10.1038/nature12820]

34 **Dawsey SM**, Fagundes RB, Jacobson BC, Kresty LA, Mallery SR, Paski S, van den Brandt PA. Diet and esophageal disease. *Ann N Y Acad Sci* 2014; **1325**: 127-137 [PMID: 25266021 DOI: 10.1111/nyas.12528]

35 **Kubo A**, Corley DA, Jensen CD, Kaur R. Dietary factors and the risks of oesophageal adenocarcinoma and Barrett's oesophagus. *Nutr Res Rev* 2010; **23**: 230-246 [PMID: 20624335 DOI: 10.1017/S0954422410000132]

36 **Dong TS**, Gupta A. Influence of Early Life, Diet, and the Environment on the Microbiome. *Clin Gastroenterol Hepatol* 2019; **17**: 231-242 [PMID: 30196160 DOI: 10.1016/j.cgh.2018.08.067]

37 **Pannaraj PS**, Li F, Cerini C, Bender JM, Yang S, Rollie A, Adisetiyo H, Zabih S, Lincez PJ, Bittinger K, Bailey A, Bushman FD, Sleasman JW, Aldrovandi GM. Association Between Breast Milk Bacterial Communities and Establishment and Development of the Infant Gut Microbiome. *JAMA Pediatr* 2017; **171**: 647-654 [PMID: 28492938 DOI: 10.1001/jamapediatrics.2017.0378]

38 **Rudloff S**, Kunz C. Milk oligosaccharides and metabolism in infants. *Adv Nutr* 2012; **3**: 398S-405S [PMID: 22585918 DOI: 10.3945/an.111.001594]

39 **Kim SY**, Yi DY. Analysis of the human breast milk microbiome and bacterial extracellular vesicles in healthy mothers. *Exp Mol Med* 2020; **52**: 1288-1297 [PMID: 32747701 DOI: 10.1038/s12276-020-0470-5]

40 **Bezirtzoglou E**, Tsiotsias A, Welling GW. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe* 2011; **17**: 478-482 [PMID: 21497661 DOI: 10.1016/j.anaerobe.2011.03.009]

41 **Bokulich NA**, Chung J, Battaglia T, Henderson N, Jay M, Li H, D Lieber A, Wu F, Perez-Perez GI, Chen Y, Schweizer W, Zheng X, Contreras M, Dominguez-Bello MG, Blaser MJ. Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci Transl Med* 2016; **8**: 343ra82 [PMID: 27306664 DOI: 10.1126/scitranslmed.aad7121]

42 **Brugman S**, Visser JT, Hillebrands JL, Bos NA, Rozing J. Prolonged exclusive breastfeeding reduces autoimmune diabetes incidence and increases regulatory T-cell frequency in bio-breeding diabetes-prone rats. *Diabetes Metab Res Rev* 2009; **25**: 380-387 [PMID: 19334008 DOI: 10.1002/dmrr.953]

43 **Steinmetz KA**, Potter JD. Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes Control* 1991; **2**: 427-442 [PMID: 1764568 DOI: 10.1007/BF00054304]

44 **Mirvish SS**, Wallcave L, Eagen M, Shubik P. Ascorbate-nitrite reaction: possible means of blocking

the formation of carcinogenic N-nitroso compounds. *Science* 1972; **177**: 65-68 [PMID: 5041776 DOI: 10.1126/science.177.4043.65]

45 **Jakszyn P**, Gonzalez CA. Nitrosamine and related food intake and gastric and oesophageal cancer risk: a systematic review of the epidemiological evidence. *World J Gastroenterol* 2006; **12**: 4296-4303 [PMID: 16865769 DOI: 10.3748/wjg.v12.i27.4296]

46 **Chen H**, Ward MH, Graubard BI, Heineman EF, Markin RM, Potischman NA, Russell RM, Weisenburger DD, Tucker KL. Dietary patterns and adenocarcinoma of the esophagus and distal stomach. *Am J Clin Nutr* 2002; **75**: 137-144 [PMID: 11756071 DOI: 10.1093/ajcn/75.1.137]

47 **Kubo A**, Levin TR, Block G, Rumore GJ, Quesenberry CP Jr, Buffler P, Corley DA. Dietary patterns and the risk of Barrett's esophagus. *Am J Epidemiol* 2008; **167**: 839-846 [PMID: 18218607 DOI: 10.1093/aje/kwm381]

48 **Nobel YR**, Snider EJ, Compres G, Freedberg DE, Khiabanian H, Lightdale CJ, Toussaint NC, Abrams JA. Increasing Dietary Fiber Intake Is Associated with a Distinct Esophageal Microbiome. *Clin Transl Gastroenterol* 2018; **9**: 199 [PMID: 30356041 DOI: 10.1038/s41424-018-0067-7]

49 **Katelaris PH**. Proton pump inhibitors. *Med J Aust* 1998; **169**: 208-211 [PMID: 9734580 DOI: 10.5694/j.1326-5377.1998.tb140224.x]

50 **Imhann F**, Bonder MJ, Vich Vila A, Fu J, Mujagic Z, Vork L, Tigchelaar EF, Jankipersadsing SA, Cenit MC, Harmsen HJ, Dijkstra G, Franke L, Xavier RJ, Jonkers D, Wijmenga C, Weersma RK, Zhernakova A. Proton pump inhibitors affect the gut microbiome. *Gut* 2016; **65**: 740-748 [PMID: 26657899 DOI: 10.1136/gutjnl-2015-310376]

51 **Jackson MA**, Goodrich JK, Maxan ME, Freedberg DE, Abrams JA, Poole AC, Sutter JL, Welter D, Ley RE, Bell JT, Spector TD, Steves CJ. Proton pump inhibitors alter the composition of the gut microbiota. *Gut* 2016; **65**: 749-756 [PMID: 26719299 DOI: 10.1136/gutjnl-2015-310861]

52 **Macke L**, Schulz C, Koletzko L, Malfertheiner P. Systematic review: the effects of proton pump inhibitors on the microbiome of the digestive tract-evidence from next-generation sequencing studies. *Aliment Pharmacol Ther* 2020; **51**: 505-526 [PMID: 31990420 DOI: 10.1111/apt.15604]

53 **Zhou J**, Shrestha P, Qiu Z, Harman DG, Teoh WC, Al-Sohaily S, Liem H, Turner I, Ho V. Distinct Microbiota Dysbiosis in Patients with Non-Erosive Reflux Disease and Esophageal Adenocarcinoma. *J Clin Med* 2020; **9** [PMID: 32650561 DOI: 10.3390/jcm9072162]

54 **Amir I**, Konikoff FM, Oppenheim M, Gophna U, Half EE. Gastric microbiota is altered in oesophagitis and Barrett's oesophagus and further modified by proton pump inhibitors. *Environ Microbiol* 2014; **16**: 2905-2914 [PMID: 24112768 DOI: 10.1111/1462-2920.12285]

55 **Vesper BJ**, Jawdi A, Altman KW, Haines GK 3rd, Tao L, Radosevich JA. The effect of proton pump inhibitors on the human microbiota. *Curr Drug Metab* 2009; **10**: 84-89 [PMID: 19149516 DOI: 10.2174/138920009787048392]

56 **Huo X**, Zhang X, Yu C, Zhang Q, Cheng E, Wang DH, Pham TH, Spechler SJ, Souza RF. In oesophageal squamous cells exposed to acidic bile salt medium, omeprazole inhibits IL-8 expression through effects on nuclear factor-κB and activator protein-1. *Gut* 2014; **63**: 1042-1052 [PMID: 24048734 DOI: 10.1136/gutjnl-2013-305533]

57 **Konturek PC**, Nikiforuk A, Kania J, Raithel M, Hahn EG, Mühldorfer S. Activation of NFkappaB represents the central event in the neoplastic progression associated with Barrett's esophagus: a possible link to the inflammation and overexpression of COX-2, PPARgamma and growth factors. *Dig Dis Sci* 2004; **49**: 1075-1083 [PMID: 15387324 DOI: 10.1023/B:DDAS.0000037790.11724.70]

58 **Socransky SS**, Haffajee AD. Periodontal microbial ecology. *Periodontol 2000* 2005; **38**: 135-187 [PMID: 15853940 DOI: 10.1111/j.1600-0757.2005.00107.x]

59 **Chen X**, Yuan Z, Lu M, Zhang Y, Jin L, Ye W. Poor oral health is associated with an increased risk of esophageal squamous cell carcinoma - a population-based case-control study in China. *Int J Cancer* 2017; **140**: 626-635 [PMID: 27778330 DOI: 10.1002/ijc.30484]

60 **Wang TW**, Asman K, Gentzke AS, Cullen KA, Holder-Hayes E, Reyes-Guzman C, Jamal A, Neff L, King BA. Tobacco Product Use Among Adults - United States, 2017. *MMWR Morb Mortal Wkly Rep* 2018; **67**: 1225-1232 [PMID: 30408019 DOI: 10.15585/mmwr.mm6744a2]

61 **Vogtmann E**, Flores R, Yu G, Freedman ND, Shi J, Gail MH, Dye BA, Wang GQ, Klepac-Ceraj V, Paster BJ, Wei WQ, Guo HQ, Dawsey SM, Qiao YL, Abnet CC. Association between tobacco use and the upper gastrointestinal microbiome among Chinese men. *Cancer Causes Control* 2015; **26**: 581-588 [PMID: 25701246 DOI: 10.1007/s10552-015-0535-2]

62 **Kulkarni R**, Antala S, Wang A, Amaral FE, Rampersaud R, Larussa SJ, Planet PJ, Ratner AJ. Cigarette smoke increases Staphylococcus aureus biofilm formation *via* oxidative stress. *Infect Immun* 2012; **80**: 3804-3811 [PMID: 22890993 DOI: 10.1128/IAI.00689-12]

63 **Arnson Y**, Shoenfeld Y, Amital H. Effects of tobacco smoke on immunity, inflammation and autoimmunity. *J Autoimmun* 2010; **34**: J258-J265 [PMID: 20042314 DOI: 10.1016/j.jaut.2009.12.003]

64 **Dicks LMT**, Dreyer L, Smith C, van Staden AD. A Review: The Fate of Bacteriocins in the Human Gastro-Intestinal Tract: Do They Cross the Gut-Blood Barrier? *Front Microbiol* 2018; **9**: 2297 [PMID: 30323796 DOI: 10.3389/fmicb.2018.02297]

65 **Kumariya R**, Garsa AK, Rajput YS, Sood SK, Akhtar N, Patel S. Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microb Pathog* 2019; **128**: 171-177 [PMID: 30610901 DOI: 10.1016/j.micpath.2019.01.002]

66 **Mathiesen G**, Huehne K, Kroeckel L, Axelsson L, Eijsink VG. Characterization of a new bacteriocin operon in sakacin P-producing Lactobacillus sakei, showing strong translational coupling

between the bacteriocin and immunity genes. *Appl Environ Microbiol* 2005; **71**: 3565-3574 [PMID: 16000763 DOI: 10.1128/AEM.71.7.3565-3574.2005]

67 **Kelly JR**, Kennedy PJ, Cryan JF, Dinan TG, Clarke G, Hyland NP. Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. *Front Cell Neurosci* 2015; **9**: 392 [PMID: 26528128 DOI: 10.3389/fncel.2015.00392]

68 **Spadoni I**, Zagato E, Bertocchi A, Paolinelli R, Hot E, Di Sabatino A, Caprioli F, Bottiglieri L, Oldani A, Viale G, Penna G, Dejana E, Rescigno M. A gut-vascular barrier controls the systemic dissemination of bacteria. *Science* 2015; **350**: 830-834 [PMID: 26564856 DOI: 10.1126/science.aad0135]

69 **Okuda K**, Zendo T, Sugimoto S, Iwase T, Tajima A, Yamada S, Sonomoto K, Mizunoe Y. Effects of bacteriocins on methicillin-resistant Staphylococcus aureus biofilm. *Antimicrob Agents Chemother* 2013; **57**: 5572-5579 [PMID: 23979748 DOI: 10.1128/AAC.00888-13]

70 **Corr SC**, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG. Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. *Proc Natl Acad Sci USA* 2007; **104**: 7617-7621 [PMID: 17456596 DOI: 10.1073/pnas.0700440104]

71 **Joo NE**, Ritchie K, Kamarajan P, Miao D, Kapila YL. Nisin, an apoptogenic bacteriocin and food preservative, attenuates HNSCC tumorigenesis *via* CHAC1. *Cancer Med* 2012; **1**: 295-305 [PMID: 23342279 DOI: 10.1002/cam4.35]

72 **Kamarajan P**, Hayami T, Matte B, Liu Y, Danciu T, Ramamoorthy A, Worden F, Kapila S, Kapila Y. Nisin ZP, a Bacteriocin and Food Preservative, Inhibits Head and Neck Cancer Tumorigenesis and Prolongs Survival. *PLoS One* 2015; **10**: e0131008 [PMID: 26132406 DOI: 10.1371/journal.pone.0131008]

73 **Machado LR**, Ottolini B. An evolutionary history of defensins: a role for copy number variation in maximizing host innate and adaptive immune responses. *Front Immunol* 2015; **6**: 115 [PMID: 25852686 DOI: 10.3389/fimmu.2015.00115]

74 **Raschig J**, Mailänder-Sánchez D, Berscheid A, Berger J, Strömstedt AA, Courth LF, Malek NP, Brötz-Oesterhelt H, Wehkamp J. Ubiquitously expressed Human Beta Defensin 1 (hBD1) forms bacteria-entrapping nets in a redox dependent mode of action. *PLoS Pathog* 2017; **13**: e1006261 [PMID: 28323883 DOI: 10.1371/journal.ppat.1006261]

75 **Schroeder S**, Robinson ZD, Masterson JC, Hosford L, Moore W, Pan Z, Harris R, Souza RF, Spechler SJ, Fillon SA, Furuta GT. Esophageal human β-defensin expression in eosinophilic esophagitis. *Pediatr Res* 2013; **73**: 647-654 [PMID: 23385963 DOI: 10.1038/pr.2013.23]

76 **Nomura Y**, Tanabe H, Moriichi K, Igawa S, Ando K, Ueno N, Kashima S, Tominaga M, Goto T, Inaba Y, Ito T, Ishida-Yamamoto A, Fujiya M, Kohgo Y. Reduction of E-cadherin by human defensin-5 in esophageal squamous cells. *Biochem Biophys Res Commun* 2013; **439**: 71-77 [PMID: 23958301 DOI: 10.1016/j.bbrc.2013.08.026]

77 **Jung S**, Mysliwy J, Spudy B, Lorenzen I, Reiss K, Gelhaus C, Podschun R, Leippe M, Grötzinger J. Human beta-defensin 2 and beta-defensin 3 chimeric peptides reveal the structural basis of the pathogen specificity of their parent molecules. *Antimicrob Agents Chemother* 2011; **55**: 954-960 [PMID: 21189349 DOI: 10.1128/AAC.00872-10]

78 **Petersen C**, Round JL. Defining dysbiosis and its influence on host immunity and disease. *Cell Microbiol* 2014; **16**: 1024-1033 [PMID: 24798552 DOI: 10.1111/cmi.12308]

79 **Ajayi TA**, Cantrell S, Spann A, Garman KS. Barrett's esophagus and esophageal cancer: Links to microbes and the microbiome. *PLoS Pathog* 2018; **14**: e1007384 [PMID: 30571768 DOI: 10.1371/journal.ppat.1007384]

80 **Yang L**, Francois F, Pei Z. Molecular pathways: pathogenesis and clinical implications of microbiome alteration in esophagitis and Barrett esophagus. *Clin Cancer Res* 2012; **18**: 2138-2144 [PMID: 22344232 DOI: 10.1158/1078-0432.CCR-11-0934]

81 **Orlando RC**. The integrity of the esophageal mucosa. Balance between offensive and defensive mechanisms. *Best Pract Res Clin Gastroenterol* 2010; **24**: 873-882 [PMID: 21126700 DOI: 10.1016/j.bpg.2010.08.008]

82 **Hershcovici T**, Fass R. Nonerosive Reflux Disease (NERD) - An Update. *J Neurogastroenterol Motil* 2010; **16**: 8-21 [PMID: 20535321 DOI: 10.5056/jnm.2010.16.1.8]

83 **Winkelstein A**. Peptic esophagitis. *J Am Med Assoc* 1935; **104**: 906-909 [DOI: 10.1001/jama.1935.02760110034008]

84 **Dunbar KB**, Agoston AT, Odze RD, Huo X, Pham TH, Cipher DJ, Castell DO, Genta RM, Souza RF, Spechler SJ. Association of Acute Gastroesophageal Reflux Disease With Esophageal Histologic Changes. *JAMA* 2016; **315**: 2104-2112 [PMID: 27187303 DOI: 10.1001/jama.2016.5657]

85 **Souza RF**, Huo X, Mittal V, Schuler CM, Carmack SW, Zhang HY, Zhang X, Yu C, Hormi-Carver K, Genta RM, Spechler SJ. Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. *Gastroenterology* 2009; **137**: 1776-1784 [PMID: 19660463 DOI: 10.1053/j.gastro.2009.07.055]

86 **Ustaoglu A**, Nguyen A, Spechler S, Sifrim D, Souza R, Woodland P. Mucosal pathogenesis in gastro-esophageal reflux disease. *Neurogastroenterol Motil* 2020; **32**: e14022 [PMID: 33118247 DOI: 10.1111/nmo.14022]

87 **Cheng L**, Harnett KM, Cao W, Liu F, Behar J, Fiocchi C, Biancani P. Hydrogen peroxide reduces lower esophageal sphincter tone in human esophagitis. *Gastroenterology* 2005; **129**: 1675-1685 [PMID: 16285965 DOI: 10.1053/j.gastro.2005.09.008]

88 **Blackett KL**, Siddhi SS, Cleary S, Steed H, Miller MH, Macfarlane S, Macfarlane GT, Dillon JF.

Oesophageal bacterial biofilm changes in gastro-oesophageal reflux disease, Barrett's and oesophageal carcinoma: association or causality? *Aliment Pharmacol Ther* 2013; **37**: 1084-1092 [PMID: 23600758 DOI: 10.1111/apt.12317]

89 **Marsh PD**. Dental plaque as a biofilm and a microbial community - implications for health and disease. *BMC Oral Health* 2006; **6** Suppl 1: S14 [PMID: 16934115 DOI: 10.1186/1472-6831-6-S1-S14]

90 **Dejea CM**, Fathi P, Craig JM, Boleij A, Taddese R, Geis AL, Wu X, DeStefano Shields CE, Hechenbleikner EM, Huso DL, Anders RA, Giardiello FM, Wick EC, Wang H, Wu S, Pardoll DM, Housseau F, Sears CL. Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. *Science* 2018; **359**: 592-597 [PMID: 29420293 DOI: 10.1126/science.aah3648]

91 **D'Souza SM**, Levitan O, Tieu AH. Bio-Geographic Variation of Colonic Biome and Biofilm Consequences on Disease: An Update for Clinicians. *World J Gastroenterol Hepatol Endosc Research* 2020; **1**: 1-11

92 **Münch NS**, Fang HY, Ingermann J, Maurer HC, Anand A, Kellner V, Sahm V, Wiethaler M, Baumeister T, Wein F, Einwächter H, Bolze F, Klingenspor M, Haller D, Kavanagh M, Lysaght J, Friedman R, Dannenberg AJ, Pollak M, Holt PR, Muthupalani S, Fox JG, Whary MT, Lee Y, Ren TY, Elliot R, Fitzgerald R, Steiger K, Schmid RM, Wang TC, Quante M. High-Fat Diet Accelerates Carcinogenesis in a Mouse Model of Barrett's Esophagus *via* Interleukin 8 and Alterations to the Gut Microbiome. *Gastroenterology* 2019; **157**: 492-506. e2 [PMID: 30998992 DOI: 10.1053/j.gastro.2019.04.013]

93 **Ropert A**, Cherbut C, Rozé C, Le Quellec A, Holst JJ, Fu-Cheng X, Bruley des Varannes S, Galmiche JP. Colonic fermentation and proximal gastric tone in humans. *Gastroenterology* 1996; **111**: 289-296 [PMID: 8690193 DOI: 10.1053/gast.1996.v111.pm8690193]

94 **Shaheen NJ**, Falk GW, Iyer PG, Gerson LB; American College of Gastroenterology. ACG Clinical Guideline: Diagnosis and Management of Barrett's Esophagus. *Am J Gastroenterol* 2016; **111**: 30-50; quiz 51 [PMID: 26526079 DOI: 10.1038/ajg.2015.322]

95 **Naini BV**, Souza RF, Odze RD. Barrett's Esophagus: A Comprehensive and Contemporary Review for Pathologists. *Am J Surg Pathol* 2016; **40**: e45-e66 [PMID: 26813745 DOI: 10.1097/PAS.0000000000000598]

96 **May M**, Abrams JA. Emerging Insights into the Esophageal Microbiome. *Curr Treat Options Gastroenterol* 2018; **16**: 72-85 [PMID: 29350339 DOI: 10.1007/s11938-018-0171-5]

97 **Souza RF**. Reflux esophagitis and its role in the pathogenesis of Barrett's metaplasia. *J Gastroenterol* 2017; **52**: 767-776 [PMID: 28451845 DOI: 10.1007/s00535-017-1342-1]

98 **Quante M**, Bhagat G, Abrams JA, Marache F, Good P, Lee MD, Lee Y, Friedman R, Asfaha S, Dubeykovskaya Z, Mahmood U, Figueiredo JL, Kitajewski J, Shawber C, Lightdale CJ, Rustgi AK, Wang TC. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia. *Cancer Cell* 2012; **21**: 36-51 [PMID: 22264787 DOI: 10.1016/j.ccr.2011.12.004]

99 **Sollberger G**, Strittmatter GE, Garstkiewicz M, Sand J, Beer HD. Caspase-1: the inflammasome and beyond. *Innate Immun* 2014; **20**: 115-125 [PMID: 23676582 DOI: 10.1177/1753425913484374]

100 **Guo H**, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med* 2015; **21**: 677-687 [PMID: 26121197 DOI: 10.1038/nm.3893]

101 **Nadatani Y**, Huo X, Zhang X, Yu C, Cheng E, Zhang Q, Dunbar KB, Theiss A, Pham TH, Wang DH, Watanabe T, Fujiwara Y, Arakawa T, Spechler SJ, Souza RF. NOD-Like Receptor Protein 3 Inflammasome Priming and Activation in Barrett's Epithelial Cells. *Cell Mol Gastroenterol Hepatol* 2016; **2**: 439-453 [PMID: 27777967 DOI: 10.1016/j.jcmgh.2016.03.006]

102 **Snider EJ**, Compres G, Freedberg DE, Giddins MJ, Khiabanian H, Lightdale CJ, Nobel YR, Toussaint NC, Uhlemann AC, Abrams JA. Barrett's esophagus is associated with a distinct oral microbiome. *Clin Transl Gastroenterol* 2018; **9**: 135 [PMID: 29491399 DOI: 10.1038/s41424-018-0005-8]

103 **Yang L**, Chaudhary N, Baghdadi J, Pei Z. Microbiome in reflux disorders and esophageal adenocarcinoma. *Cancer J* 2014; **20**: 207-210 [PMID: 24855009 DOI: 10.1097/PPO.0000000000000044]

104 **Liu N**, Ando T, Ishiguro K, Maeda O, Watanabe O, Funasaka K, Nakamura M, Miyahara R, Ohmiya N, Goto H. Characterization of bacterial biota in the distal esophagus of Japanese patients with reflux esophagitis and Barrett's esophagus. *BMC Infect Dis* 2013; **13**: 130 [PMID: 23496929 DOI: 10.1186/1471-2334-13-130]

105 **Macfarlane S**, Furrie E, Macfarlane GT, Dillon JF. Microbial colonization of the upper gastrointestinal tract in patients with Barrett's esophagus. *Clin Infect Dis* 2007; **45**: 29-38 [PMID: 17554697 DOI: 10.1086/518578]

106 **Lopetuso LR**, Severgnini M, Pecere S, Ponziani FR, Boskoski I, Larghi A, Quaranta G, Masucci L, Ianiro G, Camboni T, Gasbarrini A, Costamagna G, Consolandi C, Cammarota G. Esophageal microbiome signature in patients with Barrett's esophagus and esophageal adenocarcinoma. *PLoS One* 2020; **15**: e0231789 [PMID: 32369505 DOI: 10.1371/journal.pone.0231789]

107 **Kamangar F**, Chow WH, Abnet CC, Dawsey SM. Environmental causes of esophageal cancer. *Gastroenterol Clin North Am* 2009; **38**: 27-57, vii [PMID: 19327566 DOI: 10.1016/j.gtc.2009.01.004]

108 **Engel LS**, Chow WH, Vaughan TL, Gammon MD, Risch HA, Stanford JL, Schoenberg JB, Mayne ST, Dubrow R, Rotterdam H, West AB, Blaser M, Blot WJ, Gail MH, Fraumeni JF Jr. Population

attributable risks of esophageal and gastric cancers. *J Natl Cancer Inst* 2003; **95**: 1404-1413 [PMID: 13130116 DOI: 10.1093/jnci/djg047]

109 **Bhat S**, Coleman HG, Yousef F, Johnston BT, McManus DT, Gavin AT, Murray LJ. Risk of malignant progression in Barrett's esophagus patients: results from a large population-based study. *J Natl Cancer Inst* 2011; **103**: 1049-1057 [PMID: 21680910 DOI: 10.1093/jnci/djr203]

110 **Snider EJ**, Compres G, Freedberg DE, Khiabanian H, Nobel YR, Stump S, Uhlemann AC, Lightdale CJ, Abrams JA. Alterations to the Esophageal Microbiome Associated with Progression from Barrett's Esophagus to Esophageal Adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 2019; **28**: 1687-1693 [PMID: 31466948 DOI: 10.1158/1055-9965.EPI-19-0008]

111 **Snider EJ**, Freedberg DE, Abrams JA. Potential Role of the Microbiome in Barrett's Esophagus and Esophageal Adenocarcinoma. *Dig Dis Sci* 2016; **61**: 2217-2225 [PMID: 27068172 DOI: 10.1007/s10620-016-4155-9]

112 **Narikiyo M**, Tanabe C, Yamada Y, Igaki H, Tachimori Y, Kato H, Muto M, Montesano R, Sakamoto H, Nakajima Y, Sasaki H. Frequent and preferential infection of Treponema denticola, Streptococcus mitis, and Streptococcus anginosus in esophageal cancers. *Cancer Sci* 2004; **95**: 569-574 [PMID: 15245592 DOI: 10.1111/j.1349-7006.2004.tb02488.x]

113 **Peters BA**, Wu J, Pei Z, Yang L, Purdue MP, Freedman ND, Jacobs EJ, Gapstur SM, Hayes RB, Ahn J. Oral Microbiome Composition Reflects Prospective Risk for Esophageal Cancers. *Cancer Res* 2017; **77**: 6777-6787 [PMID: 29196415 DOI: 10.1158/0008-5472.CAN-17-1296]

114 **Zaidi AH**, Kelly LA, Kreft RE, Barlek M, Omstead AN, Matsui D, Boyd NH, Gazarik KE, Heit MI, Nistico L, Kasi PM, Spirk TL, Byers B, Lloyd EJ, Landreneau RJ, Jobe BA. Associations of microbiota and toll-like receptor signaling pathway in esophageal adenocarcinoma. *BMC Cancer* 2016; **16**: 52 [PMID: 26841926 DOI: 10.1186/s12885-016-2093-8]

115 **Abnet CC**, Arnold M, Wei WQ. Epidemiology of Esophageal Squamous Cell Carcinoma. *Gastroenterology* 2018; **154**: 360-373 [PMID: 28823862 DOI: 10.1053/j.gastro.2017.08.023]

116 **Lu CL**, Lang HC, Luo JC, Liu CC, Lin HC, Chang FY, Lee SD. Increasing trend of the incidence of esophageal squamous cell carcinoma, but not adenocarcinoma, in Taiwan. *Cancer Causes Control* 2010; **21**: 269-274 [PMID: 19866363 DOI: 10.1007/s10552-009-9458-0]

117 **Sepehr A**, Kamangar F, Fahimi S, Saidi F, Abnet CC, Dawsey SM. Poor oral health as a risk factor for esophageal squamous dysplasia in northeastern Iran. *Anticancer Res* 2005; **25**: 543-546 [PMID: 15816626]

118 **Wang Q**, Rao Y, Guo X, Liu N, Liu S, Wen P, Li S, Li Y. Oral Microbiome in Patients with Oesophageal Squamous Cell Carcinoma. *Sci Rep* 2019; **9**: 19055 [PMID: 31836795 DOI: 10.1038/s41598-019-55667-w]

119 **Chen X**, Winckler B, Lu M, Cheng H, Yuan Z, Yang Y, Jin L, Ye W. Oral Microbiota and Risk for Esophageal Squamous Cell Carcinoma in a High-Risk Area of China. *PLoS One* 2015; **10**: e0143603 [PMID: 26641451 DOI: 10.1371/journal.pone.0143603]

120 **Huang C**, Shi G. Smoking and microbiome in oral, airway, gut and some systemic diseases. *J Transl Med* 2019; **17**: 225 [PMID: 31307469 DOI: 10.1186/s12967-019-1971-7]

121 **Bishehsari F**, Magno E, Swanson G, Desai V, Voigt RM, Forsyth CB, Keshavarzian A. Alcohol and Gut-Derived Inflammation. *Alcohol Res* 2017; **38**: 163-171 [PMID: 28988571]

122 **Bull-Otterson L**, Feng W, Kirpich I, Wang Y, Qin X, Liu Y, Gobejishvili L, Joshi-Barve S, Ayvaz T, Petrosino J, Kong M, Barker D, McClain C, Barve S. Metagenomic analyses of alcohol induced pathogenic alterations in the intestinal microbiome and the effect of Lactobacillus rhamnosus GG treatment. *PLoS One* 2013; **8**: e53028 [PMID: 23326376 DOI: 10.1371/journal.pone.0053028]

123 **Mutlu E**, Keshavarzian A, Engen P, Forsyth CB, Sikaroodi M, Gillevet P. Intestinal dysbiosis: a possible mechanism of alcohol-induced endotoxemia and alcoholic steatohepatitis in rats. *Alcohol Clin Exp Res* 2009; **33**: 1836-1846 [PMID: 19645728 DOI: 10.1111/j.1530-0277.2009.01022.x]

124 **Liu Y**, Lin Z, Lin Y, Chen Y, Peng XE, He F, Liu S, Yan S, Huang L, Lu W, Xiang Z, Hu Z. Streptococcus and Prevotella are associated with the prognosis of oesophageal squamous cell carcinoma. *J Med Microbiol* 2018; **67**: 1058-1068 [PMID: 29923815 DOI: 10.1099/jmm.0.000754]

125 **Dellon ES**, Gonsalves N, Hirano I, Furuta GT, Liacouras CA, Katzka DA; American College of Gastroenterology. ACG clinical guideline: Evidenced based approach to the diagnosis and management of esophageal eosinophilia and eosinophilic esophagitis (EoE). *Am J Gastroenterol* 2013; **108**: 679-92; quiz 693 [PMID: 23567357 DOI: 10.1038/ajg.2013.71]

126 **O'Shea KM**, Aceves SS, Dellon ES, Gupta SK, Spergel JM, Furuta GT, Rothenberg ME. Pathophysiology of Eosinophilic Esophagitis. *Gastroenterology* 2018; **154**: 333-345 [PMID: 28757265 DOI: 10.1053/j.gastro.2017.06.065]

127 **Kitajima M**, Lee HC, Nakayama T, Ziegler SF. TSLP enhances the function of helper type 2 cells. *Eur J Immunol* 2011; **41**: 1862-1871 [PMID: 21484783 DOI: 10.1002/eji.201041195]

128 **Benitez AJ**, Hoffmann C, Muir AB, Dods KK, Spergel JM, Bushman FD, Wang ML. Inflammation-associated microbiota in pediatric eosinophilic esophagitis. *Microbiome* 2015; **3**: 23 [PMID: 26034601 DOI: 10.1186/s40168-015-0085-6]

129 **Jensen ET**, Kuhl JT, Martin LJ, Langefeld CD, Dellon ES, Rothenberg ME. Early-life environmental exposures interact with genetic susceptibility variants in pediatric patients with eosinophilic esophagitis. *J Allergy Clin Immunol* 2018; **141**: 632-637. e5 [PMID: 29029802 DOI: 10.1016/j.jaci.2017.07.010]

130 **Radano MC**, Yuan Q, Katz A, Fleming JT, Kubala S, Shreffler W, Keet CA. Cesarean section and antibiotic use found to be associated with eosinophilic esophagitis. *J Allergy Clin Immunol Pract*

2014; **2**: 475-477. e1 [PMID: 25017541 DOI: 10.1016/j.jaip.2014.02.018]

131 **Harris JK**, Fang R, Wagner BD, Choe HN, Kelly CJ, Schroeder S, Moore W, Stevens MJ, Yeckes A, Amsden K, Kagalwalla AF, Zalewski A, Hirano I, Gonsalves N, Henry LN, Masterson JC, Robertson CE, Leung DY, Pace NR, Ackerman SJ, Furuta GT, Fillon SA. Esophageal microbiome in eosinophilic esophagitis. *PLoS One* 2015; **10**: e0128346 [PMID: 26020633 DOI: 10.1371/journal.pone.0128346]

132 **Kashyap PC**, Johnson S, Geno DM, Lekatz HR, Lavey C, Alexander JA, Chen J, Katzka DA. A decreased abundance of clostridia characterizes the gut microbiota in eosinophilic esophagitis. *Physiol Rep* 2019; **7**: e14261 [PMID: 31650712 DOI: 10.14814/phy2.14261]

133 **Stefka AT**, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, Tjota MY, Seo GY, Cao S, Theriault BR, Antonopoulos DA, Zhou L, Chang EB, Fu YX, Nagler CR. Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci USA* 2014; **111**: 13145-13150 [PMID: 25157157 DOI: 10.1073/pnas.1412008111]

134 **Selling J**, Swann P, Madsen LR 2nd, Oswald J. Improvement in Gastroesophageal Reflux Symptoms From a Food-grade Maltosyl-isomaltooligosaccharide Soluble Fiber Supplement: A Case Series. *Integr Med (Encinitas)* 2018; **17**: 40-42 [PMID: 31043918]

135 **Beckett JM**, Singh NK, Phillips J, Kalpurath K, Taylor K, Stanley RA, Eri RD. Anti-Heartburn Effects of Sugar Cane Flour: A Double-Blind, Randomized, Placebo-Controlled Study. *Nutrients* 2020; **12** [PMID: 32570710 DOI: 10.3390/nu12061813]

136 **Cheng J**, Ouwehand AC. Gastroesophageal Reflux Disease and Probiotics: A Systematic Review. *Nutrients* 2020; **12** [PMID: 31906573 DOI: 10.3390/nu12010132]

137 **Gomi A**, Yamaji K, Watanabe O, Yoshioka M, Miyazaki K, Iwama Y, Urita Y. Bifidobacterium bifidum YIT 10347 fermented milk exerts beneficial effects on gastrointestinal discomfort and symptoms in healthy adults: A double-blind, randomized, placebo-controlled study. *J Dairy Sci* 2018; **101**: 4830-4841 [PMID: 29573807 DOI: 10.3168/jds.2017-13803]

138 **Waller PA**, Gopal PK, Leyer GJ, Ouwehand AC, Reifer C, Stewart ME, Miller LE. Dose-response effect of Bifidobacterium lactis HN019 on whole gut transit time and functional gastrointestinal symptoms in adults. *Scand J Gastroenterol* 2011; **46**: 1057-1064 [PMID: 21663486 DOI: 10.3109/00365521.2011.584895]

139 **Nakae H**, Tsuda A, Matsuoka T, Mine T, Koga Y. Gastric microbiota in the functional dyspepsia patients treated with probiotic yogurt. *BMJ Open Gastroenterol* 2016; **3**: e000109 [PMID: 27752337 DOI: 10.1136/bmjgast-2016-000109]

140 **Ohtsu T**, Takagi A, Uemura N, Inoue K, Sekino H, Kawashima A, Uchida M, Koga Y. The Ameliorating Effect of Lactobacillus gasseri OLL2716 on Functional Dyspepsia in Helicobacter pylori-Uninfected Individuals: A Randomized Controlled Study. *Digestion* 2017; **96**: 92-102 [PMID: 28768250 DOI: 10.1159/000479000]

141 **Su GL**, Ko CW, Bercik P, Falck-Ytter Y, Sultan S, Weizman AV, Morgan RL. AGA Clinical Practice Guidelines on the Role of Probiotics in the Management of Gastrointestinal Disorders. *Gastroenterology* 2020; **159**: 697-705 [PMID: 32531291 DOI: 10.1053/j.gastro.2020.05.059]

142 **Rao SSC**, Bhagatwala J. Small Intestinal Bacterial Overgrowth: Clinical Features and Therapeutic Management. *Clin Transl Gastroenterol* 2019; **10**: e00078 [PMID: 31584459 DOI: 10.14309/ctg.0000000000000078]

143 **Acharya C**, Bajaj JS. Current Management of Hepatic Encephalopathy. *Am J Gastroenterol* 2018; **113**: 1600-1612 [PMID: 30002466 DOI: 10.1038/s41395-018-0179-4]

144 **McDonald LC**, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* 2018; **66**: e1-e48 [PMID: 29462280 DOI: 10.1093/cid/cix1085]

145 **Fahim HA**, Khairalla AS, El-Gendy AO. Nanotechnology: A Valuable Strategy to Improve Bacteriocin Formulations. *Front Microbiol* 2016; **7**: 1385 [PMID: 27695440 DOI: 10.3389/fmicb.2016.01385]

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REVIEW

Immune disorders and rheumatologic manifestations of viral hepatitis

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Abstract

Infection with hepatotropic viruses is not limited to the liver and can lead to the development of various immunological disorders (the formation of cryoglobulins, rheumatoid factor, antinuclear antibodies, autoantibodies specific for autoimmune hepatitis and primary biliary cholangitis, and others), which can manifest as glomerulonephritis, arthritis, uveitis, vasculitis (cryoglobulinemic vasculitis, polyarteritis nodosa, Henoch-Schonlein purpura, isolated cutaneous necrotizing vasculitis), and other rheumatologic disorders, and be a trigger for the subsequent development of autoimmune hepatitis and primary biliary cholangitis. A further study of the association between autoimmune liver diseases and hepatotropic virus infection would be useful to assess the results of treatment of these associated diseases with antiviral drugs. The relationship of these immune disorders and their manifestations with hepatotropic viruses is best studied for chronic hepatitis B and C. Only isolated cases of these associations are described for hepatitis A. These links are least studied, and are often controversial for hepatitis E, possibly due to their relatively rare diagnoses. Patients with uveitis, glomerulonephritis, arthritis, vasculitis, autoimmune liver diseases should be tested for biomarkers of viral hepatitis, and if present, these patients should be treated with antiviral drugs.

Key Words: Hepatitis A; Hepatitis B; Hepatitis C; Hepatitis E; Vasculitis; Rheumatoid factor

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Core Tip: Infection with hepatotropic viruses is not limited to the liver and can lead to the development of various immunological disorders, which can manifest itself as glomerulonephritis, arthritis, uveitis, vasculitis, and other rheumatologic disorders, and be a trigger for the subsequent development of autoimmune hepatitis and primary biliary cholangitis. These associations are best studied for chronic hepatitis B and C. Only isolated cases of these are described for hepatitis A. These links are least studied, and are often controversial for hepatitis E. Patients with uveitis, glomerulonephritis, arthritis, vasculitis, autoimmune liver diseases should be tested for biomarkers of viral hepatitis.

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INTRODUCTION

Viruses, unlike bacteria and fungi, cannot reproduce on their own and need the resources of a host cell as they are obligate intracellular parasites[1]. The viral proteins produced by the cell undergo partial proteolysis in the proteosomes, after which their fragments in conjunction with the molecules of the major histocompatibility complex type 1, are exposed on the cell surface to stimulate the immune system[2]. The partial proteolytic products of the cell's own proteins are exposed in a similar way. If these peptides have sufficient similarity to the partial proteolytic peptides of viral proteins, it is possible to develop an autoimmune cross-reaction in response to a viral infection[3]. Antiviral immunity is directed not only and not so much against the virus itself, but against the host cell infected with it, which serves as another predictor for the development of autoimmune aggression[4,5]. Among the autoantibodies produced in response to a viral infection, rheumatoid factor [(RF) an autoantibody against IgG], and cryoglobulins (antibodies that precipitate in the cold), are of utmost importance. The release of viral antigens into the bloodstream also plays a major role. Damage to the tissues, both directly by viruses and as a result of immune aggression against infected cells, also results in the release of a large number of tissue antigens. Tissue and viral antigens interact with antibodies to form immune complexes, which are fixed in the synovial membrane of the joints, glomerular membrane, and vascular wall, including the choroid. This leads to the development of arthritis, glomerulonephritis, vasculitis, and uveitis. Thus, viral infections can be triggers for autoimmune reactions and other immune disorders, which predispose to the development of rheumatologic and immunological complications of viral infections (Figure 1). However, the exact mechanisms of the development of autoimmune reactions in viral infections are yet to be established.

Many viruses can infect the liver, but only for five of them it is the main target organ: hepatitis A viruses (HAV), hepatitis B viruses (HBV), hepatitis C viruses (HCV), hepatitis D viruses (HDV) and hepatitis E viruses (HEV)[6]. HDV is a defective virus and can only replicate when co-infected with HBV[7].

Our review is devoted to describe the development of the aforementioned immunological disorders and caused by them rheumatologic and other extrahepatic manifestations in viral hepatitis. Knowing these complications is very important as infection with hepatotropic viruses can manifest only in the form of these disorders, leading to misdiagnosis and inadequate treatment.

HEPATITIS A

Hepatitis A occurs only in an acute form[8]. Perhaps due to the short-term contact of the macroorganism with the virus, immune and rheumatologic disorders rarely develop.

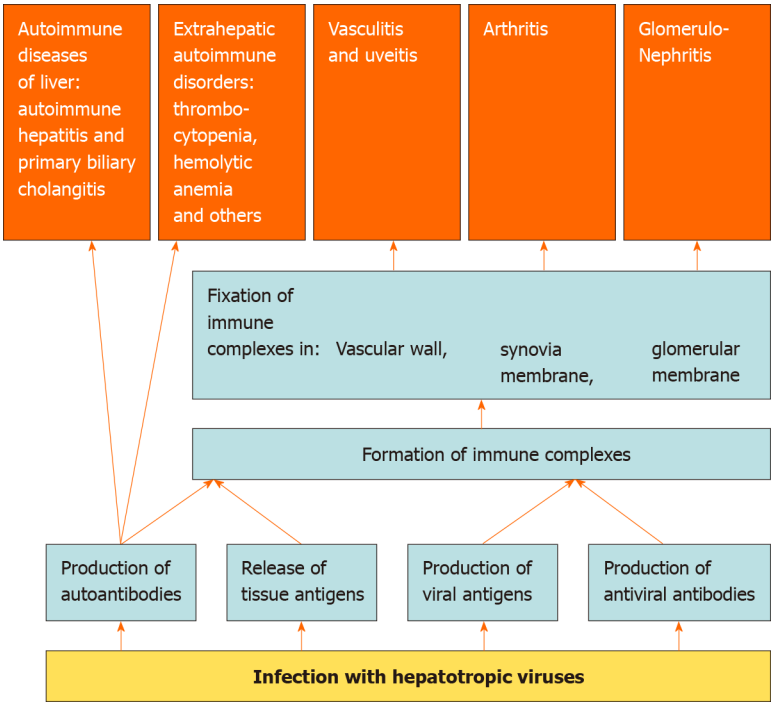


Figure 1 Scheme of the development of immunological disorders and caused by them rheumatologic and other extrahepatic manifestations of viral hepatitis.

Joint pain without arthritis occurs on average in 10%-20% of patients with hepatitis A[9-11]. True arthritis in hepatitis A develops within vasculitis (see below).

RF was found in only two patients with hepatitis A who had cryoglobulinemic vasculitis[12].

Cryoglobulinemia is observed in 95% of patients with hepatitis A in one study. Cryoprecipitate was represented by IgM, including antibodies against HAV. IgA and/or IgG were also represented in the cryoprecipitate of 15% of patients. After recovery, the cryoglobulin content decreased to normal. Interestingly, the cryoglubulin level in hepatitis A was higher than in acute hepatitis B[13].

Mesangioproliferative glomerulonephritis with deposits of IgM and complement components was found in a 7-year-old boy with hepatitis A. This was accompanied by the development of severe nephrotic syndrome, neutrophilic leukocytosis, and a decrease in the blood complement (components C3 and C4) levels. The disease ended in complete recovery[14].

In another case, mesangioproliferative glomerulonephritis in a young woman led to deposits of all three types of immunoglobulins and complement component C1q, and was accompanied by the development of acute renal failure, but not nephrotic syndrome; blood complement levels were normal. Complete recovery was observed here also[15].

The development of IgA nephropathy in hepatitis A has also been reported[16,17].

Polymyositis with myoglobulinuria, increased creatine kinase activity and electromyographic changes was verified by muscle biopsy in a patient with hepatitis A[18].

A 23-year-old man with hepatitis A developed adult-onset Still's syndrome: Fever, maculopapular rash on the trunk and legs, generalized arthralgia, severe neutrophilic leukocytosis, and hyperferritinemia. The disease was successfully controlled by glucocorticoids[19].

A case of unilateral autoimmune parotitis with left-sided pain and swelling in the face, generalized arthralgia, and rash was reported. Biopsy revealed mononuclear infiltration of the affected parotid gland. The disease was resistant to antibiotics, but was quickly treated with prednisolone[20].

We found 13 published cases of hepatitis A-associated vasculitis (Tables 1 and 2) [12,21-30]. These were Henoch-Schonlein purpura (HSP) (53.8%), cryoglobulinemic vasculitis (CGV) (30.8%), and isolated cutaneous necrotizing vasculitis (15.4%). Most of them (76.9%) occurred in children. The pathological process involved the skin (92.3%), joints (69.2%), intestines (46.2%) and kidneys (7.7%). Arthralgia without synovitis was observed in 15.4% of cases and arthritis in 54.8% of cases (mainly in the knees and

Table 1 Cases of vasculitis in hepatitis A

Case	Dan *et al*[21]	Press *et al*[22]	Nassih *et al*[23]	Chemli *et al*[24]	Mohan *et al*[25]	Altınkaynak *et al*[26]
Age, yr	30	2	8	10	< 1	10
Sex	Female	Female	Female	Male	Female	Male
Diagnosis	CNV	CNV	CGV	HSP	HSP	HSP
Body temperature	N	N/A	N	N/A	Mild fever	N
Time of the onset after the onset of hepatitis	2 wk	N/A	8 wk	3 d after admission to hospital	During the first month of illness	2 wk
Second wave of hepatitis	-	N/A	-	-	-	-
Pruritus	+	N/A	+	+	-	-
Jaundice	+	N/A	+	N/A	+	+
Rash	EPR over the hips, but also involving the buttocks and arms, and rare petechiae	Ecchymotic lesions	PP on the legs, forearms, and the back	PP on the declivous regions	Bluish PP on both lower limbs, swelling over dorsum of hands and feet	PP on the legs and on the gluteal regions.
Joints	N	N/A	Arthritis in the knees	Arthralgia	Arthritis in the right knee	Arthralgia
GN	No	N/A	Dipstick test was positive to proteins (2+) and blood (3+)	No	No	No
Gut	N	N/A	N	Abdominal pain	Small amount of blood in stool	Abdominal pain
RF	N/A	N/A	Negative	N/A	Negative	Negative
ANA	N/A	N/A	Negative	N/A	Negative	Negative
WBC, 10^9/L	6.3	N/A	7.2	N/A	↑	9.9
CRP, mg/L	N/A	N/A	38	N/A	N/A	N
Platelets, 10^9/L	408	N/A	N/A	N/A	N	416
ESR, mm/h	80	N/A	80	N/A	N/A	N/A
Cryoglobulins	N/A	N/A	IgM, IgA, and IgG	N/A	N/A	N/A
Transaminases	↑	N/A	↑	↑	↑	↑
Complement	N	N/A	N/A	N/A	N/A	N
Skin biopsy	LCV with deposition of IgM and C3	Necrotizing vasculitis with fibrin thrombi	LCV	N/A	LCV	N/A
Treatment	N/A	GC	GC	N/A	Analgetics	N/A

ANA: Antinuclear antibodies; CNV: Cutaneous necrotizing vasculitis; CRP: C-reactive protein; CGV: Cryoglobulinemic vasculitis; EPR: Erythematous papular rash; ESR: Erythrocyte sedimentation rate; GN: Glomerulonephritis; GC: Glucocorticoids; HSP: Henoch-Schonlein purpura; LCV: Leukocytoclastic vasculitis; N: Normal; N/A: No available; PP: Palpable purpura; RF: Rheumatoid factor.

ankles). Vasculitis developed about 5wk after the onset of the disease, and was accompanied by a second wave of hepatitis in 30.8% of cases and a protracted course (more than one month) of hepatitis in 23.1% of cases. All patients experienced complete recovery.

Experimental infection of monkeys marmosets with HAV led to the development of proliferative glomerulonephritis with deposits of IgM, component C3 (less often other immunoglobulins), and vasculitis[31].

Isolated cases of uveitis associated with hepatitis A have also been described[32,33]. Moreover, uveitis appeared before the symptoms of hepatitis[32].

One patient with hepatitis A developed lupus-like syndrome: symmetrical arthralgia in the wrists, metacarpophalangeal and proximal interphalangeal joints, left-sided pleurisy, Lupus cells, and antinuclear antibodies (ANA), antibodies against

Table 2 Cases of vasculitis in hepatitis A

Case	Garty *et al*[27]	Sasan *et al*[28]	Islek *et al*[29]	Islek *et al*[29]	Bozaykut *et al*[30]	Inman *et al*[12]	Inman *et al*[12]
Age, yr	8	8	13	11	< 1	26	26
Sex	Male	Male	Male	Female	Female	Female	Female
Diagnosis	HSP	HSP	HSP	HSP	CGV	CGV	CGV
Body temperature	N/A	37.2	N	N	N	N	N
Time of the onset after the onset of hepatitis	5 wk	5 wk	13 wk	5 wk	N/A	20 wk	19 wk
Second wave of hepatitis	-	-	+	+	N/A	+	+
Pruritus	-	-	-	+	-	-	-
Jaundice	-	-	-	+	-	+	-
Rash	PP on on the buttocks, penis and legs, which began with darkening of the right half of the scrotum	Non blanching red-brown papules over both thighs and legs	PP on the legs	PP on the legs	Oedema and ecchymosis on the dorsum of the hands and from feet to the knees	PP on on the legs, buttocks, arms, which began with ankles	No rash
Joint	N	Arthritis in the knees and ankles	Arthritis in the right knee and ankle	Arthritis in unspecified joints	N	Arthritis in the knees and ankles	Arthritis in the ankles, right fourth and fifth right metatarso-phalangeal joints
GN	No	No	No	No	No	No	No
Gut	N	Abdominal pain	Abdominal pain	Abdominal pain	N	N	N
RF	N/A	N/A	Negative	Negative	N/A	1:160	1:320
ANA	N/A	N/A	Negative	Negative	N/A	N/A	N/A
WBC, 10^9/L	N/A	10.1	4.6	7.2	12	N/A	N/A
CRP, mg/L	N/A	N/A	N	N/A	N/A	N/A	N/A
Platelets, 10^9/L	N/A	516	250	407	300	N/A	N/A
ESR, mm/h	N/A	34	22	42	N/A	N/A	N/A
Cryoglobulins	N/A	N/A	N/A	N/A	Positive	Anti-HAV IgG	Anti-HAV IgG
Transaminases	↑	N	↑	↑	N	↑	↑
Complement	N/A	N/A	N	C3↑, C4 - N	N	N/A	N/A
Skin biopsy	N/A	N/A	LCV with deposition of IgM in the dermo-epidermal junction	LCV	LCV	LCV	N/A
Treatment	N/A	GC	Sympto-matically	N/A	No	No	NSAID

ANA: Antinuclear antibodies; CRP: C-reactive protein; CGV: Cryoglobulinemic vasculitis; ESR: Erythrocyte sedimentation rate; GN: Glomerulonephritis; GC: Glucocorticoids; HSP: Henoch-schonlein purpura; LCV: Leukocytoclastic vasculitis; N: Normal; N/A: No available; NSAID: Nonsteroidal anti-inflammatory drugs; PP: Palpable purpura; RF: Rheumatoid factor.

double-stranded deoxyribonucleic acid (DNA) and cardiolipin, which disappeared within a few months[34].

Autoimmune hepatitis (AIH) was triggered by hepatitis A in 11 published cases[35-44]. In some of these patients, this was accompanied by the appearance of ANA. A case of a 24-year-old woman with an autoimmune hepatitis/primary biliary cirrhosis overlap syndrome triggered by an acute hepatitis A infection was

reported[45]. No case of isolated primary biliary cholangitis (PBC) associated with HAV was found.

Thus, immunological disorders and rheumatologic manifestations in hepatitis A are rare, have a favorable prognosis, and run their course without treatment or after treatment with glucocorticoids or non-steroidal anti-inflammatory drugs.

HEPATITIS B

Hepatitis B is characterized by lifelong infection, which predisposes to more frequent immunological disorders and caused by them rheumatologic and other extrahepatic manifestations.

Among patients with chronic hepatitis B, Raynaud's phenomenon occurs in 2%, arthralgia or arthritis in 3%, myalgia in 3%, Sjogren's syndrome in 3%, glomerulonephritis in 3%, uveitis in 2%, cryoglobulins in 2%[46].

RF is detected more often in asymptomatic carriers of HBV surface antigen (HBsAg) than in healthy individuals (11.8% *vs* 3.4%). RF positive rate was not significantly associated with the level of alanine aminotransferase or C-reactive protein in individuals with HBsAg[47]. HBV DNA levels significantly correlated with the titers of RF[48]. Antibodies against cyclic citrullated peptide (anti-CCP), a more specific marker of rheumatoid arthritis, were detected in individuals with HBsAg less often than was RF (4.6% *vs* 11.8%). Among patients with HBsAg, RF is found in 46% of patients with arthritis, 5% of patients with arthralgia, and in 8% of patients without rheumatologic complaints. Anti-CCP is found in 36%, 0% and less than 1% of these patients, respectively. Joint disorders and biochemical changes classified as rheumatoid arthritis were observed in 4.1% of patients with HBsAg. These patients accounted for 32.1% of HBV infected individuals with RF, 81.8% of those with anti-CCP and 90% of them have both RF and anti-CCP[49].

Patients with HBV infection accounted for 6.3% of patients with complaints of pain in joints and/or muscles. Moreover, only 26.3% of them had true arthritis[50].

HBV-associate non-rheumatoid arthritis can develop simultaneously in all affected joints, be migratory, or have an additive pattern. Synovitis develops abruptly and is severe. Arthritis develops within 12 wk after the onset of the disease, but in some cases, it is the first manifestation of hepatitis B. The age of patients with arthritis is 14-35 years in 82.8% of cases. Monoarthritis of a large joint is observed in about 40% of cases, polyarthritis of the small joints of the fingers in 10%, and a combined lesion of large and small joints in 50%. Among the large joints, the knee joints are most often affected, followed by the wrist, ankle, elbow, shoulder and hip joints. The metacarpophalangeal and proximal interphalangeal joints of the hands are affected as often as the knee joints, whereas the small joints of the feet are less frequently affected. Synovitis is usually symmetrical. The cervical and lumbar intervertebral joints are involved in about 10% of cases, usually together with other joints. ANA is determined in approximately 10% of such patients, and RF in approximately 25%, anti-CCP in approximately 5%. Complement is reduced in almost half of these patients. In all cases, arthritis resolved spontaneously or after treatment with non-steroidal anti-inflammatory drugs within 3-7 d. No development of chronic arthritis or recurrence of arthritis has been observed[49,51].

On average, 3% of glomerulonephritis is associated with the presence of HBsAg. It is membranous glomerulonephritis in 40% of cases, membranoproliferative glomerulonephritis in 20%, focal segmental glomerulosclerosis in 20%, IgA nephropathy in 10%; the remaining 10% account for the other forms. There are nephrotic syndrome in 60% of cases of HBV-associated glomerulonephritis, and nephritic syndrome or isolated changes in urine analysis in other cases[52].

Fibromyalgia is more often detected in patients with hepatitis B than in those without infection (32% *vs* 5%). Moreover, there was no difference in the incidence of this disease between patients with untreated active chronic hepatitis B, an inactive hepatitis B virus infection, and patients receiving treatment for this infection[53].

Autoantibodies are detected on average in 60% of patients with chronic hepatitis B. Most often, these are ANA (approximately 25%), anti-Ro52 (approximately 30%), anti-gp210 and anti-PML (approximately 10%), AMA-M2 (approximately 7%), anti-Sp100, anti-SMA, anti-LC-1 and anti-SLA/LP (all constituting approximately 3%), and anti-LKM-1 (< 1%). The frequency of detection of ANA was higher in the pre-cirrhotic stage than in cirrhosis (30% *vs* 20%). The frequency of detection of other autoantibodies did not differ significantly between these stages of the disease[54].

The high percentage of autoantibodies that are specific for PBC (anti-gp210, anti-PML, and AMA-M2) in HBV infection may indicate that HBV could be trigger for the development of this disease. Thus, in one study, signs of silent HBV infection (anti-HBc without HBsAg) were found in 40% of patients with PBC. Bilirubin level is higher and the degree of fibrosis is greater in these patients than in those with idiopathic PBC[55]. PBC will be diagnosed in 2%-3% of patients with hepatitis B over the next 15 years[56].

Specific biomarkers of AIH (anti-SMA, anti-LC-1, anti-SLA/LP, and anti-LKM-1) have been identified in 1%-3% of patients with hepatitis B[54]. Among patients with AIH, HBV DNA was detected in almost 25%, and serological biomarkers of HBV infection without signs of viral replication were found in another 30%[57].

About 35% of polyarteritis nodosa (PAN) cases are associated with hepatitis B. For this variant of the disease, neuropathies (approximately 85% *vs* 65%), arterial hypertension (49% *vs* 27%), abdominal pain (50% *vs* 28%), testicular involvement (24% *vs* 13%), cardiomyopathy (13% *vs* 4%) were more characteristic than for idiopathic PAN unlike livedo (10% *vs* 20%). The prognosis in patients with HBV-associated PAN was worse than in idiopathic PAN: 60% of patients from the first group and 74% from the second were alive after 10 years[58]. Hepatitis B was diagnosed before the development of PAN in approximately 30% of these patients. Vasculitis developed before the end of the clinical manifestations of hepatitis or in the next few days after this in half of these cases, and within the first 6 mo after this in the others. Transaminases are normal in 33%-50% of patients at the time of the onset of PAN[59,60].

Biomarkers of hepatitis B are detected in 2%-6% of cases with mixed cryoglobulinemia[61-63] and in almost 10% of cases with non-HCV CGV[64]. Manifestations of HBV-associated CGV are: Purpura (100%), arthralgias (71%), peripheral neuropathy (29%), glomerulonephritis (18%), Raynaud phenomenon (18%), and leg ulcer (6%)[65]. Cryoglobulins disappear in the serum leading to the regression of vasculitis in the majority of patients treated with entecavir, adefovir, and lamivudine. Corticosteroid therapy is effective for clinical symptoms of vasculitis, but ineffective for suppression of HBV and immunological features. Immunosuppressive agents are not recommended because of possible flare-up of viral replication. The use of interferons in these cases does not always lead to a positive effect[66].

There are only a few published cases of the association of HSP with hepatitis B[67-71].

Interestingly, according to a meta-analysis, biomarkers of hepatitis B are less common in systemic lupus erythematosus than in the general population: Odds ratio of HBsAg was 0.24 [95% confidence interval (CI): 0.17-0.33] and odds ratio of anti-HBc was 0.4 (95%CI: 0.31 - 0.50)[72].

HEPATITIS C

HCV (like HBV) persists for a long time in the body; therefore, the frequency of immunological disorders and rheumatologic manifestations is also quite high[73].

Patients with HCV infection accounted for 5.3% of patients with complaints of pain in the joints and/or muscles. Moreover, 40% of them had true arthritis, 50% had arthralgia, and 10% had no joint disorders[50].

Pain in the joints and/or muscles are noted in 70% of patients with chronic hepatitis C if the medical history was collected carefully. Backache is the most common complaint (54%), followed by early morning stiffness (45%), arthralgia (42%), myalgia (38%), neck pain (33%), generalized pain (21%), and subjective joint swelling (20%). Diffuse pain was present in 23% of patients, non-diffuse regional plus axial pain in 18%, axial pain in 17%, and regional pain in 12%[74].

Among patients with HCV having no rheumatologic complaints, RF is detected more than in the general population (15% *vs* 5%), unlike anti-CCP with the same incidence (about 5%)[75]. Patients with HCV and joint involvement have RF in 60% of cases, cryoglobulins in almost 50%, and both in 40%. Anti-CCP was detected only in patients without RF and cryoglobulins[76].

Cryoglobulins are found in almost 65% of a general population of HCV patients, but they clinically manifest only in 5% of them. The achievement of a stable viral response leads to a decrease in the detection rate of cryoglobulins from 57% to 33%, whereas the detection rate remains unchanged among those in whom it was not achieved. In approximately 20% of patients with cryoglobulinemia, it persists for 8 years after achieving a stable viral response. Moreover, 80% of these patients with persisted cryoglobulinemia have its clinical manifestations during this time[77]. The high

incidence of cryoglobulinemia in chronic hepatitis C can be explained by the fact that HCV can replicate in lymphocytes, protecting them from apoptosis and resulting in polyclonal proliferation, including clones that produce cryoglobulins[78].

Anti-HCV antibodies are detected in 92%-95% of patients with mixed cryoglobulinemia, and HCV RNA in 90%[61,63].

Arthritis in HCV infection develops in 5%-10% of cases[79]: Symmetric polyarthritis in 2/3 of cases, and oligo- or monoarthritis in the rest 1/3 of cases. Morning stiffness for more than 1 h occurs in 70% of HCV infected patients with arthritis, whereas erosion in the joints and subcutaneous nodules are not observed. Cryoglobulins, RF and ANA are detected in 43%, 60% and 20% of these cases, respectively. The level of complement C3 is reduced in 15% of these patients, and C4 in 30% of these patients. In addition, 86% of them had elevated transaminases. The use of anti-inflammatory or disease-modifying drugs is not effective[80], but the use of direct-acting antivirals (DAA) drugs is the most promising[81]. This arthritis is very similar to rheumatoid arthritis, and anti-CCP (unlike RF) should be used to differentiate between these diseases[82,83].

Autoantibodies are detected in 66% of patients with chronic hepatitis C. These are ANA (20%-32%), anti-LKM-1 (1%-22%), anti-Ro52 (about 15%), anti-SMA (3%-8%), AMA-M2 (approximately 3%), and anti-LC-1 (approximately 1%)[54,84].

Furthermore, 8%-12% of patients with PBC had biomarkers of HCV-infection (anti-HCV antibodies or RNA HCV)[85-87]. Despite the frequent detection of AIH-specific antibodies, it is very rarely diagnosed in HCV-infection[88]. HCV biomarkers are also rarely detected in AIH[89]. However, AIH was successfully treated with DAA in a patient with concomitant HCV infection[90].

Antibodies against HCV are detected in 40% of patients with glomerulonephritis[91]. Occult HCV infection (presence of HCV RNA by an ultrasensitive method in the absence of antibodies against HCV) is detected in 40% of the rest part of these patients: in 40% with membranous glomerulonephritis, 30% with membranoproliferative glomerulonephritis, 50% with IgA nephropathy, 30% with idiopathic nephrotic syndrome (including minimal change disease, focal segmental glomerulosclerosis, and IgM nephropathy), 50% with lupus nephropathy, and 40% with antineutrophil cytoplasmic antibody (ANCA) positive glomerulonephritis and 4% in the control group (hereditary glomerular nephropathy)[92]. The most common form of HCV-nephropathy is membranoproliferative glomerulonephritis, followed by focal segmental glomerulosclerosis, mesangioproliferative, and membranous glomer ulonephritis[91]. Anti-HCV is detected in 98% of cases of glomerulonephritis with mixed cryoglobulinemia and in 2% glomerulonephritis without cryoglobulinemia[93]. Autopsies of patients with hepatitis C revealed glomerulopathies: Mesangioproliferative glomerulonephritis (18%), membranoproliferative glomerulonephritis (11%), membranous glomerulonephritis (3%), and mesangial expansion without hypercellularity (23%). No glomerular pathology was observed in 45% of autopsies in hepatitis C[94].

The HCV antigen is detected in the glomeruli in almost 30% glomerulonephritis with antibodies against HCV in the blood and in almost 60% in glomerulonephritis with HCV RNA in the blood[95].

Extrarenal manifestations of HCV-glomerulopathy were absent in 80% of patients even though 54% had cryoglobulinemia. Electron microscopy revealed virus-like particles in 50% of renal biopsies[91].

There are publications showing the effectiveness of DAA in the treatment of HCV-nephropathy[96-98].

Among rheumatologic diseases in patients with HCV infection, Sjogren's syndrome is most often detected (almost 50% of cases) followed by rheumatoid arthritis (15%), systemic lupus erythematosus (11%), PAN (8%), antiphospholipid syndrome (6%), inflammatory myopathies (4%), systemic sclerosis (1%). The rest of rheumatic diseases, including HSP, accounted for less than 1%[99]. A meta-analysis showed that in patients with HCV infection, cryoglobulinemia [30% *vs* 2%, odd ratio (OR) is 11.5], CGV (5%), Sjogren's syndrome (12% *vs* 0.7%, odd ratio is 2.3) and arthritis (1% *vs* 0.1%, OR is 2.4) are significantly more often detected than in the general population[100]. Raynaud phenomenon was found in 8% of patients with HCV[101].

Vasculitis associated with HCV-infection are CGV in approximately 80% of cases and PAN in approximately 20% of cases[102]. Other vasculitis, including HSP, are rare[99].

Among patients with PAN, antibodies against HCV were detected in 20%, and HCV RNA in 5%[103]. PAN was diagnosed on average 2 years after the diagnosis of HCV infection. These patients had purpura (68%), livedo reticularis (20%-60%), arthralgia (61%), weight loss (60%), multiplex mononeuritis (70%), myalgias or weakness (58%),

altered arteriography (49%), hypertension (37%-55%), abdominal pain (30%), raised creatinine (26%), fever (20%), polyneuropathy (16%), proteinuria (16%), hematuria (16%), intestinal bleeding (16%), diarrhea (13%), orchitis (0%-7%)[99,102].

CGV was also diagnosed on average 2 years after the diagnosis of HCV infection. The most common manifestations of HCV-associated CGV are purpura (67%), polyneuropathy (65%), arthralgia (50%), proteinuria (30%), hematuria (22%), and arterial hypertension (22%). Myalgia (9%), multiplex mononeuritis (9%), livedo reticularis (3%), weight loss (4%), and abdominal pain (1.5%) are less common. Fever, intestinal bleeding, diarrhea, and orchitis are usually absent[102].

The differential diagnoses of vasculitis in HCV infection are presented in Table 3.

Rituximab allows to achieve remission in HCV-associated CGV in 87% of cases[104]. The effectiveness of DAA in the treatment of CGV in HCV-infection is under study. Experts currently recommend DAAs as first line treatment for mild to moderate CGV and rituximab with or without aphaeresis for severe cases[105].

Patients with HCV-associated Sjogren's syndrome present with xerophthalmia (97%), xerostomia (97%), positive Schirmer test (98%), altered salivary flow (81%), ANA (68%), RF (53%), and anti-Ro/La (25%)[99].

Very few cases of uveitis in HCV-infection were noted[106].

HEPATITIS E

Hepatitis E, as a rule, is an acute infection but it can become chronic in persons with immunodeficiency[107].

Joint or/and muscle pain without arthritis and myositis is present in approximately 60% of patients with acute hepatitis E[108]. Arthralgias were observed in 5% of patients with chronic hepatitis E[109].

Two cases of arthritis development in acute hepatitis E have been described (Table 4)[110,111].

ANA were found in 9% of patients with acute hepatitis E and in 24% of patients with chronic hepatitis E. Cryoglobulins were found in 7% and 27%, respectively. Cryoglobulinemia persisted for a median of 4 mo (range 3–15 mo). Patients with cryoglobulins had higher levels of creatinine, IgM, and HEV RNA in the blood. There were no clinical manifestations of cryoglobulinemia in all patients in this study except for a patient with neuralgic amyotrophy[112]. In another study, ANA was detected in 37% of patients with acute hepatitis E, and anti-SMA test was positive in 23% of them. These antibodies remained in the blood for more than 1 year in 37% of these cases. Moreover, no patients developed AIH during this follow-up period[113]. Similar results were published in the third study[114]. Seroprevalence of HEV in patients with AIH did not differ from that of the general population[115,116]. Another study showed that antibodies against HEV were detected slightly more often in AIH than the average for the population, but the detection rate was significantly lower than in the previous study[117]. In general, the relationship between HEV and AIH remains unclear.

Antibodies against HEV were not found in any of the 25 patients with PBC; thus, the relationship between these diseases is extremely unlikely[118].

ANCA was positive in 15% of patients with acute hepatitis E[114].

Most cases of glomerulonephritis and manifested cryoglobulinemia in hepatitis E have been described after transplantation[119,120]. Therefore, the exact cause of their development is unclear: HEV infection, the consequences of transplantation, or their combination. In any case, testing for HEV biomarkers should be performed in these patients. A cure for cryoglobulinemic membranoproliferative glomerulonephritis has been described in such a patient after treatment with ribavirin[121].

Thus far, only one case of HEV-associated cryoglobulinemic membranoproliferative glomerulonephritis in a non-transplanted person has been published. The condition appeared a month after the onset of acute hepatitis E. The activity of the inflammatory process in the liver remained elevated. This patient had RF in the blood and was successfully treated with plasmaphoresis and pulse glucocorticoids[122].

The only case of HSP triggered by acute hepatitis E virus infection has been described[123].

There are no published studies on the frequency of detection of HEV biomarkers in patients with uveitis or PAN.

Table 3 Differential diagnosis of vasculitis in hepatitis C viruses-infection (modification from[102])

	Cryoglobulinemic vasculitis	Polyarteritis nodosa
Weight loss	+/-	+++
Fever	-	++
Myalgia	+	++
Polyneuropathy	+++	++
Mononeuritis multiplex	+	+++
Livedo	+/-	++
Arterial hypertension	++	+++
Orchitis	-	+
Abdominal pain	+/-	++
Diarrhea	-	+
Intestinal bleeding	-	+
Microaneurysms or stenosis	-	+++
C-reactive protein level	Normal	↑

Table 4 Cases of arthritis in hepatitis E

Case	Al-Shukri *et al*[110]	Serratrice *et al*[111]
Age	52	51
Sex	Female	Female
Joints	Shoulders, elbows, hips, knees, ankles, left second and third metacarpophalangeal	Ankles and knees followed by the wrists and fingers
Duration of arthritis	No data	3 mo
Rash	Maculopapular, non-itchy rash all over her body	No
Fever	No	No
Jaundice	No	No
Pruritus	No	No
Other symptoms and signs	Retroorbital pain, eye discharge, headache, and loss of appetite	No
Rheumatoid factor	No	No
Antinuclear antibodies	No	No
White blood cells, 10^9/L	Normal	2.8
Erythrocyte sedimentation rate, mm/h	35	24
C-reactive protein, mg/L	25	3
Transaminases	↑	↑
Complement	C3 - ↑; C4 -Normal	No data
Treatment	No data	No specific treatment

CONCLUSION

There is a wide array of extrahepatic manifestations potentially associated with hepatitis viruses. These viruses can lead to the development of various immunological disorders (the formation of autoantibodies and cryoglobulins), which can manifest as glomerulonephritis, arthritis, uveitis, vasculitis, and other rheumatologic disorders. In addition, it is quite possible that this infection could be a trigger for the subsequent development of AIH and PBC.

Table 5 A summary of the main immunological and rheumatologic manifestation in viral hepatitis

	Hepatitis A	Hepatitis B	Hepatitis C	Hepatitis E
Rheumatoid factor	Only 2 cases	Approximately 12% of patients	15% of patients	1 case in a patient without transplant
Antinuclear antibodies	In a case of lupus-like reaction and some cases of AIH triggered by HAV infection	Approximately 25% of patients	20%-30% of patients	9%-37% (acute) and 24% (chronic) of patients
Cryoglobulins	95% of patients in 1 study	2% of patients	65% of patients	7% (acute) and 27% (chronic) of patients
Arthralgia	10%-20% of patients	3% of patients	42% of patients	Approximately 60% of patients
Arthritis	Only in patients with vasculitis (7 cases)	About a quarter of patients with pain in joints	5%-10% of patients	2 cases
GN	4 cases of mesangio-proliferative GN	3% of GN: membranous GN (40%); membrano-proliferative GN (20%); focal segmental glomerulo-sclerosis (20%); mesangio-proliferative GN (10%), others (10%)	Approximately 50% of GN: membranous GN (5%); membrano-proliferative GN (55%); focal segmental glomerulo-sclerosis (25%); mesangio-proliferative GN (20%), others (5%)	1 case of membrano-proliferative GN; some cases of GN after transplantation
Henoch-Schonlein purpura	7 cases	Some cases	Some cases	1 case
CGV	4 cases	10% of non-HCV CGV cases	90%-95% of CGV cases	Some cases
PAN	No cases	35% of PAN cases	20% of PAN cases	No cases
Uveitis	Some cases	2% of patients	Some cases	No cases
AIH	11 cases	55% of AIH cases	Very rare	No cases
PBC	1 case of overlap syndrome	40% of PBC cases	Approximately 10% of PBC cases	No cases

AIH: Autoimmune hepatitis; CGV: Cryoglobulinemic vasculitis; GN: Glomerulonephritis; HCV: Hepatitis C viruses; PAN: Polyarteritis nodosa; PBC: Primary biliary cholangitis.

The relationship between autoimmune liver diseases and hepatotropic virus infection is very interesting, especially when treated with antiviral drugs. A further study of this field would be useful to test for biomarkers of hepatotropic viruses in these diseases and to analyze the results of their treatment with antiviral drugs.

In addition to the manifestations described above, immunological disorders due to infection with hepatotropic viruses can lead to the development of many other autoimmune diseases (autoimmune thyroiditis, thrombocytopenia, hemolytic anemia, diabetes mellitus, pulmonary fibrosis, and others), the consideration of which is beyond the scope of this review.

These associations are best shown for chronic viral hepatitis B and C. Only isolated cases of these are described for hepatitis A. These links are least studied, and often controversial for hepatitis E, possibly due to its relatively rare diagnoses.

We have summarized the data presented in the review in Table 5.

To date, few studies have been published on the effectiveness of modern DAA in the treatment of patients with rheumatologic and autoimmune manifestations of hepatotropic virus infection, which represents a large field for future research.

Nevertheless, patients with uveitis, glomerulonephritis, arthritis, vasculitis, autoimmune liver diseases should be tested for biomarkers of viral hepatitis, and if these infections are present, they should be treated.

REFERENCES

1 **Yin J**, Redovich J. Kinetic Modeling of Virus Growth in Cells. *Microbiol Mol Biol Rev* 2018; **82**: e00066-17 [PMID: 29592895 DOI: 10.1128/MMBR.00066-17]

2 **Hewitt EW**. The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology* 2003; **110**: 163-169 [PMID: 14511229 DOI: 10.1046/j.1365-2567.2003.01738.x]

3 **Getts DR**, Chastain EM, Terry RL, Miller SD. Virus infection, antiviral immunity, and

autoimmunity. *Immunol Rev* 2013; **255**: 197-209 [PMID: 23947356 DOI: 10.1111/imr.12091]
4 **Panoutsakopoulou V**, Cantor H. On the relationship between viral infection and autoimmunity. *J Autoimmun* 2001; **16**: 341-345 [PMID: 11334502 DOI: 10.1006/jaut.2000.0480]
5 **Jara LJ**, Medina G, Saavedra MA. Autoimmune manifestations of infections. *Curr Opin Rheumatol* 2018; **30**: 373-379 [PMID: 29528865 DOI: 10.1097/BOR.0000000000000505]
6 **Gallegos-Orozco JF**, Rakela-Brödner J. Hepatitis viruses: not always what it seems to be. *Rev Med Chil* 2010; **138**: 1302-1311 [PMID: 21279280]
7 **Abbas Z**, Afzal R. Life cycle and pathogenesis of hepatitis D virus: A review. *World J Hepatol* 2013; **5**: 666-675 [PMID: 24409335 DOI: 10.4254/wjh.v5.i12.666]
8 **Jeong SH**, Lee HS. Hepatitis A: clinical manifestations and management. *Intervirology* 2010; **53**: 15-19 [PMID: 20068336 DOI: 10.1159/000252779]
9 **Routenberg JA**, Dienstag JL, Harrison WO, Kilpatrick ME, Hooper RR, Chisari FV, Purcell RH, Fornes MF. Foodborne outbreak of hepatitis A: clinical and laboratory features of acute and protracted illness. *Am J Med Sci* 1979; **278**: 123-137 [PMID: 517565 DOI: 10.1097/00000441-197909000-00003]
10 **Tong MJ**, el-Farra NS, Grew MI. Clinical manifestations of hepatitis A: recent experience in a community teaching hospital. *J Infect Dis* 1995; **171** Suppl 1: S15-S18 [PMID: 7876641 DOI: 10.1093/infdis/171.supplement_1.s15]
11 **Koff RS**. Clinical manifestations and diagnosis of hepatitis A virus infection. *Vaccine* 1992; **10** Suppl 1: S15-S17 [PMID: 1335649 DOI: 10.1016/0264-410x(92)90533-p]
12 **Inman RD**, Hodge M, Johnston ME, Wright J, Heathcote J. Arthritis, vasculitis, and cryoglobulinemia associated with relapsing hepatitis A virus infection. *Ann Intern Med* 1986; **105**: 700-703 [PMID: 3021038 DOI: 10.7326/0003-4819-105-5-700]
13 **Shalit M**, Wollner S, Levo Y. Cryoglobulinemia in acute type-A hepatitis. *Clin Exp Immunol* 1982; **47**: 613-616 [PMID: 7083634]
14 **Mathur RC**, Mathur NC. Mesangial proliferative glomerulonephritis and nephrotic syndrome with hepatitis A virus infection. *Indian Pediatr* 1996; **33**: 1051-1053 [PMID: 9141810]
15 **McCann UG 2nd**, Rabito F, Shah M, Nolan CR 3rd, Lee M. Acute renal failure complicating nonfulminant hepatitis A. *West J Med* 1996; **165**: 308-310 [PMID: 8993209]
16 **al-Homrany M**. Immunoglobulin A nephropathy associated with hepatitis A virus infection. *J Nephrol* 2001; **14**: 115-119 [PMID: 11411012]
17 **Cheema SR**, Arif F, Charney D, Meisels IS. IgA-dominant glomerulonephritis associated with hepatitis A. *Clin Nephrol* 2004; **62**: 138-143 [PMID: 15356971 DOI: 10.5414/cnp62138]
18 **Aggarwal SP**, Khurana SB, Sabharwal BD. Hepatitis A associated with myoglobinuria. *Indian J Gastroenterol* 1996; **15**: 107 [PMID: 8840643]
19 **Seo SR**, Kim SS, Lee SJ, Kim TJ, Park YW, Lee SS. Adult-onset Still disease in a patient with acute hepatitis A. *J Clin Rheumatol* 2011; **17**: 444-445 [PMID: 22089987 DOI: 10.1097/RHU.0b013e31823ac4ac]
20 **Bhatt G**, Sandhu VS, Mitchell CK. A rare presentation of hepatitis a infection with extrahepatic manifestations. *Case Rep Gastrointest Med* 2014; **2014**: 286914 [PMID: 25295197 DOI: 10.1155/2014/286914]
21 **Dan M**, Yaniv R. Cholestatic hepatitis, cutaneous vasculitis, and vascular deposits of immunoglobulin M and complement associated with hepatitis A virus infection. *Am J Med* 1990; **89**: 103-104 [PMID: 2368780 DOI: 10.1016/0002-9343(90)90107-o]
22 **Press J**, Maslovitz S, Avinoach I. Cutaneous necrotizing vasculitis associated with hepatitis A virus infection. *J Rheumatol* 1997; **24**: 965-967 [PMID: 9150090]
23 **Nassih H**, Bourrahouat A, Sab IA. Hepatitis A Virus Infection Associated with Cryoglobulinemic Vasculitis. *Indian Pediatr* 2020; **57**: 71-72 [PMID: 31937705 DOI: 10.1007/s13312-020-1709-x]
24 **Chemli J**, Zouari N, Belkadhi A, Abroug S, Harbi A. [Hepatitis A infection and Henoch-Schonlein purpura: a rare association]. *Arch Pediatr* 2004; **11**: 1202-1204 [PMID: 15475276 DOI: 10.1016/j.arcped.2004.06.014]
25 **Mohan N**, Karkra S. Henoch schonlein purpura as an extra hepatic manifestation of hepatitis A. *Indian Pediatr* 2010; **47**: 448 [PMID: 20519793]
26 **Altinkaynak S**, Ertekin V, Selimoglu MA. Association of Henoch-Schonlein purpura and hepatitis A. *J Emerg Med* 2006; **30**: 219-220 [PMID: 16567262 DOI: 10.1016/j.jemermed.2005.12.011]
27 **Garty BZ**, Danon YL, Nitzan M. Schoenlein-Henoch purpura associated with hepatitis A infection. *Am J Dis Child* 1985; **139**: 547 [PMID: 4003354 DOI: 10.1001/archpedi.1985.02140080017017]
28 **Sasan MS**, Doghaee MA. Association of henoch-schoenlein purpura with hepatitis a. *Iran J Pediatr* 2012; **22**: 571-572 [PMID: 23429757]
29 **Islek I**, Kalayci AG, Gok F, Muslu A. Henoch-Schönlein purpura associated with hepatitis A infection. *Pediatr Int* 2003; **45**: 114-116 [PMID: 12654084 DOI: 10.1046/j.1442-200x.2003.01657.x]
30 **Bozaykut A**, Atay E, Atay Z, Ipek IO, Akin M, Dursun E. Acute infantile haemorrhagic oedema associated with hepatitis A. *Ann Trop Paediatr* 2002; **22**: 59-61 [PMID: 11926052 DOI: 10.1179/027249302125000175]
31 **Morita M**, Kitajima K, Yoshizawa H, Itoh Y, Iwakiri S, Shibata C, Mayumi M. Glomerulonephritis associated with arteritis in marmosets infected with hepatitis A virus. *Br J Exp Pathol* 1981; **62**: 103-113 [PMID: 6452891]
32 **Azimi A**, Shirvani M, Hosseini S, Bazojoo V, Masihpoor N, Mohaghegh S, Sadeghi SM. Acute

bilateral granulomatous anterior uveitis as an extra-hepatic manifestation of hepatitis A virus (HAV) infection: a case report. *J Ophthalmic Inflamm Infect* 2020; **10**: 18 [PMID: 32851489 DOI: 10.1186/s12348-020-00210-6]

33 **Tien PT**, Lin CJ, Tsai YY, Chen HS, Hwang DK, Muo CH, Lin JM, Chen WL. Relationship Between Uveitis, Different Types Of Viral Hepatitis, And Liver Cirrhosis: A 12-Year Nationwide Population-Based Cohort Study. *Retina* 2016; **36**: 2391-2398 [PMID: 27870801 DOI: 10.1097/IAE.0000000000001103]

34 **Segev A**, Hadari R, Zehavi T, Schneider M, Hershkoviz R, Mekori YA. Lupus-like syndrome with submassive hepatic necrosis associated with hepatitis A. *J Gastroenterol Hepatol* 2001; **16**: 112-114 [PMID: 11206308 DOI: 10.1046/j.1440-1746.2001.02314.x]

35 **S-Are V**, Yoder L, Samala N, Nephew L, Lammert C, Vuppalanchi R. An Outbreak Presents An Opportunity to Learn About A Rare Phenotype: Autoimmune Hepatitis After Acute Hepatitis A. *Ann Hepatol* 2020; **19**: 694-696 [PMID: 32927125 DOI: 10.1016/j.aohep.2020.08.069]

36 **Subramanian SK**, Patel JM, Younes M, Nevah Rubin MI. Postinfectious Autoimmune Hepatitis-Induced Liver Failure: A Consequence of Hepatitis A Virus Infection. *ACG Case Rep J* 2020; **7**: e00441 [PMID: 32821769 DOI: 10.14309/crj.0000000000000441]

37 **Grave A**, Juel J, Vyberg M, Olesen SS, Hansen JB. [Autoimmune hepatitis preceded by hepatitis A]. *Ugeskr Laeger* 2015; **177**: V12140669 [PMID: 25822815]

38 **Tanaka H**, Tujioka H, Ueda H, Hamagami H, Kida Y, Ichinose M. Autoimmune hepatitis triggered by acute hepatitis A. *World J Gastroenterol* 2005; **11**: 6069-6071 [PMID: 16273628 DOI: 10.3748/wjg.v11.i38.6069]

39 **Kim YD**, Kim KA, Rou WS, Lee JS, Song TJ, Bae WK, Kim NH. [A case of autoimmune hepatitis following acute hepatitis A]. *Korean J Gastroenterol* 2011; **57**: 315-318 [PMID: 21623141 DOI: 10.4166/kjg.2011.57.5.315]

40 **Mikata R**, Yokosuka O, Imazeki F, Fukai K, Kanda T, Saisho H. Prolonged acute hepatitis A mimicking autoimmune hepatitis. *World J Gastroenterol* 2005; **11**: 3791-3793 [PMID: 15968741 DOI: 10.3748/wjg.v11.i24.3791]

41 **Huppertz HI**, Treichel U, Gassel AM, Jeschke R, Meyer zum Büschenfelde KH. Autoimmune hepatitis following hepatitis A virus infection. *J Hepatol* 1995; **23**: 204-208 [PMID: 7499793 DOI: 10.1016/0168-8278(95)80336-x]

42 **Singh G**, Palaniappan S, Rotimi O, Hamlin PJ. Autoimmune hepatitis triggered by hepatitis A. *Gut* 2007; **56**: 304 [PMID: 17303607 DOI: 10.1136/gut.2006.111864]

43 **Bouyahia O**, Naija O, Matoussi N, Khemiri M, Ben Mansour F, Khaldi F. [Autoimmune hepatitis following acute hepatitis A: a care report]. *Tunis Med* 2008; **86**: 87-88 [PMID: 19472711]

44 **Skoog SM**, Rivard RE, Batts KP, Smith CI. Autoimmune hepatitis preceded by acute hepatitis A infection. *Am J Gastroenterol* 2002; **97**: 1568-1569 [PMID: 12094893 DOI: 10.1111/j.1572-0241.2002.05751.x]

45 **Heurgué A**, Bernard-Chabert B, Picot R, Cadiot G, Thiéfin G. Overlap syndrome triggered by acute viral hepatitis A. *Eur J Gastroenterol Hepatol* 2009; **21**: 708-709 [PMID: 19282766 DOI: 10.1097/MEG.0b013e3282ffda23]

46 **Cacoub P**, Saadoun D, Bourlière M, Khiri H, Martineau A, Benhamou Y, Varastet M, Pol S, Thibault V, Rotily M, Halfon P. Hepatitis B virus genotypes and extrahepatic manifestations. *J Hepatol* 2005; **43**: 764-770 [PMID: 16087273 DOI: 10.1016/j.jhep.2005.05.029]

47 **Shim CN**, Hwang JW, Lee J, Koh EM, Cha HS, Ahn JK. Prevalence of rheumatoid factor and parameters associated with rheumatoid factor positivity in Korean health screening subjects and subjects with hepatitis B surface antigen. *Mod Rheumatol* 2012; **22**: 885-891 [PMID: 22327743 DOI: 10.1007/s10165-012-0603-3]

48 **Choi ST**, Lee HW, Song JS, Lee SK, Park YB. Analysis of rheumatoid factor according to various hepatitis B virus infectious statuses. *Clin Exp Rheumatol* 2014; **32**: 168-173 [PMID: 24143967]

49 **Lim MK**, Sheen DH, Lee YJ, Mun YR, Park M, Shim SC. Anti-cyclic citrullinated peptide antibodies distinguish hepatitis B virus (HBV)-associated arthropathy from concomitant rheumatoid arthritis in patients with chronic HBV infection. *J Rheumatol* 2009; **36**: 712-716 [PMID: 19286846 DOI: 10.3899/jrheum.080653]

50 **Oliveira ÍMX**, Silva RDSUD. Rheumatological Manifestations Associated with Viral Hepatitis B or C. *Rev Soc Bras Med Trop* 2019; **52**: e20180407 [PMID: 31800917 DOI: 10.1590/0037-8682-0407-2018]

51 **Duffy J**, Lidsky MD, Sharp JT, Davis JS, Person DA, Hollinger FB, Min KW. Polyarthritis, polyarteritis and hepatitis B. *Medicine (Baltimore)* 1976; **55**: 19-37 [PMID: 1629 DOI: 10.1097/00005792-197601000-00002]

52 **Raveendran N**, Beniwal P, D'Souza AV, Tanwar RS, Kimmatkar P, Agarwal D, Malhotra V. Profile of glomerular diseases associated with hepatitis B and C: A single-center experience from India. *Saudi J Kidney Dis Transpl* 2017; **28**: 355-361 [PMID: 28352020 DOI: 10.4103/1319-2442.202761]

53 **Yazmalar L**, Deveci Ö, Batmaz İ, İpek D, Çelepkolu T, Alpaycı M, Hattapoğlu E, Akdeniz D, Sarıyıldız MA. Fibromyalgia incidence among patients with hepatitis B infection. *Int J Rheum Dis* 2016; **19**: 637-643 [PMID: 26133007 DOI: 10.1111/1756-185X.12593]

54 **Li BA**, Liu J, Hou J, Tang J, Zhang J, Xu J, Song YJ, Liu AX, Zhao J, Guo JX, Chen L, Wang H, Yang LH, Lu J, Mao YL. Autoantibodies in Chinese patients with chronic hepatitis B: prevalence and clinical associations. *World J Gastroenterol* 2015; **21**: 283-291 [PMID: 25574103 DOI: 10.3748/wjg.v21.i1.283]

55 **Zhang Y**, Shi Y, Wu R, Wang X, Gao X, Niu J. Primary biliary cholangitis is more severe in previous hepatitis B virus infection patients. *Eur J Gastroenterol Hepatol* 2018; **30**: 682-686 [PMID: 29462025 DOI: 10.1097/MEG.0000000000001100]

56 **Rigopoulou EI**, Zachou K, Gatselis NK, Papadamou G, Koukoulis GK, Dalekos GN. Primary biliary cirrhosis in HBV and HCV patients: Clinical characteristics and outcome. *World J Hepatol* 2013; **5**: 577-583 [PMID: 24179617 DOI: 10.4254/wjh.v5.i10.577]

57 **Chen XX**, Xiang KH, Zhang HP, Kong XS, Huang CY, Liu YM, Lou JL, Gao ZH, Yan HP. Occult HBV infection in patients with autoimmune hepatitis: A virological and clinical study. *J Microbiol Immunol Infect* 2020; **53**: 946-954 [PMID: 31153830 DOI: 10.1016/j.jmii.2019.04.009]

58 **Pagnoux C**, Seror R, Henegar C, Mahr A, Cohen P, Le Guern V, Bienvenu B, Mouthon L, Guillevin L; French Vasculitis Study Group. Clinical features and outcomes in 348 patients with polyarteritis nodosa: a systematic retrospective study of patients diagnosed between 1963 and 2005 and entered into the French Vasculitis Study Group Database. *Arthritis Rheum* 2010; **62**: 616-626 [PMID: 20112401 DOI: 10.1002/art.27240]

59 **Guillevin L**, Mahr A, Callard P, Godmer P, Pagnoux C, Leray E, Cohen P; French Vasculitis Study Group. Hepatitis B virus-associated polyarteritis nodosa: clinical characteristics, outcome, and impact of treatment in 115 patients. *Medicine (Baltimore)* 2005; **84**: 313-322 [PMID: 16148731 DOI: 10.1097/01.md.0000180792.80212.5e]

60 **Guillevin L**, Lhote F, Jarrousse B, Bironne P, Barrier J, Deny P, Trepo C, Kahn MF, Godeau P. Polyarteritis nodosa related to hepatitis B virus. A retrospective study of 66 patients. *Ann Med Interne (Paris)* 1992; **143** Suppl 1: 63-74 [PMID: 1363769]

61 **Ferri C**, Sebastiani M, Giuggioli D, Cazzato M, Longombardo G, Antonelli A, Puccini R, Michelassi C, Zignego AL. Mixed cryoglobulinemia: demographic, clinical, and serologic features and survival in 231 patients. *Semin Arthritis Rheum* 2004; **33**: 355-374 [PMID: 15190522 DOI: 10.1016/j.semarthrit.2003.10.001]

62 **Monti G**, Galli M, Invernizzi F, Pioltelli P, Saccardo F, Monteverde A, Pietrogrande M, Renoldi P, Bombardieri S, Bordin G. Cryoglobulinaemias: a multi-centre study of the early clinical and laboratory manifestations of primary and secondary disease. GISC. Italian Group for the Study of Cryoglobulinaemias. *QJM* 1995; **88**: 115-126 [PMID: 7704562]

63 **Mazzaro C**, Maso LD, Mauro E, Gattei V, Ghersetti M, Bulian P, Moratelli G, Grassi G, Zorat F, Pozzato G. Survival and Prognostic Factors in Mixed Cryoglobulinemia: Data from 246 Cases. *Diseases* 2018; **6** [PMID: 29751499 DOI: 10.3390/diseases6020035]

64 **Galli M**, Oreni L, Saccardo F, Castelnovo L, Filippini D, Marson P, Mascia MT, Mazzaro C, Origgi L, Ossi E, Pietrogrande M, Pioltelli P, Quartuccio L, Scarpato S, Sollima S, Riva A, Fraticelli P, Zani R, Giuggioli D, Sebastiani M, Sarzi Puttini P, Gabrielli A, Zignego AL, Scaini P, Ferri C, De Vita S, Monti G. HCV-unrelated cryoglobulinaemic vasculitis: the results of a prospective observational study by the Italian Group for the Study of Cryoglobulinaemias (GISC). *Clin Exp Rheumatol* 2017; **35** Suppl 103: 67-76 [PMID: 28466806]

65 **Mazzaro C**, Dal Maso L, Urraro T, Mauro E, Castelnovo L, Casarin P, Monti G, Gattei V, Zignego AL, Pozzato G. Hepatitis B virus related cryoglobulinemic vasculitis: A multicentre open label study from the Gruppo Italiano di Studio delle Crioglobulinemie - GISC. *Dig Liver Dis* 2016; **48**: 780-784 [PMID: 27106525 DOI: 10.1016/j.dld.2016.03.018]

66 **Mazzaro C**, Dal Maso L, Visentini M, Gitto S, Andreone P, Toffolutti F, Gattei V. Hepatitis B virus-related cryogobulinemic vasculitis. The role of antiviral nucleot(s)ide analogues: a review. *J Intern Med* 2019; **286**: 290-298 [PMID: 31124596 DOI: 10.1111/joim.12913]

67 **Kurokawa M**, Hisano S, Ueda K. Hepatitis B virus and Schönlein-Henoch purpura. *Am J Dis Child* 1985; **139**: 861-862 [PMID: 4036912 DOI: 10.1001/archpedi.1985.02140110015013]

68 **Maggiore G**, Martini A, Grifeo S, De Giacomo C, Scotta MS. Hepatitis B virus infection and Schönlein-Henoch purpura. *Am J Dis Child* 1984; **138**: 681-682 [PMID: 6731386 DOI: 10.1001/archpedi.1984.02140450063019]

69 **Ergin S**, Sanli Erdoğan B, Turgut H, Evliyaoğlu D, Yalçin AN. Relapsing Henoch-Schönlein purpura in an adult patient associated with hepatitis B virus infection. *J Dermatol* 2005; **32**: 839-842 [PMID: 16361739 DOI: 10.1111/j.1346-8138.2005.tb00856.x]

70 **Stemerowicz R**, Möller B, Lobeck H, Oertel J, Hopf U. [Schoenlein-Henoch purpura in chronic HBsAG-positive hepatitis]. *Immun Infekt* 1988; **16**: 12-15 [PMID: 3360462]

71 **Shin JI**, Lee JS. Hepatitis B virus infection and Henoch-Schönlein purpura. *J Dermatol* 2007; **34**: 156; author reply 157 [PMID: 17239160 DOI: 10.1111/j.1346-8138.2006.00240.x]

72 **Wang S**, Chen Y, Xu X, Hu W, Shen H, Chen J. Prevalence of hepatitis B virus and hepatitis C virus infection in patients with systemic lupus erythematosus: a systematic review and meta-analysis. *Oncotarget* 2017; **8**: 102437-102445 [PMID: 29254259 DOI: 10.18632/oncotarget.22261]

73 **Kuna L**, Jakab J, Smolic R, Wu GY, Smolic M. HCV Extrahepatic Manifestations. *J Clin Transl Hepatol* 2019; **7**: 172-182 [PMID: 31293918 DOI: 10.14218/JCTH.2018.00049]

74 **Barkhuizen A**, Rosen HR, Wolf S, Flora K, Benner K, Bennett RM. Musculoskeletal pain and fatigue are associated with chronic hepatitis C: a report of 239 hepatology clinic patients. *Am J Gastroenterol* 1999; **94**: 1355-1360 [PMID: 10235218 DOI: 10.1111/j.1572-0241.1999.01087.x]

75 **Orge E**, Cefle A, Yazici A, Gürel-Polat N, Hulagu S. The positivity of rheumatoid factor and anti-cyclic citrullinated peptide antibody in nonarthritic patients with chronic hepatitis C infection. *Rheumatol Int* 2010; **30**: 485-488 [PMID: 19547976 DOI: 10.1007/s00296-009-0997-1]

76 **Liu FC**, Chao YC, Hou TY, Chen HC, Shyu RY, Hsieh TY, Chen CH, Chang DM, Lai JH.

Usefulness of anti-CCP antibodies in patients with hepatitis C virus infection with or without arthritis, rheumatoid factor, or cryoglobulinemia. *Clin Rheumatol* 2008; **27**: 463-467 [PMID: 17876647 DOI: 10.1007/s10067-007-0729-4]

77 **Cheng YT**, Cheng JS, Lin CH, Chen TH, Lee KC, Chang ML. Rheumatoid factor and immunoglobulin M mark hepatitis C-associated mixed cryoglobulinaemia: an 8-year prospective study. *Clin Microbiol Infect* 2020; **26**: 366-372 [PMID: 31229596 DOI: 10.1016/j.cmi.2019.06.018]

78 **Fuentes A**, Mardones C, Burgos PI. Understanding the Cryoglobulinemias. *Curr Rheumatol Rep* 2019; **21**: 60 [PMID: 31741077 DOI: 10.1007/s11926-019-0859-0]

79 **Ferucci ED**, Choromanski TL, Varney DT, Ryan HS, Townshend-Bulson LJ, McMahon BJ, Wener MH. Prevalence and correlates of hepatitis C virus-associated inflammatory arthritis in a population-based cohort. *Semin Arthritis Rheum* 2017; **47**: 445-450 [PMID: 28532574 DOI: 10.1016/j.semarthrit.2017.04.004]

80 **Zuckerman E**, Keren D, Rozenbaum M, Toubi E, Slobodin G, Tamir A, Naschitz JE, Yeshurun D, Rosner I. Hepatitis C virus-related arthritis: characteristics and response to therapy with interferon alpha. *Clin Exp Rheumatol* 2000; **18**: 579-584 [PMID: 11072597]

81 **Koga T**, Kawashiri SY, Nakao K, Kawakami A. Successful ledipasvir + sofosbuvir treatment of active synovitis in a rheumatoid arthritis patient with hepatitis C virus-related mixed cryoglobulinemia. *Mod Rheumatol* 2017; **27**: 917-918 [PMID: 27848256 DOI: 10.1080/14397595.2016.1253814]

82 **Siloşi I**, Boldeanu L, Biciuşcă V, Bogdan M, Avramescu C, Taisescu C, Padureanu V, Boldeanu MV, Dricu A, Siloşi CA. Serum Biomarkers for Discrimination between Hepatitis C-Related Arthropathy and Early Rheumatoid Arthritis. *Int J Mol Sci* 2017; **18** [PMID: 28629188 DOI: 10.3390/ijms18061304]

83 **Ezzat WM**, Raslan HM, Aly AA, Emara NA, El Menyawi MM, Edrees A. Anti-cyclic citrullinated peptide antibodies as a discriminating marker between rheumatoid arthritis and chronic hepatitis C-related polyarthropathy. *Rheumatol Int* 2011; **31**: 65-69 [PMID: 19882340 DOI: 10.1007/s00296-009-1225-8]

84 **Amin K**, Rasool AH, Hattem A, Al-Karboly TA, Taher TE, Bystrom J. Autoantibody profiles in autoimmune hepatitis and chronic hepatitis C identifies similarities in patients with severe disease. *World J Gastroenterol* 2017; **23**: 1345-1352 [PMID: 28293081 DOI: 10.3748/wjg.v23.i8.1345]

85 **Chen HW**, Huang HH, Lai CH, Chang WE, Shih YL, Chang WK, Hsieh TY, Chu HC. Hepatitis C virus infection in patients with primary biliary cirrhosis. *Ann Hepatol* 2013; **12**: 78-84 [PMID: 23293197 DOI: 10.1016/S1665-2681(19)31388-2]

86 **Floreani A**, Baragiotta A, Leone MG, Baldo V, Naccarato R. Primary biliary cirrhosis and hepatitis C virus infection. *Am J Gastroenterol* 2003; **98**: 2757-2762 [PMID: 14687829 DOI: 10.1111/j.1572-0241.2003.08717.x]

87 **Bertolini E**, Battezzati PM, Zermiani P, Bruno S, Moroni GA, Marelli F, Villa E, Manenti F, Zuin M, Crosignani A. Hepatitis C virus testing in primary biliary cirrhosis. *J Hepatol* 1992; **15**: 207-210 [PMID: 1324271 DOI: 10.1016/0168-8278(92)90037-p]

88 **Badiani RG**, Becker V, Perez RM, Matos CA, Lemos LB, Lanzoni VP, Andrade LE, Dellavance A, Silva AE, Ferraz ML. Is autoimmune hepatitis a frequent finding among HCV patients with intense interface hepatitis? *World J Gastroenterol* 2010; **16**: 3704-3708 [PMID: 20677344 DOI: 10.3748/wjg.v16.i29.3704]

89 **Dvir R**, Sautto GA, Mancini N, Racca S, Diotti RA, Clementi M, Memoli M. Autoimmune hepatitis and occult HCV infection: A prospective single-centre clinical study. *Autoimmun Rev* 2017; **16**: 323-325 [PMID: 28161557 DOI: 10.1016/j.autrev.2017.01.015]

90 **López Couceiro L**, Gómez Domínguez E, Muñoz Gómez R, Castellano Tortajada G, Ibarrola de Andrés C, Fernández Vázquez I. Healing of autoimmune hepatitis associated with hepatitis C virus infection treated with direct-acting antivirals. *Rev Esp Enferm Dig* 2019; **111**: 159-161 [PMID: 30449122 DOI: 10.17235/reed.2018.5528/2018]

91 **Sabry AA**, Sobh MA, Irving WL, Grabowska A, Wagner BE, Fox S, Kudesia G, El Nahas AM. A comprehensive study of the association between hepatitis C virus and glomerulopathy. *Nephrol Dial Transplant* 2002; **17**: 239-245 [PMID: 11812873 DOI: 10.1093/ndt/17.2.239]

92 **Castillo I**, Martinez-Ara J, Olea T, Bartolomé J, Madero R, Hernández E, Bernis C, Aguilar A, Quiroga JA, Carreño V, Selgas R. High prevalence of occult hepatitis C virus infection in patients with primary and secondary glomerular nephropathies. *Kidney Int* 2014; **86**: 619-624 [PMID: 24646855 DOI: 10.1038/ki.2014.68]

93 **Misiani R**, Bellavita P, Fenili D, Borelli G, Marchesi D, Massazza M, Vendramin G, Comotti B, Tanzi E, Scudeller G. Hepatitis C virus infection in patients with essential mixed cryoglobulinemia. *Ann Intern Med* 1992; **117**: 573-577 [PMID: 1326246 DOI: 10.7326/0003-4819-117-7-573]

94 **Arase Y**, Ikeda K, Murashima N, Chayama K, Tsubota A, Koida I, Suzuki Y, Saitoh S, Kobayashi M, Kumada H. Glomerulonephritis in autopsy cases with hepatitis C virus infection. *Intern Med* 1998; **37**: 836-840 [PMID: 9840704 DOI: 10.2169/internalmedicine.37.836]

95 **Cao Y**, Zhang Y, Wang S, Zou W. Detection of the hepatitis C virus antigen in kidney tissue from infected patients with various glomerulonephritis. *Nephrol Dial Transplant* 2009; **24**: 2745-2751 [PMID: 19377056 DOI: 10.1093/ndt/gfp167]

96 **Nayak S**, Kataria A, Sharma MK, Rastogi A, Gupta E, Singh A, Tiwari SC. Hepatitis C Virus-associated Membranoproliferative Glomerulonephritis Treated with Directly Acting Antiviral Therapy. *Indian J Nephrol* 2018; **28**: 462-464 [PMID: 30647501 DOI: 10.4103/ijn.IJN_235_17]

97 **Shimada M**, Nakamura N, Endo T, Yamabe H, Nakamura M, Murakami R, Narita I, Tomita H. Daclatasvir/asunaprevir based direct-acting antiviral therapy ameliorate hepatitis C virus-associated cryoglobulinemic membranoproliferative glomerulonephritis: a case report. *BMC Nephrol* 2017; **18**: 109 [PMID: 28356063 DOI: 10.1186/s12882-017-0534-5]

98 **Obata F**, Murakami T, Miyagi J, Ueda S, Inagaki T, Minato M, Ono H, Nishimura K, Shibata E, Tamaki M, Yoshimoto S, Kishi F, Kishi S, Matsuura M, Nagai K, Abe H, Doi T. A case of rapid amelioration of hepatitis C virus-associated cryoglobulinemic membranoproliferative glomerulonephritis treated by interferon-free directly acting antivirals for HCV in the absence of immunosuppressant. *CEN Case Rep* 2017; **6**: 55-60 [PMID: 28509128 DOI: 10.1007/s13730-016-0244-z]

99 **Ramos-Casals M**, Muñoz S, Medina F, Jara LJ, Rosas J, Calvo-Alen J, Brito-Zerón P, Forns X, Sánchez-Tapias JM; HISPAMEC Study Group. Systemic autoimmune diseases in patients with hepatitis C virus infection: characterization of 1020 cases (The HISPAMEC Registry). *J Rheumatol* 2009; **36**: 1442-1448 [PMID: 19369460 DOI: 10.3899/jrheum.080874]

100 **Younossi Z**, Park H, Henry L, Adeyemi A, Stepanova M. Extrahepatic Manifestations of Hepatitis C: A Meta-analysis of Prevalence, Quality of Life, and Economic Burden. *Gastroenterology* 2016; **150**: 1599-1608 [PMID: 26924097 DOI: 10.1053/j.gastro.2016.02.039]

101 **Lee YH**, Ji JD, Yeon JE, Byun KS, Lee CH, Song GG. Cryoglobulinaemia and rheumatic manifestations in patients with hepatitis C virus infection. *Ann Rheum Dis* 1998; **57**: 728-731 [PMID: 10070272 DOI: 10.1136/ard.57.12.728]

102 **Saadoun D**, Terrier B, Semoun O, Sene D, Maisonobe T, Musset L, Amoura Z, Rigon MR, Cacoub P. Hepatitis C virus-associated polyarteritis nodosa. *Arthritis Care Res (Hoboken)* 2011; **63**: 427-435 [PMID: 20981809 DOI: 10.1002/acr.20381]

103 **Carson CW**, Conn DL, Czaja AJ, Wright TL, Brecher ME. Frequency and significance of antibodies to hepatitis C virus in polyarteritis nodosa. *J Rheumatol* 1993; **20**: 304-309 [PMID: 8097250]

104 **Sneller MC**, Hu Z, Langford CA. A randomized controlled trial of rituximab following failure of antiviral therapy for hepatitis C virus-associated cryoglobulinemic vasculitis. *Arthritis Rheum* 2012; **64**: 835-842 [PMID: 22147444 DOI: 10.1002/art.34322]

105 **Mazzaro C**, Mauro E, Ermacora A, Doretto P, Fumagalli S, Tonizzo M, Toffolutti F, Gattei V. Hepatitis C virus- related cryoglobulinemic vasculitis. *Minerva Med* 2020 [PMID: 33198444 DOI: 10.23736/S0026-4806.20.07120-7]

106 **Disdier P**, Bolla G, Veit V, Ridings B, Gambarelli-Mouillac N, Harle JR, Weiller PJ. [Association of uveitis and hepatitis C. 5 cases]. *Presse Med* 1994; **23**: 541 [PMID: 8022745]

107 **European Association for the Study of the Liver**. ; European Association for the Study of the Liver. EASL Clinical Practice Guidelines on hepatitis E virus infection. *J Hepatol* 2018; **68**: 1256-1271 [PMID: 29609832 DOI: 10.1016/j.jhep.2018.03.005]

108 **Shinde N**, Patil T, Deshpande A, Gulhane R, Patil M, Bansod Y. Clinical profile, maternal and fetal outcomes of acute hepatitis e in pregnancy. *Ann Med Health Sci Res* 2014; **4**: S133-S139 [PMID: 25184080 DOI: 10.4103/2141-9248.138033]

109 **Kamar N**, Garrouste C, Haagsma EB, Garrigue V, Pischke S, Chauvet C, Dumortier J, Cannesson A, Cassuto-Viguier E, Thervet E, Conti F, Lebray P, Dalton HR, Santella R, Kanaan N, Essig M, Mousson C, Radenne S, Roque-Afonso AM, Izopet J, Rostaing L. Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. *Gastroenterology* 2011; **140**: 1481-1489 [PMID: 21354150 DOI: 10.1053/j.gastro.2011.02.050]

110 **Al-Shukri I**, Davidson E, Tan A, Smith DB, Wellington L, Johannessen I, Ramalingam S. Rash and arthralgia caused by hepatitis E. *Lancet* 2013; **382**: 1856 [PMID: 24290589 DOI: 10.1016/S0140-6736(13)62074-7]

111 **Serratrice J**, Disdier P, Colson P, Ene N, de Roux CS, Weiller PJ. Acute polyarthritis revealing hepatitis E. *Clin Rheumatol* 2007; **26**: 1973-1975 [PMID: 17340044 DOI: 10.1007/s10067-007-0595-0]

112 **Horvatits T**, Schulze Zur Wiesch J, Polywka S, Buescher G, Lütgehetmann M, Hussey E, Horvatits K, Peine S, Haag F, Addo MM, Lohse AW, Weiler-Normann C, Pischke S. Significance of Anti-Nuclear Antibodies and Cryoglobulins in Patients with Acute and Chronic HEV Infection. *Pathogens* 2020; **9** [PMID: 32947995 DOI: 10.3390/pathogens9090755]

113 **Wu J**, Guo N, Zhu L, Zhang X, Xiong C, Liu J, Xu Y, Fan J, Yu J, Pan Q, Yang J, Liang H, Jin X, Ye S, Wang W, Liu C, Zhang J, Li G, Jiang B, Cao H, Li L. Seroprevalence of AIH-related autoantibodies in patients with acute hepatitis E viral infection: a prospective case-control study in China. *Emerg Microbes Infect* 2020; **9**: 332-340 [PMID: 32037983 DOI: 10.1080/22221751.2020.1722759]

114 **Terziroli Beretta-Piccoli B**, Ripellino P, Gobbi C, Cerny A, Baserga A, Di Bartolomeo C, Bihl F, Deleonardi G, Melidona L, Grondona AG, Mieli-Vergani G, Vergani D, Muratori L; Swiss Autoimmune Hepatitis Cohort Study Group. Autoimmune liver disease serology in acute hepatitis E virus infection. *J Autoimmun* 2018; **94**: 1-6 [PMID: 30336842 DOI: 10.1016/j.jaut.2018.07.006]

115 **Llovet LP**, Gratacós-Ginés J, Ortiz O, Rodriguez-Tajes S, Lens S, Reverter E, Ruiz-Ortiz E, Costa J, Viñas O, Forns X, Parés A, Londoño MC. Higher seroprevalence of hepatitis E virus in autoimmune hepatitis: Role of false-positive antibodies. *Liver Int* 2020; **40**: 558-564 [PMID: 31863722 DOI: 10.1111/Liv.14332]

116 **van Gerven NM**, van der Eijk AA, Pas SD, Zaaijer HL, de Boer YS, Witte BI, van Nieuwkerk CM,

Mulder CJ, Bouma G, de Man RA; Dutch Autoimmune Hepatitis Study Group. Seroprevalence of Hepatitis E Virus in Autoimmune Hepatitis Patients in the Netherlands. *J Gastrointestin Liver Dis* 2016; **25**: 9-13 [PMID: 27014749 DOI: 10.15403/jgld.2014.1121.251.hpe]

117 **Pischke S**, Gisa A, Suneetha PV, Wiegand SB, Taubert R, Schlue J, Wursthorn K, Bantel H, Raupach R, Bremer B, Zacher BJ, Schmidt RE, Manns MP, Rifai K, Witte T, Wedemeyer H. Increased HEV seroprevalence in patients with autoimmune hepatitis. *PLoS One* 2014; **9**: e85330 [PMID: 24465537 DOI: 10.1371/journal.pone.0085330]

118 **Le Cann P**, Tong MJ, Werneke J, Coursaget P. Detection of antibodies to hepatitis E virus in patients with autoimmune chronic active hepatitis and primary biliary cirrhosis. *Scand J Gastroenterol* 1997; **32**: 387-389 [PMID: 9140163 DOI: 10.3109/00365529709007689]

119 **Bazerbachi F**, Leise MD, Watt KD, Murad MH, Prokop LJ, Haffar S. Systematic review of mixed cryoglobulinemia associated with hepatitis E virus infection: association or causation? *Gastroenterol Rep (Oxf)* 2017; **5**: 178-184 [PMID: 28852522 DOI: 10.1093/gastro/gox021]

120 **Fousekis FS**, Mitselos IV, Christodoulou DK. Extrahepatic manifestations of hepatitis E virus: An overview. *Clin Mol Hepatol* 2020; **26**: 16-23 [PMID: 31601068 DOI: 10.3350/cmh.2019.0082]

121 **Del Bello A**, Guilbeau-Frugier C, Josse AG, Rostaing L, Izopet J, Kamar N. Successful treatment of hepatitis E virus-associated cryoglobulinemic membranoproliferative glomerulonephritis with ribavirin. *Transpl Infect Dis* 2015; **17**: 279-283 [PMID: 25708383 DOI: 10.1111/tid.12353]

122 **Guinault D**, Ribes D, Delas A, Milongo D, Abravanel F, Puissant-Lubrano B, Izopet J, Kamar N. Hepatitis E Virus-Induced Cryoglobulinemic Glomerulonephritis in a Nonimmunocompromised Person. *Am J Kidney Dis* 2016; **67**: 660-663 [PMID: 26682764 DOI: 10.1053/j.ajkd.2015.10.022]

123 **Thapa R**, Biswas B, Mallick D. Henoch-Schönlein purpura triggered by acute hepatitis E virus infection. *J Emerg Med* 2010; **39**: 218-219 [PMID: 19201130 DOI: 10.1016/j.jemermed.2008.10.004]

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MINIREVIEWS

Neurological manifestations of hepatitis E virus infection: An overview

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Abstract

Hepatitis E virus (HEV) is an important cause of repeated waterborne outbreaks of acute hepatitis. Recently, several extrahepatic manifestations (EHMs) have been described in patients with HEV infection. Of these, neurological disorders are the most common EHM associated with HEV. The involvement of both the peripheral nervous system and central nervous system can occur together or in isolation. Patients can present with normal liver function tests, which can often be misleading for physicians. There is a paucity of data on HEV-related neurological manifestations; and these data are mostly described as case reports and case series. In this review, we analyzed data of 163 reported cases of HEV-related neurological disorders. The mechanisms of pathogenesis, clinico-demographic profile, and outcomes of the HEV-related neurological disorders are described in this article. Nerve root and plexus disorder were found to be the most commonly reported disease, followed by meningoencephalitis.

Key Words: Hepatitis E virus; Acute hepatitis E; Extrahepatic manifestations; Neurological manifestations; Neuralgic amyotrophy; Guillain-Barré syndrome

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Core Tip: Neurological involvement in patients with hepatitis E virus (HEV) infection is rare. There is a paucity of data on HEV-related neurological manifestations. This

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review comprehensively describes the mechanisms of pathogenesis, clinico-demographic profile, and outcomes of HEV-related neurological disorders. Nerve root and plexus disorder were the most commonly reported diseases followed by meningo-encephalitis. These patients can present with normal liver function tests, which can often be misleading for physicians.

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INTRODUCTION

The hepatitis E virus (HEV) is a non-enveloped, positive-sense, single-stranded ribonucleic acid (RNA) virus. It belongs to the *Hepeviridae* family and is one of the most common causes of acute hepatitis globally. Currently, HEV is represented by one serotype and eight genotypes (GTs)[1]; of these, four GTs (GT1-4) are mainly responsible for human infection. Epidemiological characteristics of GT1-4 of HEV have been summarized in Table 1.

HEV is an important cause of repeated waterborne outbreaks of acute hepatitis. While HEV outbreaks have mainly been reported in Asia and Africa, it has recently been identified as an emerging issue in public health in Western countries. However, the global burden of HEV infection remains unclear. The recent meta-analysis estimated that approximately 939 million of the global population have experienced HEV infection once, and 15-110 million individuals have had a recent or have an ongoing infection. The seroprevalence rates of HEV infection in Africa, Asia, Europe, and America are 21.76%, 15.80%, 9.31%, and 7%-8% respectively[2]. The World Health Organization (WHO) estimated 14 million symptomatic cases of HEV infection, with 0.3 million deaths and 5.2 thousand stillbirths each year worldwide[3]. More than 50% of global HEV deaths were recorded in the WHO South-East Asia Region (SEAR), which includes India, Bangladesh, Thailand, the Democratic People's Republic of Korea, Myanmar, Nepal, Bhutan, Sri Lanka, Indonesia, Maldives, and Timor-Leste. In the WHO SEAR countries, annual symptomatic cases of HEV are estimated to be 6.5 million, with 0.16 million deaths and 2.7 thousand stillbirths[3].

Symptomatic HEV infection can present with anicteric or icteric acute hepatitis. It can also rarely lead to fulminant liver failure, and HEV infection further carries high mortality (25%) in pregnancy. While most cases of HEV-related acute hepatitis spontaneously resolve within 4-8 wk, HEV sometimes causes chronic infection, especially in immunosuppressed patients[4]. Chronic HEV infection has been defined as the persistence of HEV replication for more than 6 mo[5]. GT3 and GT4 (mainly GT3) can cause chronic infection, resulting in liver fibrosis and cirrhosis. HEV infection is also an important cause of acute-on-chronic liver failure (ACLF), especially in South-East Asian countries. In our prospective study, we observed that the hepatitis virus infection is the most common acute insult identified in patients with ACLF. Overall, the most common hepatitis virus superinfection was HEV (23% cases)[6]. In another study, Kumar *et al*[7] demonstrated a 44% HEV superinfection rate in a sample of patients with chronic liver disease.

Recently, several extrahepatic manifestations (EHMs) were described in patients with HEV infection (Table 2). These EHMs were reported as case–control studies and case series or reports during the last decades. In a recent systemic review, Rawla *et al*[8] analyzed data of 324 reported cases of EHM patients with HEV infection. The most common EHMs were neurological (55%), cardiovascular or hematological (35%), and gastrointestinal manifestations (7%). Rare manifestations included renal (1.24%), endocrine (0.31%), and skin disease (0.31%), as well as manifestations in the respiratory (0.31%), muscular (0.31%), and immune system (0.31%).

This review is summarizes the available evidence regarding neurological manifestations in patients with HEV infection. To this end, we analyzed the literature related to the neurological manifestations associated with HEV. These reported manifestations were classified as central nervous system (CNS) disorders, nerve root and plexus disorders, neuropathy, cranial neuropathy, neuromuscular junction disorders, muscle

Table 1 Hepatitis E virus: Epidemiological features

Genotype	Hosts/reservoir	Mode of transmission	Geographic distribution	Remarks	Reported neurological manifestations[1]
GT1	Only humans	Fecal-oral transmission	Mostly Asia and Africa	Large outbreaks; Self-limiting; High mortality in pregnancy	Yes (2 cases)
GT2	Only humans	Fecal-oral transmission	Africa and Mexico	Large outbreaks; Self-limiting; High mortality in pregnancy	None
GT3	Human, swine, wild boar, goat, cattle, deer, camel and yak	Zoonotic infections in humans; Blood transfusion	Worldwide including Europe and America	Can cause chronic infection in organ transplant patients	Yes (56 cases)
GT4	Human, swine, wild boar, goat, cattle, deer, camel and yak	Zoonotic infections in humans; Blood transfusion	China, Japan, South-east Asia	Can cause chronic infection in organ transplant patients	Yes (4 cases)

[1]See Table 3 for reported neurological manifestations. GT: Genotype.

Table 2 Relationship of hepatitis E virus infection with extrahepatic manifestations

Extrahepatic manifestations	Likelihood of causal relationship, Hill criteria[9,97]	Evidence to support a casual role
Neurological disorders		
Neuralgic amyotrophy	Very probable	Good
Guillain-Barré syndrome	Very probable	Good
Meningoencephaltis	Very probable	Good
Others[1]	Possible/under debate	Remains to be established
Kidney disorders		
Membranoproliferative glomerulonephritis	Very probable	Good
Membranous glomerulonephritis	Very probable	Good
IgA nephropathy	Very probable	Good
Gastrointestinal disorder		
Acute pancreatitis	Very probable	Remains to be established
Hematological diseases		
Thrombocytopenia, monoclonal gammopathy of uncertain significance, Cryoglobulinemia, hemolytic anemia, aplastic anemia	Possible	Remains to be established
Miscellaneous		
Autoimmune hepatitis, myocarditis, thyroiditis	Doubtful/under debate	Remains to be established

[1]See Table 3 for other neurological disorders. IgA: Immunoglobulin A.

disorders, and other miscellaneous manifestations.

RELATIONSHIP OF HEV INFECTION WITH EHMs

Various EHMs have a temporal relationship with HEV infections; however, whether it is a coincidence or an actual causal relationship remains unclear. A causal relationship between HEV and neurological has been proposed on the basis of a few well-described case-control studies, which showed a significantly higher seroprevalence of HEV-associated neuralgic amyotrophy (NA) and Guillain-Barré syndrome (GBS) compared to non-HEV-associated cases. Moreover, several case studies have documented the presence of HEV RNA in the cerebrospinal fluid (CSF). Although the authors

explained this association on the basis of existing evidence, the pathophysiological basis of a causal relationship remains unknown. There is good evidence to support a causal role of HEV in associated conditions such as NA, GBS, meningoencephalitis, membranoproliferative glomerulonephritis, membranous glomerulonephritis, and immunoglobulin (Ig) A nephropathy[5,9]. Relationships between other EHMs and HEV are based on case reports only; therefore, causality has yet to be established (Table 2).

MECHANISMS OF PATHOGENESIS

The pathogenesis of EHMs in HEV infection is unclear. The mechanisms by which HEV can induce EHM may be caused by either direct or indirect mechanisms. Direct mechanisms involve HEV replication in affected tissues, resulting in cellular damage, whereas indirect mechanisms are caused by cross-reactive immune triggers, the formation of immune complexes, or by secondary infection[10]. Direct mechanisms are supported by *in vitro* studies. HEV can infect neuronal cells *in vitro* and human neuronal-derived cells support the full-length replication of viral RNA and translation of viral capsid protein[11,12]. Indirect immune trigger-mediated mechanisms appear to be more relevant in pathogenesis. Both humoral and cellular immune responses are likely to be involved in the pathogenesis of hepatic and EHMs. Furthermore, the prevalence of EHM is more common in immunocompetent patients than immunocompromised patients. In a study, the neurological manifestations were found to be significantly more common (22.6% as against 3.2%) in immunocompetent patients (n = 137) compared to immunocompromised patients (n = 63). The higher frequency of neurological disorders in immunocompetent patients suggests immune-mediated mechanisms causing EHMs[13]. In another study, all patients with NA were immunocompetent[14]. A schematic diagram of the potential mechanisms of EHM is shown in Figure 1.

NEUROLOGICAL MANIFESTATIONS

The neurologic manifestations of HEV infection are being increasingly recognized. Retrospective studies from Europe revealed 5.5%-7.5% of neurological manifestations in HEV-infected patients[15,16]. Furthermore, two recent European prospective studies showed an even higher prevalence of neurological symptoms (16.5%-31%) in HEV-infected patients. Ripellino *et al*[14] indicated 31% prevalence of neurological symptoms in HEV-infected patients. However, two-third of patients had myalgia only, and none of these patients underwent brachial plexus MRI or muscle biopsy. In another recent prospective case-control study, out of 200 HEV-infected patients, 33 (16.5%) showed neurological symptoms. The most frequent manifestation was neuropathic pain (42%), which suggests small fiber neuropathy. However, the results of their neurological examination were normal, and extensive investigations were not performed to detect small fiber neuropathy[13]. The prevalence of HEV infection in patients with non-traumatic neurological injury ranges from 2.4% to 6.9%. In a multicentric European study, 2.4% of 464 patients with non-traumatic neurological injury showed evidence of recent HEV infection. Symptoms of hepatitis were mild or absent and all patients were anicteric[17]. Another study from France demonstrated a high seroprevalence (6.9%) of positive anti-HEV IgM in a cohort (n = 159) of patients with acute non-traumatic, non-vascular neurological injuries as compared to seroprevalence in blood donors (0.4%) at the same period of time[18,19]. However, a recent study from China showed a similar HEV seroprevalence in patients with acute non-traumatic neuropathy (n = 1117) and healthy controls (n = 1415) (0.54% *vs* 0.68%; P = 0.65)[20].

Involvement of both the peripheral nervous system and CNS can occur together or in isolation. The various neurologic syndromes that have been reported in patients with HEV infection are shown in Table 3. Approximately 163 cases have been described to date, wherein the mean age of patients was 53.9 years with a male to female ratio (M:F) of 2.84:1. Neurological disorders are most commonly reported in Europe (74%) and SEAR nations (15%), mainly France, India, and Bangladesh. Nerve root and plexus disorders [NA (39%) and GBS (37%)] were the most commonly reported HEV-related neurological disorders, followed by CNS disorder [meningoencephalitis (4%)]. The majority of patients (88%) had normal bilirubin levels. Alanine aminotransferase (ALT) levels were widely variable [median (range): 345.5 (16-4502)

Table 3 Hepatitis E virus-related neurological manifestations

Diseases	*n* (%)	Geographic distribution	Mean age in yr	M:F ratio	Serological diagnosis	Serum HEV-RNA	Genotype	CSF HEV-RNA	Elevated bilirubin, *n*	Median (range) ALT in IU/L	Use of anti-viral	Recovery
Nerve root and plexus disorders												
Neuralgic amyotrophy[13,15-17,28-56]	64 (39)	Europe: 62; SEAR: 1; United States: 1	45.7	6.5:1	51	44	GT3: 28	0	4	1007 (22-2579)	9	CR: 9; PR: 47; NM: 8
Guillain-Barré syndrome[13,15,16,20,36,58-85]	61 (37)	SEAR: 21; Europe: 30; China/Hong Kong: 5; Japan: 4; Iraq: 1	51.16	2.42:1	46	20	GT3: 10; GT4: 1; GT1: 2	3	11	1950 (57-4502)	1	CR: 23; PR: 14; Died: 1; NM: 23
Central nervous system disorder												
Meningoencephalitis[17,20,35,86,87]	6 (4)	Europe: 2; China: 2; SEAR: 1; United States: 1	44	1:1	6	5	GT 3: 2	2	0	142 (20-479)	1	CR: 5; NM: 1
Cerebral ischemia[17,20]	5 (3)	Europe: 3; China: 2	66	4:1	5	4	GT3: 2	0	0	18 (8-28)	0	CR: 3; PR: 2
Seizures[17]	2 (1)	Europe	71	1:1	2	1	GT3: 1	0	0	19 (10-28)	0	CR: 2
Transverse myelitis[88]	1 (0.6)	Europe	62	0:1	1	1	GT3: 1	1	0	1152	0	PR: 1
Neuropathy												
Mononeuritis multiplex[36]	6 (4)	Europe	53.16	1:1	6	4	GT3: 3	0	1	188 (118-3641)	1	CR: 5; PR: 1
Peripheral neuropathy[16,20,89]	5 (3)	Europe: 4; China: 1	57.4	3:2	5	5	GT3: 2	2	1	285 (30-1606)	1	CR: 2; PR: 3
Cranial neuropathy												
Cranial neuropathy[16,17,90-92]	5 (3)	SEAR: 2; Europe: 2; Japan: 1	53	4:1	**2**	2	GT3: 1; GT4: 3; ND	0	0	1200 (60-3866)	0	CR: 5
Neuromuscular junction and muscle disorders												
Myositis[93]	1 (0.6)	Europe	57	1:0	1	1	ND	0	1	1030	1	CR:1
Myasthenia gravis[18]	1 (0.6)	Europe	33	0:1	1	1	GT3: 1	0	0	190	1	NM: 1
Others neurological disorders												
Meningoradiculitis[36,94,95]	6 (4)	Europe	53.33	1:1	6	6	GT3: 5	4	1	406 (40-822)	1	CR: 5; Died: 1
Total cases, *n* (%)												
Total	163	Europe: 120 (74); WHO SEAR: 25 (15);	53.9	2.84:1	132 (81)	94 (58)	GT3: 56 (90);	12 (7)	19 (12)	345.5 (16-4502)	Ribavirin:	CR: 60 (46); PR: 68

	China/Hong Kong: 10 (6), Japan: 4; United States: 2; Iraq: 1	GT4: 4; GT1: 2	16 (10)	(53) Died:2; NM 33 (20)

ALT: Alanine aminotransferase; CR: Complete recovery; CSF: Cerebrospinal fluid; GT: Genotype; HEV: Hepatitis E virus; M:F ratio: Male to female ratio; NM: Not mention; PR: Partial recovery; SEAR: South-East Asia Region; WHO: World Health Organization.

IU/L]. HEV RNA was detected in serum in 94 (58%) cases. Genotyping was performed in 62 (38%) cases, which revealed that GT3 is the main GT (90%) associated with neurological disorders. HEV RNA was identified in CSF in 12 (7%) cases. Patients were mainly treated with supportive measures, intravenous Ig (IVIG), plasmapheresis, steroids, and physiotherapy. Antiviral treatment (ribavirin) was given to 16 (10%) patients only. Of the 163 patients, the follow-up details of 130 patients were available. Complete recovery was observed in 60 (46%) patients, while partial recovery or long-term disability and death were noted in 68 (53%) and 2 patients respectively.

Neurological disorders have also been described in cases of viral hepatitis A, B, and C[21-26]. Hepatitis C virus infection is often associated with neuropsychiatric disorders including peripheral neuropathy (mainly sensory), cognitive impairment, and cerebrovascular accidents. Encephalitis, myelitis, encephalomyelitis, and GBS are rarely described in patients with hepatitis C virus infection[21,22]. Hepatitis A virus infection is rarely associated with neurological disorders, especially GBS and encephalitis. Other hepatitis A-related neurological disorders include meningo-encephalitis, meningitis, transverse myelitis, peripheral neuropathy, optic neuritis, and neuromuscular junction and muscular disorders[23-25]. Hepatitis B virus infection is rarely associated with neurological disorders, especially GBS and peripheral neuropathy[26]. GBS and peripheral neuropathy have been reported with viral hepatitis A, B, C, and E. It is interesting to note that NA is described in HEV infection alone.

The causal association of HEV infection with GBS, NA, and meningoencephalitis is supported by good evidence, including well-described case cohorts and a large number of case reports. However, the association of HEV infection with other neurological diseases is primarily based on a small number of case reports without sufficient evidence to establish a causal relationship.

NERVE ROOT AND PLEXUS DISORDERS

NA

NA — also known as Parsonage-Turner syndrome or brachial plexus neuritis—is a distinct disorder, with core features that include episodes of severe shoulder and arm pain at symptom onset, rapid multifocal paresis, and atrophy of the upper extremity muscles, and a slow recovery, requiring a few months to several years. The disease is

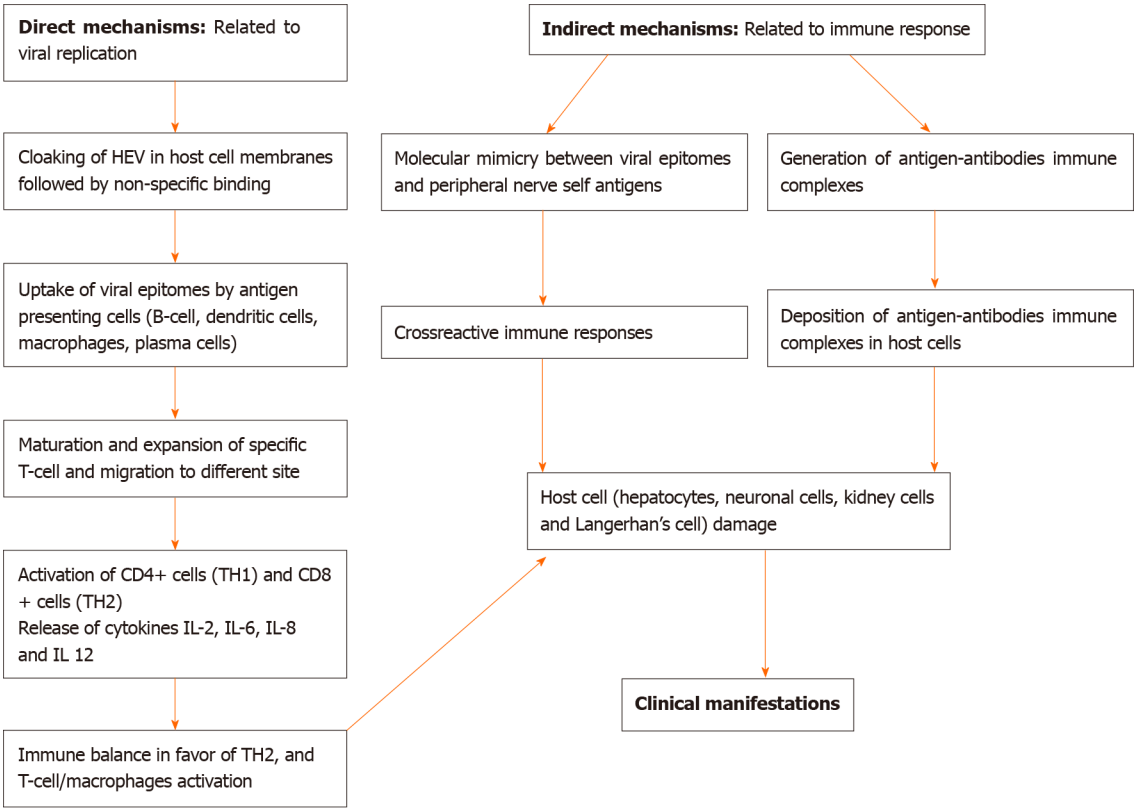


Figure 1 Schematic diagram demonstrating the potential mechanisms of extrahepatic manifestations of hepatitis E virus infection. HEV: Hepatitis E virus; IL: Interleukin.

characterized by the patchy distribution of motor, sensory, and autonomic symptoms. Any part of the brachial plexus and the lumbosacral plexus can be involved, with any combination of motor and sensory impairment. Involvement of nerves from regions other than the brachial plexus is noted in about one-fourth of patients. The pathogenesis of NA is unknown, but it may share its pathogenesis mechanism with post-infectious GBS. No currently available tests can unequivocally confirm or exclude NA, although needle electromyography (EMG) can help in estimating axonal damage and reinnervation. Exclusion of other disorders such as cervical disc herniation and neoplasms of the superior sulcus region are usually required[27].

NA is likely to be causally associated with HEV infection. In a study, acute HEV infection was identified in 10.6% of NA patients (n = 47)[28]. Another study demonstrated a high seroprevalence (6.9%) of acute HEV infection in a cohort of patients with acute non-traumatic, non-vascular neurological injuries (n = 159). Notably, more than half of the NA patients (57%) showed positive anti-HEV IgM assays[18]. Recently, a multi-center retrospective data analysis was performed for the comparison of HEV-associated NA (n = 57) and NA without HEV infection (n = 61). HEV-NA patients were found to have significantly more bilateral involvement (80% *vs* 9%), damage outside the brachial plexus (59% *vs* 11%), including phrenic nerve and lumbosacral plexus injury (25% *vs* 4% and 26% *vs* 7.0% respectively), sensory symptoms, and reduced reflexes. Despite more extensive damage to the brachial plexus in HEV-associated NA, the outcome was similar in the two groups. About 90% of patients with HEV-associated NA were anicteric[29].

Approximately 64 cases of HEV-associated NA have been reported to date[13,15-17,28-56]. The salient clinico-demographic features of NA are summarized in Tables 3 and 4. HEV-associated NA is predominantly seen in middle-aged males (mean age: 45.7 years; M:F = 6.5:1). Genotyping was conducted in 28 cases; all were found to be GT3. All patients were immunocompetent. All patients, except two, were from European countries. About 90% of the cases had bilateral involvement (89% *vs* 11% unilateral). Predominantly right-sided involvement was observed in 60% of the patients with bilateral involvement, whereas right-sided involvement was seen in 83% of the patients with unilateral involvement. An EMG was performed in 34 of the 64 cases and revealed denervation and/or damage of brachial plexus in 31 cases. Almost all patients (94%) had normal bilirubin levels and ALT levels were variable [median

Table 4 Clinical description of nerve root and plexus disorders, *n* = 125

Nerve root and plexus disorders	Source of infection	Hepatitis-neurological involvement interval	Type of involvement, *n* (%)	Specific remarks, *n* (%)
Neuralgic amyotrophy (*n* = 64)	Described in 9. Sausage figatelli: 3; Vegetables: 2; Uncooked pork: 1; Manipulation of horse manure: 1; Travel history: 2	Described in 29. Mean delay: 8.4 d	Described in 54. Brachial plexus involvement: Bilateral 48 (89) [right > left: 15 (60); left > right: 10 (40); not described: 23]; Unilateral 6 (11) [right: 5 (83); left: 1]	94% had normal bilirubin levels. EMG findings mentioned: 34; Denervation and/or damage of brachial plexus: 31 (91)
Guillain-Barré syndrome (*n* = 61)	Described in 5. Sausage figatelli: 2; Uncooked pork: 1; Dear meat: 1; Contact with farm animals: 1	Described in 51. Mean delay:15 d; Concomitant: 6 patients	Described in 38. Acute inflammatory demyelinating polyneuropathy: 28 (74); Acute motor-sensory axonal neuropathy: 5 (13); Acute motor axonal neuropathy: 4 (11); Miller Fisher syndrome: 1 (3)	82% had normal bilirubin levels; Anti-ganglioside GM1 antibodies: 4; Anti-ganglioside GM 2 antibodies: 4; Anti-GQ1b ganglioside antibody: 1

EMG: Electromyography.

(range): 1007 (22-2579) IU/L]. HEV RNA was detected in serum in 44 cases but not detected in CSF. Patients were primarily treated with supportive measures, including physiotherapy. A total of nine patients were treated with ribavirin and IVIG. Complete recovery was seen in six patients only. Partial recovery and long-term disability was noted in 40 and 7 patients respectively.

GBS

GBS is an inflammatory disease of the nerve root and plexus and is the most common cause of acute flaccid paralysis. It should be considered a diagnosis in patients who have experienced rapidly progressive bilateral weakness of the legs and/or arms, in the absence of CNS involvement or other obvious causes. Patients with the classic sensorimotor form of GBS present with distal paresthesia or sensory loss, accompanied or followed by weakness that starts in the legs and progresses to the arms and cranial muscles. Reflexes are decreased or absent in most patients at presentation and in almost all patients at nadir. GBS is a clinical diagnosis, but additional investigations are mostly performed for confirmation. The diagnosis of GBS can be supported by a CSF examination finding of classical cytoalbuminologic dissociation—the combination of a normal cell count and increased protein level. Nerve conduction studies can be helpful but are generally not required to diagnose GBS[57].

Prior respiratory or gastrointestinal tract infection has been reported in approximately two-thirds of GBS patients. A specific pathogen can be identified in about 50% of cases of GBS with a suspected infectious precipitant. Epidemiological studies have shown that *Campylobacter jejuni*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *Cytomegalovirus*, and Epstein-Barr virus infections are strongly associated with GBS. These pathogens appear to act as potential triggers of a postinfectious immune-mediated process leading to GBS. Molecular mimicry and cross-reactive immune triggers play an important role in the immunopathogenesis of GBS. Antibodies to gangliosides following infection with *C. jejuni* have been demonstrated in patients with GBS.

Recently, case-control studies and several case reports reported an association between acute HEV infection and GBS. The seroprevalence of acute HEV infection in patients with GBS has been found to range from 5% to 11%[57-61]. In a retrospective cohort study, 8% of patients (*n* = 73) with GBS showed positive IgM assays for HEV[58]. The association of HEV infection with GBS has been described in at least three well-described case-control studies. A study conducted in the Netherlands indicated a significantly higher frequency of acute HEV infection in GBS patients (5%) compared to healthy controls (0.5%)[59]. Another study from Japan demonstrated that 4.8% of the patients (*n* = 63) with GBS showed an association with acute HEV infection[60]. A study in Bangladesh documented that 11% of GBS patients (*n* = 100) were associated with acute HEV infection and seroprevalence was significantly higher for this group compared to patients with other neurological disorders as well as healthy controls[61]. In all studies, no clinical differences were observed between HEV-associated GBS and other GBS cases[58-61].

We found 61 cases of HEV-associated GBS reported in the medical literature[13,15,16,20,36,58-85]. Salient clinico-demographic features of GBS are

summarized in Tables 3 and 4. The mean age of the patients was 51.16 years with an M:F of 2.42:1. Genotyping was performed in 13 cases. All patients, except three, were GT3. HEV-associated GBS is most commonly reported in Europe and Southeast Asia, followed by East Asia and China. The patients most commonly presented with acute inflammatory demyelinating polyneuropathy. The majority of patients (82%) had normal bilirubin levels, and ALT levels widely varied (median [range]: 1950 [57-4502] IU/L). HEV RNA was detected in serum in 20 cases, while HEV RNA was detected in CSF in 3 cases. Further, anti-ganglioside antibodies were documented in nine patients. Patients were mainly treated with supportive measures, including IVIG and physiotherapy. The follow-up details of 38 of 61 patients were available. Complete recovery was seen in only 23 patients. Partial recovery was noted in 14 patients and 1 patient died.

CNS DISORDERS

The association between HEV infection and CNS disorders, including meningoencephalitis, cerebral ischemia, seizure, and transverse myelitis was reported in a few case reports. Meningoencephalitis was reported in six cases with acute HEV infection (mean age: 44 years; M:F = 1:1)[17,20,35,86,87]. Genotyping done in two cases revealed GT3, and all patients were anicteric. The median (range) ALT was 142 (20-479) IU/L. HEV RNA was detected in serum in all patients, except one. CSF examination for HEV RNA performed in four cases revealed the presence of HEV RNA in two cases. Of the six cases, complete recovery was seen in five.

Cerebral ischemia was reported in six cases[17,20], and all patients were anicteric. Serum HEV RNA was detected in four patients with cerebral ischemia. However, HEV RNA was not detected in CSF. Seizures were reported in two cases with HEV infection[17]. Transverse myelitis was reported in an anicteric patient with acute HEV (GT3) infection, and HEV RNA was detected in both serum and CSF[88].

NEUROPATHIES

The association between HEV infection and neuropathy was reported in case series and reports. Mononeuritis multiplex, defined by asymmetric, asynchronous involvement of the noncontiguous nerve trunks has been documented in six patients with acute HEV infection (GT3) from France (mean age: 53.16 years; M:F = 1:1)[36]. All patients presented with neuropathic pain and paresthesia in multiple sites with hyporeflexia or areflexia, and all patients, except one, were anicteric. The median (range) ALT was 188 (118-3641) IU/L. HEV RNA was detected in serum in four cases. All patients, except one, had a complete recovery.

Peripheral neuropathy and small fiber neuropathy were reported in five cases, mostly from Europe (4/5; mean age: 57.4 years; M:F = 3:2)[16,20,89]. Genotyping done in two cases revealed GT3. Patients presented with neuropathic pain, paresthesia and weakness of limbs. All patients, except one, were anicteric. Median (range) ALT was 285 (30-1606) IU/L. HEV RNA was detected in serum in all five cases. HEV RNA was detected in CSF in two cases. Out of five, complete recovery was seen in two-cases only.

CRANIAL NEUROPATHIES

Cranial nerve involvement was seen in five patients with acute HEV infection (mean age: 53 years; M:F = 4:1)[16,17,90-92]. Cases were reported from Europe (n = 2), India (n = 2), and Japan (n = 1) respectively. Isolated facial nerve palsy (Bell's palsy) and vestibular nerve involvement were seen in three and one patient respectively. Combined facial nerve and vestibular nerve involvement was noted in one patient. All patients were anicteric. Median (range) ALT was 1200 (60-3866) IU/L. HEV RNA was detected in serum in two cases. Genotyping was done in two cases and revealed GT3 and GT4 (Europe: GT1; Japan: GT4). CSF examination for HEV RNA was performed in none of the patients, and complete recovery was observed in all.

NEUROMUSCULAR JUNCTION AND MUSCLE DISORDERS

Cases of myasthenia gravis (n = 1) and myositis (n = 1) were also reported in serum HEV RNA positive patients[18,93]. However, HEV RNA was not detected in CSF.

OTHER NEUROLOGICAL DISORDERS

Meningoradiculitis refers to the combined involvement of meninges and nerve roots, and the lumbosacral region is the most common site of involvement. Etiology includes inflammatory, infectious, and neoplastic disorders. Recently, meningoradiculitis was reported in six patients with acute HEV infection from Europe[36,94,95]. The mean age of patients was 53.33 years with M:F of 1:1. Genotyping was done in five cases and showed GT3. Patients mainly presented with arthromyalgia and asthenia. All patients, except one, were anicteric. Median (range) ALT was 406 (40-822) IU/L. HEV RNA was detected in serum in all six cases. HEV RNA was detected in CSF in four cases. Of the six, complete recovery was noted in five cases.

DIAGNOSIS OF HEV INFECTION

Acute HEV infection is diagnosed through the detection of serum and/or stool HEV RNA by polymerase chain reaction (PCR), serum anti-HEV immunoglobulin M, HEV antigen, and rising anti-HEV immunoglobulin G titer. A negative PCR does not exclude acute infection, and serology sometimes gives false negatives in the case of immunocompromised patients. Therefore, serology and PCR testing are best used in combination. Testing for HEV should be conducted in all patients with suspicion of acute hepatitis, immunosuppressed patients with unexplained deranged liver function tests (LFTs), and in patients with NA and GBS. Testing is also suggested in patients with unexplained ACLF, and encephalitis or myelitis. Moreover, testing for proteinuria is suggested in HEV-infected patients[5].

TREATMENT

The HEV is spontaneously cleared in almost all patients with acute infection. Therefore, acute hepatitis does not require antiviral therapy. In a few case studies, ribavirin treatment of severe acute HEV infection showed rapid normalization of liver enzymes and viremia. Therefore, ribavirin treatment may be considered in cases of severe acute hepatitis E or ACLF[5,96]. Antiviral treatment is also suggested for patients with chronic HEV infection, and HEV-associated glomerular disease. There is no sufficient evidence of ribavirin use in HEV-associated neurological disorders.

PREVENTION

Several epidemiological factors associated with HEV infection are described in Table 1. HEV is principally transmitted *via* the fecal-oral route due to fecal contamination of drinking water. As such, it has been detected in sewage water, rivers, pork liver sausages, shellfish, cattle milk, unpeeled fruit, strawberries, berries, salads, and leafy green vegetables. Poor hygiene, exposure to contaminated environments, consumption of raw meat, exposure to soil, travel to endemic areas, contact with farm animals and pets, living in rural areas, and receiving lower levels of education were identified as risk factors for HEV infection. Transmission from infected blood products and vertical transmission are other modes of transmission[13]. The spread of HEV can be reduced by maintaining quality standards for public water supplies, ensuring proper disposal of human faces, maintaining individual hygienic practices, and avoiding consumption of foods of unknown purity. A recombinant subunit vaccine demonstrated efficacy against HEV infection. However, no vaccine is commercially available yet for this infection, except in China.

CONCLUSION

The neurologic manifestations of HEV infection are increasingly recognized worldwide, especially in Europe and Southeast Asia. Nerve root and plexus disorder (NA and GBS) are the most commonly reported diseases followed by meningoencephalitis. NA, GBS, and meningoencephalitis appear to be causally associated with HEV-infection. Further, GT3 is the most common GT identified in these patients. The majority of patients present without jaundice. However, the absence of jaundice and normal LFTs can mislead the physician. Thus, detailed neurological evaluation is warranted in patients with HEV infection and neurological symptoms. Patients with neurological disorders and deranged LFTs require further investigation to diagnose HEV infection. Testing for HEV in patients with neurological disorders and unexplained etiology can also be rewarding. Finally, there is no sufficient evidence of routine use of an anti-viral agent in HEV-related neurological disorders.

REFERENCES

1 **Purdy MA**, Harrison TJ, Jameel S, Meng XJ, Okamoto H, Van der Poel WHM, Smith DB; Ictv Report Consortium. ICTV Virus Taxonomy Profile: Hepeviridae. *J Gen Virol* 2017; **98**: 2645-2646 [PMID: 29022866 DOI: 10.1099/jgv.0.000940]

2 **Li P**, Liu J, Li Y, Su J, Ma Z, Bramer WM, Cao W, de Man RA, Peppelenbosch MP, Pan Q. The global epidemiology of hepatitis E virus infection: A systematic review and meta-analysis. *Liver Int* 2020; **40**: 1516-1528 [PMID: 32281721 DOI: 10.1111/liv.14468]

3 **World Health Organization**. Viral hepatitis in the WHO South-East Asia Region. [cited 15 November 2020]. In: World Health Organization [Internet]. Available from: https://apps.who.int/iris/handle/10665/206521

4 **Kamar N**, Selves J, Mansuy JM, Ouezzani L, Péron JM, Guitard J, Cointault O, Esposito L, Abravanel F, Danjoux M, Durand D, Vinel JP, Izopet J, Rostaing L. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. *N Engl J Med* 2008; **358**: 811-817 [PMID: 18287603 DOI: 10.1056/NEJMoa0706992]

5 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines on hepatitis E virus infection. *J Hepatol* 2018; **68**: 1256-1271 [PMID: 29609832 DOI: 10.1016/j.jhep.2018.03.005]

6 **Jha AK**, Nijhawan S, Rai RR, Nepalia S, Jain P, Suchismita A. Etiology, clinical profile, and inhospital mortality of acute-on-chronic liver failure: a prospective study. *Indian J Gastroenterol* 2013; **32**: 108-114 [PMID: 23526372 DOI: 10.1007/s12664-012-0295-9]

7 **Kumar A**, Aggarwal R, Naik SR, Saraswat V, Ghoshal UC, Naik S. Hepatitis E virus is responsible for decompensation of chronic liver disease in an endemic region. *Indian J Gastroenterol* 2004; **23**: 59-62 [PMID: 15176538]

8 **Rawla P**, Raj JP, Kannemkuzhiyil AJ, Aluru JS, Thandra KC, Gajendran M. A Systematic Review of the Extra-Hepatic Manifestations of Hepatitis E Virus Infection. *Med Sci (Basel)* 2020; **8** [PMID: 32033102 DOI: 10.3390/medsci8010009]

9 **Pischke S**, Hartl J, Pas SD, Lohse AW, Jacobs BC, Van der Eijk AA. Hepatitis E virus: Infection beyond the liver? *J Hepatol* 2017; **66**: 1082-1095 [PMID: 27913223 DOI: 10.1016/j.jhep.2016.11.016]

10 **Feng Z**. Causation by HEV of extrahepatic manifestations remains unproven. *Liver Int* 2016; **36**: 477-479 [PMID: 27005694 DOI: 10.1111/liv.13085]

11 **Zhou X**, Huang F, Xu L, Lin Z, de Vrij FMS, Ayo-Martin AC, van der Kroeg M, Zhao M, Yin Y, Wang W, Cao W, Wang Y, Kushner SA, Marie Peron J, Alric L, de Man RA, Jacobs BC, van Eijk JJ, Aronica EMA, Sprengers D, Metselaar HJ, de Zeeuw CI, Dalton HR, Kamar N, Peppelenbosch MP, Pan Q. Hepatitis E Virus Infects Neurons and Brains. *J Infect Dis* 2017; **215**: 1197-1206 [PMID: 28199701 DOI: 10.1093/infdis/jix079]

12 **Drave SA**, Debing Y, Walter S, Todt D, Engelmann M, Friesland M, Wedemeyer H, Neyts J, Behrendt P, Steinmann E. Extra-hepatic replication and infection of hepatitis E virus in neuronal-derived cells. *J Viral Hepat* 2016; **23**: 512-521 [PMID: 26891712 DOI: 10.1111/jvh.12515]

13 **Abravanel F**, Pique J, Couturier E, Nicot F, Dimeglio C, Lhomme S, Chiabrando J, Saune K, Péron JM, Kamar N, Evrard S, de Valk H, Cintas P, Izopet J; HEV study group. Acute hepatitis E in French patients and neurological manifestations. *J Infect* 2018; **77**: 220-226 [PMID: 29966614 DOI: 10.1016/j.jinf.2018.06.007]

14 **Ripellino P**, Pasi E, Melli G, Staedler C, Fraga M, Moradpour D, Sahli R, Aubert V, Martinetti G, Bihl F, Bernasconi E, Terziroli Beretta-Piccoli B, Cerny A, Dalton HR, Zehnder C, Mathis B, Zecca C, Disanto G, Kaelin-Lang A, Gobbi C. Neurologic complications of acute hepatitis E virus infection. *Neurol Neuroimmunol Neuroinflamm* 2020; **7** [PMID: 31806684 DOI: 10.1212/NXI.0000000000000643]

15 **Kamar N**, Bendall RP, Peron JM, Cintas P, Prudhomme L, Mansuy JM, Rostaing L, Keane F, Ijaz S, Izopet J, Dalton HR. Hepatitis E virus and neurologic disorders. *Emerg Infect Dis* 2011; **17**: 173-179 [PMID: 21291585 DOI: 10.3201/eid1702.100856]

16 **Woolson KL**, Forbes A, Vine L, Beynon L, McElhinney L, Panayi V, Hunter JG, Madden RG, Glasgow T, Kotecha A, Dalton HC, Mihailescu L, Warshow U, Hussaini HS, Palmer J, Mclean BN, Haywood B, Bendall RP, Dalton HR. Extra-hepatic manifestations of autochthonous hepatitis E infection. *Aliment Pharmacol Ther* 2014; **40**: 1282-1291 [PMID: 25303615 DOI: 10.1111/apt.12986]

17 **Dalton HR**, van Eijk JJJ, Cintas P, Madden RG, Jones C, Webb GW, Norton B, Pique J, Lutgens S, Devooght-Johnson N, Woolson K, Baker J, Saunders M, Househam L, Griffiths J, Abravanel F, Izopet J, Kamar N, van Alfen N, van Engelen BGM, Hunter JG, van der Eijk AA, Bendall RP, Mclean BN, Jacobs BC. Hepatitis E virus infection and acute non-traumatic neurological injury: A prospective multicentre study. *J Hepatol* 2017; **67**: 925-932 [PMID: 28734938 DOI: 10.1016/j.jhep.2017.07.010]

18 **Belbézier A**, Deroux A, Sarrot-Reynauld F, Colombe B, Bosseray A, Wintenberger C, Dumanoir P, Lugosi M, Boccon-Gibod I, Leroy V, Maignan M, Collomb-Muret R, Viglino D, Vaillant M, Minotti L, Lagrange E, Epaulard O, Dumestre-Perard C, Lhomme S, Lupo J, Larrat S, Morand P, Schwebel C, Vilotitch A, Bosson JL, Bouillet L. Screening of hepatitis E in patients presenting for acute neurological disorders. *J Infect Public Health* 2020; **13**: 1047-1050 [PMID: 32224109 DOI: 10.1016/j.jiph.2019.12.012]

19 **Mansuy JM**, Gallian P, Dimeglio C, Saune K, Arnaud C, Pelletier B, Morel P, Legrand D, Tiberghien P, Izopet J. A nationwide survey of hepatitis E viral infection in French blood donors. *Hepatology* 2016; **63**: 1145-1154 [PMID: 27008201 DOI: 10.1002/hep.28436]

20 **Wang Y**, Wang S, Wu J, Jiang Y, Zhang H, Li S, Liu H, Yang C, Tang H, Guo N, Peppelenbosch MP, Wei L, Pan Q, Zhao J. Hepatitis E virus infection in acute non-traumatic neuropathy: A large prospective case-control study in China. *EBioMedicine* 2018; **36**: 122-130 [PMID: 30190208 DOI: 10.1016/j.ebiom.2018.08.053]

21 **Adinolfi LE**, Nevola R, Lus G, Restivo L, Guerrera B, Romano C, Zampino R, Rinaldi L, Sellitto A, Giordano M, Marrone A. Chronic hepatitis C virus infection and neurological and psychiatric disorders: an overview. *World J Gastroenterol* 2015; **21**: 2269-2280 [PMID: 25741133 DOI: 10.3748/wjg.v21.i8.2269]

22 **Mapoure NY**, Budzi MN, Eloumou SAFB, Malongue A, Okalla C, Luma HN. Neurological manifestations in chronic hepatitis C patients receiving care in a reference hospital in sub-Saharan Africa: A cross-sectional study. *PLoS One* 2018; **13**: e0192406 [PMID: 29513678 DOI: 10.1371/journal.pone.0192406]

23 **Menon D**, Jagtap SA, Nair MD. Guillain-Barré syndrome following acute viral hepatitis A. *J Neurosci Rural Pract* 2014; **5**: 204-205 [PMID: 24966576 DOI: 10.4103/0976-3147.131695]

24 **Lee JJ**, Kang K, Park JM, Kwon O, Kim BK. Encephalitis associated with acute hepatitis a. *J Epilepsy Res* 2011; **1**: 27-28 [PMID: 24649441 DOI: 10.14581/jer.11005]

25 **Chonmaitree P**, Methawasin K. Transverse Myelitis in Acute Hepatitis A Infection: The Rare Co-Occurrence of Hepatology and Neurology. *Case Rep Gastroenterol* 2016; **10**: 44-49 [PMID: 27403101 DOI: 10.1159/000444013]

26 **Kappus MR**, Sterling RK. Extrahepatic manifestations of acute hepatitis B virus infection. *Gastroenterol Hepatol (N Y)* 2013; **9**: 123-126 [PMID: 23983659]

27 **van Alfen N**, van Engelen BG. The clinical spectrum of neuralgic amyotrophy in 246 cases. *Brain* 2006; **129**: 438-450 [PMID: 16371410 DOI: 10.1093/brain/awh722]

28 **van Eijk JJ**, Madden RG, van der Eijk AA, Hunter JG, Reimerink JH, Bendall RP, Pas SD, Ellis V, van Alfen N, Beynon L, Southwell L, McLean B, Jacobs BC, van Engelen BG, Dalton HR. Neuralgic amyotrophy and hepatitis E virus infection. *Neurology* 2014; **82**: 498-503 [PMID: 24401685 DOI: 10.1212/WNL.0000000000000112]

29 **van Eijk JJJ**, Dalton HR, Ripellino P, Madden RG, Jones C, Fritz M, Gobbi C, Melli G, Pasi E, Herrod J, Lissmann RF, Ashraf HH, Abdelrahim M, Masri OABAL, Fraga M, Benninger D, Kuntzer T, Aubert V, Sahli R, Moradpour D, Blasco-Perrin H, Attarian S, Gérolami R, Colson P, Giordani MT, Hartl J, Pischke S, Lin NX, Mclean BN, Bendall RP, Panning M, Peron JM, Kamar N, Izopet J, Jacobs BC, van Alfen N, van Engelen BGM. Clinical phenotype and outcome of hepatitis E virus-associated neuralgic amyotrophy. *Neurology* 2017; **89**: 909-917 [PMID: 28768846 DOI: 10.1212/WNL.0000000000004297]

30 **Fong F**, Illahi M. Neuralgic amyotrophy associated with hepatitis E virus. *Clin Neurol Neurosurg* 2009; **111**: 193-195 [PMID: 18986757 DOI: 10.1016/j.clineuro.2008.09.005]

31 **Rianthavorn P**, Thongmee C, Limpaphayom N, Komolmit P, Theamboonlers A, Poovorawan Y. The entire genome sequence of hepatitis E virus genotype 3 isolated from a patient with neuralgic amyotrophy. *Scand J Infect Dis* 2010; **42**: 395-400 [PMID: 20100114 DOI: 10.3109/00365540903496551]

32 **Carli P**, Landais C, Poisnel E, Cournac JM, Aletti M, Paris JF, Martinez V. [Shoulder pain in a 30-year-old man]. *Rev Med Interne* 2012; **33**: 111-114 [PMID: 22192515 DOI: 10.1016/j.revmed.2011.11.004]

33 **Moisset X**, Vitello N, Bicilli E, Courtin R, Ferrier A, Taithe F, Lahaye C, Hssain AA, Garrouste C, Pierre C. Severe bilateral amyotrophic neuralgia associated with major dysphagia secondary to acute hepatitis E. *F1000Res* 2013; **2**: 259 [PMID: 24555112 DOI: 10.12688/f1000research.2-259.v2]

34 **Motte A**, Franques J, Weitten T, Colson P. Hepatitis E-associated Parsonage-Turner syndrome, France. *Clin Res Hepatol Gastroenterol* 2014; **38**: e11-e14 [PMID: 24246798 DOI: 10.1016/j.clinre.2013.08.011]

35 **Deroux A**, Brion JP, Hyerle L, Belbezier A, Vaillant M, Mosnier E, Larrat S, Morand P, Pavese P.

Association between hepatitis E and neurological disorders: two case studies and literature review. *J Clin Virol* 2014; **60**: 60-62 [PMID: 24583064 DOI: 10.1016/j.jcv.2014.01.026]

36 **Perrin HB**, Cintas P, Abravanel F, Gérolami R, d'Alteroche L, Raynal JN, Alric L, Dupuis E, Prudhomme L, Vaucher E, Couzigou P, Liversain JM, Bureau C, Vinel JP, Kamar N, Izopet J, Peron JM. Neurologic Disorders in Immunocompetent Patients with Autochthonous Acute Hepatitis E. *Emerg Infect Dis* 2015; **21**: 1928-1934 [PMID: 26490255 DOI: 10.3201/eid2111.141789]

37 **Décard BF**, Grimm A, Andelova M, Deman A, Banderet B, Garcia M, Fuhr P. Hepatitis-E virus associated neuralgic amyotrophy with sustained plexus brachialis swelling visualized by high-resolution ultrasound. *J Neurol Sci* 2015; **351**: 208-210 [PMID: 25769655 DOI: 10.1016/j.jns.2015.03.003]

38 **Theochari E**, Vincent-Smith L, Ellis C. Neuralgic amyotrophy complicating acute hepatitis E infection: a rare association. *BMJ Case Rep* 2015; **2015** [PMID: 25739795 DOI: 10.1136/bcr-2014-207669]

39 **Martínez Rodríguez L**, Carvajal P, Morís G. [Neuralgic amyotrophy associated to hepatitis E virus infection]. *Med Clin (Barc)* 2015; **145**: 462-463 [PMID: 25817452 DOI: 10.1016/j.medcli.2015.01.021]

40 **Dartevel A**, Colombe B, Bosseray A, Larrat S, Sarrot-Reynauld F, Belbezier A, Lagrange E, Bouillet L. Hepatitis E and neuralgic amyotrophy: Five cases and review of literature. *J Clin Virol* 2015; **69**: 156-164 [PMID: 26209399 DOI: 10.1016/j.jcv.2015.06.091]

41 **Avila JD**, Lacomis D, Lam EM. Neuralgic Amyotrophy Associated With Hepatitis E Virus Infection: First Case in the United States. *J Clin Neuromuscul Dis* 2016; **18**: 96-100 [PMID: 27861224 DOI: 10.1097/CND.0000000000000137]

42 **Silva M**, Wicki B, Tsouni P, Cunningham S, Doerig C, Zanetti G, Aubert V, Sahli R, Moradpour D, Kuntzer T. Hepatitis E virus infection as a direct cause of neuralgic amyotrophy. *Muscle Nerve* 2016; **54**: 325-327 [PMID: 26939568 DOI: 10.1002/mus.25096]

43 **Altuna-Azkargorta M**, Torne-Hernandez L, Aznar-Gomez P, Ibiricu-Yanguas MA, Ducouret A. [Infection by the hepatitis E virus as a precipitating factor of Parsonage-Turner syndrome]. *Rev Neurol* 2016; **62**: 572-574 [PMID: 27270680]

44 **Pischke S**, Ryll U, De Weerth A, Ufer F, Gelderblom M. [Neuralgic amyotrophy: an extrahepatic manifestation of hepatitis E]. *Dtsch Med Wochenschr* 2016; **141**: 1239-1242 [PMID: 27557071 DOI: 10.1055/s-0042-102289]

45 **Bisciglia M**, Van den Bergh P, Duprez T, Kabamba BM, Ivanoiu A. Neuralgic amyotrophy associated with hepatitis E virus (HEV) infection: a case report. *Acta Neurol Belg* 2017; **117**: 555-557 [PMID: 27095100 DOI: 10.1007/s13760-016-0642-1]

46 **Velay A**, Kack-Kack W, Abravanel F, Lhomme S, Leyendecker P, Kremer L, Chamouard P, Izopet J, Fafi-Kremer S, Barth H. Parsonage-Turner syndrome due to autochthonous acute genotype 3f hepatitis E virus infection in a nonimmunocompromised 55-year-old patient. *J Neurovirol* 2017; **23**: 615-620 [PMID: 28439773 DOI: 10.1007/s13365-017-0525-0]

47 **Scanvion Q**, Perez T, Cassim F, Outteryck O, Lanteri A, Hatron PY, Lambert M, Morell-Dubois S. Neuralgic amyotrophy triggered by hepatitis E virus: a particular phenotype. *J Neurol* 2017; **264**: 770-780 [PMID: 28247042 DOI: 10.1007/s00415-017-8433-z]

48 **Fraga M**, Doerig C, Moulin H, Bihl F, Brunner F, Müllhaupt B, Ripellino P, Semela D, Stickel F, Terziroli Beretta-Piccoli B, Aubert V, Telenti A, Greub G, Sahli R, Moradpour D. Hepatitis E virus as a cause of acute hepatitis acquired in Switzerland. *Liver Int* 2018; **38**: 619-626 [PMID: 28834649 DOI: 10.1111/liv.13557]

49 **Fritz M**, Berger B, Schemmerer M, Endres D, Wenzel JJ, Stich O, Panning M. Pathological Cerebrospinal Fluid Findings in Patients With Neuralgic Amyotrophy and Acute Hepatitis E Virus Infection. *J Infect Dis* 2018; **217**: 1897-1901 [PMID: 29547884 DOI: 10.1093/infdis/jiy128]

50 **Sánchez Azofra M**, Romero Portales M, Tortajada Laureiro L, García-Samaniego J, Mora Sanz P. Hepatitis E virus in neurological disorders: a case of Parsonage-Turner syndrome. *Rev Esp Enferm Dig* 2018; **110**: 402-403 [PMID: 29685043 DOI: 10.17235/reed.2018.5506/2018]

51 **Njabom CN**, Gilbert A, Brasseur E, Zandona R, Ghuysen A, D'Orio V. Parsonage-Turner Syndrome as a Rare Extrahepatic Complication of Hepatitis E Infection. *Eur J Case Rep Intern Med* 2019; **6**: 001208 [PMID: 31508389 DOI: 10.12890/2019_001208]

52 **Arányi Z**, Szpisjak L, Szőke K. Multiphasic presentation of neuralgic amyotrophy associated with hepatitis E virus infection. *Muscle Nerve* 2020; **61**: 108-110 [PMID: 31573093 DOI: 10.1002/mus.26722]

53 **Mendoza-Lopez C**, Lopez-Lopez P, Atienza-Ayala S, Rivero-Juarez A, Benito R. Parsonage-Turner syndrome associated with hepatitis E infection in immunocompetent patients. *Virus Res* 2020; **290**: 198165 [PMID: 33007343 DOI: 10.1016/j.virusres.2020.198165]

54 **Inghilleri ML**, Grini Mazouzi M, Juntas Morales R. [Neuralgic amyotrophy as a manifestation of hepatitis E infection]. *Rev Neurol (Paris)* 2012; **168**: 383-384 [PMID: 22398219 DOI: 10.1016/j.neurol.2011.07.014]

55 **Diebold M**, Fischer-Barnicol B, Tsagkas C, Kuhle J, Kappos L, Derfuss T, Décard BF. Hepatitis E virus infections in patients with MS on oral disease-modifying treatment. *Neurol Neuroimmunol Neuroinflamm* 2019; **6** [PMID: 31454772 DOI: 10.1212/NXI.0000000000000594]

56 **Cheung MC**, Maguire J, Carey I, Wendon J, Agarwal K. Review of the neurological manifestations of hepatitis E infection. *Ann Hepatol* 2012; **11**: 618-622 [PMID: 22947521]

57 **van den Berg B**, Walgaard C, Drenthen J, Fokke C, Jacobs BC, van Doorn PA. Guillain-Barré

syndrome: pathogenesis, diagnosis, treatment and prognosis. *Nat Rev Neurol* 2014; **10**: 469-482 [PMID: 25023340 DOI: 10.1038/nrneurol.2014.121]

58 **Stevens O**, Claeys KG, Poesen K, Saegeman V, Van Damme P. Diagnostic Challenges and Clinical Characteristics of Hepatitis E Virus-Associated Guillain-Barré Syndrome. *JAMA Neurol* 2017; **74**: 26-33 [PMID: 27820624 DOI: 10.1001/jamaneurol.2016.3541]

59 **van den Berg B**, van der Eijk AA, Pas SD, Hunter JG, Madden RG, Tio-Gillen AP, Dalton HR, Jacobs BC. Guillain-Barré syndrome associated with preceding hepatitis E virus infection. *Neurology* 2014; **82**: 491-497 [PMID: 24415572 DOI: 10.1212/WNL.0000000000000111]

60 **Fukae J**, Tsugawa J, Ouma S, Umezu T, Kusunoki S, Tsuboi Y. Guillain-Barré and Miller Fisher syndromes in patients with anti-hepatitis E virus antibody: a hospital-based survey in Japan. *Neurol Sci* 2016; **37**: 1849-1851 [PMID: 27389141 DOI: 10.1007/s10072-016-2644-4]

61 **Geurtsvankessel CH**, Islam Z, Mohammad QD, Jacobs BC, Endtz HP, Osterhaus AD. Hepatitis E and Guillain-Barre syndrome. *Clin Infect Dis* 2013; **57**: 1369-1370 [PMID: 23899686 DOI: 10.1093/cid/cit512]

62 **Sood A**, Midha V, Sood N. Guillain-Barré syndrome with acute hepatitis E. *Am J Gastroenterol* 2000; **95**: 3667-3668 [PMID: 11151929 DOI: 10.1111/j.1572-0241.2000.03409.x]

63 **Kamani P**, Baijal R, Amarapurkar D, Gupte P, Patel N, Kumar P, Agal S. Guillain-Barre syndrome associated with acute hepatitis E. *Indian J Gastroenterol* 2005; **24**: 216 [PMID: 16361768]

64 **Khanam R**, Faruq M, Basunia R, Ahsan A. Guillain-BarrÃ© Syndrome Associated with Acute HEV Hepatitis. *IMCJ* 2008; **2**: 32-34 [DOI: 10.3329/imcj.v2i1.2930]

65 **Loly JP**, Rikir E, Seivert M, Legros E, Defrance P, Belaiche J, Moonen G, Delwaide J. Guillain-Barr é syndrome following hepatitis E. *World J Gastroenterol* 2009; **15**: 1645-1647 [PMID: 19340910 DOI: 10.3748/wjg.15.1645]

66 **Cronin S**, McNicholas R, Kavanagh E, Reid V, O'Rourke K. Anti-glycolipid GM2-positive Guillain-Barre syndrome due to hepatitis E infection. *Ir J Med Sci* 2011; **180**: 255-257 [PMID: 21063804 DOI: 10.1007/s11845-010-0635-7]

67 **Maurissen I**, Jeurissen A, Strauven T, Sprengers D, De Schepper B. First case of anti-ganglioside GM1-positive Guillain-Barré syndrome due to hepatitis E virus infection. *Infection* 2012; **40**: 323-326 [PMID: 21877179 DOI: 10.1007/s15010-011-0185-6]

68 **Del Bello A**, Arné-Bes MC, Lavayssière L, Kamar N. Hepatitis E virus-induced severe myositis. *J Hepatol* 2012; **57**: 1152-1153 [PMID: 22641093 DOI: 10.1016/j.jhep.2012.05.010]

69 **Tse AC**, Cheung RT, Ho SL, Chan KH. Guillain-Barré syndrome associated with acute hepatitis E infection. *J Clin Neurosci* 2012; **19**: 607-608 [PMID: 22285113 DOI: 10.1016/j.jocn.2011.06.024]

70 **Santos L**, Mesquita JR, Rocha Pereira N, Lima-Alves C, Serrão R, Figueiredo P, Reis J, Simões J, Nascimento M, Sarmento A. Acute hepatitis E complicated by Guillain-Barre syndrome in Portugal, December 2012--a case report. *Euro Surveill* 2013; **18** [PMID: 23987830 DOI: 10.2807/1560-7917.es2013.18.34.20563]

71 **Sharma B**, Nagpal K, Bakki Sannegowda R, Prakash S. Hepatitis E with Gullain-Barré syndrome: still a rare association. *J Neurovirol* 2013; **19**: 186-187 [PMID: 23471727 DOI: 10.1007/s13365-013-0156-z]

72 **Chen XD**, Zhou YT, Zhou JJ, Wang YW, Tong DM. Guillain-Barré syndrome and encephalitis/encephalopathy of a rare case of Northern China acute severe hepatitis E infection. *Neurol Sci* 2014; **35**: 1461-1463 [PMID: 24700050 DOI: 10.1007/s10072-014-1731-7]

73 **Scharn N**, Ganzenmueller T, Wenzel JJ, Dengler R, Heim A, Wegner F. Guillain-Barré syndrome associated with autochthonous infection by hepatitis E virus subgenotype 3c. *Infection* 2014; **42**: 171-173 [PMID: 23512540 DOI: 10.1007/s15010-013-0448-5]

74 **Comont T**, Bonnet D, Sigur N, Gerdelat A, Legrand-Abravanel F, Kamar N, Alric L. [Acute hepatitis E infection associated with Guillain-Barré syndrome in an immunocompetent patient]. *Rev Med Interne* 2014; **35**: 333-336 [PMID: 24080239 DOI: 10.1016/j.revmed.2013.05.005]

75 **Bandyopadhyay D**, Ganesan V, Choudhury C, Kar SS, Karmakar P, Choudhary V, Banerjee P, Bhar D, Hajra A, Layek M, Mukhopadhyay S. Two Uncommon Causes of Guillain-Barré Syndrome: Hepatitis E and Japanese Encephalitis. *Case Rep Neurol Med* 2015; **2015**: 759495 [PMID: 26798531 DOI: 10.1155/2015/759495]

76 **Higuchi MA**, Fukae J, Tsugawa J, Ouma S, Takahashi K, Mishiro S, Tsuboi Y. Dysgeusia in a Patient with Guillain-Barré Syndrome Associated with Acute Hepatitis E: A Case Report and Literature Review. *Intern Med* 2015; **54**: 1543-1546 [PMID: 26073247 DOI: 10.2169/internalmedicine.54.3506]

77 **Ji SB**, Lee SS, Jung HC, Kim HJ, Kim TH, Jung WT, Lee OJ, Song DH. A Korean patient with Guillain-Barré syndrome following acute hepatitis E whose cholestasis resolved with steroid therapy. *Clin Mol Hepatol* 2016; **22**: 396-399 [PMID: 27572076 DOI: 10.3350/cmh.2015.0039]

78 **Lei JH**, Tian Y, Luo HY, Chen Z, Peng F. Guillain-Barré syndrome following acute co-super-infection of hepatitis E virus and cytomegalovirus in a chronic hepatitis B virus carrier. *J Med Virol* 2017; **89**: 368-372 [PMID: 27358107 DOI: 10.1002/jmv.24620]

79 **Salim OJ**, Davidson A, Li K, Leach JP, Heath C. Brainstem encephalitis and acute polyneuropathy associated with hepatitis E infection. *BMJ Case Rep* 2017; **2017** [PMID: 28899886 DOI: 10.1136/bcr-2017-220799]

80 **Troussière AC**, Sudaveschi V, Collardelle P, Marque Julliet S, Servan J, Pico F. Guillain-Barré syndrome due to hepatitis E. *Rev Neurol (Paris)* 2018; **174**: 72-74 [PMID: 29132643 DOI: 10.1016/j.neurol.2017.06.018]

81 **Zheng X**, Yu L, Xu Q, Gu S, Tang L. Guillain-Barre syndrome caused by hepatitis E infection: case report and literature review. *BMC Infect Dis* 2018; **18**: 50 [PMID: 29357816 DOI: 10.1186/s12879-018-2959-2]

82 **Choudhary MC**, Bajpai V, Anand L, Gupta E. Guillain-Barré syndrome in a patient of acute Hepatitis E virus infection associated with genotype 1: Case report and literature review. *Intractable Rare Dis Res* 2019; **8**: 43-47 [PMID: 30881857 DOI: 10.5582/irdr.2018.01099]

83 **Kumar R**, Bhoi S, Kumar M, Sharma B, Singh BM, Gupta BB. Guillain-Barré syndrome and acute hepatitis E: A rare association. *JIACM* 2002; **4**: 389-391

84 **Oh HW**, Cha RR, Lee SS, Lee CM, Kim WS, Jo YW, Kim JJ, Lee JM, Kim HJ, Ha CY, Kim TH, Jung WT, Lee OJ. Comparing the Clinical Features and Outcomes of Acute Hepatitis E Viral Infections with Those of Acute Hepatitis A, B, and C Infections in Korea. *Intervirology* 2017; **60**: 109-117 [PMID: 29145204 DOI: 10.1159/000480506]

85 **Al-Saffar A**, Al-Fatly B. Acute Motor Axonal Neuropathy in Association with Hepatitis E. *Front Neurol* 2018; **9**: 62 [PMID: 29479336 DOI: 10.3389/fneur.2018.00062]

86 **Pasha SA**, Pasha SA, Suhasini T, Rao DA. Hepatitis E Virus-Associated Acute Encephalitic Parkinsonism. *J Assoc Physicians India* 2018; **66**: 92-93 [PMID: 30341882]

87 **Murkey JA**, Chew KW, Carlson M, Shannon CL, Sirohi D, Sample HA, Wilson MR, Vespa P, Humphries RM, Miller S, Klausner JD, Chiu CY. Hepatitis E Virus-Associated Meningoencephalitis in a Lung Transplant Recipient Diagnosed by Clinical Metagenomic Sequencing. *Open Forum Infect Dis* 2017; **4**: ofx121 [PMID: 28721353 DOI: 10.1093/ofid/ofx121]

88 **Sarkar P**, Morgan C, Ijaz S. Transverse myelitis caused by hepatitis E: previously undescribed in adults. *BMJ Case Rep* 2015; **2015** [PMID: 26150621 DOI: 10.1136/bcr-2014-209031]

89 **Bennett S**, Li K, Gunson RN. Hepatitis E virus infection presenting with paraesthesia. *Scott Med J* 2015; **60**: e27-e29 [PMID: 25663032 DOI: 10.1177/0036933015572093]

90 **Dixit VK**, Abhilash VB, Kate MP, Jain AK. Hepatitis E infection with Bell's palsy. *J Assoc Physicians India* 2006; **54**: 418 [PMID: 16909746]

91 **Jha AK**, Nijhawan S, Nepalia S, Suchismita A. Association of Bell's Palsy with Hepatitis E Virus Infection: A Rare Entity. *J Clin Exp Hepatol* 2012; **2**: 88-90 [PMID: 25755411 DOI: 10.1016/S0973-6883(12)60082-6]

92 **Yazaki Y**, Sugawara K, Honda M, Ohnishi H, Nagashima S, Takahashi M, Okamoto H. Characteristics of 20 Patients with Autochthonous Acute Hepatitis E in Hokkaido, Japan: First Report of Bilateral Facial Palsy Following the Infection with Genotype 4 Hepatitis E Virus. *Tohoku J Exp Med* 2015; **236**: 263-271 [PMID: 26228039 DOI: 10.1620/tjem.236.263]

93 **Mengel AM**, Stenzel W, Meisel A, Büning C. Hepatitis E-induced severe myositis. *Muscle Nerve* 2016; **53**: 317-320 [PMID: 26514272 DOI: 10.1002/mus.24959]

94 **Despierres LA**, Kaphan E, Attarian S, Cohen-Bacrie S, Pelletier J, Pouget J, Motte A, Charrel R, Gerolami R, Colson P. Neurologic disorders and hepatitis E, France, 2010. *Emerg Infect Dis* 2011; **17**: 1510-1512 [PMID: 21801637 DOI: 10.3201/eid1708.102028]

95 **Belliere J**, Abravanel F, Nogier MB, Martinez S, Cintas P, Lhomme S, Lavayssière L, Cointault O, Faguer S, Izopet J, Kamar N. Transfusion-acquired hepatitis E infection misdiagnosed as severe critical illness polyneuromyopathy in a heart transplant patient. *Transpl Infect Dis* 2017; **19** [PMID: 28963742 DOI: 10.1111/tid.12784]

96 **Péron JM**, Dalton H, Izopet J, Kamar N. Acute autochthonous hepatitis E in western patients with underlying chronic liver disease: a role for ribavirin? *J Hepatol* 2011; **54**: 1323-4; author reply 1324 [PMID: 21281681 DOI: 10.1016/j.jhep.2011.01.009]

97 **Hill AB**. The environment and disease: Association or causation? *Proc R Soc Med* 1965; **58**: 295-300 [PMID: 14283879]

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MINIREVIEWS

Stroma-targeting strategies in pancreatic cancer: Past lessons, challenges and prospects

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is projected to emerge as the second leading cause of cancer-related death after 2030. Extreme treatment resistance is perhaps the most significant factor that underlies the poor prognosis of PDAC. To date, combination chemotherapy remains the mainstay of treatment for most PDAC patients. Compared to other cancer types, treatment response of PDAC tumors to similar chemotherapy regimens is clearly much lower and shorter-lived. Aside from typically harboring genetic alterations that to date remain un-druggable and are drivers of treatment resistance, PDAC tumors are uniquely characterized by a densely fibrotic stroma that has well-established roles in promoting cancer progression and treatment resistance. However, emerging evidence also suggests that indiscriminate targeting and near complete depletion of stroma may promote PDAC aggressiveness and lead to detrimental outcomes. These conflicting results undoubtedly warrant the need for a more in-depth understanding of the heterogeneity of tumor stroma in order to develop modulatory strategies in favor of tumor suppression. The advent of novel techniques including single cell RNA sequencing and multiplex immuno-histochemistry have further illuminated the complex heterogeneity of tumor cells, stromal fibroblasts, and immune cells. This new knowledge is instrumental for development of more refined therapeutic strategies that can ultimately defeat this disease. Here, we provide a concise review on lessons learned from past stroma-targeting strategies, new challenges revealed from recent preclinical and clinical studies, as well as new prospects in the treatment of PDAC.

Key Words: Stroma; Pancreatic cancer; Treatment resistance; Cancer-associated fibroblasts; Clinical trials

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Core Tip: Stromal desmoplasia is not only a prominent histological hallmark of pancreatic cancer, but also a biological barrier to therapies. Various strategies aimed at targeting the stroma to improve therapeutic outcomes have been largely unsuccessful. Here we comprehensively reviewed the rationales and lessons learned from various stromal-targeting strategies and provide prospects on improving these approaches in future clinical trials.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is currently the seventh leading cause of cancer related death in the industrialized world[1]. In the United States, PDAC is projected to be the second leading cause of cancer death by 2030[2]. The current 5-year survival rate of PDAC is 9%, making it one of the deadliest cancers[3].

There are several factors that contribute to the poor outcomes of PDAC. First, non-specific symptoms and lack of PDAC specific markers and screening lead to late detection[4]. Less than 10% of PDAC is resectable at the time of diagnosis. Second, PDAC cells are highly metastatic, evidenced by the fact that most patients develop local or distal recurrences even after seemingly successful surgical resection. Third, PDAC is extremely resistant to chemotherapy and radiation. For example, the triple therapy of folinic acid, 5-fluorouracil (5-FU), irinotecan, oxaliplatin (as FOLFRINOX or FOLFOXIRI) has been used as a first line regimen in the treatment of various gastrointestinal malignancies. A phase III trial conducted by The Groupo Oncoligico Nord Ovest used FOLFOXIRI as a first line treatment in patients with metastatic colorectal cancer. The objective response rate (ORR) to FOLFOXIRI in these patients was 60%[5]. A phase II trial of FOLFIRINOX in patients with advanced gastro-esophageal cancer showed an ORR of 61%[6]. This is in contrast to a response rate of only 31.6% in patients with metastatic PDAC receiving FOLFIRINOX[7]. For patients with localized PDAC who have undergone R0 or R1 surgical resection upfront and have postoperative CA19-9 of less than 180 U/mL, treatment with FOLFIRINOX resulted in a 3-year disease free survival rate of 39.7% as opposed to 21.4% with gemcitabine[8]. While these results demonstrate that strong combination chemotherapy can potentially cure additional patients, it is also worth noting that a significant subset (approximately 60%) of patients will still succumb to disease relapse despite having received adjuvant FOLFIRINOX, clearly demonstrating that PDAC tumors are highly chemo-resistant even at the micro-metastatic stage. Another regimen that is commonly used in patients with advanced inoperable PDAC is gemcitabine plus nab-paclitaxel (GnP). A phase III trial including 842 patients with metastatic PDAC (MPACT trial) in 2013 showed the benefit of GnP over gemcitabine alone [median overall survival (OS) 8.5 *vs* 6.7 mo; 95% confidence interval (CI) 0.62-0.83; $P < 0.001$], and response rate to GnP was 23%[9]. For patients with locally advanced PDAC, the response rate to FOLFIRINOX was 19% and to GnP was as low as 6%[10]. However, these two regimens are associated with significant toxicities, therefore escalation of these regimens by adding additional cytotoxic agents is expected to be clinically challenging and prohibitive.

PDAC is driven by mutations of multiple genes including *KRAS*, *TP53*, *CDKN2A* and *SMAD4*[11], which are also present in other cancer types such as non-small cell lung and colorectal cancers. However, PDAC tumors are characterized by a profound desmoplastic tumor microenvironment (TME), which accounts for 80%-90% of the tumor architecture[12]. Major components of the TME include a dense fibrotic matrix deposited by cancer-associated fibroblasts (CAFs), and significant infiltration of various subsets of immunosuppressive myeloid cells, vascular cells, and nerve cells[13-15]. The dense stroma plays an important role in tumor growth, proliferation, epithelial-mesenchymal transition (EMT), immune evasion and resistance to various therapies[16]. The low vascularity combined with elevated interstitial pressure dramatically limits vascular delivery and diffusion of therapeutic agents to tumor

cells[17-19]. Therefore, targeting the stroma to improve therapeutic response has been fervently pursued in recent years, albeit with limited success. Importantly, the role of stroma in PDAC progression and treatment resistance has become increasingly controversial. Preclinical mouse models suggest that depletion of stromal fibroblasts alone carries a risk of reverting PDAC cells to a more progenitor-like and aggressive state, with corresponding inferior outcomes[20,21]. These observations underscore the critical need to delineate the diverse interplay between different components of the tumor stroma in order to develop therapies that can modify the tumor stroma in favor of tumor suppression[22]. Herein, we focus specifically on stroma-targeting clinical trials, discuss the outcomes and provide future prospects of this strategy.

COMPONENTS OF THE PANCREATIC TME

Pancreatic stellate cells (PSCs) are star-shaped cells that resemble their hepatic counterparts and were first discovered in 1998[23]. PSCs are found in the exocrine component of healthy pancreas, which when in their quiescent state are characterized by the presence of desmin intermediary filaments, vitamin A and fat droplets. During neoplastic progression, PSCs become activated, acquire a myofibroblast-like phenotype, express α-SMA and secrete extracellular matrix (ECM) proteins (collagen I, collagen III, collagen IV and fibronectin)[24]. These activated PSC are termed CAFs. The ECM proteins secreted/deposited by CAF form a three-dimensional stiff mesh which, along with high molecular weight glycosaminoglycans such as hyaluronan (HA), raises the interstitial pressure that leads to vascular collapse, forming a hypoxic and nutrient poor TME[25,26].

Transcriptomic profiling shows that CAFs consist of at least three distinct subpopulations: Myofibroblast-like (myCAF), inflammatory (iCAF) and antigen-presenting (apCAF, Figure 1)[27,28]. The myCAFs are the most abundant subtype, characterized by high α-SMA expression and hypothesized to have a favorable tumor-restrictive role, potentially explaining paradoxical PDAC progression in genetically engineered mouse models (GEMM) when CAFs are globally depleted[29]. The iCAFs are rich in expression of multiple inflammatory chemokines including interleukin (IL)-6 and proposed to be pro-tumorigenic. The least abundant apCAFs are characterized by abundant expression of MHC class II and therefore may be able to bind and present antigens to $CD4^+$ cytotoxic T-cells. However, due to a lack of co-stimulatory molecules on apCAFs, apCAFs actually dampen, instead of activating the interacting $CD4^+$ T cells[30]. Importantly, it appears that these subtypes are interchangeable, providing an avenue to reprogram these CAFs for therapeutic purposes.

The other predominant cellular components of the pancreatic tumor stroma include endothelial cells, inflammatory cells and nerve cells which have complex interactions with CAFs. Angiogenesis in PDAC is regulated by CAFs, PDAC cells, inflammatory cells and endothelial cells. The hypoxic microenvironment of PDAC acts as a trigger for the transcription factor hypoxia induced factor 1 expression which leads to increased vascular endothelial growth factor (VEGF) production and angiogenesis[31]. VEGF has been identified as an important negative prognostic marker in PDAC which has led to multiple preclinical and clinical studies targeting this pathway[32]. In addition, PDAC stroma is rife with various types of immune cells of myeloid lineages, T cells, B cells that are collectively rendered pro-tumorigenic by various environmental clues[33,34]. Targeting the immune compartment of PDAC, especially the suppressive myeloid cells including macrophages and monocytes, is a rapidly emerging therapeutic strategy, and is outside the scope of this review.

The acellular ECM proteins include collagen I, II, IV, XI-A, fibronectin, laminin, tenascin C and HA. While the dense ECM may physically restrict PDAC progression, ECM proteins have been shown to be protective to PDAC cells. PDAC cells detached from ECM proteins, especially laminin and fibronectin, are prone to apoptosis and necrosis as a result of mitochondrial depolarization and release of cytochrome c and Smac/DIABLO[35]. Various ECM proteins are also capable of promoting EMT and hence therapeutic resistance of PDAC cells[16]. These studies provide solid rationale for targeting these acellular ECM proteins as a therapeutic strategy. The ECM also contains a group of enzymes known as matrix metalloproteinases (MMP), which play a role in degradation of ECM components. The turnover of ECM is a dynamic process and is regulated by tissue MMP inhibitors secreted by stromal cellular structures[36].

Therapies targeting PDAC cells such as chemotherapy, radiation and surgery when used alone or in combination have only provided limited benefit in patients with advanced/metastatic PDAC, rarely improving survival beyond one year. This shifts

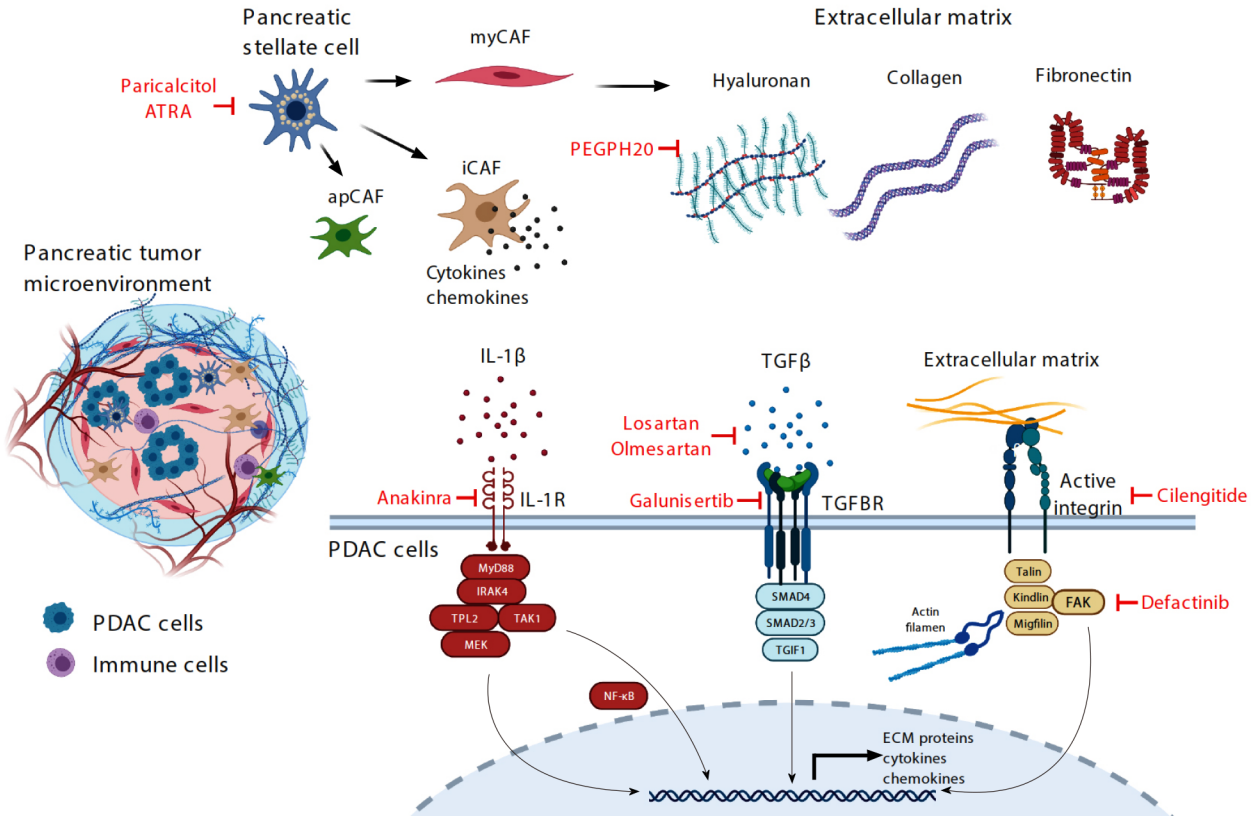


Figure 1 Illustration of the complex pancreatic ductal adenocarcinoma tumor microenvironment that consists of various cell types and acellular extracellular matrix proteins and proteoglycans. Therapeutic strategies that have recently been tested or are being tested in clinical trials are highlighted in red. PDAC: Pancreatic ductal adenocarcinoma; ATRA: All trans retinoic acid; apCAF: Antigen-presenting cancer-associated fibroblasts; iCAF: Inflammatory cancer-associated fibroblasts; myCAF: Myofibroblast-like cancer-associated fibroblasts; IL: Interleukin; TGF: Transforming growth factor; TGFBR: Transforming growth factor β receptor; PEGPH20: Pegylated PH20; NF-κB: Nuclear factor-kappa β; ECM: Extracellular matrix; FAK: Focal adhesion kinase.

the focus to the TME which plays a crucial role in inhibiting the effectiveness of cytotoxic therapies, making them less effective by providing a drug free sanctuary enabling them to thrive and proliferate[37]. A number of preclinical studies involving GEMM targeted the tumor stroma showing a beneficial effect toward better drug delivery and overall disease progression. A number of novel therapeutic strategies targeting the complex interaction of various cellular and acellular components of the PDAC microenvironment were first studied in the pre-clinical setting. The promising results of these in-vitro and in-vivo experiments resulted in clinical translation in an effort to alter the tumor micro-environment to improve outcomes of PDAC patients. Herein we review the effectiveness of these therapies and lessons learned from them, which provides guidance towards future treatments.

TARGETING STROMA-PROMOTING PATHWAYS IN PDAC CELLS

Sonic hedgehog pathway

Cancer stem cells (CSCs) are a small population of cancer cells responsible for tumor initiation, recurrence, growth and metastasis. Like normal stem cells, pancreatic CSCs are regulated by several common signaling pathways, one of which is the Sonic hedgehog (SHH) pathway. The SHH signaling pathway is regulated by a few critical nodes: the HH ligand, the Patched (PTCH) transmembrane receptor, the integral smoothened (SMO) protein and the Glioma Associated Oncogene (GLI) transcription factors. In the absence of SHH, PTCH binds and destabilizes SMO, leading to its degradation. Binding of SHH ligand to PTCH results in internalization and lysosomal degradation of both SHH and PTCH, thereby relieving SMO and allowing it to contribute to the activation of the GLI transcription factors. The GLI factors control several genes that participate in developmental patterning and also production of more SHH[38]. Compared to normal pancreatic stem cells, expression of SHH is upregulated in PDAC CSCs[39]. Olive *et al*[39] showed that targeting SMO with

sarigedib (or IPI-926) depletes the stromal tissue and transiently improves the delivery and therapeutic effect of gemcitabine in an orthotopic transplantable mouse model, suggesting that the SHH pathway may contribute to stromal fibrosis and hindrance of therapeutics in PDAC. In January 2012, a phase II trial involving the SHH pathway inhibitor saridegib (IPI-926) was halted due to detrimental outcomes of patients in the gemcitabine plus saridegib arm compared to the gemcitabine plus placebo arm (NCT01130142). This also resulted in early closure of another trial evaluating the efficacy of saridegib with modified FOLFIRINOX (NCT01383538)[40]. Vismodegib (or GDC-0449) is a competitive SMO inhibitor that is FDA-approved for treatment of advanced or metastatic basal cell carcinoma, in which loss-of-function mutations of *PTCH1* gene are common. A pilot study involving 25 patients showed downregulation of GLI and PTCH1 expression in patients receiving combination of gemcitabine and vismodegib (NCT01195415)[41]. However, a larger phase IB/II trial evaluating 106 patients showed no improvement in response rate, OS or progression free survival (PFS). Importantly, contrary to the results reported by Olive *et al*[39] (NCT01064622), vismodegib did not improve intratumoral delivery of gemcitabine, and when combined with gemcitabine also did not statistically improve the survival of autochthonous PDAC mice[42]. A later Phase II trial (NCT01088815) evaluating 67 patients with untreated metastatic PDAC showed that addition of vismodegib to GnP did not improve PFS or OS compared to historical data of chemotherapy alone[43]. Sonidegib is another orally bioavailable SMO receptor antagonist approved for the treatment of recurrent locally advanced basal cell carcinoma[44]. This drug was studied in patients with PDAC in combination with both gemcitabine and GnP. Two small phase I trials showed good tolerance but no improvement in OS or PFS compared to standard chemotherapy (NCT01487785), (NCT02358161)[45,46]. These clinical studies show that targeting the SHH pathway in combination with standard chemotherapy is ineffective in PDAC. Furthermore, these studies underscore the need to obtain on-treatment correlative data to allow investigation into mechanisms of resistance and treatment failure.

Transforming growth factor β pathway

Enhanced intratumoral transforming growth factor β (TGF-β) signaling promotes tumor fibrosis, progression and poor patient survival in PDAC[47,48]. Importantly, more than half of PDAC cases carry inactivating mutations of *SMAD4*, which encodes a growth-inhibitory transcription factor downstream of the TGF receptor. Unlike other cellular components in the PDAC stroma, pancreatic cancer cells are often deficient in *SMAD4*. This leads to upregulation of TGF-β ligand and has far reaching effects on other cellular components of PDAC that remain *SMAD4* proficient in the stroma. In these cells, TGF-β acts as a driver for desmoplasia. Galunisertib is a small molecule serine/threonine kinase inhibitor of the TGF-β receptor. A phase Ib/II study comparing galunisertib plus gemcitabine *vs* placebo plus gemcitabine was carried out in patients with advanced PDAC including 104 patients in the treatment arm and 52 patients in the placebo arm. There was a modest survival benefit in the treatment group. The median OS was 8.9 mo (95%CI: 7.3-11.1) for the galunisertib group and 7.1 mo (95%CI: 5.8-9.0) for the placebo group. The overall response rate was numerically superior in the galunisertib group, however, the differences were not statistically significant. No significant difference in PFS was observed in the two groups. Patients in the galunisertib group did not experience increased toxicity (NCT01373164)[49]. This modest but encouraging benefit of TGF-β receptor inhibition has led to further clinical trials evaluating the effect of galunisertib with the anti PD-L1 (programmed death ligand 1) monoclonal antibody durvalumab (NCT02734160). To date, the results of this trial are pending.

The angiotensin II receptor blockers losartan and olmesartan have been evaluated in animal models based on their TGF-β pathway inhibitor effects[50,51]. A retrospective study was carried out at Massachusetts General Hospital evaluating PDAC patients taking or not taking angiotensin pathway inhibitors. These investigators found that patients chronically taking angiotensin pathway inhibitors with non-metastatic PDAC had superior OS independent of chemotherapy[52]. A single arm phase II trial enrolled patients with locally advanced pancreatic cancer (LAPC) and treated with total neoadjuvant therapy of FOLFIRINOX and losartan followed by individualized chemoradiotherapy (NCT01821729). This study showed a high rate of R0 resection (69%) as well as prolonged OS among all patients (31.4 mo) including those who underwent resection (33 mo)[53]. This led to a large multicenter trial using losartan with FOLFIRINOX followed by SBRT and nivolumab in LAPC (NCT03563248). To date, the results of this clinical trial are pending.

Focal adhesion kinase

Enhanced focal adhesion kinase (FAK) activity is commonly found in PDAC cells and CAFs and is a determinant of stromal fibrosis and immune evasion[54,55]. Targeting FAK using small molecule inhibitors dramatically attenuated stromal fibrosis, greatly potentiated chemotherapy and immune checkpoint blockade in several preclinical mouse models including the highly aggressive autochthonous KPC (*p48-Cre/p53$^{f/f}$/LSL-KRAS^{G12D}*) mouse model[54]. These findings provided a solid rationale for combining a FAK inhibitor (defactinib) with gemcitabine and anti-PD-1 (programmed death 1) in a clinical trial of patients with PDAC after progression on frontline 5FU-based chemotherapy (NCT02546531). Results of this study are pending.

IL-1 receptor pathway

Constitutive activation of the canonical nuclear factor-κappa β (NF-kB) pathway is a major mechanism that contributes to stromal fibrosis, chemoresistance and poor prognosis in PDAC[56]. In PDAC, activation of the canonical NF-kB cascade is driven both by KRAS-MAPK cascades and reciprocal IL-1β signaling, which drives IRAK4-TPL2 and IKK kinases[56-58]. Targeting IKK kinases has proven to be clinically challenging due to lack of safe and effective agents, but other strategies are being developed to target this pathway. For instance, targeting IRAK4 using small molecule kinase inhibitors was shown to reduce stromal fibrosis and potentiate the efficacy of chemotherapy in preclinical mouse models[56,58]. Currently, the IL-1 receptor antagonist Anakinra is being tested in combination with nab-paclitaxel, gemcitabine and cisplatin in patients with resectable or potentially resectable PDAC (NCT 02550327). To date, the results for this clinical trial are pending.

Connective tissue growth factor

The PDAC TME is rife with a myriad of pro-tumorigenic and pro-fibrotic growth factors. One of these is connective tissue growth factor (CTGF). Interestingly, in preclinical models, treatment with pamrevlumab (or FG-3019), a humanized monoclonal antibody targeting CTGF, potentiates the effect of gemcitabine by downregulating X-linked inhibitor of apoptosis protein, rather than promoting delivery of gemcitabine[59]. In other PDAC pre-clinical studies pamrevlumab was shown to attenuate tumor growth, metastasis and angiogenesis[60]. These studies collectively suggest that the therapeutic effect of pamrevlumab is predominantly through targeting tumor cells. A phase I/II study evaluated pamrevlumab with gemcitabine and nab-paclitaxel in patients with LAPC. After 6 cycles/months of therapy, more patients treated with pamrevlumab and chemotherapy underwent resection compared to patients receiving chemotherapy only (33.3% *vs* 7.7%). The higher resection rate translated into improved OS (non-estimable *vs* 18.56 mo, P = 0.0141). However, it was unclear whether the higher resectability among patients treated with pamrevlumab and chemotherapy was due to higher response rates or lower incidence of disease progression during the six cycles of treatment. Importantly, addition of pamrevlumab did not increase perioperative adverse events or delay in surgical wound healing[61]. A phase III trial is currently underway evaluating the safety and efficacy of pamrevlumab in combination with gemcitabine and nab-paclitaxel for patients with locally advanced PDAC (NCT03941093).

TARGETING STROMA-PROMOTING PATHWAYS IN CAFs

Vitamin D receptor

The role of vitamin D in the risk of developing pancreatic cancer is highly controversial, with studies showing high serum vitamin D levels to be protective, detrimental or have no impact on pancreatic cancer development[62-64]. However, the role of vitamin D repletion in PDAC patients after initial diagnosis and during treatment is actively being pursued in clinical studies. This is based on observational studies showing that higher pre-diagnostic serum vitamin D levels were shown to be associated with better survival in PDAC patients[65], and overwhelming preclinical studies demonstrating protective effects of vitamin D. Specifically, in mouse models ligation of vitamin D receptor with the vitamin D receptor ligand calcipotriol markedly impeded PSC activation, leading to stromal remodeling that augmented intratumoral gemcitabine, reduced tumor volume and prolonged the survival of KPC mice by 57% compared to mice treated with gemcitabine alone[66]. However, it is critical to emphasize that although calcipotriol impedes activation of PSCs, it fails to

block a-SMA expression or collagen I production of fully activated PSCs[67], raising the concern for the modest efficacy of stromal effect of vitamin D receptor ligands. However, vitamin D receptor ligands could have tumor-intrinsic effects. In PDAC cells, calcipotriol lowered the expression of low-density lipoprotein receptor-related protein 6 and inhibits autocrine Wnt signaling[68]. Paricalcitol was also shown to impede PDAC cell proliferation by upregulating cell cycle inhibitors p21 (Waf1/CIP1) and p27 (Kip1)[69]. On these premises, multiple phase I or II studies testing the impact of paricalcitol are currently opened. These include in combination with cisplatin, gemcitabine and nab-paclitaxel for patients with treatment-naïve metastatic PDAC (NCT04054362); in combination with hydroxychloroquine, gemcitabine and nab-paclitaxel for patients with treatment-naïve metastatic PDAC (NCT04524702); in combination with 5-FU/Liposomal irinotecan for patients who have progressed through frontline gemcitabine-based therapies (NCT03883919); and in combination with anti-PD-1 (pembrolizumab) as maintenance treatment for patients who have achieved partial response or stable disease for at least two months on chemotherapy (NCT03331562).

All-trans retinoic acid

There is an association with lower levels of fat-soluble vitamin A and risk of pancreatic cancer, theorized to be due to impaired absorption of fat-soluble vitamins[70]. Preclinical data has shown that vitamin A deficiency leads to activation of PSC, while repletion of vitamin A in culture media converts the PSCs from an activated to a quiescent state. Using the KPC model of human PDAC, treatment with all-trans retinoic acid (ATRA) induced quiescence of PSC, reduced proliferation of PDAC cells and led to increased apoptosis of PDAC cells in part by down-regulating Wnt signaling[71]. Based on this data, a phase I clinical trial was conducted enrolling 27 patients with unresectable PDAC, treated with GnP in combination with ATRA using the established dose for acute promyelocytic leukemia. This study demonstrated the safety and tolerability of the regimen, and diffusion weighted-magnetic resonance imaging identified signals of stromal modulation. This has led to the possibility of repurposing ATRA as a stromal targeting agent in PDAC. Based on these data, the combination of ATRA along with GnP is currently being studied in the Phase II randomized STAR_PAC trial (NCT03307148) enrolling patients with locally advanced or metastatic disease[72].

Other agents targeting PSCs

Pirfenidone is an anti-inflammatory and anti-fibrotic agent that is clinically used for treatment of idiopathic pulmonary fibrosis, however with an unknown mechanism of action. In primary human lung fibroblasts, pirfenidone inhibits proliferation, TGF-β-induced myofibroblast differentiation and pro-collagen expression[73]. Pirfenidone also blocks proliferation, production of collagen, fibronectin and periostin by PSCs *in vitro* and *in vivo*, and potentiates the anti-tumor effect of gemcitabine by reducing stromal fibrosis[74].

Halofunginone (HF) is another anti-fibrotic drug that is of interest in pre-clinical studies. The exact mechanism of action of this agent is unclear however it has shown to cause resolution of pathologic liver fibrosis. In animal models, HF works by inhibiting the activation of PSC's which in-turn decreases the deposition of ECM proteins such as collagen and HA. HF inhibits the downstream signaling of TGF-β by inhibiting SMAD2 and SMAD3 receptors. HF not only improves drug delivery by decreasing fibrosis, but it also fosters favorable immune response by augmenting cytotoxic T-cells and stimulatory myeloid cells in the tumor stroma[75]. To date, no clinical trials are opened yet incorporating these two agents for PDAC patients.

TARGETING ECM

HA

HA is a high molecular weight glycosaminoglycan that is synthesized by HA synthases (HAS1, HAS2, and HAS3) in PDAC cells and CAFs and deposited into the ECM framework[76]. Intratumoral HA undergoes constant turnover *via* degradation by hyaluronidases (HYAL1-4, HYALP1, and PH20). Excessive HA deposition, which is common in PDAC tumors, causes elevated water retention and consequently high interstitial pressure that collapses tumor vasculature and limits delivery of therapeutics[19]. High expression of HA in tumors has been associated with shorter survival post-surgical resection in patients with PDAC[77]. Importantly, aside from

being an important structural component of the ECM, HA has an active signaling function. CD44 is a well-established cellular receptor for HA. Engagement of CD44 on PDAC cells enhances invasion, metastasis, angiogenesis and survival[78]. In PDAC mouse models, addition of hyaluronidase such as pegylated PH20 (PEGPH20) reduces intratumoral HA content and interstitial pressure, thereby permitting re-expansion of the microvasculature. In mouse models, this results in improved delivery of chemotherapy into the PDAC TME and prolongation of survival[19,79].

The above promising preclinical data led to incorporation of PEGPH20 with gemcitabine in a phase Ib clinical trial. Patients with high intratumoral HA content had improved PFS (7.2 mo *vs* 3.5 mo) and OS (13 mo *vs* 5.7 mo) compared to patients with low HA[80]. This encouraging data led to a larger randomized placebo-controlled phase II study (HALO 202) comparing gemcitabine/nab-paclitaxel plus either PEGPH20 (PAG arm) or placebo (AG arm). Again, patients with high HA level (defined as HA staining of > 50% of tumor surface at any intensity) had improved PFS [9.2 mo *vs* 5.2 mo, hazard ratio (HR), 0.51; 95%CI: 0.26-1.00; $P = 0.048$] and OS (11.5 mo *vs* 8.5 mo, HR, 0.96; 95%CI: 0.57-1.61) compared to patients with low HA tumors[81]. Unfortunately, these promising results failed to be recapitulated in a subsequent larger randomized phase III study (HALO 109-301). In this study, only patients with high HA tumors were enrolled, and 492 patients were included in intention-to-treat analysis. Median OS for PAG *vs* AG was 11.2 mo *vs* 11.5 mo (HR, 1.00, 95%CI: 0.80-1.27; $P = 0.97$); median PFS was 7.1 *vs* 7.1 mo (HR, 0.97, 95%CI: 0.75-1.26); confirmed ORR was 34% *vs* 27% (NCT02715804)[82]. In this study, all patients treated with PEGH20 were anticoagulated with low molecular weight heparin to prevent venous thromboembolism. In another phase Ib/II study (SWOG S1313), PEGPH20 was tested in combination with modified FOLFIRINOX *vs* modified FOLFIRINOX alone for treatment naïve PDAC patients. This study had to be halted after interim futility analysis showing inferior outcomes in the PEGPH20 group. Median OS and PFS was strikingly inferior in the PEGPH20 group with a HR of 2.07 (7.7 mo *vs* 14.4 mo, 95%CI: 1.28-3.34, $P < 0.01$) and 1.74 (4.3 mo *vs* 6.2 mo, 95%CI: 1.14-2.66, $P = 0.01$). ORR in PEGPH20 group was also lower, although this difference did not reach statistical significance (33% *vs* 45%, $P = 0.2$) (NCT01959139)[83]. A critical factor leading to the poor outcome of PEGPH20 group was the unexpectedly increased gastrointestinal toxicity and thromboembolic events, which likely resulted in dose reduction of chemotherapy or treatment interruptions[84]. These setbacks led to the cessation of further development of PEGPH20 in cancer clinical trials.

ECM-remodeling enzyme lysyl oxidase-like 2

The ECM-remodeling enzyme lysyl oxidase-like 2 (LOXL2) is a secreted enzyme that maintains the stromal microenvironment in PDAC. Preclinical data identified LOXL2 to be upregulated in cell lines with high invasive potential, and furthermore in human tissue, elevated LOXL2 expression correlates with greater depth of tumor invasion, lymph node involvement, and inferior OS[85]. Simtuzumab, an immunoglobulin G4 monoclonal antibody against LOXL2, was studied in a phase II randomized double-blind placebo-controlled study in combination with gemcitabine in metastatic PDAC patients (NCT01472198). The 240 patients were divided into three groups and received simtuzumab 700 mg, simtuzumab 200 mg or placebo along with gemcitabine. Unfortunately, despite encouraging preclinical and correlative clinical data, when prospectively evaluated, the addition of simtuzumab to gemcitabine did not prolong OS in either cohort of patients with metastatic PDAC compared to gemcitabine alone[86].

Integrins

Integrins are cell surface receptors that mediate surface adhesion of various components of ECM including fibronectin, laminin, collagen and fibrinogen[87]. They play an important role in tumor angiogenesis and lymphangiogenesis, and hence are attractive therapeutic targets[88]. Cilengitide is a low molecular weight anti-angiogenic molecule that acts by inhibiting the integrin surface receptors of endothelial cells[89]. A randomized multi-center phase II trial enrolled patients with unresectable PDAC and treated them with cilengitide and gemcitabine *vs* gemcitabine alone. There were no signs of efficacy, including a median OS of 6.7 mo for those receiving cilengitide and gemcitabine *vs* 7.7 mo for those receiving gemcitabine alone (ISRCTN13413322)[90].

Drugs targeting MMPs

The MMPs are proteolytic enzymes that play an important role in remodeling of ECM

proteins and modifying the tumor stroma in favor of tumor proliferation, invasion and metastasis. MMP inhibitors have been evaluated in multiple solid tumors based on their fundamental role in modulating tumor stroma, with encouraging preclinical data[91]. However, clinical outcomes have been disappointing in PDAC. Specifically, the MMP inhibitors marimastat and BAY12-9566 were studied in comparison to gemcitabine as first line therapy in advanced PDAC patients. These studies both demonstrated superior outcomes with gemcitabine compared to the MMP inhibitors[92,93].

LESSONS AND PROSPECTS

The histologic predominance and tumor-modulating role of stroma continues to propel preclinical and clinical research into stromal-targeting strategies for PDAC (Figure 1). However, clinical success remains limited (Table 1). Until the present time, clinical progress in PDAC has been the result of increasingly aggressive combination chemotherapies, demonstrating that targeting PDAC cells with cytotoxic agents remains a viable strategy, although not ideal, as it is no longer clinically feasible to add further cytotoxic agents to current regimens considering treatment-limiting toxicity. For this reason, stroma-targeting approaches are gaining significant attention. The excitement surrounding stromal targeting is well deserved, given that in preclinical models these approaches permit effective immunotherapy and durable tumor control. As described above, these mechanisms include re-activating T cell and myeloid compartments with or without directly altering the physical immunosuppressive stroma. In contrast to many other solid tumors, clinical efficacy has not yet been realized in PDAC. Studies to date have taught us that PDAC is a very unique tumor type, such that findings in other solid tumors cannot be extrapolated to PDAC. Thus, a more in depth understanding of the different cellular and acellular components of the pancreatic stroma is needed, in order for subsets of cellular or acellular components to be targeted. We have learned that oversimplified preclinical models, especially the widely used GEMM such as KPC mice, which consists of merely two mutations (*KRAS* and *TP53*), are clearly inadequate in PDAC, with findings that do not faithfully translate to patients.

Numerous challenges remain ahead in pancreatic cancer. In the design of future clinical trials, several factors should be taken into consideration. First, the robustness of preclinical data needs to be evaluated carefully before proceeding into the clinical setting. The most pertinent questions to ask are, what type of models were used (patient-derived xenograft, organoid, GEMM)? How many different models were studied to confirm results? How predictive is the current model, in light of prior investigations? What endpoints were considered as significant and meaningful anti-tumor activities (survival, tumor shrinkage)? How was suppressed tumor growth or tumor shrinkage in the animal models defined? Was any synergistic effect seen between the agent of interest and cytotoxic agents? How dramatic is the effect that was seen preclinically? Given that the effect seen in patients is nearly always more modest than that seen in preclinical studies, only those combinations with profound preclinical efficacy, rather than those that meet statistically significant *P* values, should be advanced into the clinic. Despite all of these considerations, the apparent discrepancies between preclinical and clinical success in PDAC research should remind us of the fact that none of the current experimental models are by themselves adequate. Multiple models, both human and mouse-based, must be tested, and ultimately better preclinical models need to be developed.

The phase I trial is a great opportunity for pharmacokinetic and pharmacodynamic (PD) investigation; however, a recent trend has arisen that shifts the focus of the phase I trial to identifying an early efficacy signal, such that subsequent investigations can directly move into the phase III setting. Tissue biopsy collection is essential for PDAC trials for two important reasons. First, rigorous collection of tissues or surrogate biospecimens for the purpose of in-human verification of PD target effects is critical. This will verify *in vivo* on-target activity of the agent being evaluated, which is necessary to confirm relative dose sufficiency and appropriate frame treatment failures. Second, tissue collection will allow an initial assessment of *in vivo* mechanisms of resistance and correlative analyses. Finally, every effort should be made to enroll PDAC patients in appropriate clinical trials to allow patient access to the most advanced therapeutics, optimizing their outcome to the greatest extent possible. Recent failed clinical trials based on impressive preclinical data have provided us with an undesired but valuable opportunity to reexamine the challenges

Table 1 Summary of stromal-targeting clinical trials in pancreatic cancer

Drug	Mechanism of action	Backbone therapy	Outcome
Targeting stroma-promoting pathways in PDAC cells			
Saridegib (IPI-926)	SHH pathway inhibitor	Gemcitabine; mFOLFIRINOX	No improvement in OS or PFS (NCT01130142); No improvement in OS or PFS (NCT01383538)
Vismodegib	SHH pathway inhibitor	Gemcitabine; Gemcitabine + nab-paclitaxel	No improvement in OS or PFS (NCT01064622); No improvement in OS or PFS (NCT01088815)
Sonidegib	SHH pathway inhibitor (SMO receptor antagonist)	Gemcitabine; Gemcitabine + nab-Paclitaxel	No improvement in OS or PFS (NCT01487785); No improvement in OS or PFS (NCT02358161)
Galunisertib	TGF-β receptor inhibitor	Gemcitabine; Gemcitabine + Durvalumab	Ongoing trial (NCT01373164); Ongoing trial (NCT02734160)
Losartan	TGF-β ligand inhibitor	FOLFRINOX; Nivolumab + FOLFRINOX	Ongoing trial (NCT01821729); Ongoing trial (NCT03563248)
Defactinib	Focal Adhesion Kinase inhibitor	Gemcitabine + Pembrolizumab	Ongoing trial (NCT02546531)
Anakinra	IL-1 receptor inhibitor	GnP + Cisplatin	Ongoing trial (NCT02550327)
Targeting stroma-promoting pathways in CAFs			
Pamrevlumab	Antibody against CTGF	Gemcitabine + nab-paclitaxel	Ongoing trial (NCT03941093)
Paricalcitol	Vitamin D agonist; Inactivation of PSC	GnP + Cisplatin; GnP + Hydroxychloroquine; 5FU/Leucovorin/liposomal Irinotecan; Pembrolizumab (2nd line)	Ongoing trial (NCT04054362); Ongoing trial (NCT04524702); Ongoing trial (NCT03883919); Ongoing trial (NCT03331562)
ATRA	Inactivation of PSC	Gemcitabine + nab-paclitaxel	Ongoing trial (NCT03307148)
Targeting ECM			
PEGPH20	Hyaluronan degradation	Gemcitabine + nab-paclitaxel; mFOLFIRINOX	No improvement in OS or PFS (NCT02715804); Poor OS due to poor tolerance (NCT01959139)
Simtuzumab	Antibody against LOXL2	Gemcitabine	No improvement in OS (NCT01472198)
Cilengitide	Integrin inhibitor	Gemcitabine	No improvement in OS (ISRCTN13413322)
Marimastat	MMP inhibitor	Gemcitabine	No improvement in OS
Bay-12-9566	MMP inhibitor	Gemcitabine	No improvement in OS
Others			
Halofunginone	PSC/CAF and SMAD2,3 inhibitor	-	Positive pre-clinical outcomes
Pirfenidone	Cell cycle inhibitor of CAF	-	Positive pre-clinical outcomes

SHH: Sonic hedgehog; OS: Overall survival; PFS: Progression free survival; SMO: Smoothened; TGF: Transforming growth factor; IL: Interleukin; GnP: Gemcitabine plus nab-paclitaxel; CTGF: Connective tissue growth factor; CAF: Cancer-associated fibroblasts; PSC: Pancreatic stellate cell; ECM: Extracellular matrix; LOXL2: Lysyl oxidase-like 2; MMP: Matrix metalloproteinases.

of PDAC and re-iterate the importance of more rigorously designed and scientifically-based clinical investigations. In summary, much progress has been made in recent years understanding the pancreatic tumor stroma, however, lack of subsequent clinical success is evidence that much work remains to be done.

CONCLUSION

The poor response of PDAC to standard cytotoxic regimens has directed attention towards the dense fibrous stroma. The stroma is thought to be a fortress that protects PDAC cells from immune invasion, leads to chemotherapeutic resistance, and thereby provides a sanctuary for these cells to proliferate[16]. There are multiple pre-clinical and *in vitro* studies wherein stroma depletion led to decreased progression and improved survival among animal models. Based on these a variety of novel approaches have been adapted in human trials for clinical translation[35,37,79]. Some of these approaches were initially promising, however, most if not all have led to negative outcomes, financial waste and frustration in the clinic. This has made stromal

targeting in PDAC a much controversial subject. It has been established time and again that indiscriminate targeting and near complete depletion of tumor stroma can cause more harm than good[40,94]. However, these attempts have enhanced our understanding of the tumor micro-environment. There is increased need for caution when targeting these matrix components as we have discovered cellular and acellular components of the stroma that in fact restrain tumor growth and progression[95].

A better understanding of plasticity of the stroma will lead to development of therapeutics that can accurately modulate the tumor micro-environment in favor of tumor suppression. The discovery of heterogeneity among CAF and their paradoxical role in tumor growth further delineates the importance of development of targeted therapies that downregulate subsets of CAF (such as iCAF or possibly apCAF) by selectively modifying the stroma[27,30]. Furthermore, vigorous evaluation of pre-clinical data, comparison of its effectiveness in multiple models and assessment of synergistic response of these novel therapeutics with existing cytotoxic therapy in both human and mouse models is vital to avoid detrimental clinical outcomes. There is a need for development of more standardized pre-clinical models and critical analysis of the data in relation to tumor response in these models before we can translate preclinical findings into clinical success. Such agents once developed may synergistically improve the efficacy of currently available cytotoxic and immune modulating therapies.

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REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Rahib L**, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; **74**: 2913-2921 [PMID: 24840647 DOI: 10.1158/0008-5472.CAN-14-0155]

3 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020; **70**: 7-30 [PMID: 31912902 DOI: 10.3322/caac.21590]

4 **Hasan S**, Jacob R, Manne U, Paluri R. Advances in pancreatic cancer biomarkers. *Oncol Rev* 2019; **13**: 410 [PMID: 31044028 DOI: 10.4081/oncol.2019.410]

5 **Falcone A**, Ricci S, Brunetti I, Pfanner E, Allegrini G, Barbara C, Crinò L, Benedetti G, Evangelista W, Fanchini L, Cortesi E, Picone V, Vitello S, Chiara S, Granetto C, Porcile G, Fioretto L, Orlandini C, Andreuccetti M, Masi G; Gruppo Oncologico Nord Ovest. Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest. *J Clin Oncol* 2007; **25**: 1670-1676 [PMID: 17470860 DOI: 10.1200/jco.2006.09.0928]

6 **Park H**, Jin RU, Wang-Gillam A, Suresh R, Rigden C, Amin M, Tan BR, Pedersen KS, Lim KH, Trikalinos NA, Acharya A, Copsey ML, Navo KA, Morton AE, Gao F, Lockhart AC. FOLFIRINOX for the Treatment of Advanced Gastroesophageal Cancers: A Phase 2 Nonrandomized Clinical Trial. *JAMA Oncol* 2020; **6**: 1231-1240 [PMID: 32469386 DOI: 10.1001/jamaoncol.2020.2020]

7 **Conroy T**, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup. FOLFIRINOX *vs* gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817-1825 [PMID: 21561347 DOI: 10.1056/NEJMoa1011923]

8 **Conroy T**, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, Choné L, Francois E, Artru P, Biagi JJ, Lecomte T, Assenat E, Faroux R, Ychou M, Volet J, Sauvanet A, Breysacher G, Di Fiore F, Cripps C, Kavan P, Texereau P, Bouhier-Leporrier K, Khemissa-Akouz F, Legoux JL, Juzyna B, Gourgou S, O'Callaghan CJ, Jouffroy-Zeller C, Rat P, Malka D, Castan F, Bachet JB; Canadian Cancer Trials Group and the Unicancer-GI–PRODIGE Group. FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer. *N Engl J Med* 2018; **379**: 2395-2406 [PMID: 30575490 DOI: 10.1056/NEJMoa1809775]

9 **Von Hoff DD**, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J,

Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* 2013; **369**: 1691-1703 [PMID: 24131140 DOI: 10.1056/NEJMoa1304369]

10 **Perri G**, Prakash L, Qiao W, Varadhachary GR, Wolff R, Fogelman D, Overman M, Pant S, Javle M, Koay EJ, Herman J, Kim M, Ikoma N, Tzeng CW, Lee JE, Katz MHG. Response and Survival Associated With First-line FOLFIRINOX *vs* Gemcitabine and nab-Paclitaxel Chemotherapy for Localized Pancreatic Ductal Adenocarcinoma. *JAMA Surg* 2020; **155**: 832-839 [PMID: 32667641 DOI: 10.1001/jamasurg.2020.2286]

11 **Bardeesy N**, DePinho RA. Pancreatic cancer biology and genetics. *Nat Rev Cancer* 2002; **2**: 897-909 [PMID: 12459728 DOI: 10.1038/nrc949]

12 **Feig C**, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res* 2012; **18**: 4266-4276 [PMID: 22896693 DOI: 10.1158/1078-0432.CCR-11-3114]

13 **Mitchem JB**, Brennan DJ, Knolhoff BL, Belt BA, Zhu Y, Sanford DE, Belaygorod L, Carpenter D, Collins L, Piwnica-Worms D, Hewitt S, Udupi GM, Gallagher WM, Wegner C, West BL, Wang-Gillam A, Goedegebuure P, Linehan DC, DeNardo DG. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. *Cancer Res* 2013; **73**: 1128-1141 [PMID: 23221383 DOI: 10.1158/0008-5472.CAN-12-2731]

14 **Goedegebuure P**, Mitchem JB, Porembka MR, Tan MC, Belt BA, Wang-Gillam A, Gillanders WE, Hawkins WG, Linehan DC. Myeloid-derived suppressor cells: general characteristics and relevance to clinical management of pancreatic cancer. *Curr Cancer Drug Targets* 2011; **11**: 734-751 [PMID: 21599634 DOI: 10.2174/156800911796191024]

15 **Bayne LJ**, Beatty GL, Jhala N, Clark CE, Rhim AD, Stanger BZ, Vonderheide RH. Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. *Cancer Cell* 2012; **21**: 822-835 [PMID: 22698406 DOI: 10.1016/j.ccr.2012.04.025]

16 **Bulle A**, Lim KH. Beyond just a tight fortress: contribution of stroma to epithelial-mesenchymal transition in pancreatic cancer. *Signal Transduct Target Ther* 2020; **5**: 249 [PMID: 33122631 DOI: 10.1038/s41392-020-00341-1]

17 **Kong X**, Li L, Li Z, Xie K. Targeted destruction of the orchestration of the pancreatic stroma and tumor cells in pancreatic cancer cases: molecular basis for therapeutic implications. *Cytokine Growth Factor Rev* 2012; **23**: 343-356 [PMID: 22749856 DOI: 10.1016/j.cytogfr.2012.06.006]

18 **Hidalgo M**. Pancreatic cancer. *N Engl J Med* 2010; **362**: 1605-1617 [PMID: 20427809 DOI: 10.1056/NEJMra0901557]

19 **Jacobetz MA**, Chan DS, Neesse A, Bapiro TE, Cook N, Frese KK, Feig C, Nakagawa T, Caldwell ME, Zecchini HI, Lolkema MP, Jiang P, Kultti A, Thompson CB, Maneval DC, Jodrell DI, Frost GI, Shepard HM, Skepper JN, Tuveson DA. Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer. *Gut* 2013; **62**: 112-120 [PMID: 22466618 DOI: 10.1136/gutjnl-2012-302529]

20 **Rhim AD**, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA, Dekleva EN, Saunders T, Becerra CP, Tattersall IW, Westphalen CB, Kitajewski J, Fernandez-Barrena MG, Fernandez-Zapico ME, Iacobuzio-Donahue C, Olive KP, Stanger BZ. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer Cell* 2014; **25**: 735-747 [PMID: 24856585 DOI: 10.1016/j.ccr.2014.04.021]

21 **Özdemir BC**, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu CC, Simpson TR, Laklai H, Sugimoto H, Kahlert C, Novitskiy SV, De Jesus-Acosta A, Sharma P, Heidari P, Mahmood U, Chin L, Moses HL, Weaver VM, Maitra A, Allison JP, LeBleu VS, Kalluri R. Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer Cell* 2014; **25**: 719-734 [PMID: 24856586 DOI: 10.1016/j.ccr.2014.04.005]

22 **Wang Z**, Li J, Chen X, Duan W, Ma Q, Li X. Disrupting the balance between tumor epithelia and stroma is a possible therapeutic approach for pancreatic cancer. *Med Sci Monit* 2014; **20**: 2002-2006 [PMID: 25327552 DOI: 10.12659/MSM.892523]

23 **Apte MV**, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut* 1998; **43**: 128-133 [PMID: 9771417 DOI: 10.1136/gut.43.1.128]

24 **Bachem MG**, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grünert A, Adler G. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 1998; **115**: 421-432 [PMID: 9679048 DOI: 10.1016/s0016-5085(98)70209-4]

25 **DuFort CC**, DelGiorno KE, Carlson MA, Osgood RJ, Zhao C, Huang Z, Thompson CB, Connor RJ, Thanos CD, Scott Brockenbrough J, Provenzano PP, Frost GI, Michael Shepard H, Hingorani SR. Interstitial Pressure in Pancreatic Ductal Adenocarcinoma Is Dominated by a Gel-Fluid Phase. *Biophys J* 2016; **110**: 2106-2119 [PMID: 27166818 DOI: 10.1016/j.bpj.2016.03.040]

26 **DuFort CC**, DelGiorno KE, Hingorani SR. Mounting Pressure in the Microenvironment: Fluids, Solids, and Cells in Pancreatic Ductal Adenocarcinoma. *Gastroenterology* 2016; **150**: 1545-1557. e2 [PMID: 27072672 DOI: 10.1053/j.gastro.2016.03.040]

27 **Öhlund D**, Handly-Santana A, Biffi G, Elyada E, Almeida AS, Ponz-Sarvise M, Corbo V, Oni TE, Hearn SA, Lee EJ, Chio II, Hwang CI, Tiriac H, Baker LA, Engle DD, Feig C, Kultti A, Egeblad M,

Fearon DT, Crawford JM, Clevers H, Park Y, Tuveson DA. Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *J Exp Med* 2017; **214**: 579-596 [PMID: 28232471 DOI: 10.1084/jem.20162024]

28 **Elyada E**, Bolisetty M, Laise P, Flynn WF, Courtois ET, Burkhart RA, Teinor JA, Belleau P, Biffi G, Lucito MS, Sivajothi S, Armstrong TD, Engle DD, Yu KH, Hao Y, Wolfgang CL, Park Y, Preall J, Jaffee EM, Califano A, Robson P, Tuveson DA. Cross-Species Single-Cell Analysis of Pancreatic Ductal Adenocarcinoma Reveals Antigen-Presenting Cancer-Associated Fibroblasts. *Cancer Discov* 2019; **9**: 1102-1123 [PMID: 31197017 DOI: 10.1158/2159-8290.CD-19-0094]

29 **Hwang RF**, Moore T, Arumugam T, Ramachandran V, Amos KD, Rivera A, Ji B, Evans DB, Logsdon CD. Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer Res* 2008; **68**: 918-926 [PMID: 18245495 DOI: 10.1158/0008-5472.CAN-07-5714]

30 **Djurec M**, Graña O, Lee A, Troulé K, Espinet E, Cabras L, Navas C, Blasco MT, Martín-Díaz L, Burdiel M, Li J, Liu Z, Vallespinós M, Sanchez-Bueno F, Sprick MR, Trumpp A, Sainz B Jr, Al-Shahrour F, Rabadan R, Guerra C, Barbacid M. Saa3 is a key mediator of the protumorigenic properties of cancer-associated fibroblasts in pancreatic tumors. *Proc Natl Acad Sci USA* 2018; **115**: E1147-E1156 [PMID: 29351990 DOI: 10.1073/pnas.1717802115]

31 **Masamune A**, Kikuta K, Watanabe T, Satoh K, Hirota M, Shimosegawa T. Hypoxia stimulates pancreatic stellate cells to induce fibrosis and angiogenesis in pancreatic cancer. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G709-G717 [PMID: 18669622 DOI: 10.1152/ajpgi.90356.2008]

32 **Ikeda N**, Adachi M, Taki T, Huang C, Hashida H, Takabayashi A, Sho M, Nakajima Y, Kanehiro H, Hisanaga M, Nakano H, Miyake M. Prognostic significance of angiogenesis in human pancreatic cancer. *Br J Cancer* 1999; **79**: 1553-1563 [PMID: 10188906 DOI: 10.1038/sj.bjc.6690248]

33 **Wahab ZA**, Metzgar RS. Human cytotoxic lymphocytes reactive with pancreatic adenocarcinoma cells. *Pancreas* 1991; **6**: 307-317 [PMID: 1862066 DOI: 10.1097/00006676-199105000-00008]

34 **Thomas SK**, Lee J, Beatty GL. Paracrine and cell autonomous signalling in pancreatic cancer progression and metastasis. *EBioMedicine* 2020; **53**: 102662 [PMID: 32139180 DOI: 10.1016/j.ebiom.2020.102662]

35 **Vaquero EC**, Edderkaoui M, Nam KJ, Gukovsky I, Pandol SJ, Gukovskaya AS. Extracellular matrix proteins protect pancreatic cancer cells from death *via* mitochondrial and nonmitochondrial pathways. *Gastroenterology* 2003; **125**: 1188-1202 [PMID: 14517801 DOI: 10.1016/s0016-5085(03)01203-4]

36 **Stetler-Stevenson WG**, Liotta LA, Kleiner DE Jr. Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. *FASEB J* 1993; **7**: 1434-1441 [PMID: 8262328 DOI: 10.1096/fasebj.7.15.8262328]

37 **Apte MV**, Xu Z, Pothula S, Goldstein D, Pirola RC, Wilson JS. Pancreatic cancer: The microenvironment needs attention too! *Pancreatology* 2015; **15**: S32-S38 [PMID: 25845856 DOI: 10.1016/j.pan.2015.02.013]

38 **Sari IN**, Phi LTH, Jun N, Wijaya YT, Lee S, Kwon HY. Hedgehog Signaling in Cancer: A Prospective Therapeutic Target for Eradicating Cancer Stem Cells. *Cells* 2018; **7** [PMID: 30423843 DOI: 10.3390/cells7110208]

39 **Olive KP**, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, Feig C, Combs C, Winter SP, Ireland-Zecchini H, Reichelt S, Howat WJ, Chang A, Dhara M, Wang L, Rückert F, Grützmann R, Pilarsky C, Izeradjene K, Hingorani SR, Huang P, Davies SE, Plunkett W, Egorin M, Hruban RH, Whitebread N, McGovern K, Adams J, Iacobuzio-Donahue C, Griffiths J, Tuveson DA. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009; **324**: 1457-1461 [PMID: 19460966 DOI: 10.1126/science.1171362]

40 **Ko AH**, LoConte N, Tempero MA, Walker EJ, Kate Kelley R, Lewis S, Chang WC, Kantoff E, Vannier MW, Catenacci DV, Venook AP, Kindler HL. A Phase I Study of FOLFIRINOX Plus IPI-926, a Hedgehog Pathway Inhibitor, for Advanced Pancreatic Adenocarcinoma. *Pancreas* 2016; **45**: 370-375 [PMID: 26390428 DOI: 10.1097/MPA.0000000000000458]

41 **Kim EJ**, Sahai V, Abel EV, Griffith KA, Greenson JK, Takebe N, Khan GN, Blau JL, Craig R, Balis UG, Zalupski MM, Simeone DM. Pilot clinical trial of hedgehog pathway inhibitor GDC-0449 (vismodegib) in combination with gemcitabine in patients with metastatic pancreatic adenocarcinoma. *Clin Cancer Res* 2014; **20**: 5937-5945 [PMID: 25278454 DOI: 10.1158/1078-0432.CCR-14-1269]

42 **Catenacci DV**, Junttila MR, Karrison T, Bahary N, Horiba MN, Nattam SR, Marsh R, Wallace J, Kozloff M, Rajdev L, Cohen D, Wade J, Sleckman B, Lenz HJ, Stiff P, Kumar P, Xu P, Henderson L, Takebe N, Salgia R, Wang X, Stadler WM, de Sauvage FJ, Kindler HL. Randomized Phase Ib/II Study of Gemcitabine Plus Placebo or Vismodegib, a Hedgehog Pathway Inhibitor, in Patients With Metastatic Pancreatic Cancer. *J Clin Oncol* 2015; **33**: 4284-4292 [PMID: 26527777 DOI: 10.1200/JCO.2015.62.8719]

43 **De Jesus-Acosta A**, Sugar EA, O'Dwyer PJ, Ramanathan RK, Von Hoff DD, Rasheed Z, Zheng L, Begum A, Anders R, Maitra A, McAllister F, Rajeshkumar NV, Yabuuchi S, de Wilde RF, Batukbhai B, Sahin I, Laheru DA. Phase 2 study of vismodegib, a hedgehog inhibitor, combined with gemcitabine and nab-paclitaxel in patients with untreated metastatic pancreatic adenocarcinoma. *Br J Cancer* 2020; **122**: 498-505 [PMID: 31857726 DOI: 10.1038/s41416-019-0683-3]

44 **Burness CB**. Sonidegib: First Global Approval. *Drugs* 2015; **75**: 1559-1566 [PMID: 26323341 DOI: 10.1007/s40265-015-0458-y]

45 **Macarulla T**, Tabernero J, Palmer DH, Sharma S, Yu KH, Sellami DB, Zhou J, Yi W, Boss H, Kwak

EL. A phase Ib dose escalation, safety, and tolerability study of sonidegib in combination with gemcitabine in patients with locally advanced or metastatic pancreatic adenocarcinoma. *J Clin Oncol* 2016; **34**: 371-371 [DOI: 10.1200/jco.2016.34.4_suppl.371]

46 **Lee K**, Molenaar RJ, Klaassen R, Bijlsma MF, Weterman MJ, Richel DJ, Wymenga M, van Laarhoven HWM, Wilmink JW. A Phase I study of LDE225 in combination with gemcitabine and nab-paclitaxel in patients with metastasized pancreatic cancer. *Ann Oncol* 2017; **28**: v260 [DOI: 10.1093/annonc/mdx369.143]

47 **Principe DR**, DeCant B, Mascariñas E, Wayne EA, Diaz AM, Akagi N, Hwang R, Pasche B, Dawson DW, Fang D, Bentrem DJ, Munshi HG, Jung B, Grippo PJ. TGFβ Signaling in the Pancreatic Tumor Microenvironment Promotes Fibrosis and Immune Evasion to Facilitate Tumorigenesis. *Cancer Res* 2016; **76**: 2525-2539 [PMID: 26980767 DOI: 10.1158/0008-5472.CAN-15-1293]

48 **Friess H**, Yamanaka Y, Büchler M, Ebert M, Beger HG, Gold LI, Korc M. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology* 1993; **105**: 1846-1856 [PMID: 8253361 DOI: 10.1016/0016-5085(93)91084-u]

49 **Melisi D**, Garcia-Carbonero R, Macarulla T, Pezet D, Deplanque G, Fuchs M, Trojan J, Oettle H, Kozloff M, Cleverly A, Smith C, Estrem ST, Gueorguieva I, Lahn MMF, Blunt A, Benhadji KA, Tabernero J. Galunisertib plus gemcitabine vs. gemcitabine for first-line treatment of patients with unresectable pancreatic cancer. *Br J Cancer* 2018; **119**: 1208-1214 [PMID: 30318515 DOI: 10.1038/s41416-018-0246-z]

50 **Masamune A**, Hamada S, Kikuta K, Takikawa T, Miura S, Nakano E, Shimosegawa T. The angiotensin II type I receptor blocker olmesartan inhibits the growth of pancreatic cancer by targeting stellate cell activities in mice. *Scand J Gastroenterol* 2013; **48**: 602-609 [PMID: 23477656 DOI: 10.3109/00365521.2013.777776]

51 **Arnold SA**, Rivera LB, Carbon JG, Toombs JE, Chang CL, Bradshaw AD, Brekken RA. Losartan slows pancreatic tumor progression and extends survival of SPARC-null mice by abrogating aberrant TGFβ activation. *PLoS One* 2012; **7**: e31384 [PMID: 22348081 DOI: 10.1371/journal.pone.0031384]

52 **Liu H**, Naxerova K, Pinter M, Incio J, Lee H, Shigeta K, Ho WW, Crain JA, Jacobson A, Michelakos T, Dias-Santos D, Zanconato A, Hong TS, Clark JW, Murphy JE, Ryan DP, Deshpande V, Lillemoe KD, Fernandez-Del Castillo C, Downes M, Evans RM, Michaelson J, Ferrone CR, Boucher Y, Jain RK. Use of Angiotensin System Inhibitors Is Associated with Immune Activation and Longer Survival in Nonmetastatic Pancreatic Ductal Adenocarcinoma. *Clin Cancer Res* 2017; **23**: 5959-5969 [PMID: 28600474 DOI: 10.1158/1078-0432.CCR-17-0256]

53 **Murphy JE**, Wo JY, Ryan DP, Clark JW, Jiang W, Yeap BY, Drapek LC, Ly L, Baglini CV, Blaszkowsky LS, Ferrone CR, Parikh AR, Weekes CD, Nipp RD, Kwak EL, Allen JN, Corcoran RB, Ting DT, Faris JE, Zhu AX, Goyal L, Berger DL, Qadan M, Lillemoe KD, Talele N, Jain RK, DeLaney TF, Duda DG, Boucher Y, Fernández-Del Castillo C, Hong TS. Total Neoadjuvant Therapy With FOLFIRINOX in Combination With Losartan Followed by Chemoradiotherapy for Locally Advanced Pancreatic Cancer: A Phase 2 Clinical Trial. *JAMA Oncol* 2019; **5**: 1020-1027 [PMID: 31145418 DOI: 10.1001/jamaoncol.2019.0892]

54 **Jiang H**, Hegde S, Knolhoff BL, Zhu Y, Herndon JM, Meyer MA, Nywening TM, Hawkins WG, Shapiro IM, Weaver DT, Pachter JA, Wang-Gillam A, DeNardo DG. Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. *Nat Med* 2016; **22**: 851-860 [PMID: 27376576 DOI: 10.1038/nm.4123]

55 **Zaghdoudi S**, Decaup E, Belhabib I, Samain R, Cassant-Sourdy S, Rochotte J, Brunel A, Schlaepfer D, Cros J, Neuzillet C, Strehaiano M, Alard A, Tomasini R, Rajeeve V, Perraud A, Mathonnet M, Pearce OM, Martineau Y, Pyronnet S, Bousquet C, Jean C. FAK activity in cancer-associated fibroblasts is a prognostic marker and a druggable key metastatic player in pancreatic cancer. *EMBO Mol Med* 2020; **12**: e12010 [PMID: 33025708 DOI: 10.15252/emmm.202012010]

56 **Zhang D**, Li L, Jiang H, Knolhoff BL, Lockhart AC, Wang-Gillam A, DeNardo DG, Ruzinova MB, Lim KH. Constitutive IRAK4 Activation Underlies Poor Prognosis and Chemoresistance in Pancreatic Ductal Adenocarcinoma. *Clin Cancer Res* 2017; **23**: 1748-1759 [PMID: 27702822 DOI: 10.1158/1078-0432.CCR-16-1121]

57 **Dodhiawala PB**, Khurana N, Zhang D, Cheng Y, Li L, Wei Q, Seehra K, Jiang H, Grierson PM, Wang-Gillam A, Lim KH. TPL2 enforces RAS-induced inflammatory signaling and is activated by point mutations. *J Clin Invest* 2020; **130**: 4771-4790 [PMID: 32573499 DOI: 10.1172/JCI137660]

58 **Zhang D**, Li L, Jiang H, Li Q, Wang-Gillam A, Yu J, Head R, Liu J, Ruzinova MB, Lim KH. Tumor-Stroma IL1β-IRAK4 Feedforward Circuitry Drives Tumor Fibrosis, Chemoresistance, and Poor Prognosis in Pancreatic Cancer. *Cancer Res* 2018; **78**: 1700-1712 [PMID: 29363544 DOI: 10.1158/0008-5472.CAN-17-1366]

59 **Neesse A**, Frese KK, Bapiro TE, Nakagawa T, Sternlicht MD, Seeley TW, Pilarsky C, Jodrell DI, Spong SM, Tuveson DA. CTGF antagonism with mAb FG-3019 enhances chemotherapy response without increasing drug delivery in murine ductal pancreas cancer. *Proc Natl Acad Sci U S A* 2013; **110**: 12325-12330 [PMID: 23836645 DOI: 10.1073/pnas.1300415110]

60 **Aikawa T**, Gunn J, Spong SM, Klaus SJ, Korc M. Connective tissue growth factor-specific antibody attenuates tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of pancreatic cancer. *Mol Cancer Ther* 2006; **5**: 1108-1116 [PMID: 16731742 DOI: 10.1158/1535-7163.Mct-05-0516]

61 **Picozzi VJ**, Pishvaian MJ, Mody K, Winter JM, Glaspy JA, Larson T, Matrana MR, Saikali K, Carney M, Porter S, Yu P, Kouchakji E, Carrier E. Effect of anti-CTGF human recombinant

monoclonal antibody pamrevlumab on resectability and resection rate when combined with gemcitabine/nab-paclitaxel in phase 1/2 clinical study for the treatment of locally advanced pancreatic cancer patients. *J Clin Oncol* 2018; **36**: 4016-4016 [DOI: 10.1200/JCO.2018.36.15_suppl.4016]

62 **van Duijnhoven FJB**, Jenab M, Hveem K, Siersema PD, Fedirko V, Duell EJ, Kampman E, Halfweeg A, van Kranen HJ, van den Ouweland JMW, Weiderpass E, Murphy N, Langhammer A, Ness-Jensen E, Olsen A, Tjønneland A, Overvad K, Cadeau C, Kvaskoff M, Boutron-Ruault MC, Katzke VA, Kühn T, Boeing H, Trichopoulou A, Kotanidou A, Kritikou M, Palli D, Agnoli C, Tumino R, Panico S, Matullo G, Peeters P, Brustad M, Olsen KS, Lasheras C, Obón-Santacana M, Sánchez MJ, Dorronsoro M, Chirlaque MD, Barricarte A, Manjer J, Almquist M, Renström F, Ye W, Wareham N, Khaw KT, Bradbury KE, Freisling H, Aune D, Norat T, Riboli E, Bueno-de-Mesquita HBA. Circulating concentrations of vitamin D in relation to pancreatic cancer risk in European populations. *Int J Cancer* 2018; **142**: 1189-1201 [PMID: 29114875 DOI: 10.1002/ijc.31146]

63 **Waterhouse M**, Risch HA, Bosetti C, Anderson KE, Petersen GM, Bamlet WR, Cotterchio M, Cleary SP, Ibiebele TI, La Vecchia C, Skinner HG, Strayer L, Bracci PM, Maisonneuve P, Bueno-de-Mesquita HB, Zaton Ski W, Lu L, Yu H, Janik-Koncewicz K, Polesel J, Serraino D, Neale RE; Pancreatic Cancer Case–Control Consortium (PanC4). Vitamin D and pancreatic cancer: a pooled analysis from the Pancreatic Cancer Case-Control Consortium. *Ann Oncol* 2015; **26**: 1776-1783 [PMID: 25977560 DOI: 10.1093/annonc/mdv236]

64 **Weinstein SJ**, Stolzenberg-Solomon RZ, Kopp W, Rager H, Virtamo J, Albanes D. Impact of circulating vitamin D binding protein levels on the association between 25-hydroxyvitamin D and pancreatic cancer risk: a nested case-control study. *Cancer Res* 2012; **72**: 1190-1198 [PMID: 22232734 DOI: 10.1158/0008-5472.CAN-11-2950]

65 **Yuan C**, Qian ZR, Babic A, Morales-Oyarvide V, Rubinson DA, Kraft P, Ng K, Bao Y, Giovannucci EL, Ogino S, Stampfer MJ, Gaziano JM, Sesso HD, Buring JE, Cochrane BB, Chlebowski RT, Snetselaar LG, Manson JE, Fuchs CS, Wolpin BM. Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival. *J Clin Oncol* 2016; **34**: 2899-2905 [PMID: 27325858 DOI: 10.1200/JCO.2015.66.3005]

66 **Sherman MH**, Yu RT, Engle DD, Ding N, Atkins AR, Tiriac H, Collisson EA, Connor F, Van Dyke T, Kozlov S, Martin P, Tseng TW, Dawson DW, Donahue TR, Masamune A, Shimosegawa T, Apte MV, Wilson JS, Ng B, Lau SL, Gunton JE, Wahl GM, Hunter T, Drebin JA, O'Dwyer PJ, Liddle C, Tuveson DA, Downes M, Evans RM. Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. *Cell* 2014; **159**: 80-93 [PMID: 25259922 DOI: 10.1016/j.cell.2014.08.007]

67 **Wallbaum P**, Rohde S, Ehlers L, Lange F, Hohn A, Bergner C, Schwarzenböck SM, Krause BJ, Jaster R. Antifibrogenic effects of vitamin D derivatives on mouse pancreatic stellate cells. *World J Gastroenterol* 2018; **24**: 170-178 [PMID: 29375203 DOI: 10.3748/wjg.v24.i2.170]

68 **Arensman MD**, Nguyen P, Kershaw KM, Lay AR, Ostertag-Hill CA, Sherman MH, Downes M, Liddle C, Evans RM, Dawson DW. Calcipotriol Targets LRP6 to Inhibit Wnt Signaling in Pancreatic Cancer. *Mol Cancer Res* 2015; **13**: 1509-1519 [PMID: 26224368 DOI: 10.1158/1541-7786.MCR-15-0204]

69 **Schwartz GG**, Eads D, Naczki C, Northrup S, Chen T, Koumenis C. 19-nor-1 alpha,25-dihydroxyvitamin D2 (paricalcitol) inhibits the proliferation of human pancreatic cancer cells *in vitro* and in vivo. *Cancer Biol Ther* 2008; **7**: 430-436 [PMID: 18094617 DOI: 10.4161/cbt.7.3.5418]

70 **Huang X**, Gao Y, Zhi X, Ta N, Jiang H, Zheng J. Association between vitamin A, retinol and carotenoid intake and pancreatic cancer risk: Evidence from epidemiologic studies. *Sci Rep* 2016; **6**: 38936 [PMID: 27941847 DOI: 10.1038/srep38936]

71 **Froeling FE**, Feig C, Chelala C, Dobson R, Mein CE, Tuveson DA, Clevers H, Hart IR, Kocher HM. Retinoic acid-induced pancreatic stellate cell quiescence reduces paracrine Wnt-β-catenin signaling to slow tumor progression. *Gastroenterology* 2011; **141**: 1486-1497, 1497.e1-1497. 14 [PMID: 21704588 DOI: 10.1053/j.gastro.2011.06.047]

72 **Kocher HM**, Basu B, Froeling FEM, Sarker D, Slater S, Carlin D, deSouza NM, De Paepe KN, Goulart MR, Hughes C, Imrali A, Roberts R, Pawula M, Houghton R, Lawrence C, Yogeswaran Y, Mousa K, Coetzee C, Sasieni P, Prendergast A, Propper DJ. Phase I clinical trial repurposing all-trans retinoic acid as a stromal targeting agent for pancreatic cancer. *Nat Commun* 2020; **11**: 4841 [PMID: 32973176 DOI: 10.1038/s41467-020-18636-w]

73 **Conte E**, Gili E, Fagone E, Fruciano M, Iemmolo M, Vancheri C. Effect of pirfenidone on proliferation, TGF-β-induced myofibroblast differentiation and fibrogenic activity of primary human lung fibroblasts. *Eur J Pharm Sci* 2014; **58**: 13-19 [PMID: 24613900 DOI: 10.1016/j.ejps.2014.02.014]

74 **Kozono S**, Ohuchida K, Eguchi D, Ikenaga N, Fujiwara K, Cui L, Mizumoto K, Tanaka M. Pirfenidone inhibits pancreatic cancer desmoplasia by regulating stellate cells. *Cancer Res* 2013; **73**: 2345-2356 [PMID: 23348422 DOI: 10.1158/0008-5472.CAN-12-3180]

75 **Elahi-Gedwillo KY**, Carlson M, Zettervall J, Provenzano PP. Antifibrotic Therapy Disrupts Stromal Barriers and Modulates the Immune Landscape in Pancreatic Ductal Adenocarcinoma. *Cancer Res* 2019; **79**: 372-386 [PMID: 30401713 DOI: 10.1158/0008-5472.CAN-18-1334]

76 **Toole BP**. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* 2004; **4**: 528-539 [PMID: 15229478 DOI: 10.1038/nrc1391]

77 **Cheng XB**, Sato N, Kohi S, Yamaguchi K. Prognostic impact of hyaluronan and its regulators in pancreatic ductal adenocarcinoma. *PLoS One* 2013; **8**: e80765 [PMID: 24244714 DOI:

10.1371/journal.pone.0080765]
78 **Toole BP**, Slomiany MG. Hyaluronan: a constitutive regulator of chemoresistance and malignancy in cancer cells. *Semin Cancer Biol* 2008; **18**: 244-250 [PMID: 18534864 DOI: 10.1016/j.semcancer.2008.03.009]
79 **Provenzano PP**, Cuevas C, Chang AE, Goel VK, Von Hoff DD, Hingorani SR. Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. *Cancer Cell* 2012; **21**: 418-429 [PMID: 22439937 DOI: 10.1016/j.ccr.2012.01.007]
80 **Hingorani SR**, Harris WP, Beck JT, Berdov BA, Wagner SA, Pshevlotsky EM, Tjulandin SA, Gladkov OA, Holcombe RF, Korn R, Raghunand N, Dychter S, Jiang P, Shepard HM, Devoe CE. Phase Ib Study of PEGylated Recombinant Human Hyaluronidase and Gemcitabine in Patients with Advanced Pancreatic Cancer. *Clin Cancer Res* 2016; **22**: 2848-2854 [PMID: 26813359 DOI: 10.1158/1078-0432.CCR-15-2010]
81 **Hingorani SR**, Zheng L, Bullock AJ, Seery TE, Harris WP, Sigal DS, Braiteh F, Ritch PS, Zalupski MM, Bahary N, Oberstein PE, Wang-Gillam A, Wu W, Chondros D, Jiang P, Khelifa S, Pu J, Aldrich C, Hendifar AE. HALO 202: Randomized Phase II Study of PEGPH20 Plus Nab-Paclitaxel/Gemcitabine Versus Nab-Paclitaxel/Gemcitabine in Patients With Untreated, Metastatic Pancreatic Ductal Adenocarcinoma. *J Clin Oncol* 2018; **36**: 359-366 [PMID: 29232172 DOI: 10.1200/JCO.2017.74.9564]
82 **Tempero MA**, Cutsem EV, Sigal D, Oh D-Y, Fazio N, Macarulla T, Hitre E, Hammel P, Hendifar AE, Bates SE, Li C-P, Fouchardiere CDL, Heinemann V, Maraveyas A, Bahary N, Layos L, Sahai V, Zheng L, Lacy J, Bullock AJ, Investigators H-. HALO 109-301: A randomized, double-blind, placebo-controlled, phase 3 study of pegvorhyaluronidase alfa (PEGPH20) + nab-paclitaxel/gemcitabine (AG) in patients (pts) with previously untreated hyaluronan (HA)-high metastatic pancreatic ductal adenocarcinoma (mPDA). *J Clin Oncol* 2020; **38**: 638-638 [DOI: 10.1200/JCO.2020.38.4_suppl.638]
83 **Ramanathan RK**, McDonough SL, Philip PA, Hingorani SR, Lacy J, Kortmansky JS, Thumar J, Chiorean EG, Shields AF, Behl D, Mehan PT, Gaur R, Seery T, Guthrie KA, Hochster HS. Phase IB/II Randomized Study of FOLFIRINOX Plus Pegylated Recombinant Human Hyaluronidase Versus FOLFIRINOX Alone in Patients With Metastatic Pancreatic Adenocarcinoma: SWOG S1313. *J Clin Oncol* 2019; **37**: 1062-1069 [PMID: 30817250 DOI: 10.1200/JCO.18.01295]
84 **Wang-Gillam A**. Targeting Stroma: A Tale of Caution. *J Clin Oncol* 2019; **37**: 1041-1043 [PMID: 30860950 DOI: 10.1200/JCO.19.00056]
85 **Peng L**, Ran YL, Hu H, Yu L, Liu Q, Zhou Z, Sun YM, Sun LC, Pan J, Sun LX, Zhao P, Yang ZH. Secreted LOXL2 is a novel therapeutic target that promotes gastric cancer metastasis *via* the Src/FAK pathway. *Carcinogenesis* 2009; **30**: 1660-1669 [PMID: 19625348 DOI: 10.1093/carcin/bgp178]
86 **Benson AB 3rd**, Wainberg ZA, Hecht JR, Vyushkov D, Dong H, Bendell J, Kudrik F. A Phase II Randomized, Double-Blind, Placebo-Controlled Study of Simtuzumab or Placebo in Combination with Gemcitabine for the First-Line Treatment of Pancreatic Adenocarcinoma. *Oncologist* 2017; **22**: 241-e15 [PMID: 28246206 DOI: 10.1634/theoncologist.2017-0024]
87 **Brooks PC**. Role of integrins in angiogenesis. *Eur J Cancer* 1996; **32A**: 2423-2429 [PMID: 9059330 DOI: 10.1016/s0959-8049(96)00381-4]
88 **Garmy-Susini B**, Varner JA. Roles of integrins in tumor angiogenesis and lymphangiogenesis. *Lymphat Res Biol* 2008; **6**: 155-163 [PMID: 19093788 DOI: 10.1089/lrb.2008.1011]
89 **Eskens FA**, Dumez H, Hoekstra R, Perschl A, Brindley C, Böttcher S, Wynendaele W, Drevs J, Verweij J, van Oosterom AT. Phase I and pharmacokinetic study of continuous twice weekly intravenous administration of Cilengitide (EMD 121974), a novel inhibitor of the integrins alphavbeta3 and alphavbeta5 in patients with advanced solid tumours. *Eur J Cancer* 2003; **39**: 917-926 [PMID: 12706360 DOI: 10.1016/s0959-8049(03)00057-1]
90 **Friess H**, Langrehr JM, Oettle H, Raedle J, Niedergethmann M, Dittrich C, Hossfeld DK, Stöger H, Neyns B, Herzog P, Piedbois P, Dobrowolski F, Scheithauer W, Hawkins R, Katz F, Balcke P, Vermorken J, van Belle S, Davidson N, Esteve AA, Castellano D, Kleeff J, Tempia-Caliera AA, Kovar A, Nippgen J. A randomized multi-center phase II trial of the angiogenesis inhibitor Cilengitide (EMD 121974) and gemcitabine compared with gemcitabine alone in advanced unresectable pancreatic cancer. *BMC Cancer* 2006; **6**: 285 [PMID: 17156477 DOI: 10.1186/1471-2407-6-285]
91 **Winer A**, Adams S, Mignatti P. Matrix Metalloproteinase Inhibitors in Cancer Therapy: Turning Past Failures Into Future Successes. *Mol Cancer Ther* 2018; **17**: 1147-1155 [PMID: 29735645 DOI: 10.1158/1535-7163.MCT-17-0646]
92 **Moore MJ**, Hamm J, Dancey J, Eisenberg PD, Dagenais M, Fields A, Hagan K, Greenberg B, Colwell B, Zee B, Tu D, Ottaway J, Humphrey R, Seymour L; National Cancer Institute of Canada Clinical Trials Group. Comparison of gemcitabine *vs* the matrix metalloproteinase inhibitor BAY 12-9566 in patients with advanced or metastatic adenocarcinoma of the pancreas: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2003; **21**: 3296-3302 [PMID: 12947065 DOI: 10.1200/jco.2003.02.098]
93 **Evans JD**, Stark A, Johnson CD, Daniel F, Carmichael J, Buckels J, Imrie CW, Brown P, Neoptolemos JP. A phase II trial of marimastat in advanced pancreatic cancer. *Br J Cancer* 2001; **85**: 1865-1870 [PMID: 11747327 DOI: 10.1054/bjoc.2001.2168]
94 **van Mackelenbergh MG**, Stroes CI, Spijker R, van Eijck CHJ, Wilmink JW, Bijlsma MF, van Laarhoven HWM. Clinical Trials Targeting the Stroma in Pancreatic Cancer: A Systematic Review

and Meta-Analysis. *Cancers (Basel)* 2019; **11** [PMID: 31035512 DOI: 10.3390/cancers11050588]

95 **Jiang H**, Torphy RJ, Steiger K, Hongo H, Ritchie AJ, Kriegsmann M, Horst D, Umetsu SE, Joseph NM, McGregor K, Pishvaian MJ, Blais EM, Lu B, Li M, Hollingsworth M, Stashko C, Volmar K, Yeh JJ, Weaver VM, Wang ZJ, Tempero MA, Weichert W, Collisson EA. Pancreatic ductal adenocarcinoma progression is restrained by stromal matrix. *J Clin Invest* 2020; **130**: 4704-4709 [PMID: 32749238 DOI: 10.1172/JCI136760]

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MINIREVIEWS

Magnetic resonance imaging-based artificial intelligence model in rectal cancer

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Abstract

Rectal magnetic resonance imaging (MRI) is the preferred method for the diagnosis of rectal cancer as recommended by the guidelines. Rectal MRI can accurately evaluate the tumor location, tumor stage, invasion depth, extramural vascular invasion, and circumferential resection margin. We summarize the progress of research on the use of artificial intelligence (AI) in rectal cancer in recent years. AI, represented by machine learning, is being increasingly used in the medical field. The application of AI models based on high-resolution MRI in rectal cancer has been increasingly reported. In addition to staging the diagnosis and localizing radiotherapy, an increasing number of studies have reported that AI models based on high-resolution MRI can be used to predict the response to chemotherapy and prognosis of patients.

Key Words: Artificial intelligence; Deep learning; Colorectal cancer; Magnetic resonance imaging; Radiomics

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Core Tip: Recently, there has been an increase in the use of artificial intelligence (AI) models based on magnetic resonance (MR) images in locally advanced rectal cancer. MR imaging based on big datasets helps in the provision of systematic and precise medical services to patients with rectal cancer in the treatment process. This review summarizes recent research progress on the application of AI based on MR images in rectal cancer.

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INTRODUCTION

Over 1 million people die from colorectal cancer (CRC) every year worldwide. According to the statistics by the United States National Cancer Institute, CRC is the third most common cancer in both men and women[1]. The risk of lymph node metastasis in patients with rectal cancer is 29%-52%[2-4]. Lymph node metastasis has predictive value for postoperative local recurrence and distant metastasis. High-resolution magnetic resonance imaging (MRI) is the preferred clinical imaging method to evaluate lymph node metastasis of rectal cancer[5]. Rectal MRI can accurately evaluate the tumor location, tumor stage, invasion depth, extramural vascular invasion (EMVI), and circumferential resection margin[6-8]. Accurate MRI assessment is of great significance to patients with rectal cancer, as it is related to the patients' prognosis[9]. It can be used to make a reasonable clinical treatment plan.

Nowadays, with the rapid development of advanced technology, artificial intelligence (AI) is being used in many fields. AI, represented by machine learning, is being increasingly used in the medical field too[10-12]. Machine learning-based AI uses big data collection and analysis to make effective decisions and predictions about new situations. In terms of algorithm application, artificial neural networks, support vector machines (SVM), and kernel methods are what are mainly being used[13-18] (Figure 1). With the increasing application of AI in the medical field, MRI based on big datasets helps in the provision of systematic and precise medical services to patients with rectal cancer in the treatment process.

MRI-BASED AI MODEL IMPROVES THE ACCURACY OF STAGING RECTAL CANCER

Lymphatic spread is an important metastatic route of rectal cancer, and many studies have reported that lymph node metastasis increases the risk of local recurrence in patients. Only 10% of the patients with stage T1 rectal cancer had lymph node metastasis. More than 40% of rectal cancer patients with lymph node metastasis did not have distant metastasis when local recurrence occurred[19,20]. If a metastatic lymph node can be identified before surgery, it is necessary and reasonable to perform radical surgery involving regional lymph node dissection for these patients. According to the National Comprehensive Cancer Network guidelines and clinical practice, neoadjuvant chemoradiotherapy is recommended for patients with locally advanced rectal cancer. Therefore, accurate evaluation by using MRI to stage the disease is crucial for clinical decision-making.

High-resolution MRI has many advantages for clinically staging rectal cancer[21]. The images are reviewed by two radiologists who are experts in clinical work. It is time-consuming to make an accurate diagnosis. There is an inevitable subjective bias among different physicians[22]. Girshick *et al* [23] put forward faster region-based convolutional neural network ("Faster R-CNN") in 2016, which was widely used in the simulation of medical image diagnosis. Ding *et al*[24] developed an AI model based on the "Faster R-CNN" algorithm to detect the lymph nodes of patients with rectal cancer. The accuracy of the model was verified by multicenter clinical practice. The MRI data of 414 CRC patients with pelvic lymph node metastasis were first identified using the R-CNN system, and the results were compared to those of radiologists and pathologists. The consistency between radiologists and the AI model was 0.912. The consistency of the AI model and pathology diagnosis was 0.448. The consistency between radiologists and pathologists was only 0.134. The conclusion was that the AI model based on "Faster R-CNN" was superior to the radiologists in predicting pelvic lymph node metastasis in rectal cancer patients, but its accuracy was inferior to that of the pathologists.

Lymph node detection is more complicated due to obvious individual differences in lymph node locations and sizes. He *et al*[25] proposed the "Mask R-CNN" that has excellent data processing ability. Multiparametric MRI (mpMRI) includes images such as T2WI and DWI. Zhao *et al*[26] attempted to combine mpMRI and "Mask R-CNN"

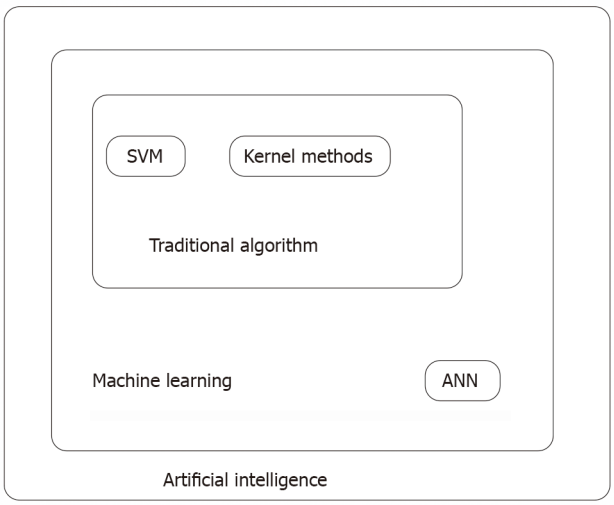


Figure 1 Relationship between artificial neural networks and support vector machines in artificial intelligence. ANN: Artificial neural networks; SVM: Support vector machines.

into an automatic lymph node detection system. The sensitivity for lymph node detection in this model was 80%. The results are similar to those of Barbu *et al* (80%) and Feuersteins *et al* (82.1%), which were higher than those of Kitasaka *et al* (57%) and Feulner *et al* (65.4%)[27-29]. The positive predictive value of the AI model for lymph node detection was 73.5%, which was significantly higher than that of the other four studies. The average time required by the AI model to detect a patient was 1.43 s. The average time required for a radiologist with nine years of experience at the center was 134.4 s. The AI model reduces the time cost to some extent. The conclusion is that the AI detection system can improve the efficiency of lymph node detection, especially for lymph nodes ≥ 3 mm. Based on this study, we can infer that R-CNN combined with multi-parameter MRI can better improve the efficiency of lymph node detection.

A single-center retrospective study reported that high-resolution MRI based on an AI model can be used to evaluate the involvement of circumferential resection margins in rectal cancer[30]. A total of 240 CRC patients with positive circumferential resection margins were included, and image training was carried out based on the regional convolutional neural network AI model. In the training set, the ratio of positive to negative images was 1:2. The diagnosis results of the AI platform were compared with those of a senior radiologist. The accuracy, sensitivity, and specificity of the model were 0.932, 0.838, and 0.956, respectively. The area under the curve (AUC) was 0.953. This study suggested high-resolution MRI based on an AI model has a good prospect of application in evaluation for the involvement of circumferential resection margins in rectal cancer.

Concurrent peritoneal metastasis (PM) may occur in 5%–15% of CRC patients, and heterochronous peritoneal metastasis may occur in approximately 20% of CRC patients. The sensitivity of computed tomography (CT) to PM was positively correlated with lesion size, especially for lesions < 0.5 cm. The sensitivity was between 11% and 70%[31]. It is difficult for radiologists to detect simultaneous peritoneal metastasis in CRC. Yuan *et al*[32] constructed an AI model based on an SVM classifier using the RESnet-3D algorithm to evaluate simultaneous peritoneal metastatic carcinoma of CRC. In this model, CT images of 54 patients with peritoneal metastatic cancer and 76 patients with CRC without peritoneal metastatic cancer were included in the training set. The RESnet-3D features were combined with 12 specific signs of peritoneal metastatic cancer in order to construct an SVM classifier. The sensitivity, specificity, and accuracy of the AI model for PM detection were 93.75%, 94.44%, and 94.11%, respectively. It is therefore much better than conventional enhanced CT. It is believed that there will be a more developed image diagnosis system based on the AI model in the future that will make staging diagnosis in MR images more efficient, accurate, and stable, and reduce the judgment error caused by the differences in radiologists' diagnostic abilities to a certain extent.

APPLICATION OF AN MRI-BASED AI MODEL IN RADIOTHERAPY

It is a very important yet time-consuming process to manually draw tumor volume contours in rectal cancer radiotherapy. Wang *et al*[33] aimed to develop an automatic tumor segmentation algorithm based on deep learning to segment rectal tumors using T2-weighted MRI images. This study included 93 patients with locally advanced rectal cancer (cT3-4 and/or cN1-2) who underwent surgical treatment following neoadjuvant chemoradiotherapy (nCRT). The model was trained in two stages: Tumor recognition and tumor segmentation. The data sets were randomly divided into training sets (90%) and validation sets (10%). Four indices, namely, the Hausdorff distance (HD), average surface distance (ASD), Dice index (DSC), and Jaccard index (JSC), were calculated to evaluate the advantages and disadvantages of automatic and manual segmentation. The DSC, JSC, HD, and ASD results of the AI model and those of radiologists (manual segmentation) were compared as follows: DSC [(0.74 ± 0.14) *vs* (0.71 ± 0.13)] (P = 0.42), JSC [0.60 ± 0.16 *vs* 0.57 ± 0.15] (P = 0.35), HD [20.44 ± 13.35 *vs* 14.91 ± 7.62] (P = 0.35), and ASD [3.25 ± 1.69 mm *vs* 2.67 ± 1.46] (P = 0.16), which showed no statistically significant difference between the automatic segmentation group and the manual segmentation group. A total of 140 patients with locally advanced rectal cancer were included in another study[34]. The multi-parameter MRI images of patients were identified using an AI model based on a deep learning algorithm. Its deep learning algorithm was mainly based on a convolutional neural network. The model was used to segment the tumor after recognition of the MRI image. In this study, the results of the AI model were compared with those of two senior radiologists, and the AUC was 0.99. The AI model can be accurate in tumor location and tumor segmentation in CRC patients.

CNNs are an advanced algorithm applied at present. However, repeated pooling and undersampling can reduce the resolution of the features and may result in the loss of detailed information. Men *et al*[35] combined cascaded atrous convolution (CAC) and spatial pyramid pooling module (SPP), and developed a new-type CNN (CAC-SPP) that could further improve the accuracy of tumor segmentation. The detection efficiency of the CAC-SPP model was superior to that of the algorithm models U-NET and RESnet-101 (P < 0.05). The detection speed of the CAC-SPP model was similar to that of RESnet-101, which was faster than that of U-NET. In short, the CAC-SPP model with high-resolution MRI can improve the accuracy of rectal tumor segmentation by obtaining more tumor features.

APPLICATION OF AN MRI-BASED AI MODEL IN THE EVALUATION OF STAGE AFTER NEOADJUVANT THERAPY FOR RECTAL CANCER

Currently, total mesorectal excision has become the standard treatment for locally advanced rectal cancer[36]. In clinical practice, there are often patients with rectal cancer of the same stage for whom nCRT has different curative effects. Some patients achieved a pathological complete response (PCR) after nCRT, while others did not respond to treatment and some even had progressive disease. Over 30% of the patients could not benefit from nCRT[37]. For the patients who had a PCR after neoadjuvant therapy, the risk of local recurrence was relatively low. For these patients, minimally invasive and anal sphincter sparing surgery, or even the "watch and see" strategy is the mainstream opinion at present. However, due to the limitations of the current diagnostic methods, clinical complete response is not equivalent to PCR. Clinicians should focus on improving the accuracy of MRI by using high-resolution MRI and selecting a more effective MR contrast agent. The increase in the MR sequence increases the workload of the radiologists and leads to the consumption of imaging examination time. In addition, different hospitals have different clinical diagnostic abilities and different radiologists are inconsistent in making imaging diagnoses. Combined with radiomics and convolutional neural networks, increasing research has been conducted on construction of image recognition models based on AI in order to solve these problems. The use of MR images based on AI models to evaluate the response to neoadjuvant therapy in patients with locally advanced rectal cancer and identify patients with a complete pathological response in the early stages will be of great significance for the realization of individualized precision therapy for CRC in the future.

Ferrari *et al*[38] developed an AI model based on high-resolution MRI, hoping that this model can help identify patients with a complete pathological response after neoadjuvant therapy in locally advanced rectal cancer, and can identify the non-

responders after neoadjuvant therapy in the early stages. This study included 55 patients with locally advanced rectal cancer who received surgical treatment after neoadjuvant therapy. The patients were randomly divided into two groups, one for the training cohort and the other for the validation cohort. In the training cohort, the MRI images of patients were combined with the histopathological results using the algorithm, and the model performance was verified in the validation cohort. The AUC for identifying complete reaction in the validation set was 0.86 (95%CI: 0.70. 0.94), and the AUC for identifying the non-responders was 0.83 (95%CI: 0.71, 0.92). In conclusion, the AI model based on high-resolution MR images has achieved good results, which is helpful in identifying patients with complete remission after neoadjuvant therapy and patients with no response to neoadjuvant therapy in the early stages.

Zhang *et al*[39] developed a deep learning algorithm model based on T2 weighting and diffusion kurtosis MR images. A prospective study was carried out based on this model, involving 383 patients with 308 as the training set and 104 as the verification set. The model aimed to evaluate PCR, tumor regression grade, and down-staging of locally advanced rectal cancer after neoadjuvant therapy. The study results showed that the AUC of the validation set in predicting PCR was 0.99. The AUC for T-cell receptor gamma and T staging assessments was 0.70 and 0.79, respectively. With the assistance of the deep learning algorithm model, the error rate of radiology physicians in reading films was significantly reduced. A deep learning model based on diffusion kurtosis MR can evaluate the PCR of patients after nCRT and assist radiologists in evaluating the chemotherapy responses after neoadjuvant therapy in locally advanced rectal cancer.

Shi *et al*[40] developed an AI model based on a deep learning algorithm using MRI images before and during neoadjuvant therapy. The study aimed to evaluate and predict the patient's response to neoadjuvant therapy. It was found that the AI image recognition model based solely on MR images before nCRT or MRI images during neoadjuvant therapy was less effective than that combining two MR images in terms of PCR or good response after neoadjuvant therapy.

APPLICATION OF AN MRI-BASED AI MODEL IN PREDICTING CHEMORADIOTHERAPY RESPONSE IN PATIENTS WITH RECTAL CANCER

Fu *et al*[41] designed a study using experienced radiation oncologists and a deep learning algorithm model to map tumor volume contours on DWI images after neoadjuvant therapy in patients with locally advanced rectal cancer. In this study, a least absolute shrinkage and selection operator-logistic regression model was used to predict treatment response. The results of the two models were verified and compared with regard to the AUC. The AUC of the manual drawing group was 0.64, and that of the deep learning group was 0.73 ($P < 0.05$). On analysis of the findings from this study, the performance of the deep learning model was found to be better than that of manual mapping in predicting the response of rectal cancer to neoadjuvant therapy. A similar retrospective study was recently published by Yi *et al*[42]. A total of 134 patients were included, and T2-weighted MRI images were extracted using machine learning models to predict the patients' chemotherapeutic response and tumor downstaging. The results were verified using the subject work curve. The AUC of the PCR group was 0.91 (95%CI: 0.83-0.98), that of the good response group was 0.90 (95%CI: 0.83-0.97), and the tumor downstage AUC was 0.93 (95%CI: 0.87-0.98). The results suggest that radiomics based on machine learning has promising application prospects in predicting the response of patients to chemotherapy. A study compared a Bayesian network algorithm model with a model combining SVM, Bayesian networks, convolutional neural networks, and the K-nearest algorithm in predicting CRC patients' responses to neoadjuvant therapy[43]. The shape feature set, texture feature set, intensity feature set, and the other information of the MR images were extracted by the two models for comprehensive evaluation. The AUC and accuracy of the Bayesian network algorithm model in the validation queue were 74% and 79%, respectively, while the AUC and accuracy of the combined model were 95% and 90%, respectively. The deep learning model integrated with multiple algorithms showed better performance in predicting neoadjuvant therapy responsiveness in rectal cancer patients.

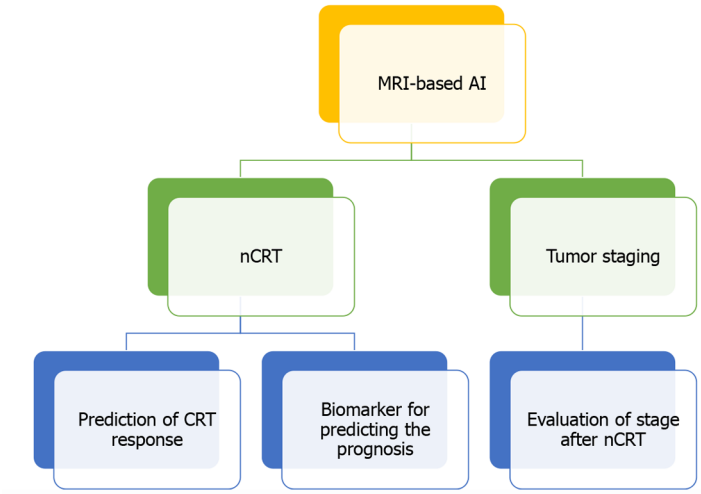


Figure 2 Application of artificial intelligence based on magnetic resonance images in rectal cancer. MRI: Magnetic resonance imaging; AI: Artificial intelligence; nCRT: Neoadjuvant chemoradiotherapy.

MRI-BASED AI MODEL MAY BE AN EFFECTIVE BIOMARKER FOR PREDICTING THE PROGNOSIS OF PATIENTS

Many scholars have realized the role of radiological characteristics in predicting the mutation status of the *KRAS* gene in patients. Recently, Cui *et al*[44] used an AI model based on MR images to predict individuals with *KRAS* gene mutations among CRC patients. In this study, 304 patients were included, and the model predicted performance with an AUC of 0.722 (95%CI, 0.654-0.790) in the training set and 0.714 (95%CI, 0.602-0.827) in the validation set.

Radiomics features based on T2-weighted images are effective in predicting the *KRAS* status in patients with rectal cancer to determine the expression of *KRAS* in patients with rectal cancer, which may be helpful in supplementing genomic analysis of patients. In the future, more MR images should be included, analyzed, and trained to improve the predictive efficiency of this model.

MrT staging, mrN staging, positive EMVI, and peritoneal reflection invasion were all associated with heterochronic distant metastasis after surgery. It has been confirmed that histological invasion of the vessels around the intestinal wall is an independent predictor of distant metastasis for rectal cancer[45]. Clinically, if the high-risk population of postoperative local recurrence and distant metastasis of rectal cancer can be identified and screened in the early stages, targeted systematic treatment can be administered to high-risk populations before surgery to adjust the treatment strategy and improve the prognosis of patients. It has been reported that the radiomics model based on MRI can be used to predict lymph node metastasis of bladder cancer and breast cancer[46,47]. The results suggest that the radiological information contained in MRI images may serve as an effective biomarker for predicting the prognosis of patients. In other words, the MR-based AI models use automated, high-throughput technologies to extract image information and features to capture intra-tumor heterogeneity in a non-invasive manner to guide personalized medicine and precision therapy.

CONCLUSION

AI is a new technical science. AI methods represented by machine learning have been applied more and more widely in the field of medicine (Figure 2). Rectal MRI is considered the preferred method for the diagnosis of rectal cancer as recommended by national guidelines. Therefore, its importance is self-evident. Recently, there has been an increase in the use of AI models based on MR images in locally advanced rectal cancer. MRI combined with AI can not only improve the accuracy of staging and the efficiency of lymph node detection but also effectively evaluate the patients' response to neoadjuvant chemoradiotherapy. Through continuous optimization of the deep learning algorithm, MRI combined with a deep learning image recognition system can effectively predict the prognosis and treatment response of patients. It is believed that

in the near future, AI models based on MRI will be vital in the provision of systematic and precise medical services for the treatment of CRC patients.

REFERENCES

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020; **70**: 7-30 [PMID: 31912902 DOI: 10.3322/caac.21590]

2 **Ogura A**, Konishi T, Cunningham C, Garcia-Aguilar J, Iversen H, Toda S, Lee IK, Lee HX, Uehara K, Lee P, Putter H, van de Velde CJH, Beets GL, Rutten HJT, Kusters M; Lateral Node Study Consortium. Neoadjuvant (Chemo)radiotherapy With Total Mesorectal Excision Only Is Not Sufficient to Prevent Lateral Local Recurrence in Enlarged Nodes: Results of the Multicenter Lateral Node Study of Patients With Low cT3/4 Rectal Cancer. *J Clin Oncol* 2019; **37**: 33-43 [PMID: 30403572 DOI: 10.1200/JCO.18.00032]

3 **Ngamruengphong S**, Kamal A, Akshintala V, Hajiyeva G, Hanada Y, Chen YI, Sanaei O, Fluxa D, Haito Chavez Y, Kumbhari V, Singh VK, Lennon AM, Canto MI, Khashab MA. Prevalence of metastasis and survival of 788 patients with T1 rectal carcinoid tumors. *Gastrointest Endosc* 2019; **89**: 602-606 [PMID: 30447216 DOI: 10.1016/j.gie.2018.11.010]

4 **Cordero-Gallardo F**, Lee Burnett O 3rd, McNamara MM, Weber TM, Zarzour J, Bae S, Jang S, Barrett OC, McDonald A, Kim RY. Incidence of mesorectal node metastasis in locally advanced cervical cancer: its therapeutic implications. *Int J Gynecol Cancer* 2019; **29**: 48-52 [PMID: 30640683 DOI: 10.1136/ijgc-2018-000031]

5 **Taylor FG**, Quirke P, Heald RJ, Moran B, Blomqvist L, Swift I, Sebag-Montefiore DJ, Tekkis P, Brown G; MERCURY study group. Preoperative high-resolution magnetic resonance imaging can identify good prognosis stage I, II, and III rectal cancer best managed by surgery alone: a prospective, multicenter, European study. *Ann Surg* 2011; **253**: 711-719 [PMID: 21475011 DOI: 10.1097/SLA.0b013e31820b8d52]

6 **Dieguez A**. Rectal cancer staging: focus on the prognostic significance of the findings described by high-resolution magnetic resonance imaging. *Cancer Imaging* 2013; **13**: 277-297 [PMID: 23876415 DOI: 10.1102/1470-7330.2013.0028]

7 **Horvat N**, Veeraraghavan H, Khan M, Blazic I, Zheng J, Capanu M, Sala E, Garcia-Aguilar J, Gollub MJ, Petkovska I. MR Imaging of Rectal Cancer: Radiomics Analysis to Assess Treatment Response after Neoadjuvant Therapy. *Radiology* 2018; **287**: 833-843 [PMID: 29514017 DOI: 10.1148/radiol.2018172300]

8 **Chen Y**, Yang X, Wen Z, Liu Y, Lu B, Yu S, Xiao X. Association between high-resolution MRI-detected extramural vascular invasion and tumour microcirculation estimated by dynamic contrast-enhanced MRI in rectal cancer: preliminary results. *BMC Cancer* 2019; **19**: 498 [PMID: 31133005 DOI: 10.1186/s12885-019-5732-z]

9 **Lambregts DM**, Beets GL, Maas M, Kessels AG, Bakers FC, Cappendijk VC, Engelen SM, Lahaye MJ, de Bruïne AP, Lammering G, Leiner T, Verwoerd JL, Wildberger JE, Beets-Tan RG. Accuracy of gadofosveset-enhanced MRI for nodal staging and restaging in rectal cancer. *Ann Surg* 2011; **253**: 539-545 [PMID: 21239980 DOI: 10.1097/SLA.0b013e31820b01f1]

10 **Thrall JH**, Li X, Li Q, Cruz C, Do S, Dreyer K, Brink J. Artificial Intelligence and Machine Learning in Radiology: Opportunities, Challenges, Pitfalls, and Criteria for Success. *J Am Coll Radiol* 2018; **15**: 504-508 [PMID: 29402533 DOI: 10.1016/j.jacr.2017.12.026]

11 **Hecht J**. Managing expectations of artificial intelligence. *Nature* 2018; **563**: S141-S143 [PMID: 30487629 DOI: 10.1038/d41586-018-07504-9]

12 **Kohli M**, Geis R. Ethics, Artificial Intelligence, and Radiology. *J Am Coll Radiol* 2018; **15**: 1317-1319 [PMID: 30017625 DOI: 10.1016/j.jacr.2018.05.020]

13 **Ahmed FE**. Artificial neural networks for diagnosis and survival prediction in colon cancer. *Mol Cancer* 2005; **4**: 29 [PMID: 16083507 DOI: 10.1186/1476-4598-4-29]

14 **Nartowt BJ**, Hart GR, Roffman DA, Llor X, Ali I, Muhammad W, Liang Y, Deng J. Scoring colorectal cancer risk with an artificial neural network based on self-reportable personal health data. *PLoS One* 2019; **14**: e0221421 [PMID: 31437221 DOI: 10.1371/journal.pone.0221421]

15 **Zhao D**, Liu H, Zheng Y, He Y, Lu D, Lyu C. Whale optimized mixed kernel function of support vector machine for colorectal cancer diagnosis. *J Biomed Inform* 2019; **92**: 103124 [PMID: 30796977 DOI: 10.1016/j.jbi.2019.103124]

16 **Zhi J**, Sun J, Wang Z, Ding W. Support vector machine classifier for prediction of the metastasis of colorectal cancer. *Int J Mol Med* 2018; **41**: 1419-1426 [PMID: 29328363 DOI: 10.3892/ijmm.2018.3359]

17 **Yang K**, Zhou B, Yi F, Chen Y. Colorectal Cancer Diagnostic Algorithm Based on Sub-Patch Weight Color Histogram in Combination of Improved Least Squares Support Vector Machine for Pathological Image. *J Med Syst* 2019; **43**: 306 [PMID: 31410693 DOI: 10.1007/s10916-019-1429-8]

18 **Alam MA**, Shahjaman M, Rahman MF, Hossain F, Deng HW. Gene shaving using a sensitivity analysis of kernel based machine learning approach, with applications to cancer data. *PLoS One* 2019; **14**: e0217027 [PMID: 31120939 DOI: 10.1371/journal.pone.0217027]

19 **Ishihara S**, Kawai K, Tanaka T, Kiyomatsu T, Hata K, Nozawa H, Morikawa T, Watanabe T. Oncological Outcomes of Lateral Pelvic Lymph Node Metastasis in Rectal Cancer Treated With

Preoperative Chemoradiotherapy. *Dis Colon Rectum* 2017; **60**: 469-476 [PMID: 28383446 DOI: 10.1097/DCR.0000000000000752]

20 **Sato H**, Maeda K, Maruta M, Masumori K, Koide Y. Who can get the beneficial effect from lateral lymph node dissection for Dukes C rectal carcinoma below the peritoneal reflection? *Dis Colon Rectum* 2006; **49**: S3-12 [PMID: 17106812 DOI: 10.1007/s10350-006-0699-7]

21 **Kennedy ED**, Simunovic M, Jhaveri K, Kirsch R, Brierley J, Drolet S, Brown C, Vos PM, Xiong W, MacLean T, Kanthan S, Stotland P, Raphael S, Chow G, O'Brien CA, Cho C, Streutker C, Wong R, Schmocker S, Liberman S, Reinhold C, Kopek N, Marcus V, Bouchard A, Lavoie C, Morin S, Périgny M, Wright A, Neumann K, Clarke S, Patil NG, Arnason T, Williams L, McLeod R, Brown G, Mathieson A, Pooni A, Baxter NN. Safety and Feasibility of Using Magnetic Resonance Imaging Criteria to Identify Patients With "Good Prognosis" Rectal Cancer Eligible for Primary Surgery: The Phase 2 Nonrandomized QuickSilver Clinical Trial. *JAMA Oncol* 2019; **5**: 961-966 [PMID: 30973610 DOI: 10.1001/jamaoncol.2019.0186]

22 **Ogawa S**, Hida J, Ike H, Kinugasa T, Ota M, Shinto E, Itabashi M, Kameoka S, Sugihara K. Selection of Lymph Node-Positive Cases Based on Perirectal and Lateral Pelvic Lymph Nodes Using Magnetic Resonance Imaging: Study of the Japanese Society for Cancer of the Colon and Rectum. *Ann Surg Oncol* 2016; **23**: 1187-1194 [PMID: 26671038 DOI: 10.1245/s10434-015-5021-2]

23 **Ren S**, He K, Girshick R, Sun J. Faster R-CNN: Towards Real-Time Object Detection with Region Proposal Networks. *IEEE Trans Pattern Anal Mach Intell* 2017; **39**: 1137-1149 [PMID: 27295650 DOI: 10.1109/TPAMI.2016.2577031]

24 **Ding L**, Liu GW, Zhao BC, Zhou YP, Li S, Zhang ZD, Guo YT, Li AQ, Lu Y, Yao HW, Yuan WT, Wang GY, Zhang DL, Wang L. Artificial intelligence system of faster region-based convolutional neural network surpassing senior radiologists in evaluation of metastatic lymph nodes of rectal cancer. *Chin Med J (Engl)* 2019; **132**: 379-387 [PMID: 30707177 DOI: 10.1097/CM9.0000000000000095]

25 **He K**, Gkioxari G, Dollar P, Girshick R. Mask R-CNN. *IEEE Trans Pattern Anal Mach Intell* 2020; **42**: 386-397 [PMID: 29994331 DOI: 10.1109/TPAMI.2018.2844175]

26 **Zhao X**, Xie P, Wang M, Li W, Pickhardt PJ, Xia W, Xiong F, Zhang R, Xie Y, Jian J, Bai H, Ni C, Gu J, Yu T, Tang Y, Gao X, Meng X. Deep learning-based fully automated detection and segmentation of lymph nodes on multiparametric-mri for rectal cancer: A multicentre study. *EBioMedicine* 2020; **56**: 102780 [PMID: 32512507 DOI: 10.1016/j.ebiom.2020.102780]

27 **Barbu A**, Suehling M, Xu X, Liu D, Zhou SK, Comaniciu D. Automatic detection and segmentation of lymph nodes from CT data. *IEEE Trans Med Imaging* 2012; **31**: 240-250 [PMID: 21968722 DOI: 10.1109/TMI.2011.2168234]

28 **Kitasaka T**, Tsujimura Y, Nakamura Y, Mori K, Suenaga Y, Ito M, Nawano S. Automated extraction of lymph nodes from 3-D abdominal CT images using 3-D minimum directional difference filter. *Med Image Comput Comput Assist Interv* 2007; **10**: 336-343 [PMID: 18044586 DOI: 10.1007/978-3-540-75759-7_41]

29 **Feulner J**, Zhou SK, Hammon M, Hornegger J, Comaniciu D. Lymph node detection and segmentation in chest CT data using discriminative learning and a spatial prior. *Med Image Anal* 2013; **17**: 254-270 [PMID: 23246185 DOI: 10.1016/j.media.2012.11.001]

30 **Wang D**, Xu J, Zhang Z, Li S, Zhang X, Zhou Y, Lu Y. Evaluation of Rectal Cancer Circumferential Resection Margin Using Faster Region-Based Convolutional Neural Network in High-Resolution Magnetic Resonance Images. *Dis Colon Rectum* 2020; **63**: 143-151 [PMID: 31842158 DOI: 10.1097/DCR.0000000000001519]

31 **Koumpa FS**, Xylas D, Konopka M, Galea D, Veselkov K, Antoniou A, Mehta A, Mirnezami R. Colorectal Peritoneal Metastases: A Systematic Review of Current and Emerging Trends in Clinical and Translational Research. *Gastroenterol Res Pract* 2019; **2019**: 5180895 [PMID: 31065262 DOI: 10.1155/2019/5180895]

32 **Yuan Z**, Xu T, Cai J, Zhao Y, Cao W, Fichera A, Liu X, Yao J, Wang H. Development and Validation of an Image-based Deep Learning Algorithm for Detection of Synchronous Peritoneal Carcinomatosis in Colorectal Cancer. *Ann Surg* 2020 [PMID: 32694449 DOI: 10.1097/SLA.0000000000004229]

33 **Wang J**, Lu J, Qin G, Shen L, Sun Y, Ying H, Zhang Z, Hu W. Technical Note: A deep learning-based autosegmentation of rectal tumors in MR images. *Med Phys* 2018; **45**: 2560-2564 [PMID: 29663417 DOI: 10.1002/mp.12918]

34 **Trebeschi S**, van Griethuysen JJM, Lambregts DMJ, Lahaye MJ, Parmar C, Bakers FCH, Peters NHGM, Beets-Tan RGH, Aerts HJWL. Deep Learning for Fully-Automated Localization and Segmentation of Rectal Cancer on Multiparametric MR. *Sci Rep* 2017; **7**: 5301 [PMID: 28706185 DOI: 10.1038/s41598-017-05728-9]

35 **Men K**, Boimel P, Janopaul-Naylor J, Zhong H, Huang M, Geng H, Cheng C, Fan Y, Plastaras JP, Ben-Josef E, Xiao Y. Cascaded atrous convolution and spatial pyramid pooling for more accurate tumor target segmentation for rectal cancer radiotherapy. *Phys Med Biol* 2018; **63**: 185016 [PMID: 30109986 DOI: 10.1088/1361-6560/aada6c]

36 **Kapiteijn E**, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, Rutten HJ, Pahlman L, Glimelius B, van Krieken JH, Leer JW, van de Velde CJ; Dutch Colorectal Cancer Group. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001; **345**: 638-646 [PMID: 11547717 DOI: 10.1056/NEJMoa010580]

37 **O'Neill BD**, Brown G, Heald RJ, Cunningham D, Tait DM. Non-operative treatment after neoadjuvant chemoradiotherapy for rectal cancer. *Lancet Oncol* 2007; **8**: 625-633 [PMID: 17613424

DOI: 10.1016/S1470-2045(07)70202-4]
38 **Ferrari R**, Mancini-Terracciano C, Voena C, Rengo M, Zerunian M, Ciardiello A, Grasso S, Mare' V, Paramatti R, Russomando A, Santacesaria R, Satta A, Solfaroli Camillocci E, Faccini R, Laghi A. MR-based artificial intelligence model to assess response to therapy in locally advanced rectal cancer. *Eur J Radiol* 2019; **118**: 1-9 [PMID: 31439226 DOI: 10.1016/j.ejrad.2019.06.013]
39 **Zhang XY**, Wang L, Zhu HT, Li ZW, Ye M, Li XT, Shi YJ, Zhu HC, Sun YS. Predicting Rectal Cancer Response to Neoadjuvant Chemoradiotherapy Using Deep Learning of Diffusion Kurtosis MRI. *Radiology* 2020; **296**: 56-64 [PMID: 32315264 DOI: 10.1148/radiol.2020190936]
40 **Shi L**, Zhang Y, Nie K, Sun X, Niu T, Yue N, Kwong T, Chang P, Chow D, Chen JH, Su MY. Machine learning for prediction of chemoradiation therapy response in rectal cancer using pre-treatment and mid-radiation multi-parametric MRI. *Magn Reson Imaging* 2019; **61**: 33-40 [PMID: 31059768 DOI: 10.1016/j.mri.2019.05.003]
41 **Fu J**, Zhong X, Li N, Van Dams R, Lewis J, Sung K, Raldow AC, Jin J, Qi XS. Deep learning-based radiomic features for improving neoadjuvant chemoradiation response prediction in locally advanced rectal cancer. *Phys Med Biol* 2020; **65**: 075001 [PMID: 32092710 DOI: 10.1088/1361-6560/ab7970]
42 **Yi X**, Pei Q, Zhang Y, Zhu H, Wang Z, Chen C, Li Q, Long X, Tan F, Zhou Z, Liu W, Li C, Zhou Y, Song X, Li Y, Liao W, Li X, Sun L, Pei H, Zee C, Chen BT. MRI-Based Radiomics Predicts Tumor Response to Neoadjuvant Chemoradiotherapy in Locally Advanced Rectal Cancer. *Front Oncol* 2019; **9**: 552 [PMID: 31293979 DOI: 10.3389/fonc.2019.00552]
43 **Shayesteh SP**, Alikhassi A, Fard Esfahani A, Miraie M, Geramifar P, Bitarafan-Rajabi A, Haddad P. Neo-adjuvant chemoradiotherapy response prediction using MRI based ensemble learning method in rectal cancer patients. *Phys Med* 2019; **62**: 111-119 [PMID: 31153390 DOI: 10.1016/j.ejmp.2019.03.013]
44 **Cui Y**, Liu H, Ren J, Du X, Xin L, Li D, Yang X, Wang D. Development and validation of a MRI-based radiomics signature for prediction of KRAS mutation in rectal cancer. *Eur Radiol* 2020; **30**: 1948-1958 [PMID: 31942672 DOI: 10.1007/s00330-019-06572-3]
45 **Chand M**, Swift RI, Tekkis PP, Chau I, Brown G. Extramural venous invasion is a potential imaging predictive biomarker of neoadjuvant treatment in rectal cancer. *Br J Cancer* 2014; **110**: 19-25 [PMID: 24300971 DOI: 10.1038/bjc.2013.603]
46 **Wu S**, Zheng J, Li Y, Yu H, Shi S, Xie W, Liu H, Su Y, Huang J, Lin T. A Radiomics Nomogram for the Preoperative Prediction of Lymph Node Metastasis in Bladder Cancer. *Clin Cancer Res* 2017; **23**: 6904-6911 [PMID: 28874414 DOI: 10.1158/1078-0432.CCR-17-1510]
47 **Dong Y**, Feng Q, Yang W, Lu Z, Deng C, Zhang L, Lian Z, Liu J, Luo X, Pei S, Mo X, Huang W, Liang C, Zhang B, Zhang S. Preoperative prediction of sentinel lymph node metastasis in breast cancer based on radiomics of T2-weighted fat-suppression and diffusion-weighted MRI. *Eur Radiol* 2018; **28**: 582-591 [PMID: 28828635 DOI: 10.1007/s00330-017-5005-7]

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MINIREVIEWS

Remaining issues of recommended management in current guidelines for asymptomatic common bile duct stones

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Abstract

Current guidelines for treating asymptomatic common bile duct stones (CBDS) recommend stone removal, with endoscopic retrograde cholangiopan-creatography (ERCP) being the first treatment choice. When deciding on ERCP treatment for asymptomatic CBDS, the risk of ERCP-related complications and outcome of natural history of asymptomatic CBDS should be compared. The incidence rate of ERCP-related complications, particularly of post-ERCP pancreatitis for asymptomatic CBDS, was reportedly higher than that of symptomatic CBDS, increasing the risk of ERCP-related complications for asymptomatic CBDS compared with that previously reported for biliopancreatic diseases. Although studies have reported short- to middle-term outcomes of natural history of asymptomatic CBDS, its long-term natural history is not well known. Till date, there are no prospective studies that determined whether ERCP has a better outcome than no treatment in patients with asymptomatic CBDS or not. No randomized controlled trial has evaluated the risk of early and late ERCP-related complications *vs* the risk of biliary complications in the wait-and-see approach, suggesting that a change is needed in our perspective on endoscopic treatment for asymptomatic CBDS. Further studies examining long-term complication risks of ERCP and wait-and-see groups for asymptomatic CBDS are warranted to discuss

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whether routine endoscopic treatment for asymptomatic CBDS is justified or not.

Key Words: Asymptomatic common bile duct stone; Endoscopic retrograde cholangiopancreatography; Complication; Natural history of asymptomatic common bile duct stone; Guideline; Recommendation

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Core Tip: Current guidelines recommend endoscopic stone removal for asymptomatic common bile duct stones (CBDS). However, the risks of endoscopic retrograde cholangiopancreatography (ERCP)-related complication and natural history outcome of asymptomatic CBDS should be compared to decide on endoscopic treatment by ERCP. ERCP for asymptomatic CBDS reportedly has a high risk of ERCP-related complications; post-ERCP pancreatitis risk being the most common. The long-term natural history of asymptomatic CBDS is not well known. Further studies examining long-term complication risks of ERCP and wait-and-see groups for asymptomatic CBDS are warranted to evaluate whether routine endoscopic stone removal for asymptomatic CBDS is justified or not.

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INTRODUCTION

Common bile duct stones (CBDS) are a common biliary tract disease worldwide with secondary CBDS migrated from gallbladder into CBD, being one of major causes of CBDS[1]. The prevalence of gallstones has increased in the elderly population[2], and for this reason, the number of patients with CBDS is likely to increase in the future as the world's population ages. Owing to diagnostic modalities, such as computed tomography (CT), endoscopic ultrasonography (EUS) or magnetic resonance cholangio-pancreatography (MRCP) recently developed, there is greater probability for asymptomatic CBDS to be discovered incidentally.

Endoscopic management by endoscopic retrograde cholangiopancreatography (ERCP) is widely accepted as a first choice of treatment for CBDS[3]. For asymptomatic CBDS, endoscopic treatment is consistently recommended in current guidelines[1,3-5], and removal of asymptomatic CBDS is a common practice as asymptomatic CBDS have a potential risk of causing obstructive jaundice, acute cholangitis, and biliary pancreatitis.

ERCP is however an endoscopic procedure with high risk of procedure-related complications. In general, an overall incidence of ERCP-related complications, including post-ERCP pancreatitis (PEP), cholangitis, bleeding, and perforation has been reported in 4.0%-15.9% of patients[6,7], PEP being the most common. A systematic review of randomized controlled trials revealed an overall PEP incidence of 9.7%[8].

When determining the indication of ERCP for asymptomatic CBDS, the risk of ERCP-related complications should be focused on asymptomatic CBDS rather than on average risk of ERCP-related complications of biliopancreatic diseases.

Some investigators recently reported the risk of ERCP-related complications focusing on asymptomatic CBDS. An overall incidence rate of ERCP-related complication for asymptomatic CBDS was reported to be approximately 15%-25%, with a PEP incidence of approximately 12%-20%[9-13]. The risk of ERCP-related complications, in particular the risk of PEP, for asymptomatic CBDS appeared to be higher than that previously reported. The risk of ERCP-related complications for asymptomatic common bile duct (CBD) should therefore not be the same as the previously reported risk of ERCP-related complications for biliopancreatic diseases.

When determining the indication of ERCP for asymptomatic CBDS, the risk of biliary complications in wait-and-see approach should be considered. Previous studies on the natural history of CBDS reported the biliary complication rates of wait-and-see approach for asymptomatic CBDS to vary between 0-25.3% during a follow-up period of 30 d to 4.8 years[14-18]. Endoscopic sphincterotomy, endoscopic papillary balloon dilation, or endoscopic large papillary balloon dilation was performed for endoscopic stone removal bearing in mind the risk of stone recurrence as these procedures can cause papillary dysfunction[19,20].

We clarified the remaining issues of recommended management in current guidelines for asymptomatic CBDS by reviewing the current guidelines for asymptomatic CBDS and previous studies on the risk of ERCP-related complications and natural history of asymptomatic CBDS.

EPIDEMIOLOGY OF CBDS

The origin of CBDS is different in Asian populations and Western population. Primary CBDS, which are more common in Asian populations, form de novo in the intra- and extrahepatic ducts. Primary CBDS are associated with biliary infection and cholestasis[21-24]. Secondary CBDS, which are more prevalent in Western populations, typically originate in the gallbladder and migrate into CBD[1].

The prevalence of CBDS in patients with symptomatic gallstones is estimated to be 10%-20%[25-29]. In patients without jaundice and dilated CBD on trans-abdominal ultrasound, the prevalence of CBDS during cholecystectomy is reported to be < 5%[15]. It has been reported that the rate of coexisting CBDS was increased in elderly populations with symptomatic gallstones[2].

However, to best of our knowledge, there are no studies focusing on the prevalence of CBDS in patients with asymptomatic gallstones, as most studies are based on intraoperative cholangiography during cholecystectomy for symptomatic gallstones. Although the prevalence of CBDS is expected to increase due to the aging of world population, the prevalence of CBDS is still unknown.

Further studies are warranted to clarify the prevalence of asymptomatic CBDS for a better understanding of the natural history of asymptomatic CBDS.

DIAGNOSTIC MODALITIES OF CBDS

Recent guidelines recommend the use of either EUS or MRCP to diagnose CBDS in patients with suspected CBDS. A recent meta-analysis of five head-to-head studies revealed that the sensitivity and specificity of EUS and MRCP are 97% *vs* 90%, and specificity 87% *vs* 92%, respectively. However, the overall diagnostic odds ratio of EUS was significantly higher than that of MRCP because EUS had a higher detection rate of small CBDS compared with that of MRCP, while the specificity showed no significant differences between the two modalities[30]. Another meta-analysis demonstrated high diagnostic accuracy for EUS and MRCP. This meta-analysis reported that the sensitivity and specificity for EUS were 95% and 97%, and 93% and 96% for MRCP[31].

Although CT is a diagnostic modality for CBDS, routine CT scanning for suspected CBDS is not recommend in current guidelines because of some disadvantages, such as radiation exposure, side effects of contrast agent, and lower diagnostic ability for CBDS compared with MRCP and EUS[3,32]. Several studies examining the diagnostic ability of multi-slice CT scanner for CBDS showed that conventional CT scanning had reasonable sensitivity (69%-87%) and specificity (68%-96%) for diagnostic CBDS[33-36]. Recently, dual-energy CT scanner and dual-layer spectral CT (DLCT) scanner, which can perform a multiparameter approach, are commercially available. These CT scanners can detect noncalcified stones, which cannot be detected in conventional CT. A recent retrospective study comparing the diagnostic ability between DLCT and modern MRCP suggested that the diagnostic ability of DLCT for biliary stones was comparable to that of modern MRCP[37]; the detection of noncalcified small stones < 9 mm in diameter seem to be a limitation of dual-energy CT and DLCT scanners[37-39].

Diagnostic modalities for CBDS, such as EUS, MRCP, and CT have recently been developed increasing the chance of detecting incidentally discovered asymptomatic CBD.

RECOMMENDED MANAGEMENT IN CURRENT GUIDELINES FOR ASYMPTOMAIC CBDS

Recommended management of current guidelines is presented in Table 1. Current guidelines published by European Society of Gastrointestinal Endoscopy, The British Society of Gastroenterology, American Society for Gastrointestinal Endoscopy, and The Japanese Society of Gastroenterology strongly recommend bile duct stone removal[1,3-5], with ERCP as the first choice of treatment for asymptomatic CBDS removal.

Although the lifetime risk of untreated CBDS is unknown, complication of CBDS, such as pain, obstructive jaundice, acute cholangitis, hepatic abscesses, biliary pancreatitis, secondary biliary cirrhosis, and portal hypertension due to biliary obstruction are potentially life threatening. Available guidelines recommend treatment for asymptomatic CBDS. A conservative approach can only be considered in patients in whom the risks of CBDS removal are higher than the risks of leaving CBDS in situ[1,3,4].

Current guidelines strongly recommended stone removal for asymptomatic CBDS even though the evidence quality is low.

RISK OF ERCP-RELATED COMPLICATIONS FOR ASYMPTOMATIC CBDS

It has been recognized that the risk of ERCP-related complications of CBDS was 6%-15%[6,7]. However, the risk of ERCP focusing on asymptomatic CBDS is important to determine the indication of ERCP for asymptomatic CBDS.

Recently, several reports revealed that the risk of ERCP-related complications for patients with asymptomatic CBDS was higher than that for symptomatic patients (Table 2). A multicenter retrospective study including 164 patients with asymptomatic CBDS and 949 patients with symptomatic CBDS reported that the incidence rate of ERCP-related complications was 19.5% in asymptomatic patients and 6.2% in symptomatic patients. In particular, PEP was significantly higher in asymptomatic patients than symptomatic patients (14.6% *vs* 3.0%)[11]. A prospective study including 53 asymptomatic patients and 274 symptomatic patients reported the similar results. The rate of ERCP-related complications in asymptomatic patients and symptomatic patients were 26.4% *vs* 11.7%, respectively, and the rate of PEP was 20.8% *vs* 6.9%, respectively[12]. The possible explanation for a higher incidence of PEP in asymptomatic patients is that the number of asymptomatic patients with normal serum bilirubin, nondilated CBD, and difficult cannulation, which are patient- and procedure-related risk factors of PEP, was higher than that of symptomatic patients because of no cholestasis in patients with asymptomatic CBDS. Furthermore, asymptomatic CBDS itself may be an important clinical risk factor of PEP[11].

Although a single-center retrospective study, in which ERCP was performed by skilled endoscopists, reported that the incidence of both overall ERCP-related complications and PEP in asymptomatic patients were comparable with those of symptomatic patients[13], other studies reported that the incidence rate of overall ERCP-related complication was approximately 15%-25% in asymptomatic patients and 4%-12% in symptomatic patients, and the incidence of PEP was approximately 12%-20% in asymptomatic patients and 2%-7% in symptomatic patients[9-12]. Therefore, the risk of ERCP-related complications, in particular the risk of PEP, in asymptomatic patients with CBDS is possibly higher than that of symptomatic patients.

NATURAL HISTORY OF ASYMPTOMAIC CBDS

About 2%-4% of patients with asymptomatic gallstones becomes symptomatic over the years. Multiple gallstones, negative cholecystography findings, and young age are the risk factors for transition from asymptomatic to symptomatic[40-43]. The potential risk of intraoperative and postoperative complications related to surgery explains why current guidelines are against laparoscopic cholecystectomy for patients with asymptomatic gallstones in a normal gallbladder[4,32].

Although the long-term natural history of CBDS is less understood than that of gallstones, several studies have examined the short- to middle-term natural history of asymptomatic CBDS[14-18] (Table 3).

Table 1 Recommended management and evidence level in current guidelines for asymptomatic common bile duct stones

Guideline	Recommendation
ESGE[1]	ESGE recommends stone extraction in all patients with CBDS, regardless of being symptomatic or not, who are fit enough to tolerate the intervention. (Strong recommendation, low quality evidence)
BSG[3]	Stone extraction is recommended in patients diagnosed with CBDS if possible. Evidence of benefit of stone extraction is greatest for symptomatic patients (Low quality evidence; strong recommendation)
JGES[4]	Asymptomatic choledocholithiasis should be treated because of a risk of developing biliary complications. [Evidence level: A; Strength of recommendation (agreement rate): 2 (100%)]
ASGE[5]	CBDS should be treated if detected regardless of the presence or absence of significant mitigating clinical circumstances (Moderate quality)

CBDS: Common bile duct stones; ESGE: European Society of Gastrointestinal Endoscopy; BSG: The British Society of Gastroenterology; ASGE: American Society for Gastrointestinal Endoscopy; JSGE: The Japanese Society of Gastroenterology.

Table 2 Summary of studies comparing the risks of endoscopic retrograde cholangiopancreatography-related complications for asymptomatic and symptomatic patients with common bile duct stones

Ref.	Study design	Patients, *n*		Overall, complications (%)		PEP (%)	
		Asymptomatic group	Symptomatic group	Asymptomatic group	Symptomatic group	Asymptomatic group	Symptomatic group
Kim *et al*[9], 2016	Single-center retrospective	32	536	15.6	10.4	12.5[a]	3.9
Saito *et al*[10], 2017	Multicenter retrospective	67	536	26.9[a]	3.9	16.4[a]	2.2
Saito *et al*[11], 2019	Multicenter retrospective	164	949	19.5[a]	6.2	14.6[a]	3.0
Xu *et al*[12], 2019	Single-center prospective	53	274	26.4[a]	11.7	20.8[a]	6.9
Xiao *et al*[13], 2021	Single-center retrospective	79	795	13.3	9.7	7.6	6.9

[a]Statistically significant difference. ERCP: Endoscopic retrograde cholangiopancreatography; PEP: Postendoscopic retrograde cholangiopancreatography pancreatitis.

Table 3 Summary of studies reporting natural history of asymptomatic common bile duct stones

Ref.	Study design	Patients, *n*	Median follow-up period	Cumulative incidence of biliary complications, *n* (%)
Ammori *et al*[14], 2000	Prospective	14	1.4 yr	4 (29)
Collins *et al*[15], 2004	Prospective	46	6 wk	0
Caddy *et al*[16], 2005	Retrospective	59	4.8 yr	0
Moller *et al*[17], 2014	Retrospective	594	30 d	150 (25)
Hakuta *et al*[18], 2020	Retrospective	114	3.2 yr	20 (18)

Data from the GallRiks study, including 594 patients who had CBDS incidentally discovered by intraoperative cholangiography and were untreated, suggest that 25.3% (150/594) of patients developed unfavorable outcomes defined as incomplete clearance of CBDS and/or complications within 30 d post cholecystectomy. Among the 3234 patients who underwent any procedure of CBDS removal, including post- or intraoperative ERCP, laparoscopic or open choledochotomy, transcystic stone extraction, or flushing/manipulation, 12.7% (411/3234) developed unfavorable

outcomes. This led to conclusion that if CBDS are detected, they should be removed to reduce the risk of CBDS-related complications over time[17].

However, there are several noteworthy aspects in the results of GallRiks study. First, long-term outcomes of patients in the untreated asymptomatic CBDS group and treated asymptomatic CBDS group were unclear. Second, patients who underwent postoperative ERCP had a high unfavorable outcome rate of 18% (103/572), suggesting that ERCP for asymptomatic CBDS had a high risk of ERCP-related complications. Third, although the unfavorable outcome rate of no treatment for CBDS also included post-ERCP complications in patients who became symptomatic and underwent ERCP within 30 d post cholecystectomy, this unfavorable outcome should also be included in the unfavorable outcomes of treatment strategies. Therefore, the risk of ERCP-related complications for CBDS removal may be underestimated in the GallRiks study.

Hakuta *et al*[18] recently reported that out of the 114 patients with asymptomatic CBDS who underwent wait-and-see approach, 18% developed CBDS-related complications, which were cholangitis in 16 patients (14%), cholecystitis in 1 patient (0.9%), and cholestasis in 4 patients (3.5%), and no biliary pancreatitis during the average follow-up period of 3.2 years.

In some patients with asymptomatic small CBDS, the stones can be drained into duodenum spontaneously without the need for an intervention[44-48]. Collins *et al*[15] demonstrated the spontaneous passage of small CBDS without serious complications in 24 of 46 patients with a filling defect observed by intraoperative cholangiography during cholecystectomy within 6 wk of surgery. Asymptomatic spontaneous passage of small CBDS less than 8 mm in size has also been found in approximately 20% CBDS in the interval between diagnosis at EUS and ERCP[49].

Clarifying the long-term natural history of asymptomatic CBDS is essential to determine whether intervention by ERCP or wait-and-see approach for asymptomatic CBDS has a better outcome. A large-scale prospective study examining long-term natural history of asymptomatic CBDS is therefore warranted.

RECCURENCE OF CBDS AFTER ENDOSCOPIC STONE REMOVAL

The Korean nationwide study including 46181 patients with CBDS demonstrated that 5228 (11.3%) patients experienced first CBDS recurrence at mean follow-up of 4.3 years. The cumulative second and third recurrence rates after the initial CBD recurrence were 23.4% and 33.4%. Therefore, the higher the frequency of recurrence of stones, the higher the recurrence rate of the stones[19]. Another retrospective study revealed that CBDS recurrence was observed in 121 patients (12.4%) out of 976 patients. Multiple recurrence after first recurrence was observed in 26 patients (21.5%) of 121 patients with stone recurrence during a median follow-up period of 5.1 years. The risk factors for single recurrence were CBD size, gallbladder left in situ with gallstones, and pneumobilia after ERCP, and the risk factor for multiple recurrence was the number of CBDS at the first recurrence[20].

Stone recurrence should be considered when balancing stone removal by ERCP *vs* wait-and-see approach for asymptomatic CBDS.

REMAINING ISSUES IN CURRENT GUIDELINES FOR ASYMPTOMATIC CBDS

When determining the indication of ERCP for patient with asymptomatic CBDS, the risk of early and late ERCP-related complications and long-term natural history of asymptomatic CBDS should be considered.

Recently, the risk of ERCP-related complications for asymptomatic CBDS and the short- to middle-term natural history of this disease have been reported as above[9-18].

Based on the previous reports, the risk of early ERCP-related complications, including PEP, cholangitis, bleeding, and perforation can be estimated to approximately 15%-25% with the incidence of PEP of 12%-20% in patients with asymptomatic CBDS[9-14]. The risk of late ERCP-related complications including stone recurrence and cholangitis can be estimated to approximately 10%[18-20], with the risk of early and late ERCP-related complications for asymptomatic CBDS being approximately 25%-35%. The risk of biliary complications in wait-and-see approach during median follow-up period of 30 d to 4.9 years was estimated to be approxim-ately 0%-

25%[14-18]; ERCP for asymptomatic CBDS may therefore have poorer outcomes than that of wait-and-see approach. A retrospective longitudinal cohort study revealed that out of 77 who underwent ERCP, 16 patients (21%) experienced PEP and 25 patients (32%) experienced early ERCP-related complications and late biliary complications, such as cholangitis and cholecystitis; further, recurrence of CBDS was observed in 7 patients (9.6%) out of 73 patients during a median follow-up period of 1.9 years. They reported that out of the 114 patients with asymptomatic CBDS who underwent wait-and-see approach, 18% developed CBDS-related complications, among which 16 patients (14%) developed cholangitis, 1 patient (0.9%) developed cholecystitis, 4 patients (3.5%) developed cholestasis, and no patients developed biliary pancreatitis during an average follow-up period of 3.2 years. The outcome of intervention group by ERCP was poorer than that of wait-and-see group for patients with asymptomatic CBDS[18].

The fact that there are no randomized prospective studies to compare the risk of ERCP and wait-and-see approach for asymptomatic CBDS is a serious issue as it is important to determine the indication of ERCP for asymptomatic CBDS. Although current guidelines for asymptomatic CBDS recommend stone removal for asymptomatic CBDS, the risk of ERCP and the risk of wait-and-see approach for patients with asymptomatic CBDS cannot be balanced currently because of the absence of randomized controlled studies. Patients should be told that current guidelines' recommendation is to undergo stone extraction in patients with asymptomatic CBDS based on the evidence from the symptomatic patients and expert opinion[1,3].

Further large-scale prospective studies comparing the risk of ERCP and the risk of wait-and-see approach for patients with asymptomatic CBDS are warranted. The indication for preoperative or postoperative ERCP for cholelithiasis should equally be discussed after clarifying the risk and benefit of ERCP for asymptomatic CBDS.

STRATEGY OF ERCP FOR ASYMPTOMAIC CBDS TO REDUCE ERCP-RELATED COMPLICATIONS

Because asymptomatic CBDS are a benign disease with no noticeable symptoms, endoscopists must try to reduce ERCP-related complications particularly when performing ERCP for asymptomatic CBDS. A previous study reported that precut sphincterotomy, biliary sphincter dilation, and involvement of trainees were significant risk factors for developing PEP in asymptomatic CBDS[50]. A propensity-score matched study reported that ERCP for asymptomatic CBDS conducted by experienced endoscopists was of comparable safety as ERCP for symptomatic CBDS[13]. ERCP for asymptomatic CBDS should therefore be performed by experienced endoscopists. Prophylactic procedure such as prophylactic pancreatic stent placement should be considered in asymptomatic patients with risk factors of PEP including precut sphincterotomy and biliary sphincter dilation[50].

Single-stage CBDS removal following endoscopic sphincterotomy at first ERCP session in patients with mild-to-moderate cholangitis is accepted in the revised Tokyo guidelines for the management of acute cholangitis and cholecystitis[51], making patients with asymptomatic CBDS a candidate for single-stage CBDS removal. A retrospective study revealed that single-stage CBDS removal with difficult biliary cannulation requiring > 15 min is a significant risk factor of PEP. It may be preferable to remove CBDS at second session ERCP in asymptomatic patients requiring > 15 min for biliary cannulation in order to reduce the development of PEP[52].

CONCLUSION

Although current guidelines for asymptomatic CBDS recommend stone removal for asymptomatic CBDS, there is need for a change in perspective on ERCP for asymptomatic CBDS. To best of our knowledge, no prospective studies exist as to whether ERCP has a better outcome than no treatment in patients with asymptomatic CBDS. The risk of inducing early and late ERCP-related complications *vs* the risk of complication related to the natural history of asymptomatic CBDS cannot be balanced currently.

Further studies examining long-term complication risks of ERCP group and wait-and-see group is warranted to discuss whether routine treatment of asymptomatic CBDS is justified or not.

REFERENCES

1 **Manes G**, Paspatis G, Aabakken L, Anderloni A, Arvanitakis M, Ah-Soune P, Barthet M, Domagk D, Dumonceau JM, Gigot JF, Hritz I, Karamanolis G, Laghi A, Mariani A, Paraskeva K, Pohl J, Ponchon T, Swahn F, Ter Steege RWF, Tringali A, Vezakis A, Williams EJ, van Hooft JE. Endoscopic management of common bile duct stones: European Society of Gastrointestinal Endoscopy (ESGE) guideline. *Endoscopy* 2019; **51**: 472-491 [PMID: 30943551 DOI: 10.1055/a-0862-0346]

2 **Siegel JH**, Kasmin FE. Biliary tract diseases in the elderly: management and outcomes. *Gut* 1997; **41**: 433-435 [PMID: 9391238 DOI: 10.1136/gut.41.4.433]

3 **Williams E**, Beckingham I, El Sayed G, Gurusamy K, Sturgess R, Webster G, Young T. Updated guideline on the management of common bile duct stones (CBDS). *Gut* 2017; **66**: 765-782 [PMID: 28122906 DOI: 10.1136/gutjnl-2016-312317]

4 **Tazuma S**, Unno M, Igarashi Y, Inui K, Uchiyama K, Kai M, Tsuyuguchi T, Maguchi H, Mori T, Yamaguchi K, Ryozawa S, Nimura Y, Fujita N, Kubota K, Shoda J, Tabata M, Mine T, Sugano K, Watanabe M, Shimosegawa T. Evidence-based clinical practice guidelines for cholelithiasis 2016. *J Gastroenterol* 2017; **52**: 276-300 [PMID: 27942871 DOI: 10.1007/s00535-016-1289-7]

5 **ASGE Standards of Practice Committee**, Maple JT, Ikenberry SO, Anderson MA, Appalaneni V, Decker GA, Early D, Evans JA, Fanelli RD, Fisher D, Fisher L, Fukami N, Hwang JH, Jain R, Jue T, Khan K, Krinsky ML, Malpas P, Ben-Menachem T, Sharaf RN, Dominitz JA. The role of endoscopy in the management of choledocholithiasis. *Gastrointest Endosc* 2011; **74**: 731-744 [PMID: 21951472 DOI: 10.1016/j.gie.2011.04.012]

6 **Andriulli A**, Loperfido S, Napolitano G, Niro G, Valvano MR, Spirito F, Pilotto A, Forlano R. Incidence rates of post-ERCP complications: a systematic survey of prospective studies. *Am J Gastroenterol* 2007; **102**: 1781-1788 [PMID: 17509029 DOI: 10.1111/j.1572-0241.2007.01279.x]

7 **Freeman ML**, Nelson DB, Sherman S, Haber GB, Herman ME, Dorsher PJ, Moore JP, Fennerty MB, Ryan ME, Shaw MJ, Lande JD, Pheley AM. Complications of endoscopic biliary sphincterotomy. *N Engl J Med* 1996; **335**: 909-918 [PMID: 8782497 DOI: 10.1056/NEJM199609263351301]

8 **Kochar B**, Akshintala VS, Afghani E, Elmunzer BJ, Kim KJ, Lennon AM, Khashab MA, Kalloo AN, Singh VK. Incidence, severity, and mortality of post-ERCP pancreatitis: a systematic review by using randomized, controlled trials. *Gastrointest Endosc* 2015; **81**: 143-149. e9 [PMID: 25088919 DOI: 10.1016/j.gie.2014.06.045]

9 **Kim SB**, Kim KH, Kim TN. Comparison of Outcomes and Complications of Endoscopic Common Bile Duct Stone Removal Between Asymptomatic and Symptomatic Patients. *Dig Dis Sci* 2016; **61**: 1172-1177 [PMID: 26589817 DOI: 10.1007/s10620-015-3965-5]

10 **Saito H**, Kakuma T, Kadono Y, Urata A, Kamikawa K, Imamura H, Tada S. Increased risk and severity of ERCP-related complications associated with asymptomatic common bile duct stones. *Endosc Int Open* 2017; **5**: E809-E817 [PMID: 28879226 DOI: 10.1055/s-0043-107615]

11 **Saito H**, Koga T, Sakaguchi M, Kadono Y, Kamikawa K, Urata A, Imamura H, Tada S, Kakuma T, Matsushita I. Post-endoscopic retrograde cholangiopancreatography pancreatitis in patients with asymptomatic common bile duct stones. *J Gastroenterol Hepatol* 2019; **34**: 1153-1159 [PMID: 30650203 DOI: 10.1111/jgh.14604]

12 **Xu XD**, Qian JQ, Dai JJ, Sun ZX. Endoscopic treatment for choledocholithiasis in asymptomatic patients. *J Gastroenterol Hepatol* 2020; **35**: 165-169 [PMID: 31334888 DOI: 10.1111/jgh.14790]

13 **Xiao L**, Geng C, Li X, Li Y, Wang C. Comparable safety of ERCP in symptomatic and asymptomatic patients with common bile duct stones: a propensity-matched analysis. *Scand J Gastroenterol* 2021; **56**: 111-117 [PMID: 33295209 DOI: 10.1080/00365521.2020.1853222]

14 **Ammori BJ**, Birbas K, Davides D, Vezakis A, Larvin M, McMahon MJ. Routine *vs* "on demand" postoperative ERCP for small bile duct calculi detected at intraoperative cholangiography. Clinical evaluation and cost analysis. *Surg Endosc* 2000; **14**: 1123-1126 [PMID: 11148780 DOI: 10.1007/s004640000146]

15 **Collins C**, Maguire D, Ireland A, Fitzgerald E, O'Sullivan GC. A prospective study of common bile duct calculi in patients undergoing laparoscopic cholecystectomy: natural history of choledocholithiasis revisited. *Ann Surg* 2004; **239**: 28-33 [PMID: 14685097 DOI: 10.1097/01.sla.0000103069.00170.9c]

16 **Caddy GR**, Kirby J, Kirk SJ, Allen MJ, Moorehead RJ, Tham TC. Natural history of asymptomatic bile duct stones at time of cholecystectomy. *Ulster Med J* 2005; **74**: 108-112 [PMID: 16235763]

17 **Möller M**, Gustafsson U, Rasmussen F, Persson G, Thorell A. Natural course *vs* interventions to clear common bile duct stones: data from the Swedish Registry for Gallstone Surgery and Endoscopic Retrograde Cholangiopancreatography (GallRiks). *JAMA Surg* 2014; **149**: 1008-1013 [PMID: 25133326 DOI: 10.1001/jamasurg.2014.249]

18 **Hakuta R**, Hamada T, Nakai Y, Oyama H, Kanai S, Suzuki T, Sato T, Ishigaki K, Saito K, Saito T, Takahara N, Mizuno S, Kogure H, Watadani T, Tsujino T, Tada M, Abe O, Isayama H, Koike K. Natural history of asymptomatic bile duct stones and association of endoscopic treatment with clinical outcomes. *J Gastroenterol* 2020; **55**: 78-85 [PMID: 31473828 DOI: 10.1007/s00535-019-01612-7]

19 **Park BK**, Seo JH, Jeon HH, Choi JW, Won SY, Cho YS, Lee CK, Park H, Kim DW. A nationwide population-based study of common bile duct stone recurrence after endoscopic stone removal in Korea. *J Gastroenterol* 2018; **53**: 670-678 [PMID: 29192348 DOI: 10.1007/s00535-017-1419-x]

20 **Kawaji Y**, Isayama H, Nakai Y, Saito K, Sato T, Hakuta R, Saito T, Takahara N, Mizuno S, Kogure H, Matsubara S, Tada M, Kitano M, Koike K. Multiple recurrences after endoscopic removal of

common bile duct stones: A retrospective analysis of 976 cases. *J Gastroenterol Hepatol* 2019; **34**: 1460-1466 [PMID: 30761603 DOI: 10.1111/jgh.14630]

21 **Kaufman HS**, Magnuson TH, Lillemoe KD, Frasca P, Pitt HA. The role of bacteria in gallbladder and common duct stone formation. *Ann Surg* 1989; **209**: 584-91; discussion 591 [PMID: 2705823 DOI: 10.1097/00000658-198905000-00011]

22 **Cetta FM**. Bile infection documented as initial event in the pathogenesis of brown pigment biliary stones. *Hepatology* 1986; **6**: 482-489 [PMID: 3519417 DOI: 10.1002/hep.1840060327]

23 **Tazuma S**. Gallstone disease: Epidemiology, pathogenesis, and classification of biliary stones (common bile duct and intrahepatic). *Best Pract Res Clin Gastroenterol* 2006; **20**: 1075-1083 [PMID: 17127189 DOI: 10.1016/j.bpg.2006.05.009]

24 **Tsui WM**, Lam PW, Lee WK, Chan YK. Primary hepatolithiasis, recurrent pyogenic cholangitis, and oriental cholangiohepatitis: a tale of 3 countries. *Adv Anat Pathol* 2011; **18**: 318-328 [PMID: 21654363 DOI: 10.1097/PAP.0b013e318220fb75]

25 **Neuhaus H**, Feussner H, Ungeheuer A, Hoffmann W, Siewert JR, Classen M. Prospective evaluation of the use of endoscopic retrograde cholangiography prior to laparoscopic cholecystectomy. *Endoscopy* 1992; **24**: 745-749 [PMID: 1468389 DOI: 10.1055/s-2007-1010576]

26 **Saltzstein EC**, Peacock JB, Thomas MD. Preoperative bilirubin, alkaline phosphatase and amylase levels as predictors of common duct stones. *Surg Gynecol Obstet* 1982; **154**: 381-384 [PMID: 6175028]

27 **Lacaine F**, Corlette MB, Bismuth H. Preoperative evaluation of the risk of common bile duct stones. *Arch Surg* 1980; **115**: 1114-1116 [PMID: 7416958 DOI: 10.1001/archsurg.1980.01380090080019]

28 **Houdart R**, Perniceni T, Darne B, Salmeron M, Simon JF. Predicting common bile duct lithiasis: determination and prospective validation of a model predicting low risk. *Am J Surg* 1995; **170**: 38-43 [PMID: 7793492 DOI: 10.1016/s0002-9610(99)80249-9]

29 **Welbourn CR**, Mehta D, Armstrong CP, Gear MW, Eyre-Brook IA. Selective preoperative endoscopic retrograde cholangiography with sphincterotomy avoids bile duct exploration during laparoscopic cholecystectomy. *Gut* 1995; **37**: 576-579 [PMID: 7489949 DOI: 10.1136/gut.37.4.576]

30 **Meeralam Y**, Al-Shammari K, Yaghoobi M. Diagnostic accuracy of EUS compared with MRCP in detecting choledocholithiasis: a meta-analysis of diagnostic test accuracy in head-to-head studies. *Gastrointest Endosc* 2017; **86**: 986-993 [PMID: 28645544 DOI: 10.1016/j.gie.2017.06.009]

31 **Giljaca V**, Gurusamy KS, Takwoingi Y, Higgie D, Poropat G, Štimac D, Davidson BR. Endoscopic ultrasound versus magnetic resonance cholangiopancreatography for common bile duct stones. *Cochrane Database Syst Rev* 2015; CD011549 [PMID: 25719224 DOI: 10.1002/14651858.CD011549]

32 **Internal Clinical Guidelines Team (UK)**. Gallstone Disease: Diagnosis and Management of Cholelithiasis, Cholecystitis and Choledocholithiasis. London: National Institute for Health and Care Excellence (UK); 2014 [PMID: 25473723]

33 **Anderson SW**, Lucey BC, Varghese JC, Soto JA. Accuracy of MDCT in the diagnosis of choledocholithiasis. *AJR Am J Roentgenol* 2006; **187**: 174-180 [PMID: 16794173 DOI: 10.2214/AJR.05.0459]

34 **Anderson SW**, Rho E, Soto JA. Detection of biliary duct narrowing and choledocholithiasis: accuracy of portal venous phase multidetector CT. *Radiology* 2008; **247**: 418-427 [PMID: 18372450 DOI: 10.1148/radiol.2472070473]

35 **Kim CW**, Chang JH, Lim YS, Kim TH, Lee IS, Han SW. Common bile duct stones on multidetector computed tomography: attenuation patterns and detectability. *World J Gastroenterol* 2013; **19**: 1788-1796 [PMID: 23555167 DOI: 10.3748/wjg.v19.i11.1788]

36 **Tseng CW**, Chen CC, Chen TS, Chang FY, Lin HC, Lee SD. Can computed tomography with coronal reconstruction improve the diagnosis of choledocholithiasis? *J Gastroenterol Hepatol* 2008; **23**: 1586-1589 [PMID: 18713297 DOI: 10.1111/j.1440-1746.2008.05547.x]

37 **Saito H**, Iwagoi Y, Noda K, Atsuji S, Takaoka H, Kajihara H, Shono T, Nasu J, Obara H, Kakuma T, Tada S, Morishita S, Matsushita I, Katahira K. Dual-layer spectral detector computed tomography versus magnetic resonance cholangiopancreatography for biliary stones. *Eur J Gastroenterol Hepatol* 2021; **33**: 32-39 [PMID: 32639415 DOI: 10.1097/MEG.0000000000001832]

38 **Kim JE**, Lee JM, Baek JH, Han JK, Choi BI. Initial assessment of dual-energy CT in patients with gallstones or bile duct stones: can virtual nonenhanced images replace true nonenhanced images? *AJR Am J Roentgenol* 2012; **198**: 817-824 [PMID: 22451546 DOI: 10.2214/AJR.11.6972]

39 **Uyeda JW**, Richardson IJ, Sodickson AD. Making the invisible visible: improving conspicuity of noncalcified gallstones using dual-energy CT. *Abdom Radiol (NY)* 2017; **42**: 2933-2939 [PMID: 28660332 DOI: 10.1007/s00261-017-1229-x]

40 **Wittenburg H**. Hereditary liver disease: gallstones. *Best Pract Res Clin Gastroenterol* 2010; **24**: 747-756 [PMID: 20955975 DOI: 10.1016/j.bpg.2010.07.004]

41 **Ransohoff DF**, Gracie WA. Treatment of gallstones. *Ann Intern Med* 1993; **119**: 606-619 [PMID: 8363172 DOI: 10.7326/0003-4819-119-7_part_1-199310010-00010]

42 **Halldestam I**, Enell EL, Kullman E, Borch K. Development of symptoms and complications in individuals with asymptomatic gallstones. *Br J Surg* 2004; **91**: 734-738 [PMID: 15164444 DOI: 10.1002/bjs.4547]

43 **Attili AF**, De Santis A, Capri R, Repice AM, Maselli S. The natural history of gallstones: the GREPCO experience. The GREPCO Group. *Hepatology* 1995; **21**: 655-660 [PMID: 7875663 DOI: 10.1002/hep.1840210309]

44 **Murison MS**, Gartell PC, McGinn FP. Does selective peroperative cholangiography result in missed common bile duct stones? *J R Coll Surg Edinb* 1993; **38**: 220-224 [PMID: 7693932]

45 **Soper NJ**, Dunnegan DL. Routine versus selective intra-operative cholangiography during laparoscopic cholecystectomy. *World J Surg* 1992; **16**: 1133-1140 [PMID: 1455885 DOI: 10.1007/BF02067079]

46 **Nies C**, Bauknecht F, Groth C, Clerici T, Bartsch D, Lange J, Rothmund M. [Intraoperative cholangiography as a routine method? *Chirurg* 1997; **68**: 892-897 [PMID: 9410677 DOI: 10.1007/s001040050290]

47 **Khan OA**, Balaji S, Branagan G, Bennett DH, Davies N. Randomized clinical trial of routine on-table cholangiography during laparoscopic cholecystectomy. *Br J Surg* 2011; **98**: 362-367 [PMID: 21254008 DOI: 10.1002/bjs.7356]

48 **Hauer-Jensen M**, Karesen R, Nygaard K, Solheim K, Amlie EJ, Havig O, Rosseland AR. Prospective randomized study of routine intraoperative cholangiography during open cholecystectomy: long-term follow-up and multivariate analysis of predictors of choledocholithiasis. *Surgery* 1993; **113**: 318-323 [PMID: 8441966]

49 **Frossard JL**, Hadengue A, Amouyal G, Choury A, Marty O, Giostra E, Sivignon F, Sosa L, Amouyal P. Choledocholithiasis: a prospective study of spontaneous common bile duct stone migration. *Gastrointest Endosc* 2000; **51**: 175-179 [PMID: 10650260 DOI: 10.1016/s0016-5107(00)70414-7]

50 **Saito H**, Kakuma T, Matsushita I. Risk factors for the development of post-endoscopic retrograde cholangiopancreatography pancreatitis in patients with asymptomatic common bile duct stones. *World J Gastrointest Endosc* 2019; **11**: 515-522 [PMID: 31798772 DOI: 10.4253/wjge.v11.i10.515]

51 **Miura F**, Okamoto K, Takada T, Strasberg SM, Asbun HJ, Pitt HA, Gomi H, Solomkin JS, Schlossberg D, Han HS, Kim MH, Hwang TL, Chen MF, Huang WS, Kiriyama S, Itoi T, Garden OJ, Liau KH, Horiguchi A, Liu KH, Su CH, Gouma DJ, Belli G, Dervenis C, Jagannath P, Chan ACW, Lau WY, Endo I, Suzuki K, Yoon YS, de Santibañes E, Giménez ME, Jonas E, Singh H, Honda G, Asai K, Mori Y, Wada K, Higuchi R, Watanabe M, Rikiyama T, Sata N, Kano N, Umezawa A, Mukai S, Tokumura H, Hata J, Kozaka K, Iwashita Y, Hibi T, Yokoe M, Kimura T, Kitano S, Inomata M, Hirata K, Sumiyama Y, Inui K, Yamamoto M. Tokyo Guidelines 2018: initial management of acute biliary infection and flowchart for acute cholangitis. *J Hepatobiliary Pancreat Sci* 2018; **25**: 31-40 [PMID: 28941329 DOI: 10.1002/jhbp.509]

52 **Saito H**, Koga T, Sakaguchi M, Kadono Y, Kamikawa K, Urata A, Imamura H, Tada S, Kakuma T, Matsushita I. Post-endoscopic retrograde cholangiopancreatography pancreatitis in single-stage endoscopic common bile duct stone removal. *JGH Open* 2020; **4**: 394-399 [PMID: 32514443 DOI: 10.1002/jgh3.12263]

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ORIGINAL ARTICLE

Basic Study

Alleviation of acute pancreatitis-associated lung injury by inhibiting the p38 mitogen-activated protein kinase pathway in pulmonary microvascular endothelial cells

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Institutional animal care and use committee statement: The study

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Abstract

BACKGROUND

Previous reports have suggested that the p38 mitogen-activated protein kinase signaling pathway is involved in the development of severe acute pancreatitis (SAP)-related acute lung injury (ALI). Inhibition of p38 by SB203580 blocked the inflammatory responses in SAP-ALI. However, the precise mechanism associated with p38 is unclear, particularly in pulmonary microvascular endothelial cell (PMVEC) injury.

AIM

To determine its role in the tumor necrosis factor-alpha (TNF-α)-induced inflammation and apoptosis of PMVECs *in vitro*. We then conducted *in vivo* experiments to confirm the effect of SB203580-mediated p38 inhibition on SAP-ALI.

METHODS

In vitro, PMVEC were transfected with mitogen-activated protein kinase kinase 6 (Glu), which constitutively activates p38, and then stimulated with TNF-α. Flow cytometry and western blotting were performed to detect the cell apoptosis and inflammatory cytokine levels, respectively. *In vivo*, SAP-ALI was induced by 5% sodium taurocholate and three different doses of SB203580 (2.5, 5.0 or 10.0 mg/kg) were intraperitoneally injected prior to SAP induction. SAP-ALI was assessed by performing pulmonary histopathology assays, measuring myeloperoxidase activity, conducting arterial blood gas analyses and measuring TNF-α, interleukin (IL)-1β and IL-6 levels. Lung microvascular permeability was measured by determining bronchoalveolar lavage fluid protein concentration, Evans blue

extravasation and ultrastructural changes in PMVECs. The apoptotic death of pulmonary cells was confirmed by performing a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling analysis and examining the Bcl2, Bax, Bim and cle-caspase3 levels. The proteins levels of P-p38, NFκB, IκB, P-signal transducer and activator of transcription-3, nuclear factor erythroid 2-related factor 2, HO-1 and Myd88 were detected in the lungs to further evaluate the potential mechanism underlying the protective effect of SB203580.

RESULTS

In vitro, mitogen-activated protein kinase (Glu) transfection resulted in higher apoptotic rates and cytokine (IL-1β and IL-6) levels in TNF-α-treated PMVECs. *In vivo*, SB2035080 attenuated lung histopathological injury, decreased inflammatory activity (TNF-α, IL-1β, IL-6 and myeloperoxidase) and preserved pulmonary function. Furthermore, SB203580 significantly reversed changes in the bronchoalveolar lavage fluid protein concentration, Evans blue accumulation, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cell numbers, apoptosis-related proteins (cle-caspase3, Bim and Bax) and endothelial microstructure. Moreover, SB203580 significantly reduced the pulmonary P-p38, NFκB, P-signal transducer and activator of transcription-3 and Myd88 levels but increased the IκB and HO-1 levels.

CONCLUSION

p38 inhibition may protect against SAP-ALI by alleviating inflammation and the apoptotic death of PMVECs.

Key Words: Acute pancreatitis; Acute lung injury; Pulmonary microvascular endothelial cells; P38; SB203580; Apoptosis

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Core Tip: We explored the role of the p38 mitogen-activated protein kinase pathway in the tumor necrosis factor-alpha-induced inflammation and apoptosis of pulmonary microvascular endothelial cells *in vitro* and verified the effect of SB203580-mediated p38 inhibition on severe acute pancreatitis-related acute lung injury in rats. p38 mitogen-activated protein kinase overactivation promoted tumor necrosis factor-alpha-induced inflammatory cytokine expression and pulmonary microvascular endothelial cell apoptosis *in vitro*. SB203580 improved severe acute pancreatitis-related acute lung injury by downregulating P-p38, NFκB, P-SATA3 and Myd88 and upregulating IκB and HO-1, which alleviated inflammation and apoptotic cell death in rats.

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INTRODUCTION

Acute pancreatitis (AP), a common gastrointestinal disease, ranges from a self-limiting disease to a severe condition with high mortality. The overall mortality rate is approximately 2%[1], while the mortality rate of severe AP (SAP) with distant organ dysfunction increases to 37%-50%[2]. To date, the pathogenesis of SAP has not been completely clarified, and no specific pharmacological therapies for SAP or its associated organ failure are available. The management of AP is mainly limited to supportive care. The lung is the most vulnerable organ in patients with SAP. Acute lung injury (ALI) or its more severe form, acute respiratory distress syndrome, is reported to occur in 10%-25% of patients with AP and accounts for up to 60% of AP-associated deaths[3,4]. Therefore, the identification of an effective therapy for AP-

was performed according to the Guidelines of Animal Care and Use Committee of West China Hospital of Sichuan University (protocol number, 2017082A).

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associated ALI is still urgently needed to decrease AP mortality.

ALI secondary to SAP is clinically and histologically indistinguishable from other indirect (sepsis, trauma or burns) or direct (*e.g.*, toxins or infection) insults to the lungs[5]. ALI is characterized by disruption of the alveolar capillary barrier, which increases endothelial and epithelial permeability, decreases lung compliance and impairs gas exchange[6]. Multiple biological processes are involved in the mechanisms of endothelial permeability, such as cytoskeletal rearrangements, assembly/disassembly of cell junctions and survival/death[7-9]. Pulmonary microvascular endothelial cells (PMVECs) line the interior of blood vessels and are an essential part of the blood and tissue microenvironment. They are major actors responding to insults related to ALI[10,11]. PMVECs are the main targets of humoral and cellular mediators during injury[12] and actively produce proinflammatory cytokines, increase the levels of adhesion molecules and secrete prothrombotic substances, promoting injury by transforming to a prothrombotic and proinflamma-tory phenotype to further activate the immune and hemostatic systems[4,8]. PMVECs are likely to be the first-line responders in efforts to prevent the development of SAP-associated ALI. Therefore, modulation of pulmonary vascular homeostasis is crucial for the successful treatment of AP-associated ALI.

p38 mitogen-activated protein kinase (p38 MAPK), a member of the serine/threonine kinase family, is ubiquitously expressed. It plays an important role in diverse processes including endocytosis, gene transcription, cell proliferation, cytokine production, apoptosis and cytoskeletal redistribution[8]. Therefore, unsurprisingly, p38 MAPK is involved in many cascades that result in the development of mild AP with limited pancreatic injury to SAP with ALI. In an experimental AP model, p38 MAPK activation was observed in the lungs as early as 2 h after model establishment[13], while p38 MAPK inhibitors (SB203580 or CNI-1493) alleviated pancreatic and pulmonary injury, limited cytokine increases and improved survival[14-16]. Currently, the precise mechanism by which the p38 MAPK signaling pathway regulates SAP-related ALI is unclear, particularly PMVEC injury. In this study, we overactivated p38 MAPK to define its role in the tumor necrosis factor-alpha (TNF-α)-induced inflammation and apoptosis of rat PMVECs *in vitro* and then investigated the effect of SB203580, a specific p38 MAPK inhibitor, on SAP rats with ALI *in vivo*.

MATERIALS AND METHODS

Cell culture

Rat PMVECs were obtained from the BeNa Culture Collection (338210, Beijing, China). Cells were identified according to staining with rat anti–factor VIII and binding of FITC-labeled lectin (Sigma, United States)[17]. The cells were cultured in DMEM (Thermo Fisher, United States) supplemented with 100 mL/L fetal bovine serum (BI, Australia), 50 U/mL penicillin and 50 μg/mL streptomycin (Thermo Fisher, United States). PMVECs were maintained at 37 °C in a humidified atmosphere with 950 mL/L O_2 and 50 mL/L CO_2.

Mitogen-activated protein kinase kinase 6 (Glu) recombinant adenovirus construction and transduction

The MAPK pathway is comprised of three sequential dual-specificity kinases, and MAPK kinase (MKK) 6 is highly selective for p38 activation. Overexpression of activated MKK 6 (Glu) constitutively activates p38 MAPK[18,19]. Lentiviruses carrying either no inserted sequence (null) or the *MKK 6 (Glu)* gene were constructed by Wuhan PeptBio company limited China. PMVECs were infected with the lentivirus at a multiplicity of infection of 10 in the presence of 5 μg/mL polybrene (Sigma, United States). After lentivirus infection, MKK 6 (Glu)-overexpressing cells and control cells were obtained *via* puromycin (2 μg/mL) screening and analyzed using western blotting. When the cells reached 70%–80% confluence, they were treated with TNF-α (20 ng/mL) for the indicated times and then used for the subsequent analyses.

Flow cytometry

PMVEC apoptosis was assessed using an Annexin V-FITC/PI kit (4A Biotech, China). Briefly, PMVECs were detached using trypsin, washed with cold PBS, pelleted and resuspended in binding buffer. Then, 10 μL of Annexin V was added, and they were incubated for 5 min in the dark. After mixing with 5 μL of propidium iodide, the cells were analyzed using a flow cytometer (BD Biosciences, United States).

Animals

Adult male Sprague-Dawley rats (n = 25, 210-230 g) were purchased from the Experimental Animal Center of Sichuan University (Chengdu, China). The animals were housed at 22-25 °C on a 12 h day-night cycle and fed standard rodent chow and tap water ad libitum for 1 wk of acclimation. The animal experiments performed in this study were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was designed to minimize pain or discomfort to the animals and approved by the Animal Ethics Committee of West China Hospital of Sichuan University.

Induction of the SAP model and experimental groups

After fasting for 12 h, the SAP model was established by biliary-pancreatic duct injection of 50 g/L sodium taurocholate (Sigma, United States, 1 mL/kg) using a micropump at a speed of 0.2 mL/min[20]. In the sham group, the rats received the same volume of saline instead of sodium taurocholate. All rats received a saline injection subcutaneously to compensate for fluid loss during the surgery (20 mL/kg body weight). The rats were randomly divided into the SO group, SAP group and SAP + SB203580 group. The SAP + SB203580 group was further divided into low-dose (SB2.5 group), middle-dose (SB5 group) and high-dose (SB10 group) subgroups according to the dose of SB203580 (2.5 mg/kg, 5.0 mg/kg and 10.0 mg/kg, respectively, n = 5). SB203580 (Selleck, United States) was intraperitoneally administered before model establishment. The rats were sacrificed 12 h after model induction, and samples were harvested rapidly for subsequent analysis.

Arterial blood gas analysis

At the end of the experiment, arterial blood samples were collected by cardiac puncture. A blood gas analysis was performed to detect the partial pressure of oxygen (PaO_2) and oxygen saturation (SaO_2, Cobas b 123 Analyzer, Roche, Switzerland).

Bronchoalveolar lavage fluid collection

After opening the chest, the right lung was ligated. Bronchoalveolar lavage fluid (BALF) was collected through slow and careful lavage of the left lung with 2 mL of 4 °C PBS. Then, the fluid was centrifuged at 500 g for 5 min. The supernatant was collected and stored at -80 °C until the analyses of the protein concentration and cytokine levels.

Histological examination and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining

The pancreas and lung were fixed with 40 g/L formaldehyde for 24 h. The tissues were then embedded in paraffin, and continuously sliced at a 5 mm thickness for staining with hematoxylin and eosin. The pancreatic and pulmonary histological assessment was performed and scored by two pathologists using a microscope at 200 × magnification[21,22]. For the assessment of the apoptosis of lung tissue, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed with a commercially available In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Measurements of cytokines and BALF protein concentrations

Concentrations of TNF-α, interleukin (IL)-1β and IL-6 in the serum and BALF were evaluated using ELISA kits according to the manufacturer's instructions (R and D Systems, United States). The BALF protein concentration was determined using a BCA kit (Thermo, United States).

Lung microvascular permeability analysis

Evans blue extravasation was used to quantify the capillary permeability in the lungs. A 10 g/L Evans Blue solution (20 mg/kg, Sigma-Aldrich, United States) was injected into the tail vein 1 h before exsanguination. PBS was used to flush the remaining Evans blue from the pulmonary vascular system. After the complete removal of blood, the left lung was removed, weighed, homogenized in formamide and incubated at 37 °C for 24 h. Finally, the supernatant was quantified spectrophotometrically at 620 nm.

Myeloperoxidase activity

Pulmonary myeloperoxidase (MPO) activity was measured using the method described by Dawra *et al*[23]. Briefly, lung tissue was homogenized in phosphate

buffer (100 mmol/L, pH 7.4) at 4 °C and centrifuged to pellet the tissues. The samples were then resuspended in phosphate buffer (100 mmol/L, pH 5.4) containing protease inhibitors, 5 g/L hexadecyl trimethyl ammonium bromide and 10 mmol/L EDTA and subjected to three rapid freeze-thaw cycles. The suspension was sonicated and centrifuged to obtain the supernatant. Twenty microliters of the supernatant were incubated with 200 μL of phosphate buffer (pH 5.4) containing 5 g/L hexadecyl trimethyl ammonium bromide and 2 mmol/L 3,3,5,5-tetramethylbenzidine for 3 min at room temperature followed by the addition of 50 μL H_2O_2. The difference in the absorbance of this assay solution between 0 min and 3 min was measured at 655 nm at 37 °C with a microplate reader (BMG LABTECH, Germany). MPO activity was calculated from a human MPO standard curve, normalized to the protein concentration and reported as mU/mg protein.

Western blotting

Lysates from PMVECs or lung tissues were extracted with RIPA lysis buffer (Abcam, United Kingdom) supplemented with protease inhibitors (Roche, Germany). The total protein concentration was quantified using a BCA kit (Thermo, United States). The same amounts of protein were separated on 10% or 12% SDS-PAGE gels and transferred to a polyvinylidene fluoride membrane. Membranes were blocked with 50 g/L nonfat milk for 2 h at room temperature and subsequently incubated with the following primary antibodies overnight at 4 °C: P-p38 (1:2000), NFκB (1:1000), IκB (1:1000), cleaved caspase-3 (cle-casp3, 1:1000), P-signal transducer and activator of transcription-3 (P-STAT3, 1:1000), myeloid differentiation factor 88 (Myd88, 1:1000), nuclear factor erythroid 2-related factor 2 (Nrf2, 1:1000), Bax (1:1000) and Bim (1:1000) from CST, United States; IL-1β (1:1000), heme oxygenase-1 (HO-1, 1:1000) and Bcl2 (1:1000) from Abcam, United States; and TNF-α (1:1000), IL-6 (1:1000) and β-actin (1:1000) from Proteintech, China. After rinsing with Tris-buffered saline containing Tween 20 (10 min, × 3), the membranes were incubated with a secondary goat anti-rabbit (1:5000, Proteintech, China) or a goat anti-mouse IgG-HRP antibody (1:10000, Proteintech, China) for 1-2 h at room temperature. Finally, after washing, the membranes were imaged using a chemiluminescence detection system (Bio-Rad, United States) and analyzed with Image Lab software.

Transmission electron microscopy

A 2 mm^3 peripheral specimen was fixed with 25 mL/L glutaraldehyde for 2 h and then washed with 0.1 mol/L phosphate buffer (PH 7.4, 10 min × 3). After fixing the samples with 10 mL/L osmium tetroxide for 2 h, the specimens were dehydrated through a graded series of ethanol solutions and acetone and embedded in Epon 812 (Sigma, United States). Ultrathin sections were cut with an ultramicrotome (Leica, Germany). Finally, the sections were stained with uranyl acetate and lead citrate and examined using a JEM-1230 transmission electron microscope (JEOL, Japan).

Statistical analysis

All statistical tests were performed using SPSS 20.0 (SPSS, United States) by a biomedical statistician. Data are presented as the mean ± SE for continuous variables or frequencies and percentages for categorical variables. Data were analyzed using one-way analysis of variance or the Kruskal-Wallis test. A P value of < 0.05 was considered statistically significant.

RESULTS

Overactivation of p38 MAPK aggravates TNF-α-induced apoptosis and inflammatory cytokine expression in PMVECs

We overexpressed a constitutively activated MKK6 (Glu) in PMVECs, which was confirmed to over activate p38 MAPK (Figure 1A). Then, the cells were incubated in the absence or presence of TNF-α. Flow cytometry analysis showed TNF-α induced apoptosis in both null and MKK (Glu) cells. Compared with the null groups, the apoptotic rate was significantly higher in the MKK (Glu) group treated with TNF-α (Figure 1B and 1C). Meanwhile, the levels of inflammatory cytokines (IL-6 and IL-1β) were also significantly upregulated in the MKK (Glu) cells (Figure 1D and 1E).

SB203580 attenuates pancreatic damage in SAP rats

Representative images of hematoxylin and eosin staining of the pancreas were shown

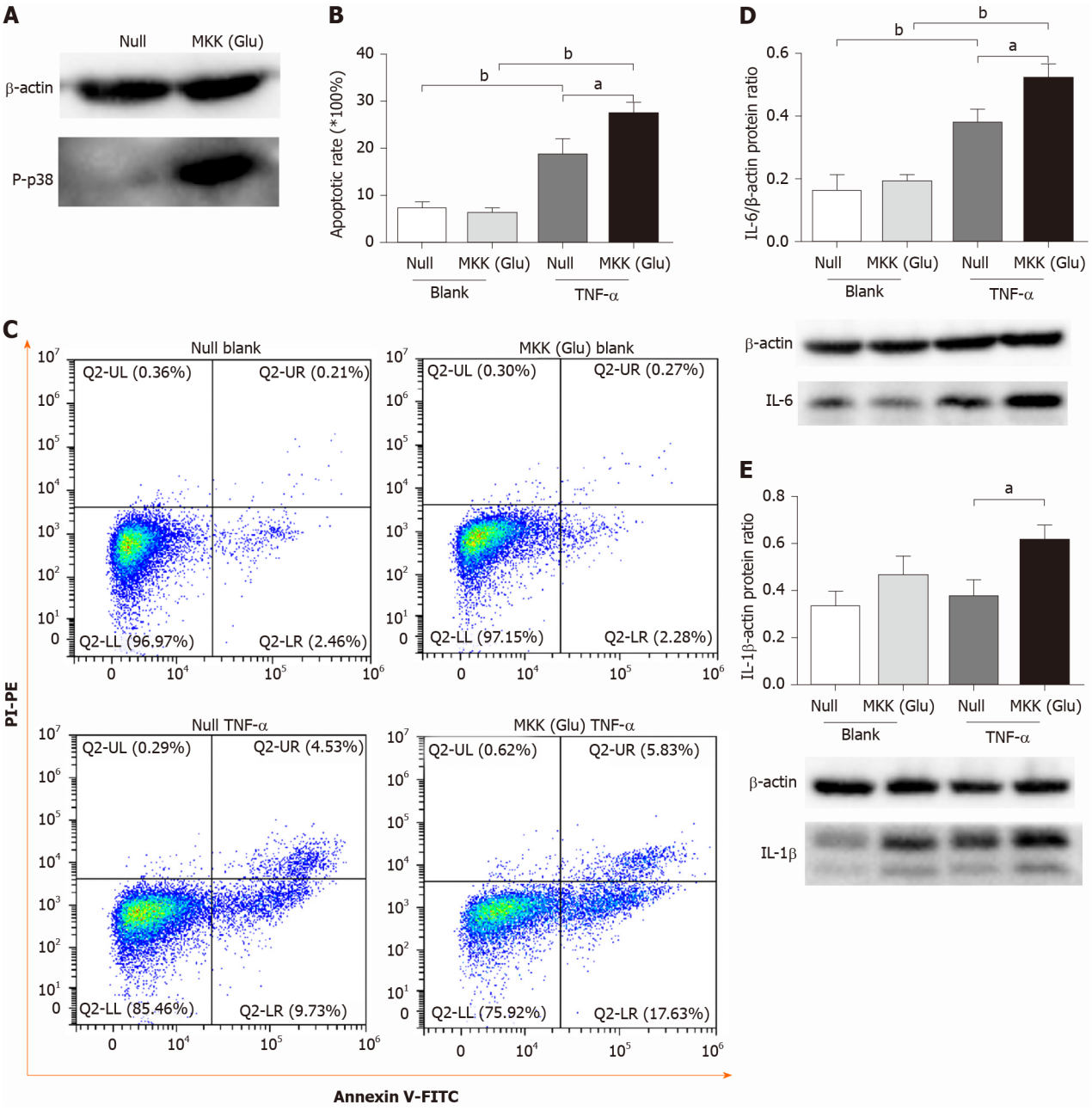


Figure 1 p38 mitogen-activated protein kinase overactivation aggravates tumor necrosis factor-alpha-induced apoptosis and inflammatory cytokine expression in pulmonary microvascular endothelial cells. A: Western blotting confirmed that mitogen-activated protein kinase kinase (Glu) transfection constitutively activated p38 mitogen-activated protein kinase in pulmonary microvascular endothelial cells; B: Apoptotic rate in null and mitogen-activated protein kinase kinase (Glu) pulmonary microvascular endothelial cells stimulated with or without tumor necrosis factor-alpha for 24 h; C: Representative images of flow cytometry analysis for apoptosis; D: The expression level of interleukin-6 in each group; E: The expression level of interleukin-1β in each group. Data are expressed as mean ± SE of 3-4 samples per group; $^{a}P < 0.05$; $^{b}P < 0.01$. IL: Interleukin; MKK: Mitogen-activated protein kinase kinase; TNF-α: Tumor necrosis factor-alpha.

in Figure 2A. Except for slight pancreatic edema, no obvious changes indicative of AP were observed in sham-operated animals. Sodium taurocholate induced typical histopathological changes associated with AP, including diffuse edema, marked neutrophil infiltration, intrapancreatic hemorrhage and acinar cell necrosis. The histopathological scores were all significantly increased (Figure 2B-F). All three tested doses of SB203580 (2.5, 5.0 and 10.0 mg/kg) alleviated injury to the pancreatic tissues (all $P < 0.05$). We explored the difference in the therapeutic effect among the three different doses; compared to the SB2.5 group, 10.0 mg/kg SB203580 further significantly decreased the histopathological inflammatory infiltration, necrosis and total scores (Figure 2B, 2D and 2F).

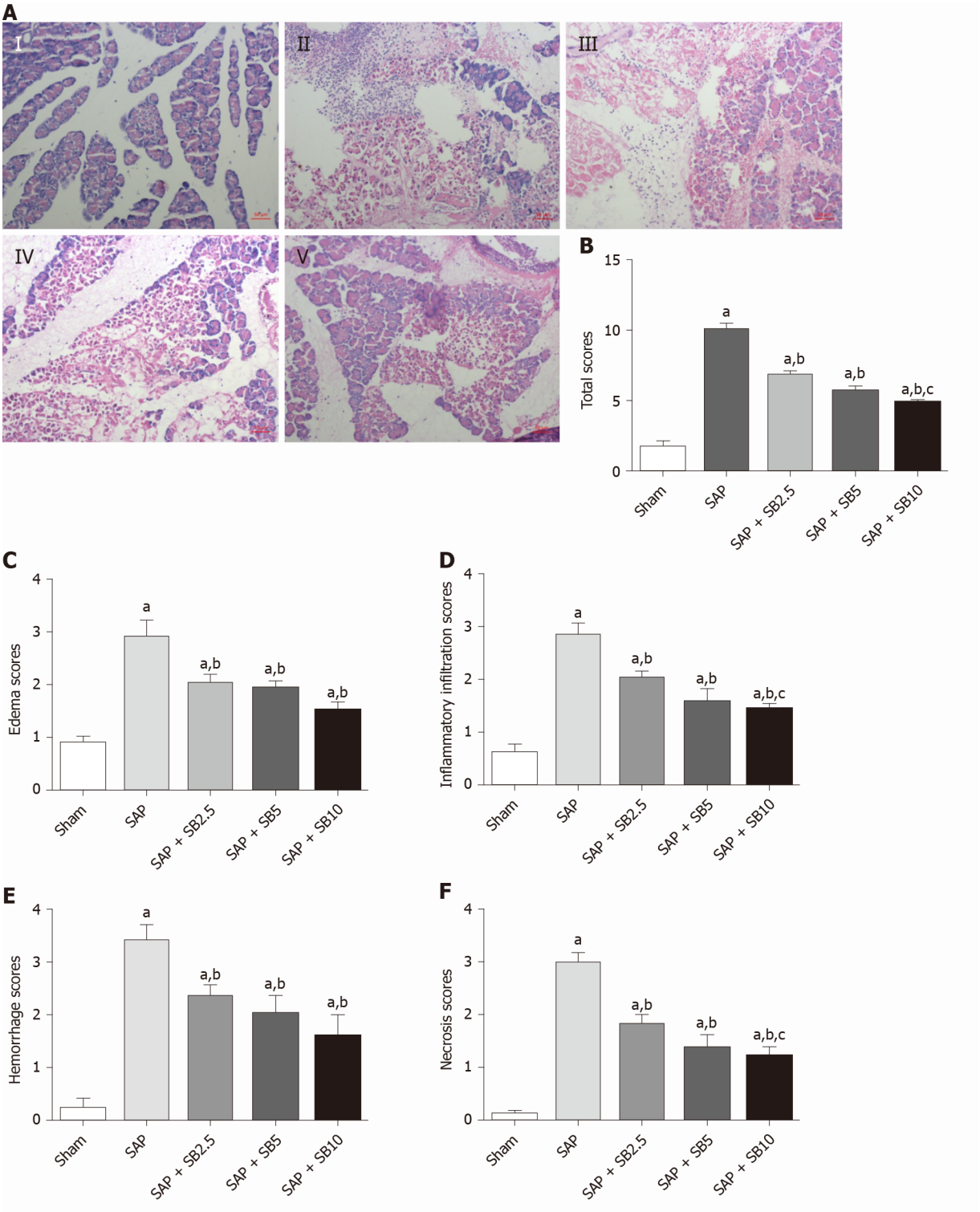


Figure 2 Effects of SB203580 on pancreatic histopathological changes and histopathological severity scores. A: Representative hematoxylin and eosin images of pancreatic sections (magnification 200 ×); I: Sham group; II: Severe acute pancreatitis (SAP) group; III: SAP + SB2.5 group; IV: SAP + SB5 group; V: SAP + SB10 group; B: Total scores; C: Edema scores; D: Inflammatory infiltration scores; E: Hemorrhage scores; F: Necrosis scores. B-F: Pancreatic histopathology scores in different groups; Data were expressed as mean ± SE; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* SAP group; $^{c}P < 0.05$ *vs* SAP + SB2.5 group. SAP: Severe acute pancreatitis.

SB203580 alleviates lung injury in SAP rats

The histopathological changes in the pulmonary tissues were shown in Figure 3A. In SAP rats, lung damage was characterized by neutrophil infiltration, thickened alveolar walls, intra-alveolar hemorrhaging and alveolar collapse. The histopathological scores were also significantly increased (Figure 3B-D). These changes were accompanied by significantly increased IL-1 and TNF-α levels in the BALF and serum (Figure 4A-D)

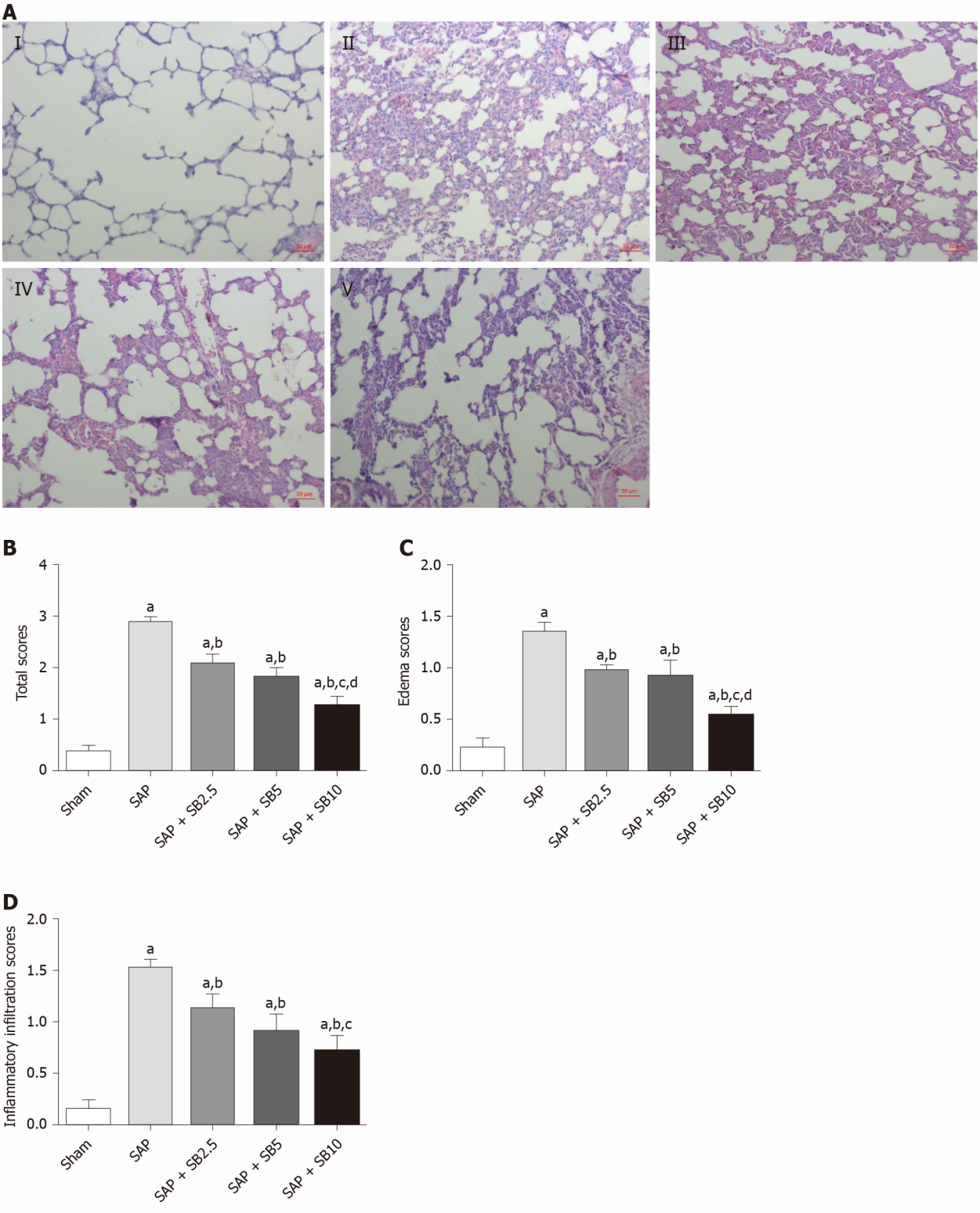
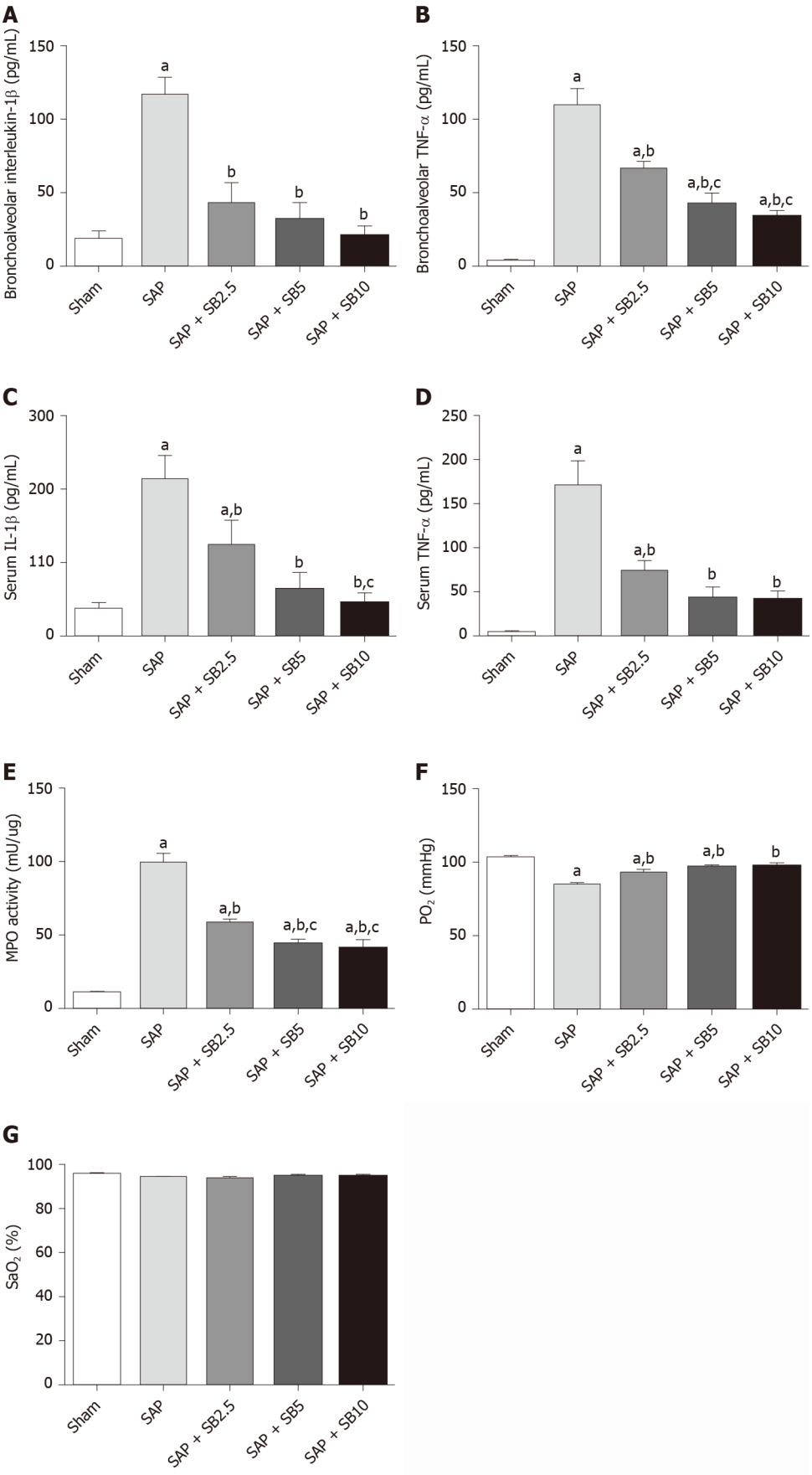


Figure 3 Effects of SB203580 on pulmonary histopathological changes and histopathological severity scores. A: Representative hematoxylin and eosin images of pulmonary sections (magnification 200 ×); I: Sham group; II: Severe acute pancreatitis (SAP) group; III: SAP + SB2.5 group; IV: SAP + SB5 group; V: SAP + SB10 group; B: Total scores. C: Edema scores; D: Inflammatory infiltration scores. B-D: Pulmonary histopathology scores in different groups; Data are expressed as mean ± SE of the mean; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* SAP group; $^{c}P < 0.05$ *vs* SAP + SB2.5 group; $^{d}P < 0.05$ *vs* SAP + SB5 group. SAP: Severe acute pancreatitis.

and pulmonary MPO activity (Figure 4E) and a decreased PaO_2 (Figure 4F). There was no change of SO_2 in the SAP rats (Figure 4G). At the same time, western blotting analysis also confirmed increased levels of inflammatory cytokines (IL-1, TNF-α, IL-6) in the SAP group compared with the sham group (Figure 4H-J). SB203580 treatment alleviated the histopathological injuries and scores, decreased the levels of inflam-


A
Bronchoalveolar interleukin-1β (pg/mL)
150
100
50
0
a
b
b
b
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
B
Bronchoalveolar TNF-α (pg/mL)
150
100
50
0
a
a,b
a,b,c
a,b,c
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
C
Serum IL-1β (pg/mL)
300
200
110
0
a
a,b
b
b,c
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
D
Serum TNF-α (pg/mL)
250
200
150
100
50
0
a
a,b
b
b
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
E
MPO activity (mU/ug)
150
100
50
0
a
a,b
a,b,c
a,b,c
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
F
PO2 (mmHg)
150
100
50
0
a
a,b
a,b
b
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
G
SaO2 (%)
100
80
60
40
20
0
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10

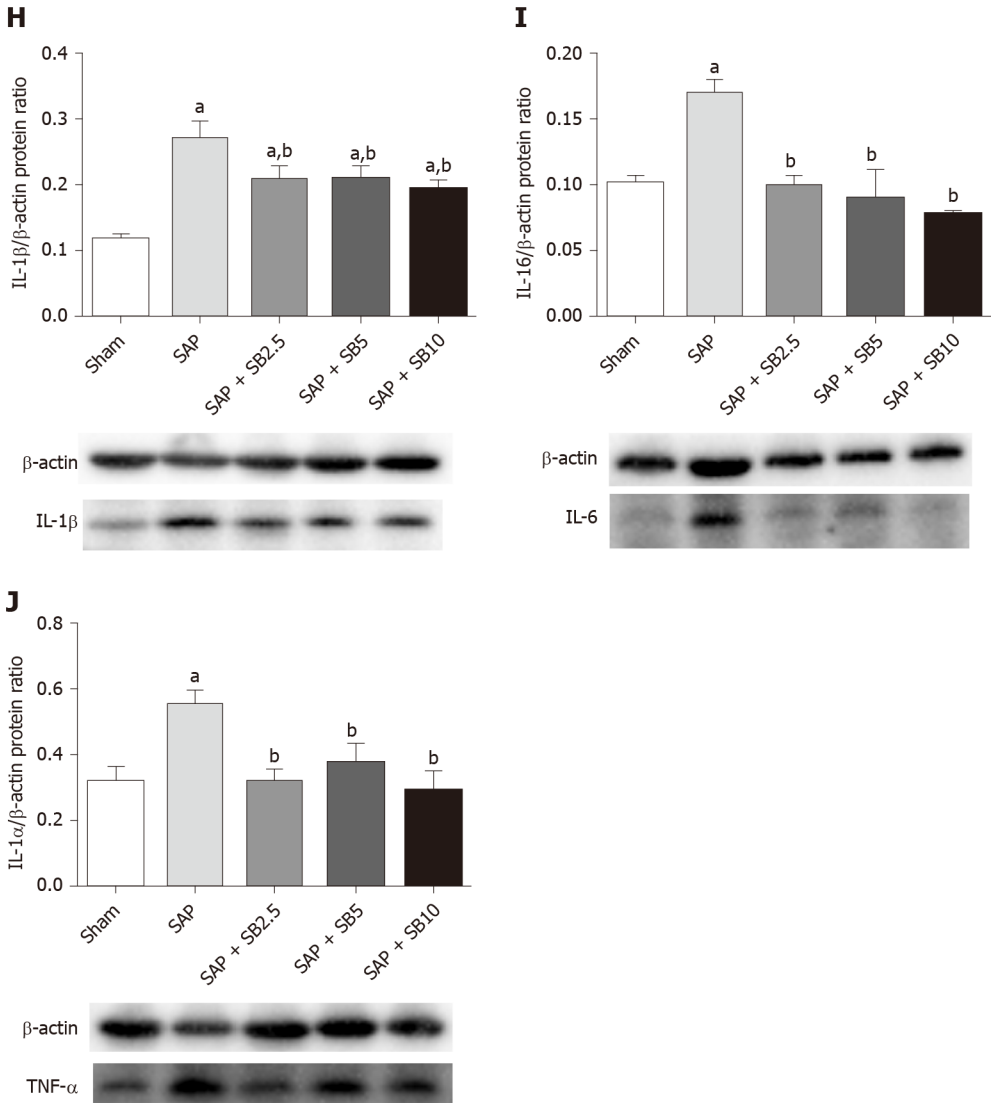


Figure 4 Effects of SB203580 on pulmonary severity indices of severe acute pancreatitis in rats. A: Bronchoalveolar interleukin-1β (IL-1β); B: Bronchoalveolar tumor necrosis factor alpha (TNF-α); C: Serum IL-1β; D: Serum TNF-α; E: Lung myeloperoxidase activity; F: Partial pressure of oxygen; G: Oxygen saturation; H: Representative western blotting analysis results for IL-1β in lung tissues; I: Representative western blotting analysis results for IL-6 in lung tissues; J: Representative western blotting analysis results for TNF-α in lung tissues. Data are expressed as mean ± SE of the mean; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* severe acute pancreatitis group; $^{c}P < 0.05$ *vs* severe acute pancreatitis + SB2.5 group. IL: Interleukin; MPO: Myeloperoxidase; PaO_2: Pressure of oxygen; SaO_2: Oxygen saturation; SAP: Severe acute pancreatitis; TNF-α: Tumor necrosis factor-alpha.

matory factors (IL-1, TNF-α, IL-6 and MPO) and preserved PaO_2. Among the three doses tested, SB203580 at 10.0 mg/kg consistently and significantly reduced all histopathological and biochemical severity indices with the highest efficiency.

Protective effects of SB203580 on capillary injury

The permeability of the PMVECs was evaluated *in vivo* by measuring the BALF protein concentration and Evans blue accumulation in the lung tissue. Quantitative analysis showed a significant increase in these two parameters in SAP rats, which were suppressed by SB203580 cotreatment (Figure 5A and 5B). They further decreased after treatment with increasing SB203580 doses. The microstructure and integrity of the endothelium were evaluated using transmission electron microscopy. In the sham group, the lung tissues showed a normal capillary endothelium, as evidenced by an intact capillary basal membrane, dense endothelial cell-cell junctions and continuous and clear delineation between cells (Figure 5C). In the SAP group, capillary endothelial dissolution and rupture were noted. In addition, low electron density clouds with an irregular thickness indicating basal membrane edema, increased vacuolization, loose cellular junctions, swollen cellular organelles and nuclear degeneration were often observed. In contrast, the swelling and injury of endothelial cells were partially alleviated in lungs from SB203580-treated rats. After treatment with 10.0 mg/kg

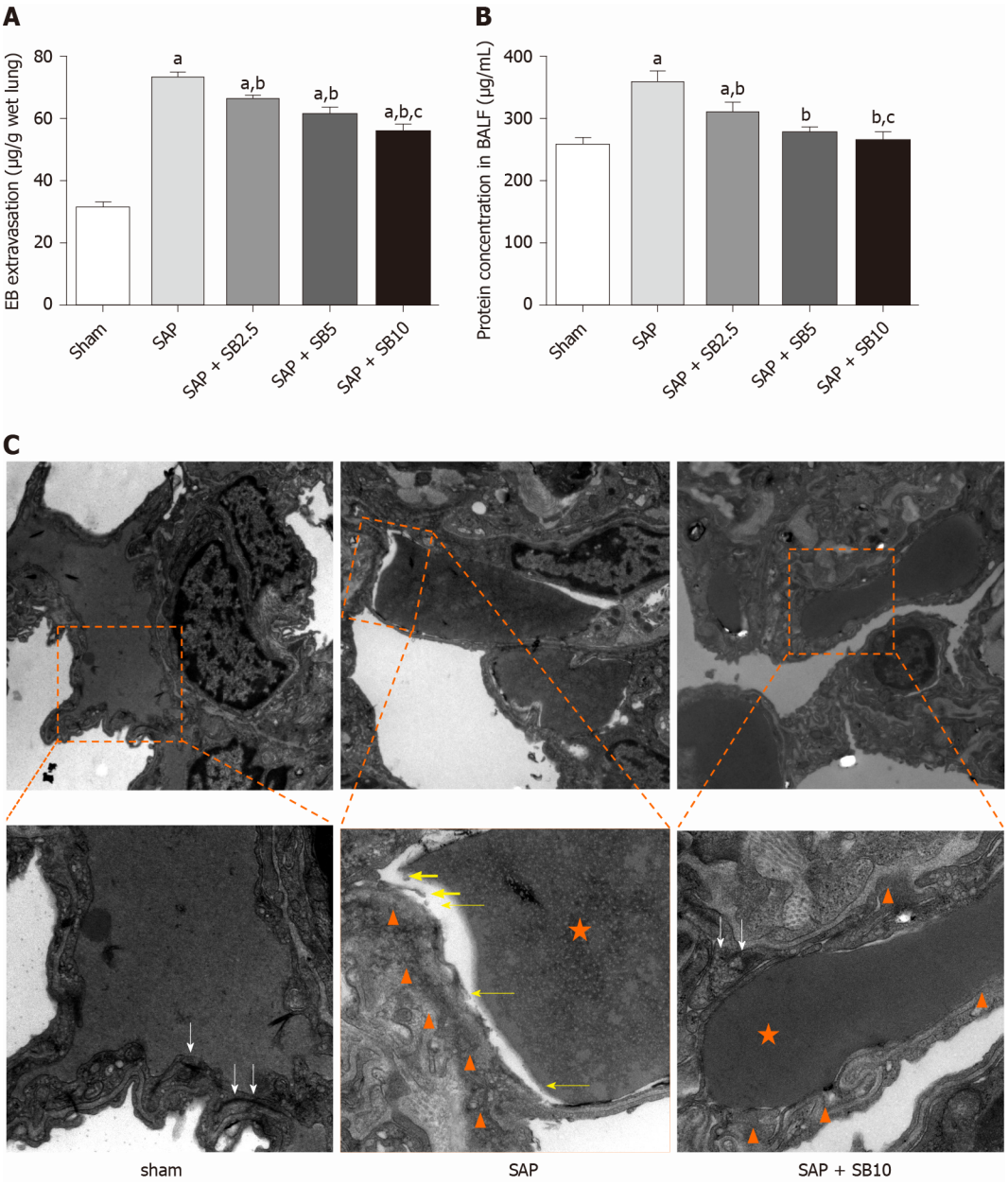
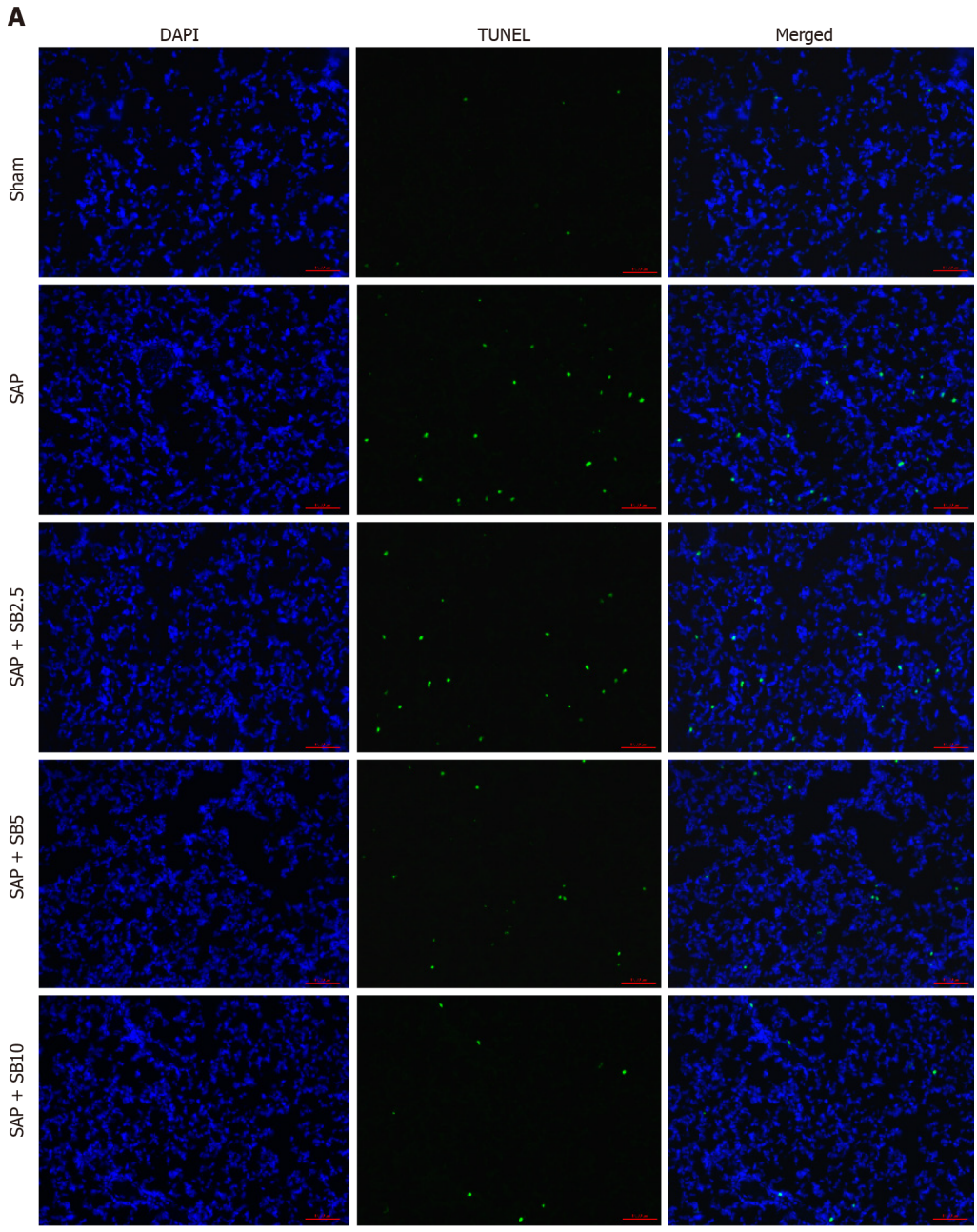


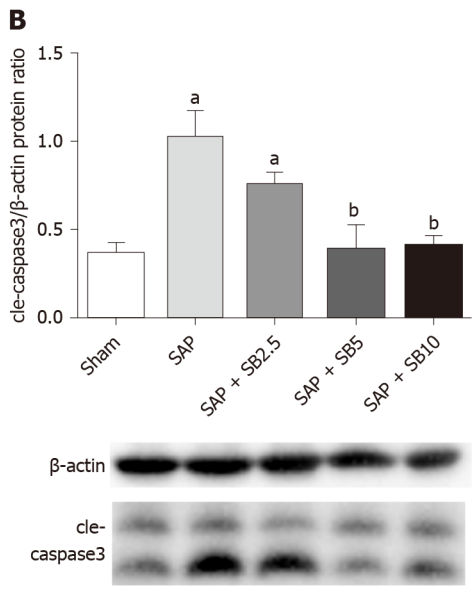
Figure 5 Effects of SB203580 on pulmonary capillary injury of severe acute pancreatitis rats. A: Evans blue extravasation in lung tissues; B: Bronchoalveolar protein concentration; C: Representative electron microscope photomicrographs of pulmonary microvascular endothelial cells in lung tissues; Sham group: Magnification 1500 × and 4000 ×, respectively; Severe acute pancreatitis (SAP) group: Magnification 1500 × and 5000 ×, respectively; SAP + SB10 group: Magnification 1500 × and 5000 ×, respectively; White arrows: Dense endothelial cell–cell junctions; White arrowheads: Low electron density clouds with an irregular thickness indicating basal membrane edema; Yellow arrows: Dissolution, rupture and debris of capillary endothelial; Orange star: Red blood cell. Data were expressed as mean ± SE; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* SAP group; $^{c}P < 0.05$ *vs* SAP + SB2.5 group. SAP: Severe acute pancreatitis.

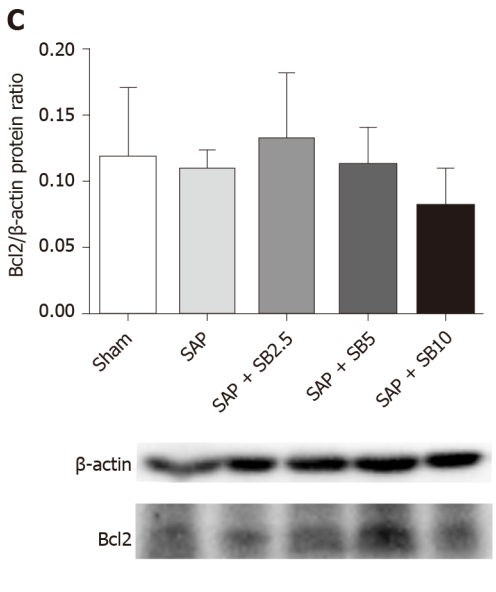
SB203580, no obvious rupture of endothelial cells or red cells extravasating from the vessels were observed.

SB203580 inhibits apoptosis in the lungs of SAP rats

TUNEL staining was used to examine cell death in lung tissues. Cell apoptosis in the lung tissues was assessed by the colocalization of TUNEL-green and DAPI-blue staining. A prominent increase in the number of positively stained cells was observed in the SAP group compared to the sham group, indicating a higher apoptotic rate (Figure 6A). Treatment of SAP rats with SB203580 significantly decreased the number of apoptotic cells, and the greatest inhibitory effect was observed in rats treated with 10.0 mg/kg SB203580. Proteins regulating the apoptotic signaling pathways were examined using western blotting to further confirm the cell death pathway. As shown in Figure 6B-E, although no change in the antiapoptotic protein Bcl2 was observed, the levels of the proapoptotic proteins Bax, Bim and cle-caspase3 were upregulated in the


A
DAPI
TUNEL
Merged
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10


B
cle-caspase3/β-actin protein ratio
1.5
1.0
0.5
0.0
a
a
b
b
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
β-actin
cle-caspase3


C
Bcl2/β-actin protein ratio
0.20
0.15
0.10
0.05
0.00
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
β-actin
Bcl2

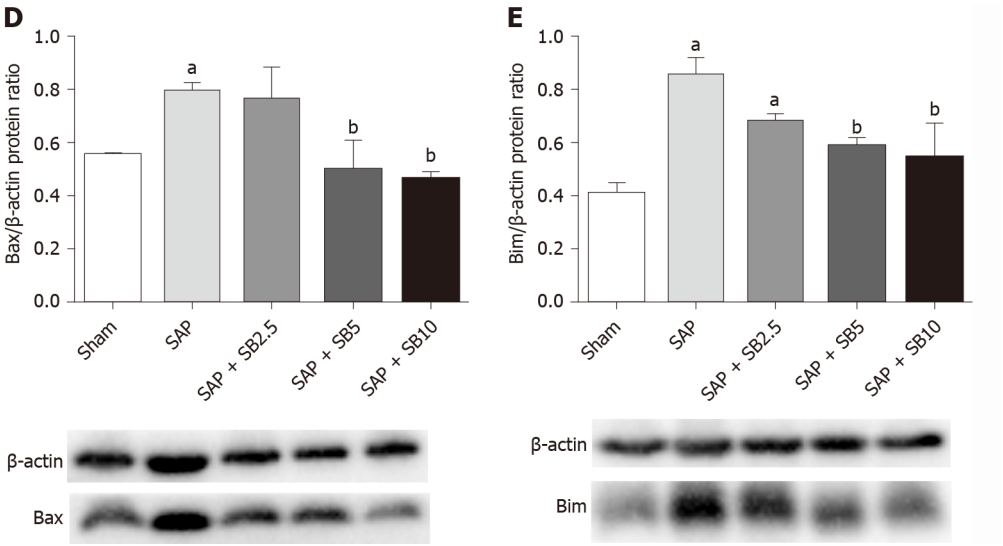


Figure 6 Effect of SB203580 on apoptosis of lung tissues in severe acute pancreatitis. A: Representative terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling images of pulmonary sections (200 ×); B: Cle-caspase3; C: Bcl2; D: Bax; E: Bim. B-E: Representative western blotting analysis results for apoptosis-related proteins in lung tissues; Data were expressed as mean ± SE; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* severe acute pancreatitis group. SAP: Severe acute pancreatitis.

SAP group compared to the sham group. SB203580 treatment reversed the changes in the levels of these proteins in the SAP group in a dose-dependent manner.

SB203580 protects against ALI by regulating proinflammatory and proapoptotic signaling pathways

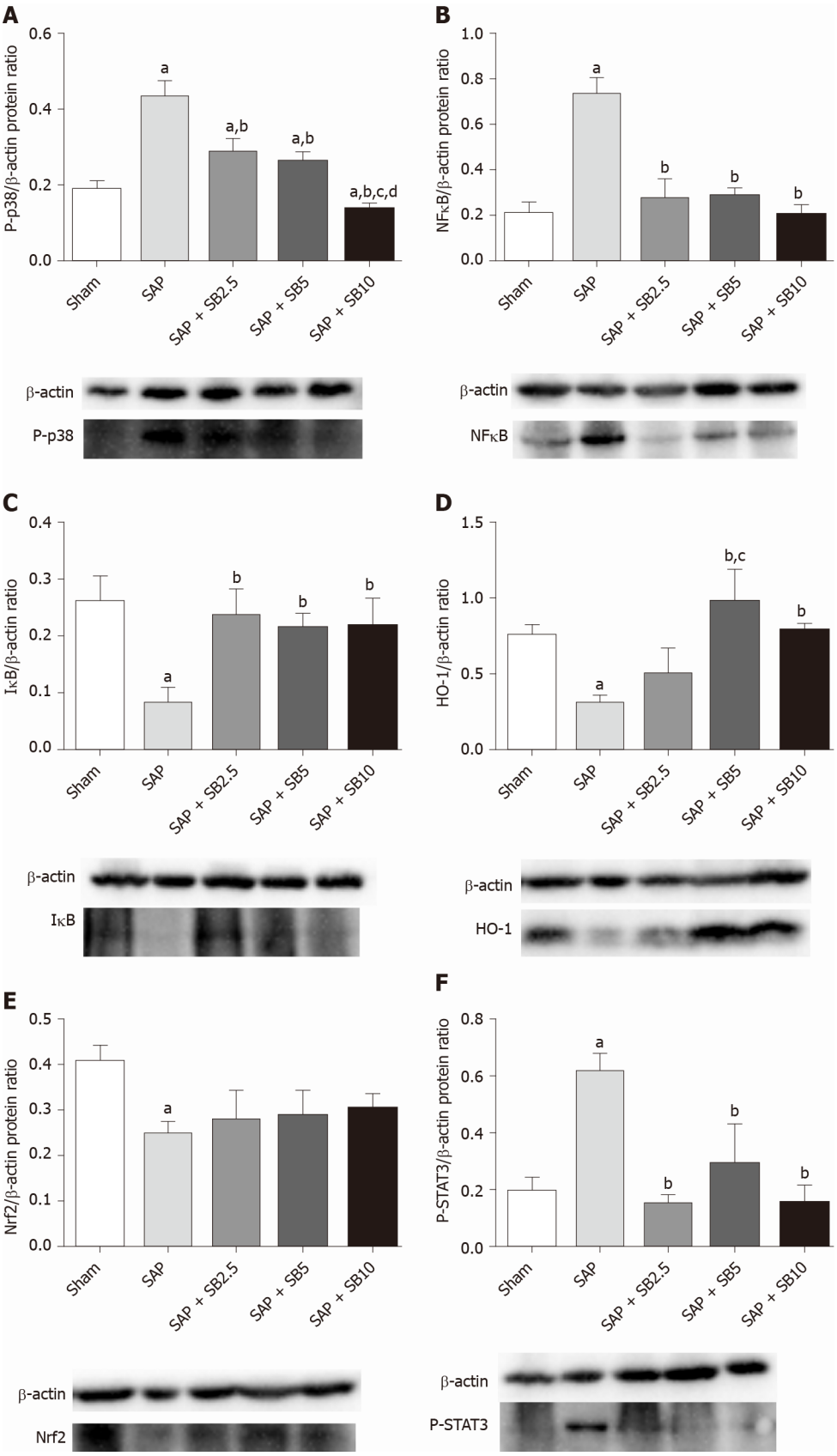
Levels of the P-p38, NFκB, IκB, Nrf2, HO-1, P-SATA3 and Myd88 proteins were detected in the lungs for further evaluation of the protective mechanism of SB203580. Figure 7 showed increased levels of P-p38, NFκB, P-SATA3 and Myd88 but decreased levels of IκB, Nrf2 and HO-1 in the SAP group compared with the sham group. Intriguingly, SB203580 reversed the changes in the levels of these proteins, reducing P-p38, NFκB, P-SATA3 and Myd88 levels but increasing IκB and HO-1 levels compared with the SAP group. However, except for P-p38 and HO-1, the improvement of these parameters was not positively related to the dose of SB203580. In addition, SB203580 slightly increased the level of Nrf2, but the difference was not statistically significant (Figure 7E).

DISCUSSION

Although the mechanisms of ALI/acute respiratory distress syndrome following SAP have not been completely elucidated, an increasing number of studies have described PMVEC injury and dysfunction as prerequisites for ALI[24]. The PMVECs are activated, participate in host defenses and result in increased microvascular permeability and damaged lung function[8,25]. Regardless of the etiology, an exaggerated inflammatory response is widely accepted to play a vital role in the transition of AP into a systemic disease[26]. Various inflammatory mediators have been related to AP severity and they induce their own or other mediators' secretion through a positive feedback mechanism, leading to amplification of the inflammatory cascade[27]. TNF-α was discovered to play a role in the early phase and cause the activation and injury of endothelial cells, functioning as a key promoter of the development of SAP-ALI[28]. In the present study, TNF-α was used to mimic SAP-related PMVEC injury *in vitro*.

p38 is activated by various insults, including cytokines, while the activation of p38 MAPK inevitably induces the expression of proinflammatory cytokines[29]. In our study, TNF-α induced higher levels of inflammatory cytokines (IL-1β and IL-6) in PMVECs with p38 overactivation (Figure 1D and 1E). These results further confirmed a positive role for the p38 MAPK signaling pathway in regulating cytokine expression.

According to previous studies, p38 MAPK is involved in both survival and apoptosis pathways. It promotes anti- or proapoptotic effects in a cell type and


A
P-p38/β-actin protein ratio
0.0
0.2
0.4
0.6
a
a,b
a,b
a,b,c,d
Sham
SAP
SAP + SB2.5
SAP + SB5
SAP + SB10
β-actin
P-p38
B
NFκB/β-actin protein ratio
0.0
0.2
0.4
0.6
0.8
1.0
a
b
b
b
β-actin
NFκB
C
IκB/β-actin ratio
0.0
0.1
0.2
0.3
0.4
a
b
b
b
β-actin
IκB
D
HO-1/β-actin ratio
0.0
0.5
1.0
1.5
a
b,c
b
β-actin
HO-1
E
Nrf2/β-actin protein ratio
0.0
0.1
0.2
0.3
0.4
0.5
a
β-actin
Nrf2
F
P-STAT3/β-actin protein ratio
0.0
0.2
0.4
0.6
0.8
a
b
b
b
β-actin
P-STAT3

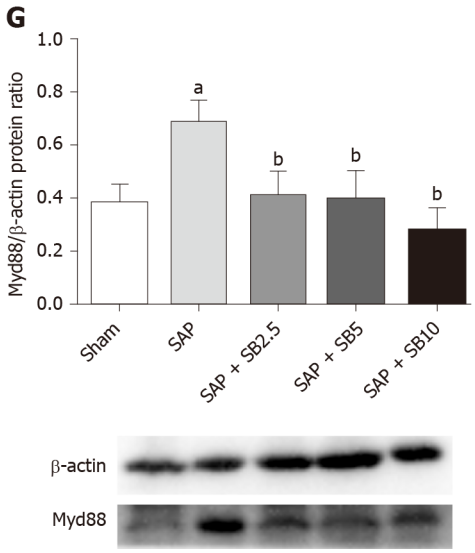


Figure 7 Effect of SB203580 on proinflammatory and proapoptotic signaling pathways. Representative Western blotting analysis results for protein expression in pulmonary tissue; A: P-p38; B: NFκB; C: IκB; D: HO-1; E: Nrf2; F: P-STAT3; G: Myd88. Data are expressed as mean ± SE; [a]P < 0.05 *vs* sham group; [b]P < 0.05 *vs* severe acute pancreatitis group; [c]P < 0.05 *vs* severe acute pancreatitis + SB2.5 group; [d]P < 0.05 *vs* severe acute pancreatitis + SB5 group. Nrf2: Nuclear factor erythroid 2-related factor 2; STAT3: Signal transducer and activator of transcription-3.

stimulus-specific manner in endothelial cells. For example, one group reported that SB203580 inhibited p38 MAPK and then decreased the endothelial cell apoptosis stimulated by TNF-α and cycloheximide[30], while another group illustrated that SB203580 enhanced endothelial cell apoptosis triggered by TNF-α alone[31]. It was reported that endothelial cells are relatively resistant to TNF-α, which induce only modest or no cell damage[31,32]. Then, we chose to overexpress activated MKK 6 (Glu) to constitutively activate p38 MAPK in PMVECs. Flow cytometry analysis revealed apoptotic cell death in null PMVECs stimulated with TNF-α, and MKK 6 (Glu) transfection resulted in a significantly higher apoptotic rate (Figure 1B). This finding indicated that persistent activation of p38 MAPK promotes TNF-α-induced PMVEC apoptosis. However, SB203580 pretreatment conversely aggravated PMVEC injury in this study (data not shown) and did not alleviate cell apoptosis. This discrepancy may be attributed to the extent or duration of p38 MAPK activation[33]. Rapid and transient p38 activation modulates inhibitors of the apoptosis protein family to prevent TNF-α-induced apoptosis[31], while sustained activation leads to apoptosis through the phosphorylation and downregulation of Bcl-x_L[30]. Therefore, SB203580 may exert a beneficial effect to maintain an appropriate level of p38 MAPK activity.

Many small-molecule inhibitors targeting p38 MAPK, such as SB203850, CNI-1493[34], SB202190[35], RWJ-67657[36] and UM101[37], have been tested in various cell injury or disease models. In AP, the p38 MAPK signaling pathway is involved not only in pancreatic acinar injury[20,38] but also in injury to distant organs (lung, liver, kidney, gut and placenta)[39-43]. SB203850 is a pyridinyl imidazole derivative that competes for ATP binding, and the inhibition of p38 MAPK has been shown to attenuate the severity of AP and its related distant organ injury[14,15,39,44]. In the present study, we treated SAP rats with SB203580 to investigate its protective effect on SAP-associated lung injury and the potential underlying mechanisms. As shown in Figure 2, SB203580 significantly alleviated pancreatic histopathological damage in SAP rats. Furthermore, it alleviated lung histopathological injuries (Figure 3), decreased the levels of inflammatory factors (IL-1β, TNF-α, IL-6 and MPO, Figure 4) and preserved PaO_2, consistent with previous reports[14,15]. Based on these results, SB203580 effectively decreased the inflammatory response in rats with SAP-ALI. In addition, by analyzing three different doses of SB203580, we observed a dose-dependent improvement in the therapeutic effects, as 10.0 mg/kg was the most effective.

Although apoptosis is regarded as a protective mechanism, excessive apoptosis is harmful to the alveolar capillary membrane and pulmonary function. As the first-line responder to systemic injurious factor insults, damage to endothelial cells is detected earlier than damage to epithelial cells[45]. PMVEC damage results in lung injury, which in turn promotes the secretion of inflammatory mediators to destroy PMVECs in individuals with SAP[46]. In the present study, increased Evans blue extravasation

and BALF protein concentrations revealed increased capillary permeability in SAP rats. Moreover, transmission electron microscopy analysis directly confirmed capillary endothelial dissolution and rupture in the lung (Figure 5). Finally, the results of the TUNEL and western blot analyses (Figure 6) suggested that an increased number of apoptotic cells was involved in the pathological process of SAP-ALI. However, SB203580 significantly reversed the changes in Evans blue accumulation, BALF protein concentration, TUNEL-positive cells, apoptosis-regulated proteins and endothelial microstructure, providing evidence for the underlying protective effects of p38 MAPK inhibitors on endothelial cell apoptosis.

Because p38 MAPK is a proximal initiator of signal transduction, various substrates are directly or indirectly activated by p38 MAPK, permitting it to perform a wide range of functions[29,47]. The biological consequences of p38 MAPK inhibition are complicated by interactions with other signaling pathways. p38 MAPK was reported to have a dual function in regulating Nrf2/HO-1 signaling[48,49]. HO-1 possesses cytoprotective properties, including anti-inflammatory, antioxidant and antiapoptotic activities. NFκB, which is downstream of p38 MAPK, represses Nrf2 transcriptional activity[50,51], while HO-1 inhibits proinflammatory mediators through the inactivation of NFκB[52]. STAT3 is activated by p38 MAPK, transferring signals to the nucleus and regulating gene expression[53,54]. MyD88 is considered a classical proinflammatory and proapoptotic upstream signaling adaptor molecule of p38 MAPK[55]. While the mechanism underlying the effects of SB203580 must be further elucidated, the levels of these key proteins involved in inflammatory, oxidative and apoptotic signaling pathways were measured. In the present study, SB203580 treatment reversed the increased levels of P-p38, NFκB, P-STAT3 and Myd88 and decreased levels of IκB and HO-1 in the SAP group (Figure 7). Therefore, the protective effects of p38 inhibition *via* SB203580 were likely partially attributed to the inhibition of pulmonary proinflammatory and proapoptotic signaling pathways.

CONCLUSION

In conclusion, p38 MAPK overactivation promoted TNF-α-induced inflammatory cytokine expression and PMVEC apoptosis *in vitro*. The inhibition of p38 MAPK with SB203580 protected against SAP-ALI by alleviating inflammation and apoptotic cell death in rats. Therefore, SB203580 may alleviate SAP-ALI by inhibiting the proinflammatory and proapoptotic effects of the p38 MAPK pathway on PMVECs. These results suggest the possibility of applying p38 MAPK inhibitors as treatments for SAP-ALI in the clinical setting.

ARTICLE HIGHLIGHTS

Research background

Acute lung injury (ALI) is the main reason for the high mortality of patients with severe acute pancreatitis (SAP). Injury and dysfunction of pulmonary microvascular endothelial cells (PMVEC) are considered prerequisites for ALI. The p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway is involved in the development of SAP-related ALI. However, the precise mechanism by which p38 MAPK regulates PMVEC injury in SAP-related ALI is unclear.

Research motivation

To date, no specific pharmacological therapies for SAP associated ALI are available. Elucidating the mechanism of PMVEC injury regulated by p38 MAPK is expected to help identify new treatments for SAP-associated ALI.

Research objectives

To determine the role of p38 MAPK in the tumor necrosis factor-alpha-induced injury of PMVECs *in vitro* and to explore the effect of SB203580-mediated p38 inhibition on SAP-ALI *in vivo*.

Research methods

In vitro, PMVECs were transfected with MAPK kinase 6 (Glu) and stimulated with tumor necrosis factor-alpha to detect cell apoptosis and inflammatory cytokine levels.

In vivo, SAP-ALI rats were treated with three different doses of SB203580 (2.5, 5.0 or 10.0 mg/kg). Blood, bronchoalveolar lavage fluid and tissue samples were harvested to assess cytokine levels, blood gas analyses, histopathological changes, myeloperoxidase activity, bronchoalveolar protein concentration, Evans blue extravasa-tion, apoptosis and ultrastructural changes of PMVECs. Then, the mechanism underlying the effects of SB203580 was also detected in the lungs.

Research results

In vitro, MAPK kinase (Glu) transfection resulted in higher apoptotic rates and cytokine levels in TNF-α-treated PMVECs. *In vivo*, SB2035080 attenuated lung histopathological injury, decreased inflammatory activity and preserved pulmonary function. Furthermore, SB203580 significantly reversed the microvascular permeability, the increase in the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells, the increased expression of apoptosis-related proteins and the endothelial microstructure changes. Moreover, SB203580 significantly reduced pulmonary P-p38, NFκB, P-SATA3 and Myd88 levels but increased the IκB and HO-1 levels.

Research conclusions

p38 inhibition may protect against SAP-ALI by alleviating inflammation and the apoptotic death of PMVECs.

Research perspectives

In this study, p38 MAPK overactivation promoted PMVEC injury *in vitro*, while p38 inhibition protected against SAP-ALI *in vivo*. These results suggest the potential of applying p38 MAPK inhibitors as treatments for SAP-ALI in the clinical setting.

REFERENCES

1 **Forsmark CE**, Vege SS, Wilcox CM. Acute Pancreatitis. *N Engl J Med* 2016; **375**: 1972-1981 [PMID: 27959604 DOI: 10.1056/NEJMra1505202]

2 **Banks PA**, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS; Acute Pancreatitis Classification Working Group. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; **62**: 102-111 [PMID: 23100216 DOI: 10.1136/gutjnl-2012-302779]

3 **Elder AS**, Saccone GT, Dixon DL. Lung injury in acute pancreatitis: mechanisms underlying augmented secondary injury. *Pancreatology* 2012; **12**: 49-56 [PMID: 22487475 DOI: 10.1016/j.pan.2011.12.012]

4 **De Campos T**, Deree J, Coimbra R. From acute pancreatitis to end-organ injury: mechanisms of acute lung injury. *Surg Infect (Larchmt)* 2007; **8**: 107-120 [PMID: 17381402 DOI: 10.1089/sur.2006.011]

5 **Matthay MA**, Zemans RL. The acute respiratory distress syndrome: pathogenesis and treatment. *Annu Rev Pathol* 2011; **6**: 147-163 [PMID: 20936936 DOI: 10.1146/annurev-pathol-011110-130158]

6 **Aldridge AJ**. Role of the neutrophil in septic shock and the adult respiratory distress syndrome. *Eur J Surg* 2002; **168**: 204-214 [PMID: 12440757 DOI: 10.1080/11024150260102807]

7 **Fang M**, Zhong WH, Song WL, Deng YY, Yang DM, Xiong B, Zeng HK, Wang HD. Ulinastatin Ameliorates Pulmonary Capillary Endothelial Permeability Induced by Sepsis Through Protection of Tight Junctions *via* Inhibition of TNF-α and Related Pathways. *Front Pharmacol* 2018; **9**: 823 [PMID: 30150933 DOI: 10.3389/fphar.2018.00823]

8 **Maniatis NA**, Kotanidou A, Catravas JD, Orfanos SE. Endothelial pathomechanisms in acute lung injury. *Vascul Pharmacol* 2008; **49**: 119-133 [PMID: 18722553 DOI: 10.1016/j.vph.2008.06.009]

9 **Lum H**, Malik AB. Mechanisms of increased endothelial permeability. *Can J Physiol Pharmacol* 1996; **74**: 787-800 [PMID: 8946065 DOI: 10.1139/y96-081]

10 **Suzuki T**, Sakata K, Mizuno N, Palikhe S, Yamashita S, Hattori K, Matsuda N, Hattori Y. Different involvement of the MAPK family in inflammatory regulation in human pulmonary microvascular endothelial cells stimulated with LPS and IFN-γ. *Immunobiology* 2018; **223**: 777-785 [PMID: 30115376 DOI: 10.1016/j.imbio.2018.08.003]

11 **Maniatis NA**, Orfanos SE. The endothelium in acute lung injury/acute respiratory distress syndrome. *Curr Opin Crit Care* 2008; **14**: 22-30 [PMID: 18195622 DOI: 10.1097/MCC.0b013e3282f269b9]

12 **Yang Y**, Li Q, Deng Z, Zhang Z, Xu J, Qian G, Wang G. Protection from lipopolysaccharide-induced pulmonary microvascular endothelial cell injury by activation of hedgehog signaling pathway. *Mol Biol Rep* 2011; **38**: 3615-3622 [PMID: 21110116 DOI: 10.1007/s11033-010-0473-8]

13 **de Campos T**, Deree J, Martins JO, Loomis WH, Shenvi E, Putnam JG, Coimbra R. Pentoxifylline attenuates pulmonary inflammation and neutrophil activation in experimental acute pancreatitis. *Pancreas* 2008; **37**: 42-49 [PMID: 18580443 DOI: 10.1097/MPA.0b013e3181612d19]

14 **Zhou Y**, Xia H, Zhao L, Mei F, Li M, You Y, Zhao K, Wang W. SB203580 attenuates acute lung injury and inflammation in rats with acute pancreatitis in pregnancy. *Inflammopharmacology* 2019; **27**: 99-107 [PMID: 30094758 DOI: 10.1007/s10787-018-0522-9]

15 **Cao MH**, Xu J, Cai HD, Lv ZW, Feng YJ, Li K, Chen CQ, Li YY. p38 MAPK inhibition alleviates experimental acute pancreatitis in mice. *Hepatobiliary Pancreat Dis Int* 2015; **14**: 101-106 [PMID: 25655298 DOI: 10.1016/s1499-3872(15)60327-7]

16 **Yang J**, Denham W, Tracey KJ, Wang H, Kramer AA, Salhab KF, Norman J. The physiologic consequences of macrophage pacification during severe acute pancreatitis. *Shock* 1998; **10**: 169-175 [PMID: 9744644 DOI: 10.1097/00024382-199809000-00004]

17 **King J**, Hamil T, Creighton J, Wu S, Bhat P, McDonald F, Stevens T. Structural and functional characteristics of lung macro- and microvascular endothelial cell phenotypes. *Microvasc Res* 2004; **67**: 139-151 [PMID: 15020205 DOI: 10.1016/j.mvr.2003.11.006]

18 **Li L**, Hu J, He T, Zhang Q, Yang X, Lan X, Zhang D, Mei H, Chen B, Huang Y. P38/MAPK contributes to endothelial barrier dysfunction *via* MAP4 phosphorylation-dependent microtubule disassembly in inflammation-induced acute lung injury. *Sci Rep* 2015; **5**: 8895 [PMID: 25746230 DOI: 10.1038/srep08895]

19 **Hu JY**, Chu ZG, Han J, Dang YM, Yan H, Zhang Q, Liang GP, Huang YS. The p38/MAPK pathway regulates microtubule polymerization through phosphorylation of MAP4 and Op18 in hypoxic cells. *Cell Mol Life Sci* 2010; **67**: 321-333 [PMID: 19915797 DOI: 10.1007/s00018-009-0187-z]

20 **Ren HB**, Li ZS, Xu GM, Tu ZX, Shi XG, Jia YT, Gong YF. Dynamic changes of mitogen-activated protein kinase signal transduction in rats with severe acute pancreatitis. *Chin J Dig Dis* 2004; **5**: 123-125 [PMID: 15612248 DOI: 10.1111/j.1443-9573.2004.00162.x]

21 **Zemans RL**, Colgan SP, Downey GP. Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury. *Am J Respir Cell Mol Biol* 2009; **40**: 519-535 [PMID: 18978300 DOI: 10.1165/rcmb.2008-0348TR]

22 **Schmidt J**, Rattner DW, Lewandrowski K, Compton CC, Mandavilli U, Knoefel WT, Warshaw AL. A better model of acute pancreatitis for evaluating therapy. *Ann Surg* 1992; **215**: 44-56 [PMID: 1731649 DOI: 10.1097/00000658-199201000-00007]

23 **Dawra R**, Ku YS, Sharif R, Dhaulakhandi D, Phillips P, Dudeja V, Saluja AK. An improved method for extracting myeloperoxidase and determining its activity in the pancreas and lungs during pancreatitis. *Pancreas* 2008; **37**: 62-68 [PMID: 18580446 DOI: 10.1097/MPA.0b013e3181607761]

24 **Ye W**, Zheng C, Yu D, Zhang F, Pan R, Ni X, Shi Z, Zhang Z, Xiang Y, Sun H, Shi K, Chen B, Zhang Q, Zhou M. Lipoxin A4 Ameliorates Acute Pancreatitis-Associated Acute Lung Injury through the Antioxidative and Anti-Inflammatory Effects of the Nrf2 Pathway. *Oxid Med Cell Longev* 2019; **2019**: 2197017 [PMID: 31781326 DOI: 10.1155/2019/2197017]

25 **Yi L**, Huang X, Guo F, Zhou Z, Chang M, Huan J. GSK-3Beta-Dependent Activation of GEF-H1/ROCK Signaling Promotes LPS-Induced Lung Vascular Endothelial Barrier Dysfunction and Acute Lung Injury. *Front Cell Infect Microbiol* 2017; **7**: 357 [PMID: 28824887 DOI: 10.3389/fcimb.2017.00357]

26 **Yang X**, Zhang X, Lin Z, Guo J, Yang X, Yao L, Wang H, Xue P, Xia Q. Chaiqin chengqi decoction alleviates severe acute pancreatitis associated acute kidney injury by inhibiting endoplasmic reticulum stress and subsequent apoptosis. *Biomed Pharmacother* 2020; **125**: 110024 [PMID: 32187959 DOI: 10.1016/j.biopha.2020.110024]

27 **Sandoval J**, Pereda J, Pérez S, Finamor I, Vallet-Sánchez A, Rodríguez JL, Franco L, Sastre J, López-Rodas G. Epigenetic Regulation of Early- and Late-Response Genes in Acute Pancreatitis. *J Immunol* 2016; **197**: 4137-4150 [PMID: 27798150 DOI: 10.4049/jimmunol.1502378]

28 **Yu S**, Xie J, Xiang Y, Dai S, Yu D, Sun H, Chen B, Zhou M. Downregulation of TNF-*α*/TNF-R1 Signals by AT-Lipoxin A4 May Be a Significant Mechanism of Attenuation in SAP-Associated Lung Injury. *Mediators Inflamm* 2019; **2019**: 9019404 [PMID: 31097921 DOI: 10.1155/2019/9019404]

29 **Viemann D**, Goebeler M, Schmid S, Klimmek K, Sorg C, Ludwig S, Roth J. Transcriptional profiling of IKK2/NF-kappa B- and p38 MAP kinase-dependent gene expression in TNF-alpha-stimulated primary human endothelial cells. *Blood* 2004; **103**: 3365-3373 [PMID: 14715628 DOI: 10.1182/blood-2003-09-3296]

30 **Grethe S**, Ares MP, Andersson T, Pörn-Ares MI. p38 MAPK mediates TNF-induced apoptosis in endothelial cells *via* phosphorylation and downregulation of Bcl-x(L). *Exp Cell Res* 2004; **298**: 632-642 [PMID: 15265709 DOI: 10.1016/j.yexcr.2004.05.007]

31 **Furusu A**, Nakayama K, Xu Q, Konta T, Kitamura M. MAP kinase-dependent, NF-kappaB-independent regulation of inhibitor of apoptosis protein genes by TNF-alpha. *J Cell Physiol* 2007; **210**: 703-710 [PMID: 17133355 DOI: 10.1002/jcp.20881]

32 **Roulston A**, Reinhard C, Amiri P, Williams LT. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. *J Biol Chem* 1998; **273**: 10232-10239 [PMID: 9553074 DOI: 10.1074/jbc.273.17.10232]

33 **Matsuzawa A**, Ichijo H. Stress-responsive protein kinases in redox-regulated apoptosis signaling. *Antioxid Redox Signal* 2005; **7**: 472-481 [PMID: 15706095 DOI: 10.1089/ars.2005.7.472]

34 **Yang J**, Murphy C, Denham W, Botchkina G, Tracey KJ, Norman J. Evidence of a central role for p38 map kinase induction of tumor necrosis factor alpha in pancreatitis-associated pulmonary injury. *Surgery* 1999; **126**: 216-222 [PMID: 10455887]

35 **Lüschen S**, Scherer G, Ussat S, Ungefroren H, Adam-Klages S. Inhibition of p38 mitogen-activated protein kinase reduces TNF-induced activation of NF-kappaB, elicits caspase activity, and enhances

cytotoxicity. *Exp Cell Res* 2004; **293**: 196-206 [PMID: 14729457 DOI: 10.1016/j.yexcr.2003.10.009]
36 **Kuldo JM**, Westra J, Asgeirsdóttir SA, Kok RJ, Oosterhuis K, Rots MG, Schouten JP, Limburg PC, Molema G. Differential effects of NF-{kappa}B and p38 MAPK inhibitors and combinations thereof on TNF-{alpha}- and IL-1{beta}-induced proinflammatory status of endothelial cells in vitro. *Am J Physiol Cell Physiol* 2005; **289**: C1229-C1239 [PMID: 15972838 DOI: 10.1152/ajpcell.00620.2004]
37 **Shah NG**, Tulapurkar ME, Ramarathnam A, Brophy A, Martinez R 3rd, Hom K, Hodges T, Samadani R, Singh IS, MacKerell AD Jr, Shapiro P, Hasday JD. Novel Noncatalytic Substrate-Selective p38α-Specific MAPK Inhibitors with Endothelial-Stabilizing and Anti-Inflammatory Activity. *J Immunol* 2017; **198**: 3296-3306 [PMID: 28298524 DOI: 10.4049/jimmunol.1602059]
38 **Twait E**, Williard DE, Samuel I. Dominant negative p38 mitogen-activated protein kinase expression inhibits NF-kappaB activation in AR42J cells. *Pancreatology* 2010; **10**: 119-128 [PMID: 20453549 DOI: 10.1159/000290656]
39 **Ouyang J**, Zhang ZH, Zhou YX, Niu WC, Zhou F, Shen CB, Chen RG, Li X. Up-regulation of Tight-Junction Proteins by p38 Mitogen-Activated Protein Kinase/p53 Inhibition Leads to a Reduction of Injury to the Intestinal Mucosal Barrier in Severe Acute Pancreatitis. *Pancreas* 2016; **45**: 1136-1144 [PMID: 27171513 DOI: 10.1097/MPA.0000000000000656]
40 **Fan HN**, Chen W, Fan LN, Wu JT, Zhu JS, Zhang J. Macrophages-derived p38α promotes the experimental severe acute pancreatitis by regulating inflammation and autophagy. *Int Immunopharmacol* 2019; **77**: 105940 [PMID: 31655340 DOI: 10.1016/j.intimp.2019.105940]
41 **Wang B**, Zhao KL, Hu WJ, Zuo T, Ding YM, Wang WX. Macrophage Migration Inhibitor Promoted the Intrahepatic Bile Duct Injury in Rats with Severe Acute Pancreatitis. *Dig Dis Sci* 2019; **64**: 759-772 [PMID: 30465176 DOI: 10.1007/s10620-018-5379-7]
42 **Li M**, Yu J, Zhao L, Mei FC, Zhou Y, Hong YP, Zuo T, Wang WX. Inhibition of macrophage migration inhibitory factor attenuates inflammation and fetal kidney injury in a rat model of acute pancreatitis in pregnancy. *Int Immunopharmacol* 2019; **68**: 106-114 [PMID: 30622028 DOI: 10.1016/j.intimp.2018.12.068]
43 **Zuo T**, Yu J, Wang WX, Zhao KL, Chen C, Deng WH, He XB, Wang P, Shi Q, Guo WY. Mitogen-Activated Protein Kinases Are Activated in Placental Injury in Rat Model of Acute Pancreatitis in Pregnancy. *Pancreas* 2016; **45**: 850-857 [PMID: 26491907 DOI: 10.1097/MPA.0000000000000528]
44 **Wan YD**, Zhu RX, Bian ZZ, Pan XT. Improvement of Gut Microbiota by Inhibition of P38 Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway in Rats with Severe Acute Pancreatitis. *Med Sci Monit* 2019; **25**: 4609-4616 [PMID: 31226101 DOI: 10.12659/MSM.914538]
45 **Wang F**, Lu F, Huang H, Huang M, Luo T. Ultrastructural changes in the pulmonary mechanical barriers in a rat model of severe acute pancreatitis-associated acute lung injury. *Ultrastruct Pathol* 2016; **40**: 33-42 [PMID: 26512751 DOI: 10.3109/01913123.2015.1088907]
46 **Han X**, Wang Y, Chen H, Zhang J, Xu C, Li J, Li M. Enhancement of ICAM-1 *via* the JAK2/STAT3 signaling pathway in a rat model of severe acute pancreatitis-associated lung injury. *Exp Ther Med* 2016; **11**: 788-796 [PMID: 26997994 DOI: 10.3892/etm.2016.2988]
47 **Hoefen RJ**, Berk BC. The role of MAP kinases in endothelial activation. *Vascul Pharmacol* 2002; **38**: 271-273 [PMID: 12487031 DOI: 10.1016/s1537-1891(02)00251-3]
48 **Naidu S**, Vijayan V, Santoso S, Kietzmann T, Immenschuh S. Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression *via* Nrf2. *J Immunol* 2009; **182**: 7048-7057 [PMID: 19454702 DOI: 10.4049/jimmunol.0900006]
49 **Zhou MM**, Zhang WY, Li RJ, Guo C, Wei SS, Tian XM, Luo J, Kong LY. Anti-inflammatory activity of Khayandirobilide A from Khaya senegalensis *via* NF-κB, AP-1 and p38 MAPK/Nrf2/HO-1 signaling pathways in lipopolysaccharide-stimulated RAW 264.7 and BV-2 cells. *Phytomedicine* 2018; **42**: 152-163 [PMID: 29655681 DOI: 10.1016/j.phymed.2018.03.016]
50 **Reboll MR**, Schweda AT, Bartels M, Franke R, Frank R, Nourbakhsh M. Mapping of NRF binding motifs of NF-kappaB p65 subunit. *J Biochem* 2011; **150**: 553-562 [PMID: 21821668 DOI: 10.1093/jb/mvr099]
51 **Niedick I**, Froese N, Oumard A, Mueller PP, Nourbakhsh M, Hauser H, Köster M. Nucleolar localization and mobility analysis of the NF-kappaB repressing factor NRF. *J Cell Sci* 2004; **117**: 3447-3458 [PMID: 15226370 DOI: 10.1242/jcs.01129]
52 **Ha YM**, Kim MY, Park MK, Lee YS, Kim YM, Kim HJ, Lee JH, Chang KC. Higenamine reduces HMGB1 during hypoxia-induced brain injury by induction of heme oxygenase-1 through PI3K/Akt/Nrf-2 signal pathways. *Apoptosis* 2012; **17**: 463-474 [PMID: 22183510 DOI: 10.1007/s10495-011-0688-8]
53 **Stephanou A**, Latchman DS. Opposing actions of STAT-1 and STAT-3. *Growth Factors* 2005; **23**: 177-182 [PMID: 16243709 DOI: 10.1080/08977190500178745]
54 **Ng DC**, Long CS, Bogoyevitch MA. A role for the extracellular signal-regulated kinase and p38 mitogen-activated protein kinases in interleukin-1 beta-stimulated delayed signal tranducer and activator of transcription 3 activation, atrial natriuretic factor expression, and cardiac myocyte morphology. *J Biol Chem* 2001; **276**: 29490-29498 [PMID: 11382751 DOI: 10.1074/jbc.M100699200]
55 **Shih JH**, Tsai YF, Li IH, Chen MH, Huang YS. Hp-s1 Ganglioside Suppresses Proinflammatory Responses by Inhibiting MyD88-Dependent NF-κB and JNK/p38 MAPK Pathways in Lipopolysaccharide-Stimulated Microglial Cells. *Mar Drugs* 2020; **18** [PMID: 33003399 DOI: 10.3390/md18100496]

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ORIGINAL ARTICLE

Basic Study

Partially hydrolyzed guar gum attenuates non-alcoholic fatty liver disease in mice through the gut-liver axis

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Abstract

BACKGROUND

The gut-liver axis has attracted much interest in the context of chronic liver disease pathogenesis. Prebiotics such as dietary fibers were shown to attenuate non-alcoholic fatty liver disease (NAFLD) by modulating gut microbiota. Partially hydrolyzed guar gum (PHGG), a water-soluble dietary fiber, has been reported to alleviate the symptoms of various intestinal diseases and metabolic syndromes. However, its effects on NAFLD remain to be fully elucidated.

AIM

To determine whether treatment with PHGG attenuates NAFLD development in mice through the gut-liver axis.

METHODS

Seven-week-old male C57BL/6J mice with increased intestinal permeability were fed a control or atherogenic (Ath) diet (a mouse model of NAFLD) for 8 wk, with or without 5% PHGG. Increased intestinal permeability was induced through chronic intermittent administration of low-dose dextran sulfate sodium. Body weight, liver weight, macroscopic findings in the liver, blood biochemistry [aspartate aminotransferase (AST) and alanine aminotransferase (ALT), total cholesterol, triglyceride, free fatty acids, and glucose levels], liver histology, myeloperoxidase activity in liver tissue, mRNA expression in the liver and intestine, serum endotoxin levels in the portal vein, intestinal permeability, and microbiota and short-chain fatty acid (SCFA) profiles in the cecal samples were investigated.

RESULTS

Mice with increased intestinal permeability subjected to the Ath diet showed significantly increased serum AST and ALT levels, liver fat accumulation, liver inflammatory (tumor necrosis factor-α and monocyte chemotactic protein-1) and fibrogenic (collagen 1a1 and α smooth muscle actin) marker levels, and liver myeloperoxidase activity, which were significantly attenuated by PHGG treatment. Furthermore, the Ath diet combined with increased intestinal permeability resulted in elevated portal endotoxin levels and activated toll-like receptor (TLR) 4 and TLR9 expression, confirming that intestinal permeability was significantly elevated, as observed by evaluating the lumen-to-blood clearance of fluorescein isothiocyanate-conjugated dextran. PHGG treatment did not affect fatty acid metabolism in the liver. However, it decreased lipopolysaccharide signaling through the gut-liver axis. In addition, it significantly increased the abundance of cecal *Bacteroides* and *Clostridium* subcluster XIVa. Treatment with PHGG markedly increased the levels of SCFAs, particularly, butyric acid, acetic acid, propionic acid, and formic acid, in the cecal samples.

CONCLUSION

PHGG partially prevented NAFLD development in mice through the gut-liver axis by modulating microbiota and downstream SCFA profiles.

Key Words: Non-alcoholic fatty liver disease; Partially hydrolyzed guar gum; Gut-liver axis; Intestinal barrier integrity; Microbiota; Short-chain fatty acids

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Core Tip: The gut-liver axis has attracted much interest in the context of chronic liver disease pathogenesis, including non-alcoholic fatty liver disease (NAFLD). Here, by using a mouse model of NAFLD induced by an atherogenic diet combined with increased intestinal permeability, we report that partially hydrolyzed guar gum (PHGG), a water-soluble dietary fiber, prevented NAFLD development in mice partially through the gut-liver axis by modulating the microbiota and downstream short-chain fatty acid profiles, highlighting that treatment with PHGG might show great promise as a therapeutic strategy for NAFLD.

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INTRODUCTION

The incidence of obesity is currently increasing owing to changing dietary habits, consequently resulting in the development of a fatty liver irrespective of alcohol consumption, a condition known as non-alcoholic fatty liver disease (NAFLD)[1]. NAFLD is an acquired metabolic stress-related liver disease that is strongly associated with obesity, dyslipidemia, hypertension, type 2 diabetes mellitus, and metabolic syndrome[2]. It was originally considered a benign disease. However, 37% of patients with NAFLD were recently reported to progress to non-alcoholic steatohepatitis (NASH) and consequent cirrhosis. Multiple studies have shown the pathogenesis and pathophysiology of NAFLD[3,4]. Various treatments such as vitamin E[5], anti-diabetic agents[6], farnesoid X receptor ligand[7], bile acid sequestrant, and apical sodium-dependent bile acid transporter inhibitors are under investigation[4], but there is no established standard treatment at present.

The mechanisms underlying NAFLD/NASH are far from being clarified and the supposed mechanisms that are at the basis of numerous attempts to cure NAFLD until now have ended, at best, in modest results[8]. The "gut-liver axis", which is potentially involved in the pathogenesis of NAFLD, has attracted much interest in the context of chronic liver disease[9]. Serum levels of lipopolysaccharide (LPS), derived from gram-negative bacteria in the gut, are elevated in patients with NASH and in animal models of NASH[10,11]. Additionally, activation of toll-like receptor (TLR) 4 in the liver plays an important role in NASH development in mice[12]. Furthermore, increased intestinal permeability is highlighted as the primary cause of liver injury in NASH[13]. Gäbele *et al*[14] reported that increased intestinal permeability *via* dextran sulfate sodium (DSS) treatment induces colitis, increases portal LPS levels, and enhances hepatic inflammation and fibrogenesis in an experimental NASH model[14]. In addition to these studies, recent evidence strongly supports a close link between gut microbiota and NAFLD. The severity of NAFLD is reportedly associated with gut dysbiosis and a shift in the metabolic function of the gut microbiota[3]. Therefore, many NAFLD studies have focused on gut dysbiosis, microbiota composition, and on treatments targeting the gut-liver axis that include antibiotics, probiotics, prebiotics, and synbiotics[15].

Probiotic and prebiotic supplements, which are inexpensive and safe for humans, may be considered as possible interventions for NAFLD. Probiotics are live microbial food supplements or bacterial components that are suggested to improve liver function and reduce liver fat in NASH patients[16,17]. In contrast, prebiotics refer to a group of non-digestible carbohydrates that alter the composition and activity of the gut microbiota[18]. The beneficial effects of prebiotics on NAFLD have been demonstrated in a few human studies[19]. Furthermore, a recent review showed that clinical trials using prebiotics such as oligofructose, oat bran, and inulin might not be applicable[4]. Therefore, further studies are required to demonstrate the effect of prebiotics on NAFLD.

Partially hydrolyzed guar gum (PHGG) is a water-soluble dietary fiber prepared from guar gum, a galactomannan obtained from the seeds of *Cyamopsis tetragonoloba* through microbial endo-β-mannanase activity to reduce its high viscosity[20]. PHGG treatment has been reported to improve the symptoms associated with both constipation- and diarrhea-predominant forms of irritable bowel syndrome[21]. The beneficial effects of dietary PHGG have also been demonstrated in the treatment of cholera[22], small intestinal bacterial overgrowth[23], pediatric functional gastrointestinal disorders[24], and metabolic syndrome-related functions such as aberrant lipid and glucose metabolism[25,26]. PHGG remains undigested in the upper gastrointestinal tract and is fermented by colonic bacteria, resulting in the production of short-chain fatty acids (SCFAs), particularly butyrate. It has recently been reported that PHGG increases butyrate-producing bacteria in the intestine[27]. More recently, PHGG treatment has been reported to alter the composition of the human microbiota, along with increasing the abundance of metabolites such as butyrate, acetate, and various amino acids[28]. To this end, PHGG should exert beneficial health effects on the host through alterations to the gut microbiota and SCFA production. However, the mechanisms by which PHGG prevents NAFLD development are yet to be elucidated. In this study, we used a murine model of NAFLD induced by an atherogenic (Ath) diet and an increase in intestinal permeability, and investigated the protective mechanisms by which PHGG modulates the gut microbiota.

MATERIALS AND METHODS

Experimental design

Seven-week-old male C57BL/6J mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). The mice were caged individually in a room maintained at 18-24 °C with 40%-70% relative humidity and a 12-h light/dark cycle. The mice were allowed free access to drinking water and were fed with rodent diet CE-2 (Nihon Clea, Tokyo, Japan) during their 1-wk acclimatization period. Mouse maintenance and all subsequent experimental procedures were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All the programs were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan, M2020-126). For NAFLD induction, mice were fed for 8 wk with an Ath diet as described by Matsuzawa *et al*[29], which resulted in hepatic steatosis and inflammation. The Ath diet was prepared by the addition of cocoa butter, cholesterol, and cholate to corticotropin-releasing factor-1 (CRF-1) (Oritental Yeast, Tokyo, Japan). Increased intestinal permeability was induced through the administration of 0.5% DSS (MW 36000-50000, MP Biomedicals, Illkirch, France) in drinking water as described previously[14]. DSS was administered in cycles, each consisting of 5-d DSS administration followed by a 9-d interval with normal drinking water. The cycles were repeated throughout the experimental period of 8 wk. Mice were randomly allocated to the following groups: (1) control mice fed standard chow (CRF-1; Control); (2) control mice fed a CRF-1 diet containing 5% PHGG (Control + PHGG); (3) NAFLD mice fed the Ath diet along with DSS induction (NAFLD); and (4) NAFLD mice supplemented with 5% PHGG (NAFLD + PHGG). Ingredient and fat contents of CRF-1 and the Ath diet are shown in Table 1. The commercial PHGG preparation (Sunfiber) used in this study was obtained from Taiyo Kagaku (Yokkaichi, Japan). The preparation was obtained by treating guar gum with β-endo-galactomannase from a strain of *Aspergillus niger*. Mice were sacrificed after being anesthetized with ketamine and xylazine. Blood samples were collected from the portal vein for serum endotoxin measurements through opposite cannulation with a 23-gauge needle or from the inferior vena cava for serum chemistry measurements followed by exsanguination, and the organs and cecal contents were harvested.

Serum biochemistry and liver histology

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using commercial assay kits (SRL, Tokyo, Japan) with an automated chemical analyzer (Olympus Automated Chemistry Analyzer AU5400, Tokyo, Japan). For hematoxylin and eosin (H&E) staining, liver samples were fixed in formalin, embedded in paraffin, sectioned at 5 μm, and stained with H&E. Histopathological changes were evaluated under a light microscope (Olympus BX50, Tokyo, Japan) in a blinded manner.

Measurement of myeloperoxidase activity

Tissue-associated myeloperoxidase (MPO) activity was measured in the liver tissue by a modified method reported by Grisham *et al*[30] as an index of neutrophil accumulation[30]. Liver tissue fragments were homogenized in 1 mL of 10 mmol/L potassium phosphate buffer (pH 7.8) containing 30 mmol/L KCl using a Teflon Potter-Elvehjem homogenizer. MPO activity was then determined. Briefly, liver homogenates were centrifuged at 20000´g for 15 min at 4 °C. The resulting pellet was then re-homogenized in 0.3 mL of 50 mmol/L potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. The mixture was then centrifuged at 20000´g for 15 min at 4 °C, and the supernatants were collected. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO that changed the absorbance by 1.0/min at 460 nm and 37 °C. Total protein in the tissue homogenates was measured using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, K. K., Tokyo, Japan) according to the manufacturer's protocol.

Analysis of mRNA expression in the liver and intestine

Hepatic and intestinal mRNA expression was determined using real-time polymerase chain reaction (PCR). Total RNA was extracted using the acid-guanidinium-phenol chloroform method (Isogen kit; Nippon Gene, Tokyo, Japan). Extracted RNA was stored at −80 °C until use in real-time PCR. For the latter, 1 μg of RNA was reverse-transcribed to first-strand complementary DNA (cDNA) using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States). Real-

Table 1 Ingredients and fat content of the experimental diets

Ingredients (g/kg)	CRF-1	Ath
Moisture (g/kg)	82	74.5
Protein (g/kg)	219	199
Fat (g/kg)	54	141.5
Crude ash (g/kg)	63	57
Crude fiber (g/kg)	29	26
Soluble nitrogen free extract (g/kg)	553	502
Total (g)	1000	1000
Fat content	**CRF-1**	**Ath**
Fatty acid (g/kg)	54	124
Cholesterol (g/kg)		12.5
Cholate (g/kg)		5

Ath: Atherogenic; CRF-1: Corticotropin-releasing factor-1.

time PCR was carried out on a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, United States) using the DNA-binding dye SYBR Green to detect the PCR products with the primers shown in Table 2. Gene expression was normalized to that of *b-actin* as an internal control.

Serum endotoxin levels in the portal vein

After the experimental period of 8 wk, blood samples were collected from the portal vein. The chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay kit (GenScript, Piscataway, NJ, United States) was used to measure serum endotoxin levels. In brief, the samples, blanks, or standards were incubated with reconstituted LAL at 37 °C for 45 min followed by incubation with a reconstituted chromogenic substrate solution at 37 °C for 6 min. After adding the stop solution, the absorbance of each reaction was recorded at 545 nm with a microplate reader (Spectramax M2; Molecular Devices, Sunnyvale, CA, United States). Distilled water was used as the blank.

Intestinal permeability assay

Intestinal permeability was determined by measuring the lumen-to-blood clearance of fluorescein isothiocyanate (FITC)-conjugated dextran according to a previously published method, with some modifications[31,32]. In brief, after the experimental period of 8 wk, mice were anesthetized with ketamine and xylazine, and FITC-conjugated dextran (1 mg/mL; molecular mass, 4 kDa; Sigma Aldrich, St. Louis, MO, United States) in poly(butylene succinate) was administered from the proximal duodenum in a total volume of 0.5 mL. One hour after administration, blood samples were collected, centrifuged, and stored at -80 °C. The serum was then examined at the excitation/emission wavelengths of 490 nm/525 nm using a spectrofluorometer (Spectramax M2; Molecular Devices, Sunnyvale, CA, United States).

DNA extraction from mouse fecal samples

After the experimental period of 8 wk, mouse fecal samples were collected from the cecum, suspended in lysis buffer [4 mol/L guanidinium thiocyanate, 100 mmol/L tris-HCl (pH 9.0), 40 mmol/L ethylenediaminetetraacetic acid, and 0.001% bromothymol blue], and shaken in the presence of zirconia beads. DNA was extracted from the resultant suspension (Magtration System 12GC and GC series MagDEA DNA 200; Precision System Science, Chiba, Japan).

PCR amplification and terminal restriction fragment length polymorphism analysis

The 16S rRNA gene was amplified from fecal DNA using the 516F (5′-TGCCAGCAGCCGCGGTA-3′; *Escherichia coli* positions 516 to 532) and 1510R (5′-GGTTACCTTGTTACGACTT-3′; *Escherichia coli* positions 1510 to 1492) primers. The 5′-ends of the forward primers were labeled with 6'-carboxyfluorescein synthesized by

Table 2 Primer sequences for the targeted mouse genes

Gene	Forward primer	Reverse primer
m_βactin	TATCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTA
m_TNF-a	ATCCGCGACGTGGAACTG	ACCGCCTGGAGTTCTGGAA
m_MCP-1	CTTCCTCCACCACCATGCA	CCAGCCGGCAACTGTGA
m_aSMA	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
m_collagen1a1	GCTTCACCTACAGCACCCTTGT	GATGACTGTCTTGCCCCAAGTT
m_Acc	CTGGCTGCATCCATTATGTCA	TGGTAGACTGCCCGTGTGAA
m_Cpt2	GGGCGAGCTTCAGCATATG	GGCCCATCGCTGCTTCTT
m_PPARa	CGATGCTGTCCTCCTTGATGA	CGCGTGTGATAAAGCCATTG
m_PPARγ	CTTGCTGTGGGGATGTCTCA	ATCTCCGCCAACAGCTTCTC
m_TLR4	CCTCTGCCTTCACTACAGAGACTTT	CACAATAACCTTCCGGCTCTTG
m_TLR9	CCTTCGTGGTGTTCGATAAGG	CACCCGCAGCTCGTTATACA

TNF-α: Tumor necrosis factor-α; MCP-1: Monocyte chemotactic protein 1; αSMA: α smooth muscle actin; ACC: Acetyl-CoA carboxylase; CPT2: Carnitine O-palmitoyltransferase II; PPAR: Peroxisome proliferator-activated receptor; TLR: Toll-like receptor.

Applied Biosystems (Life Technologies, Thermo Fisher Scientific, Waltham, MA). PCR amplification of the DNA samples (10 ng of each DNA sample) was performed according to a previously described protocol[33]. The amplified 16S rDNA genes were purified (MultiScreen PCR micro96 Plate; Merck Millipore, Tokyo, Japan) and dissolved in 40 μL of distilled water. The restriction enzymes for restriction fragment length polymorphism (RFLP) were selected according to a previously described protocol[34]. Purified PCR products were digested for 3 h with 10 U of the restriction enzyme *Bsl*I at 55 °C. The length of the terminal RF (T-RF) was determined using a genetic analyzer (ABI PRISM 3130xl; Applied Biosystems, Life Technologies, Thermo Fisher Scientific) and analysis software (GeneMapper; Applied Biosystems, Life Technologies, Thermo Fisher Scientific, Waltham, MA, United States). Standard-size markers were used (*e.g.*, MapMarker X-Rhodamine Labeled 50-1000 bp; BioVentures, Murfreesboro, TN, United States). An operational taxonomic unit (OTU) was used to describe the clusters of clone sequences that differed from the known species by approximately 2%, which was at least 98% similar to the members of their cluster[35]. As the apparent size of identical T-RFs can vary over a range of 1-3 bp, major T-RFs that were similar in size to within 1-3 bp, were grouped as OTUs[34]. The major T-RFs were identified using a computer simulation with a T-RFLP analysis program and a phylogenetic assignment database for the T-RFLP analysis of human colonic microbiota[36].

Determination of organic acid concentration in cecal contents

A portion of the cecal contents (0.5 g) was suspended in 0.5 mL of 14% perchloric acid to remove proteins. After centrifugation at 10000 × *g* for 5 min at 4 °C, the supernatant was filtered through a cellulose acetate membrane filter with a pore size of 0.45 μm, and its organic acid content was then analyzed with ion-exclusion high performance liquid chromatography as described by Ushida and Sakata[37].

Statistical analyses

Data are expressed as the mean ± SD. Overall differences between groups were determined by one-way analysis of variance (ANOVA). Differences between individual groups were determined using Tukey's multiple comparison tests when one-way ANOVA indicated a significant difference. Target gene copy numbers, estimated using real-time PCR, and the concentration of organic acids were analyzed using the Steel-Dwass method after the Friedman test. A P value < 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, United States) for Macintosh.

RESULTS

Effects of PHGG on body weight, liver weight, and biochemical parameters

Mice with increased intestinal permeability exposed to the Ath diet showed significant increases in liver weight, liver/body ratios, AST, ALT, and total cholesterol levels, as well as a significant decrease in body weight and triglyceride levels, as shown in Table 3. Treatment with PHGG did not change the NAFLD model-induced change in body weight, liver weight, liver/body ratio, and total cholesterol levels but significantly improved the increase in AST and ALT levels.

Effects of PHGG on liver damage, inflammation, and fibrosis

Macroscopic observation showed that livers from mice subjected to the Ath diet with increased intestinal permeability were visibly lighter and fatty compared to those from mice subjected to a control diet (Figure 1A). Treatment with PHGG did not induce a dramatic change, but partly reverted some morphological alterations. Histological analysis of the liver tissues indicated that the fat content in mice fed a control diet with or without PHGG treatment were normal without inflammatory infiltration (Figure 1B). In contrast, mice with increased intestinal permeability exposed to the Ath diet showed both increased fat content and inflammatory infiltration (Figure 1B). These alterations were prevented by PHGG treatment (Figure 1B). The observed inflammatory infiltration was supported by the MPO activity results, which confirmed that treatment with PHGG attenuated liver damage in the NAFLD model (Figure 1C). Histological analysis of intestine and colon tissues indicated that mice with increased intestinal permeability exposed to the Ath diet did not show any morphological changes compared to the control mice (data not shown). Figure 2 shows hepatic inflammation and fibrosis in the NAFLD model. *Tumor necrosis factor-a* (*TNF-a*; (TNF-α; Figure 2A) and *monocyte chemotactic protein-1* (*MCP-1*; (MCP-1; Figure 2B) mRNA expression was significantly higher in the liver tissue of mice with increased intestinal permeability exposed to the Ath diet than in that of control mice. This increase was significantly attenuated by treatment with PHGG. With respect to hepatic fibrosis, *collagen 1a1* (Figure 2C) and *a smooth muscle actin* (*aSMA*; (αSMA; Figure 2D) mRNA expression was significantly elevated in the liver tissue of mice with NAFLD compared to that in control mice. Treatment with PHGG clearly decreased *collagen 1a1* mRNA expression in the NAFLD model.

Effects of PHGG on lipid synthesis and degradation in the liver

Enzymes of the lipid synthesis and degradation pathways in the liver were further assessed as shown in Figure 3. Mice with increased intestinal permeability exposed to the Ath diet showed upregulation of *acetyl-CoA carboxylase* and *peroxisome proliferator-activated receptor γ* (*PPARγ*) and downregulation of *carnitine O-palmitoyltransferase II* and *PPARa* mRNA expression. Treatment with PHGG did not change these effects. Moreover, enzymes related to lipid absorption, such as acetyl-coenzyme A acetyltransferase 2, Niemann-Pick C1-like 1, and fatty acid binding protein, were evaluated in the intestine. Ath diet combined with increased intestinal permeability, and treatment with PHGG, did not affect the levels of these enzymes (data not shown).

Effects of PHGG on regulation of the gut-liver axis

The concept of the gut-liver axis has received considerable attention in recent years. In this study, chronic administration of 0.5% DSS did not result in morphological changes such as mucosal disruption. However, it induced some effects on the liver, thereby leading to NAFLD development. We therefore examined the expression of TLRs in the liver. The results showed that the Ath diet combined with increased intestinal permeability significantly increased the mRNA expression of *TLR4* and *TLR9* in the liver, and that treatment with PHGG significantly attenuated this upregulation (Figure 4A and B). These results are consistent with the endotoxin levels determined in the portal vein (Figure 4C). To determine whether our NAFLD model or PHGG treatment resulted in altered intestinal permeability, we evaluated the lumen-to-blood clearance of FITC-conjugated dextran (FD4 flux). As shown in Figure 5, the FD4 flux was significantly elevated in the NAFLD model mice, and this increase was significantly reduced by treatment with PHGG.

Effects of PHGG on SCFA profiles and cecal bacteria

The SCFA profiles of the cecal contents were determined using ion-exclusion high performance liquid chromatography, the results of which are summarized in Table 4. Under normal conditions, treatment with PHGG significantly increased the acetic acid,

Table 3 Characteristics of the experimental groups

Characteristics	Control	Control + PHGG	NAFLD	NAFLD + PHGG
Body weight (g)	25.7 ± 0.5	25.2 ± 1.1	20.6 ± 0.8[a]	20.8 ± 1.6[a]
Liver weight (g)	1.17 ± 0.05	1.03 ± 0.08	1.47 ± 0.11[a]	1.55 ± 0.13[a]
Liver weight (% body)	4.45 ± 0.13	4.13 ± 0.32	7.13 ± 0.40[a]	7.44 ± 0.61[a]
Aspartate aminotransferase (IU/L)	72.0 ± 7.1	73.1 ± 11.9	133.1 ± 69.1[a]	83.5 ± 24.1[a,b]
Alanine aminotransferase (IU/L)	23.4 ± 2.9	20.0 ± 3.4	112.9 ± 77.0[a]	46.3 ± 15.9[a,b]
Total cholesterol (mg/dL)	97.7 ± 6.6	83.7 ± 10.8	359.7 ± 95.0[a]	306.7 ± 26.5[a]
Triglyceride (mg/dL)	88.9 ± 25.4	71.1 ± 25.1	16.6 ± 4.0[a]	36.2 ± 25.7[a,b]
Free fatty acids (mEQ/L)	1668 ± 257	1334 ± 254[a]	1254 ± 201[a]	1119 ± 310[a]
Glucose (mg/dL)	70 ± 53	73 ± 16	73 ± 19	95 ± 32

Data are presented as the mean ± SD.
[a]$P < 0.05$ significant differences compared to the control group.
[b]$P < 0.05$ significant differences between the non-alcoholic fatty liver disease (NAFLD) group and the NAFLD + Partially hydrolyzed guar gum group.
Non-alcoholic fatty liver disease group was induced by high fat diet with increased intestinal permeability. NAFLD: Non-alcoholic fatty liver disease; PHGG: Partially hydrolyzed guar gum.

Table 4 Partially hydrolyzed guar gum-mediated profiles of short-chain fatty acids in cecal contents

Short chain fatty acids (mmol/g)	Control	Control + PHGG	NAFLD	NAFLD + PHGG
Succinic acid	0.82 ± 0.34	0.85 ± 0.36	5.08 ± 2.27[a]	5.28 ± 2.20[a]
Lactic acid	0.05 ± 0.08	0.09 ± 0.13	0.40 ± 0.46[a]	0.45 ± 0.39[a]
Formic acid	0.89 ± 0.09	1.90 ± 0.06[a]	0.72 ± 0.16[a]	0.83 ± 0.28[b]
Acetic acid	41.2 ± 6.0	47.9 ± 8.8[a]	32.8 ± 5.3[a]	33.1 ± 7.9[a]
Propionic acid	8.18 ± 1.14	9.16 ± 1.04[a]	9.76 ± 2.56[a]	9.82 ± 3.39[a]
Isobutyric acid	1.19 ± 0.11	1.04 ± 0.11	1.44 ± 0.20[a]	1.06 ± 0.28[b]
Butyric acid	8.86 ± 2.42	13.81 ± 5.14[a]	3.91 ± 0.58[a]	3.34 ± 1.09[a]
Isovaleric acid	0.33 ± 0.14	0.12 ± 0.11[a]	1.05 ± 0.40[a]	0.38 ± 0.40[b]
Valeric acid	0.49 ± 0.18	0.48 ± 0.14	0.50 ± 0.22	0.18 ± 0.17[a,b]

Data are presented as the mean ± SD.
[a]$P < 0.05$ significant differences compared to the control group.
[b]$P < 0.05$ significant differences between the non-alcoholic fatty liver disease (NAFLD) group and the NAFLD + Partially hydrolyzed guar gum group.
PHGG: Partially hydrolyzed guar gum; NAFLD: Non-alcoholic fatty liver disease.

formic acid, propionic acid, and butyric acid contents. In contrast, isovaleric acid content was significantly decreased after PHGG treatment. The Ath diet together with increased intestinal permeability significantly elevated succinic acid, lactic acid, propionic acid, isobutyric acid, and isovaleric acid contents and significantly reduced formic acid, acetic acid, and butyric acid contents. In the NAFLD model, PHGG treatment significantly upregulated formic acid levels and significantly downregulated isobutyric acid, isovaleric acid, and valeric acid contents.

To determine whether treatment with PHGG altered the cecal bacteria profiles, we examined the variation in eight representative bacterial groups using T-RFLP analysis. Chronic administration of 0.5% DSS caused a dramatic decrease in the number of cecal bacteria, and therefore, cecal bacterial profiles were only analyzed in the control and control + PHGG groups. As shown in Figure 6, the administration of PHGG altered the cecal bacteria profiles (Figure 6A) and significantly increased the cecal *Bacteroides* (Figure 6B) and *Clostridium* subcluster XIVa (Figure 6C) compared with that in the control mice. Conversely, PHGG treatment significantly reduced the cecal *Prevotella*, *Bifidobacterium* (Figure 6D), and *Clostridium* cluster XVIII (Figure 6E).

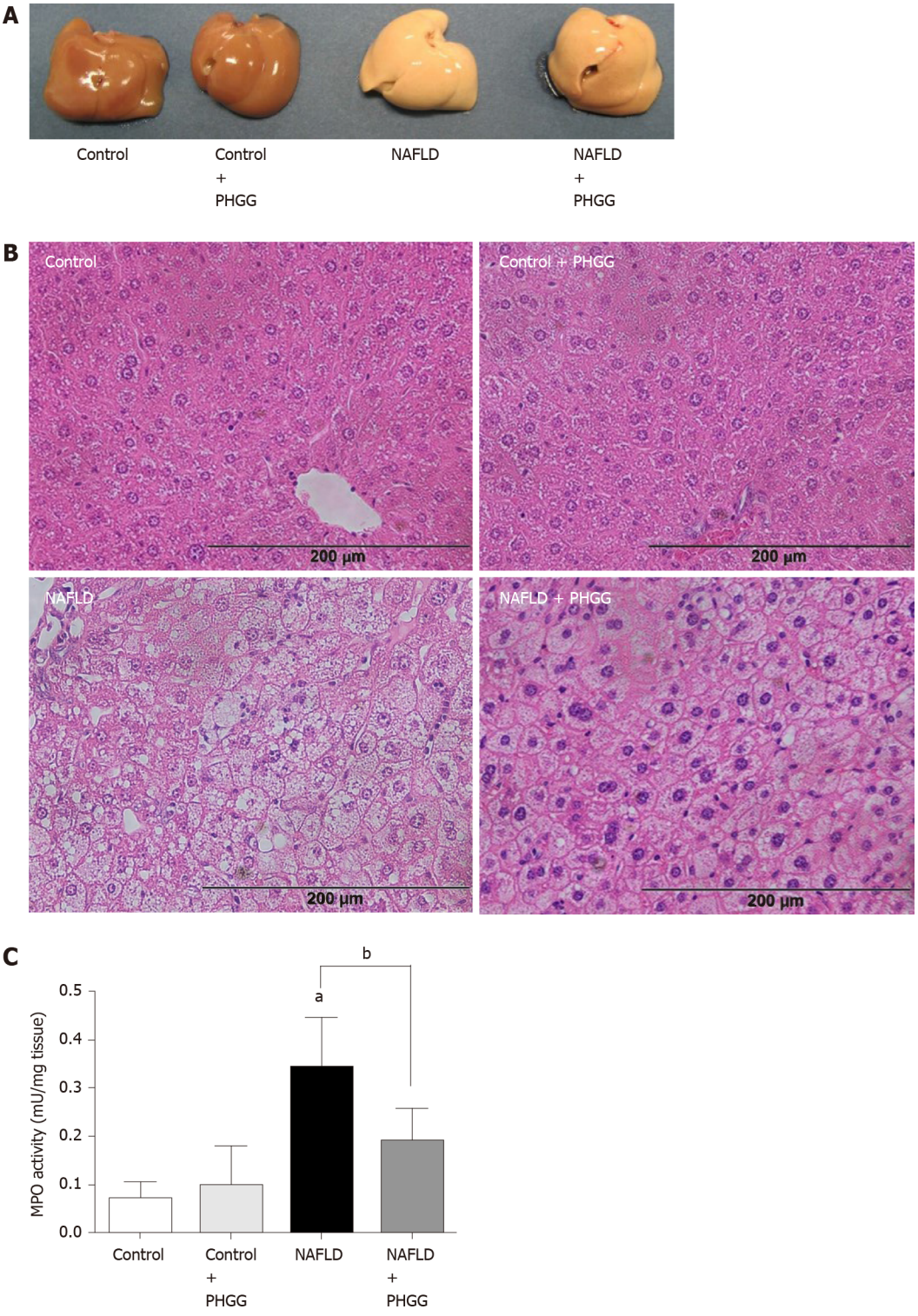


Figure 1 Effects of partially hydrolyzed guar gum on the non-alcoholic fatty liver disease model. A: Representative macroscopic findings in the liver. Non-alcoholic fatty liver disease was induced in mice subjected to the atherogenic diet with increased intestinal permeability for 8 wk; B: Representative histological findings in the liver. Magnification, × 100. Hematoxylin and eosin staining; C: Myeloperoxidase (MPO) activity. The liver was homogenized and MPO activity was determined as an index of neutrophil accumulation in the liver. Data represent the mean ± SD of seven mice. [a]$P < 0.05$ significant differences compared to the control group; [b]$P < 0.05$ significant differences between the 2 selected groups. NAFLD: Non-alcoholic fatty liver disease; PHGG: Partially hydrolyzed guar gum; MPO: Myeloperoxidase.

DISCUSSION

Our results clearly indicate that increased intestinal permeability in combination with the administration of Ath diet resulted in NAFLD development through the gut-liver axis and that treatment with PHGG significantly attenuated NAFLD development in mice. The protective effects of PHGG might result in part from the modulation of

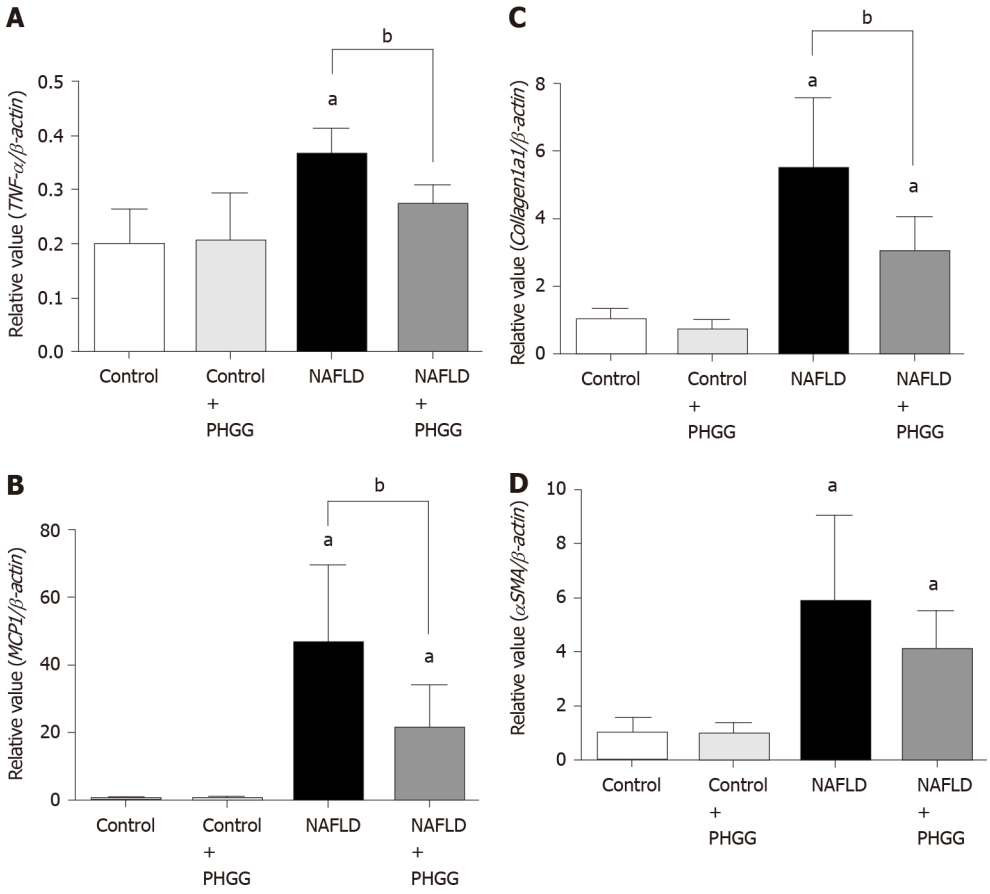


Figure 2 Effects of partially hydrolyzed guar gum on hepatic inflammation and fibrosis in the non-alcoholic fatty liver disease model. A: *Tumor necrosis factor-α*; B: *Monocyte chemotactic protein-1*; C: *Collagen 1a1*; D: *α smooth muscle actin* mRNA expression in the liver. mRNA expression was evaluated using real-time polymerase chain reaction. Data represent the mean ± SD of seven mice. [a]*P* < 0.05 significant differences compared to the control group; [b] *P* < 0.05 significant differences between the 2 selected groups. NAFLD: Non-alcoholic fatty liver disease; PHGG: Partially hydrolyzed guar gum; *TNF-α*: *Tumor necrosis factor-α*; *MCP-1*: *Monocyte chemotactic protein-1*; *αSMA*: *α smooth muscle actin*.

microbiota composition and SCFA profiles, leading to attenuation of the increased intestinal permeability. A variety of animal models for NAFLD and NASH, including models induced by high-fat, high-fructose, high-cholesterol, high-sucrose, Ath, choline- and L-amino acid-deficient, and methionine- and choline-deficient diets, have been used to investigate the pathophysiology of NAFLD and NASH, indicating the important role of diet in these clinical states. However, because of the complex, multidirectional pathophysiology involved in NAFLD, the perfect animal model representing the complete NAFLD spectrum in a workable time frame does not appear to exist[38]. Moreover, due to the substantial physiological differences between rodents and humans, animal models should be used with caution.

Accumulating evidence indicates the significance of alterations in intestinal permeability in NAFLD and NASH pathogenesis. An increase in intestinal epithelial cell permeability is reported to allow the translocation of microbial products into the portal vein, subsequently inducing hepatic inflammation[14,39]. Luther *et al*[31] demonstrated that an early phase of hepatic injury and inflammation contributes to altered intestinal permeability[31]. Furthermore, Mouries *et al*[40] demonstrated that disruption of the gut vascular barrier is an early event in NASH pathogenesis and is a consequence of diet-induced dysbiosis[40]. These data suggest that communication between the gut and liver plays an important role in NAFLD development. In this context, the murine NAFLD model described in the present study appears to be valuable for investigating the pathophysiology of this disease. Our study also clearly shows that increased intestinal permeability combined with the Ath diet resulted in increased liver weight, AST/ALT, total cholesterol levels, and lipid accumulation in the liver, as well as the upregulation of pro-inflammatory cytokines/chemokines such as TNF-α and MCP-1, neutrophil accumulation, lipid synthesis, and hepatic fibrogenesis. These results are consistent with those of a previous study[14]. Several cytokines, such as interleukin (IL)-6, IL-1b, and TNF-α, have been recently suggested to be involved in the so-called chronic low-grade inflammation or meta inflammation

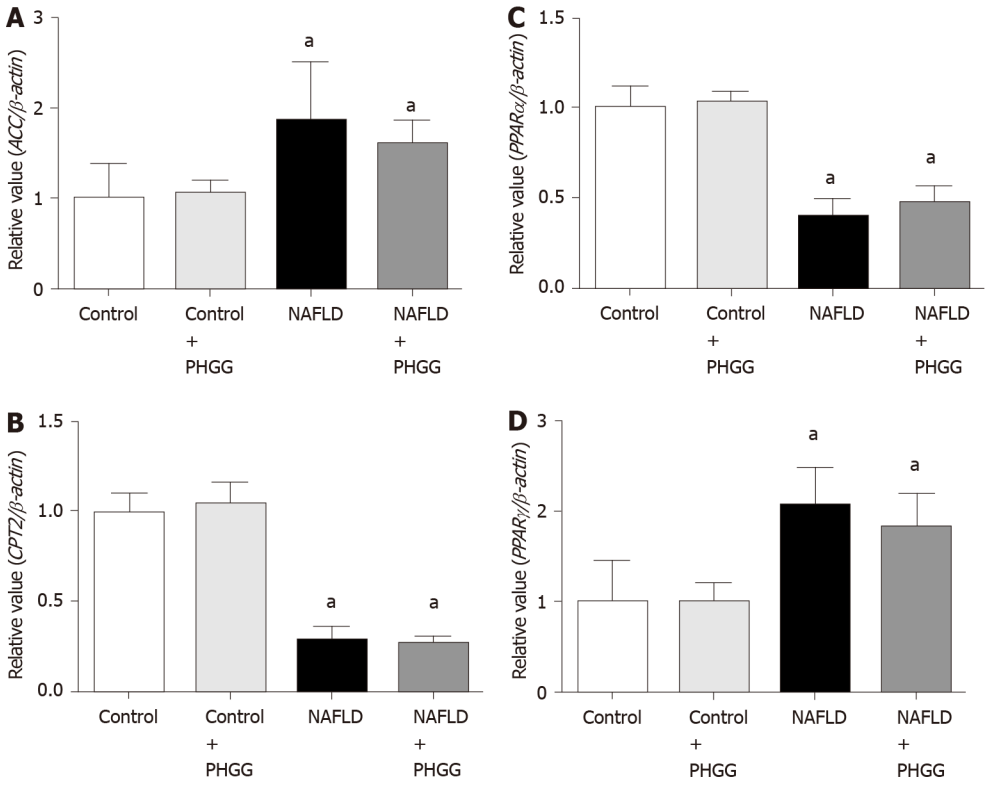


Figure 3 Effects of partially hydrolyzed guar gum on lipid synthesis and degradation in the non-alcoholic fatty liver disease model. A: *Acetyl-CoA carboxylase*; B: *Carnitine O-palmitoyltransferase II*; C: *Peroxisome proliferator-activated receptor* (*PPAR*) α; D: *PPAR*γ mRNA expression in the liver. mRNA expression was evaluated using real-time polymerase chain reaction. Data represent the mean ± SD of seven mice. $^{a}P < 0.05$ significant differences compared to the control group. NAFLD: Non-alcoholic fatty liver disease; PHGG: Partially hydrolyzed guar gum; *ACC: Acetyl-CoA carboxylase*; *PPAR*: *Peroxisome proliferator-activated receptor*; *CPT2*: *Carnitine O-palmitoyltransferase II*.

of NAFLD/NASH[41,42]. Our study further demonstrates that increased intestinal permeability in combination with the Ath diet resulted in elevated portal endotoxin levels and activated the expression of TLR4 and TLR9, confirming that intestinal permeability was significantly elevated in this NAFLD model. Indeed, the FD4 flux, a marker of lumen-to-serum permeability, was significantly increased in our NAFLD model. These findings are consistent with those of a recent review[4,15,43]. Interestingly, our study also shows that treatment with PHGG significantly attenuated the development of NAFLD by decreasing TLR signaling and its downstream molecules, such as pro-inflammatory cytokines, despite continued lipid synthesis and degradation, suggesting that treatment with PHGG might have therapeutic effects on NAFLD through the gut-liver axis. Another study has also shown that a prebiotic, fermentable, dietary fructo-oligosaccharide alleviates hepatic steatosis[44]. However, in this study, the protection appeared to be mediated by reduced fatty acid oxidation and cholesterol accumulation. Thus, the protective mechanisms by which various prebiotics modulate NAFLD remain to be fully elucidated.

Accumulating evidence suggests a relationship between the intestinal microbiota and NAFLD, and modification of the composition of the former could play a role in the development and progression of the latter[45]. Le Roy *et al*[46] have shown that in mice fed the Ath diet, the gut microbiota composition alters lipid metabolism in the liver[46]. De Minicis *et al*[47] also demonstrated that the Ath diet model subjected to bile duct ligation shows an increase in the abundance of gram-negative bacteria, a reduced Bacteroidetes/Firmicutes ratio, the complete disappearance of *Bifido-bacteriaceae*, and increased abundance of gram-negative *Proteobacteria* (especially *Enterobacteriaceae*)[47]. In our experiments, we quantified the *Clostridium* subcluster XIVa, *Clostridium* cluster IV, and *Bacteroides* as the SCFA-producing indigenous bacteria, *Prevotella* as resident microbiota, *Bifidobacterium* as a beneficial bacterium, Lactobacillales as the lactate-producing bacteria, *Clostridium* cluster XI as the harmful bacteria, including *Clostridium difficile*, and the *Clostridium* cluster XVIII as pathogenic bacteria such as *Escherichia coli* in regard to the cecal bacteria profiles. Additionally, the compositional alterations in intestinal bacteria might also change their metabolic capacity. Colonic fermentation of carbohydrates results in the generation of SCFAs.

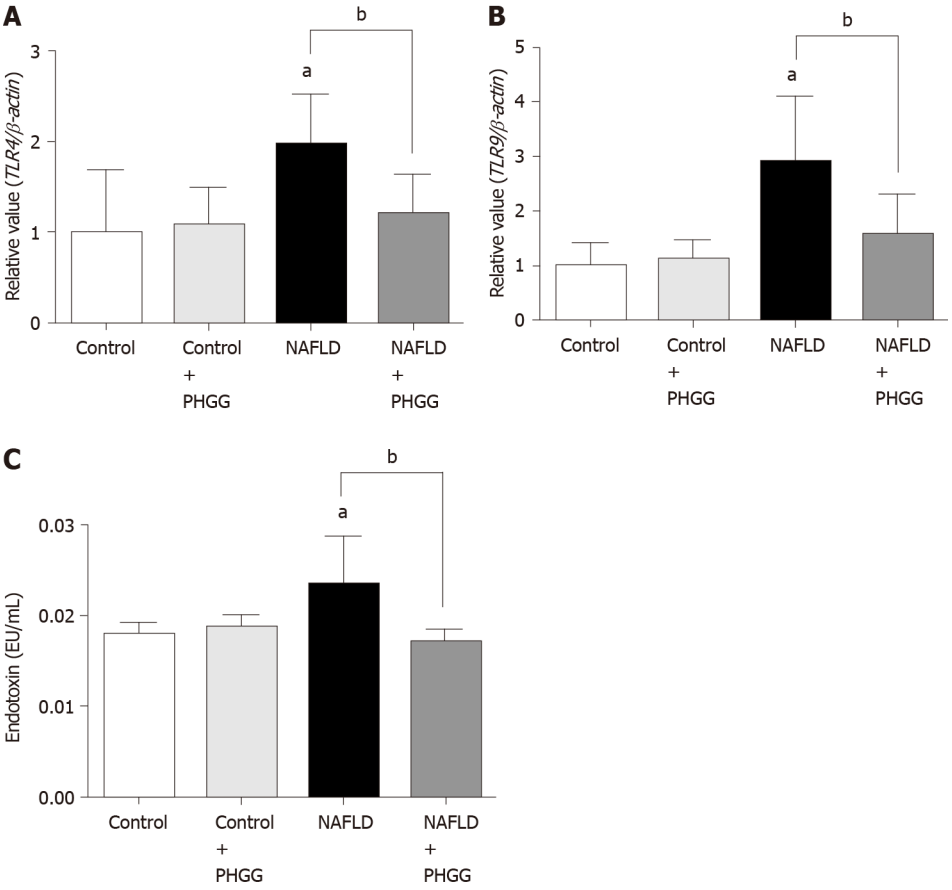


Figure 4 Effects of partially hydrolyzed guar gum on toll-like receptor signaling through the gut-liver axis. A: *Toll-like receptor* (*TLR*) *4* mRNA expression; B: *TLR9* mRNA expression in the liver. mRNA expression was evaluated using real-time polymerase chain reaction; C: Endotoxin levels in the portal vein. Endotoxin levels were measured using the chromogenic Limulus Amebocyte Lysate Endotoxin Assay kit. Data represent the mean ± SD of seven mice. $^{a}P < 0.05$ significant differences compared to the control group; $^{b}P < 0.05$ significant differences between the 2 selected groups. NAFLD: Non-alcoholic fatty liver disease; PHGG: Partially hydrolyzed guar gum; *TLR*: *Toll-like receptor*.

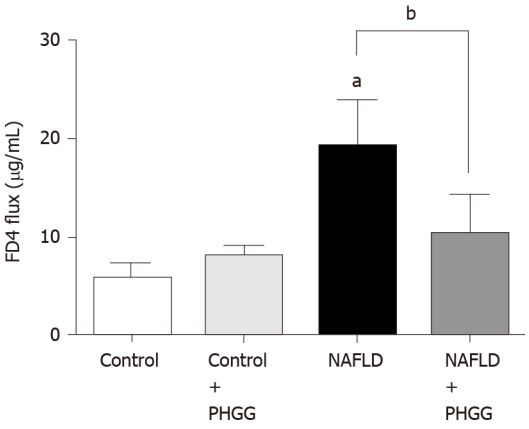


Figure 5 Effects of partially hydrolyzed guar gum on intestinal permeability in the non-alcoholic fatty liver disease model. Lumen-to-blood clearance of fluorescein isothiocyanate-conjugated dextran was evaluated by injection into the duodenum and collection of blood after 60 min. Fluorescence intensity in the serum was measured with a spectrofluorometer. Data represent the mean ± SD of seven mice. $^{a}P < 0.05$ significant differences compared to the control group; $^{b}P < 0.05$ significant differences between the 2 selected groups. NAFLD: Non-alcoholic fatty liver disease; PHGG: Partially hydrolyzed guar gum.

Recent evidence suggests that supplementation with sodium butyrate protects mice from NASH development[48]. Moreover, a recent review indicated the beneficial effects of commensal bacteria on the intestinal epithelial barrier and the importance of butyrate-producing bacteria for intestinal health[49]. A recent study also showed that SCFAs (acetate, propionate, and butyrate) stimulate and protect the intestinal barrier function[50].

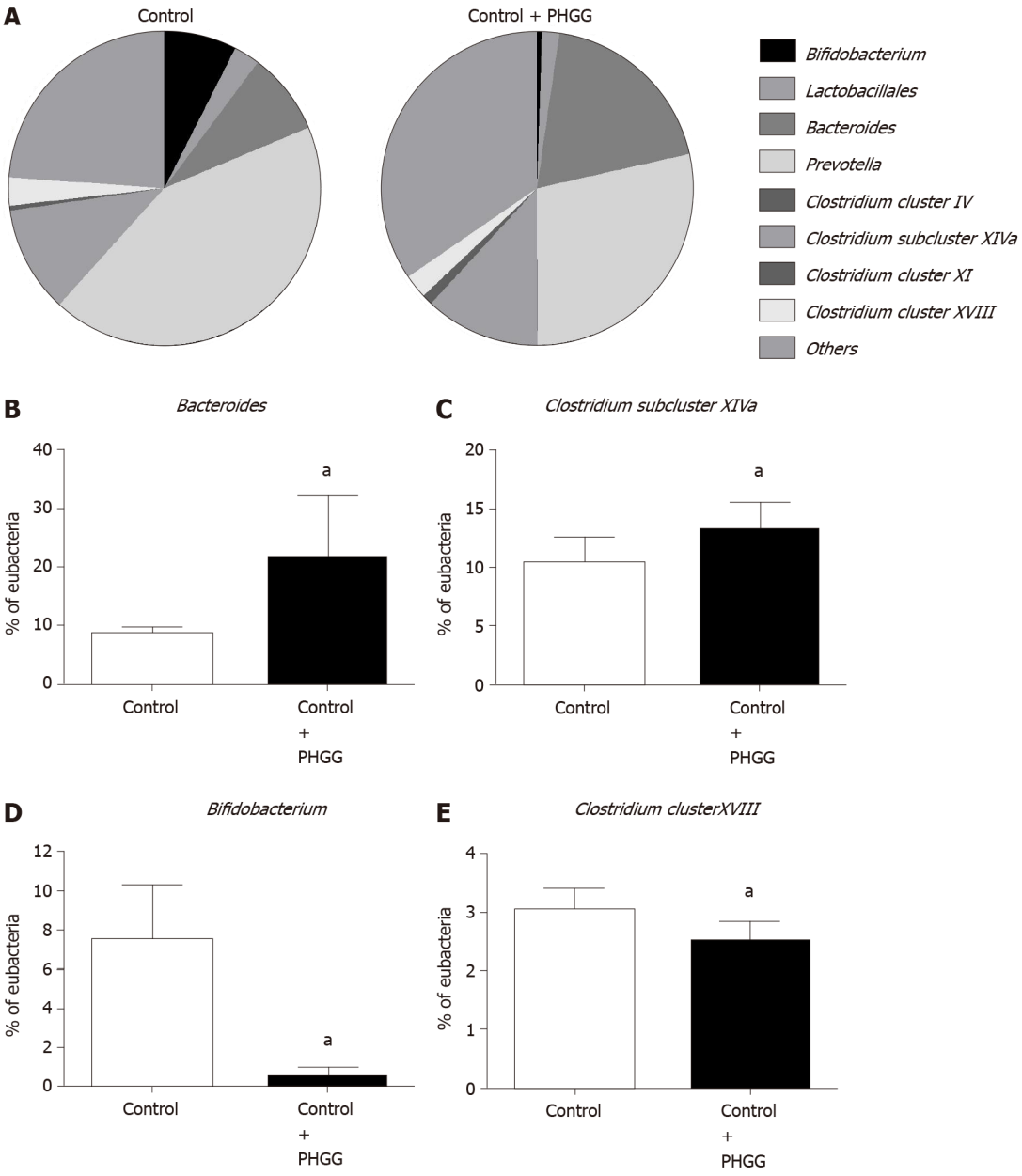


Figure 6 Effects of partially hydrolyzed guar gum on cecal microbiota after administration. A: Percentage of the total identified sequences per group. Microbiota profiles in the cecal contents were analyzed using terminal restriction fragment-length polymorphism; B–E: Changes in the microbiota composition. Bars represent the mean ± SD of seven mice. $^{a}P < 0.05$ significant differences compared to the control group. PHGG: Partially hydrolyzed guar gum.

In our study, treatment with PHGG significantly increased the abundance of cecal *Bacteroides* and *Clostridium* subcluster XIVa. Our results are partially in agreement with previous human experiments showing that PHGG increases the abundance of butyrate-producing bacteria, including *Clostridium* subcluster XIVa, in the intestine[27]. Moreover, treatment with PHGG resulted in a significant increase in SCFA levels, particularly butyric acid, acetic acid, propionic acid, and formic acid, in the cecal content. These results are consistent with those of a previous study[27]. PHGG-mediated increases in SCFA levels might exert protective effects on the intestinal barrier function. These observations are supported by a recent study[50], although it lacked direct data.

Our study has the following limitations: (1) the dramatic changes in microbiota and SCFA profiles in the current NAFLD model resulting from the adverse effects of DSS administration; (2) the method of analysis of microbiota, T-RFLP analysis; (3) the lack of determination of IL-6 and IL-1b (other markers of low-grade inflammation of NAFLD/NASH); and (4) the modest sample size of each group associated with mean differences and SD in some experiments. Further investigations are therefore required.

CONCLUSION

In summary, our data indicate that PHGG inhibits NAFLD development in mice, which is associated with decreased lipid accumulation, pro-inflammatory cytokine/chemokine production, and neutrophil infiltration in the liver. These findings might be related to the gut-liver axis, through the protection of intestinal barrier integrity, as well as cecal bacteria and SCFA profile alterations. Particularly, treatment with PHGG promotes an SCFA-producing bacterial group of *Clostridium* subcluster XIV and *Bacteroides*. Further studies are warranted to elucidate the detailed mechanisms involved in this phenomenon; however, treatment with PHGG in humans could show great promise as a therapeutic strategy for NAFLD.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) is caused by a variety of pathogenesis, and may involve the gut-liver axis, which has attracted much attention. Prebiotics such as dietary fibers were shown to attenuate NAFLD by modulating gut microbiota. Partially hydrolyzed guar gum (PHGG), a water-soluble dietary fiber, is associated with alteration of gut microbiota and the production of short-chain fatty acids (SCFAs), and has been reported to alleviate the symptoms of various intestinal diseases and metabolic syndrome.

Research motivation

PHGG should exert beneficial health effects on the host through alterations to the gut microbiota and SCFA production. However, its effects on NAFLD remain to be fully elucidated.

Research objectives

The present study aimed to determine whether treatment with PHGG attenuates NAFLD development in mice through the gut-liver axis.

Research methods

Male C57BL/6J mice with increased intestinal permeability by chronic intermittent administration of low-dose dextran sulfate sodium were fed a control or atherogenic (Ath) diet (a mouse model of NAFLD) for 8 wk, with or without 5% PHGG. Body weight, liver weight, macroscopic findings in the liver, blood biochemistry, liver histology, myeloperoxidase activity in liver tissue, mRNA expression in the liver and intestine, serum endotoxin levels in the portal vein, intestinal permeability, and microbiota and SCFA profiles in the cecal samples were investigated.

Research results

Mice subjected to a Ath diet with increased intestinal permeability showed significantly increased serum aspartate aminotransferase and alanine aminotransferase levels, liver fat accumulation, liver inflammatory (tumor necrosis factor-α and monocyte chemotactic protein-1) and fibrogenic (collagen 1a1 and α smooth muscle actin) marker levels, and liver myeloperoxidase activity, which were significantly attenuated by PHGG treatment. Moreover, increased intestinal permeability in combination with the Ath diet resulted in increased portal endotoxin levels and activated toll-like receptor (TLR) 4 and TLR9 expression. PHGG administration did not affect fatty acid metabolism in the liver, but decreased lipopolysaccharide signaling through the gut-liver axis. The administration of PHGG altered the cecal bacteria profiles and significantly increased the cecal Bacteroides and Clostridium subcluster XIVa. Treatment with PHGG markedly increased the levels of SCFAs, particularly, butyric acid, acetic acid, propionic acid, and formic acid, in the cecal samples.

Research conclusions

Treatment with PHGG attenuated NAFLD development in mice through the gut-liver axis by modulating microbiota and downstream SCFA profiles.

Research perspectives

By indicating that PHGG administration inhibits NAFLD development through the

gut-liver axis, the present study showed a possible treatment strategy of NAFLD in humans.

REFERENCES

1 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]

2 **Chalasani N**, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ; American Gastroenterological Association; American Association for the Study of Liver Diseases; American College of Gastroenterologyh. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. *Gastroenterology* 2012; **142**: 1592-1609 [PMID: 22656328 DOI: 10.1053/j.gastro.2012.04.001]

3 **Boursier J**, Mueller O, Barret M, Machado M, Fizanne L, Araujo-Perez F, Guy CD, Seed PC, Rawls JF, David LA, Hunault G, Oberti F, Calès P, Diehl AM. The severity of nonalcoholic fatty liver disease is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota. *Hepatology* 2016; **63**: 764-775 [PMID: 26600078 DOI: 10.1002/hep.28356]

4 **Hu H**, Lin A, Kong M, Yao X, Yin M, Xia H, Ma J, Liu H. Intestinal microbiome and NAFLD: molecular insights and therapeutic perspectives. *J Gastroenterol* 2020; **55**: 142-158 [PMID: 31845054 DOI: 10.1007/s00535-019-01649-8]

5 **Lavine JE**, Schwimmer JB, Van Natta ML, Molleston JP, Murray KF, Rosenthal P, Abrams SH, Scheimann AO, Sanyal AJ, Chalasani N, Tonascia J, Ünalp A, Clark JM, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR; Nonalcoholic Steatohepatitis Clinical Research Network. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. *JAMA* 2011; **305**: 1659-1668 [PMID: 21521847 DOI: 10.1001/jama.2011.520]

6 **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR; NASH CRN. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]

7 **Neuschwander-Tetri BA**, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarathy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, Doo E; NASH Clinical Research Network. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* 2015; **385**: 956-965 [PMID: 25468160 DOI: 10.1016/S0140-6736(14)61933-4]

8 **Tarantino G**, Citro V, Capone D. Nonalcoholic Fatty Liver Disease: A Challenge from Mechanisms to Therapy. *J Clin Med* 2019; **9** [PMID: 31861591 DOI: 10.3390/jcm9010015]

9 **Zeuzem S**. Gut-liver axis. *Int J Colorectal Dis* 2000; **15**: 59-82 [PMID: 10855547]

10 **Cani PD**, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmée E, Cousin B, Sulpice T, Chamontin B, Ferrières J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007; **56**: 1761-1772 [PMID: 17456850 DOI: 10.2337/db06-1491]

11 **Miele L**, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Mascianà R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 1877-1887 [PMID: 19291785 DOI: 10.1002/hep.22848]

12 **Csak T**, Velayudham A, Hritz I, Petrasek J, Levin I, Lippai D, Catalano D, Mandrekar P, Dolganiuc A, Kurt-Jones E, Szabo G. Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G433-G441 [PMID: 21233280 DOI: 10.1152/ajpgi.00163.2009]

13 **Seki E**, Schnabl B. Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut. *J Physiol* 2012; **590**: 447-458 [PMID: 22124143 DOI: 10.1113/jphysiol.2011.219691]

14 **Gäbele E**, Dostert K, Hofmann C, Wiest R, Schölmerich J, Hellerbrand C, Obermeier F. DSS induced colitis increases portal LPS levels and enhances hepatic inflammation and fibrogenesis in experimental NASH. *J Hepatol* 2011; **55**: 1391-1399 [PMID: 21703208 DOI: 10.1016/j.jhep.2011.02.035]

15 **Safari Z**, Gérard P. The links between the gut microbiome and non-alcoholic fatty liver disease (NAFLD). *Cell Mol Life Sci* 2019; **76**: 1541-1558 [PMID: 30683985 DOI: 10.1007/s00018-019-03011-w]

16 **Aller R**, De Luis DA, Izaola O, Conde R, Gonzalez Sagrado M, Primo D, De La Fuente B, Gonzalez J. Effect of a probiotic on liver aminotransferases in nonalcoholic fatty liver disease patients: a double blind randomized clinical trial. *Eur Rev Med Pharmacol Sci* 2011; **15**: 1090-1095 [PMID: 22013734]

17 **Malaguarnera M**, Vacante M, Antic T, Giordano M, Chisari G, Acquaviva R, Mastrojeni S, Malaguarnera G, Mistretta A, Li Volti G, Galvano F. Bifidobacterium longum with fructo-oligosaccharides in patients with non alcoholic steatohepatitis. *Dig Dis Sci* 2012; **57**: 545-553 [PMID: 21901256 DOI: 10.1007/s10620-011-1887-4]

18 **Roberfroid M**, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco MJ, Léotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A. Prebiotic effects: metabolic and health benefits. *Br J Nutr* 2010; **104** Suppl 2: S1-63 [PMID: 20920376 DOI: 10.1017/S0007114510003363]

19 **Parnell JA**, Raman M, Rioux KP, Reimer RA. The potential role of prebiotic fibre for treatment and management of non-alcoholic fatty liver disease and associated obesity and insulin resistance. *Liver Int* 2012; **32**: 701-711 [PMID: 22221818 DOI: 10.1111/j.1478-3231.2011.02730.x]

20 **Yoon SJ**, Chu DC, Raj Juneja L. Chemical and physical properties, safety and application of partially hydrolized guar gum as dietary fiber. *J Clin Biochem Nutr* 2008; **42**: 1-7 [PMID: 18231623 DOI: 10.3164/jcbn.2008001]

21 **Giannini EG**, Mansi C, Dulbecco P, Savarino V. Role of partially hydrolyzed guar gum in the treatment of irritable bowel syndrome. *Nutrition* 2006; **22**: 334-342 [PMID: 16413751 DOI: 10.1016/j.nut.2005.10.003]

22 **Alam NH**, Ashraf H, Sarker SA, Olesen M, Troup J, Salam MA, Gyr N, Meier R. Efficacy of partially hydrolyzed guar gum-added oral rehydration solution in the treatment of severe cholera in adults. *Digestion* 2008; **78**: 24-29 [PMID: 18769066 DOI: 10.1159/000152844]

23 **Furnari M**, Parodi A, Gemignani L, Giannini EG, Marenco S, Savarino E, Assandri L, Fazio V, Bonfanti D, Inferrera S, Savarino V. Clinical trial: the combination of rifaximin with partially hydrolysed guar gum is more effective than rifaximin alone in eradicating small intestinal bacterial overgrowth. *Aliment Pharmacol Ther* 2010; **32**: 1000-1006 [PMID: 20937045 DOI: 10.1111/j.1365-2036.2010.04436.x]

24 **Romano C**, Comito D, Famiani A, Calamarà S, Loddo I. Partially hydrolyzed guar gum in pediatric functional abdominal pain. *World J Gastroenterol* 2013; **19**: 235-240 [PMID: 23345946 DOI: 10.3748/wjg.v19.i2.235]

25 **Dall'Alba V**, Silva FM, Antonio JP, Steemburgo T, Royer CP, Almeida JC, Gross JL, Azevedo MJ. Improvement of the metabolic syndrome profile by soluble fibre - guar gum - in patients with type 2 diabetes: a randomised clinical trial. *Br J Nutr* 2013; **110**: 1601-1610 [PMID: 23551992 DOI: 10.1017/S0007114513001025]

26 **Yasukawa Z**, Naito Y, Takagi T, Mizushima K, Tokunaga M, Ishihara N, R Juneja L, Yoshikawa T. Partially hydrolyzed guar gum affects the expression of genes involved in host defense functions and cholesterol absorption in colonic mucosa of db/db male mice. *J Clin Biochem Nutr* 2012; **51**: 33-38 [PMID: 22798710 DOI: 10.3164/jcbn.11-104]

27 **Ohashi Y**, Sumitani K, Tokunaga M, Ishihara N, Okubo T, Fujisawa T. Consumption of partially hydrolysed guar gum stimulates Bifidobacteria and butyrate-producing bacteria in the human large intestine. *Benef Microbes* 2015; **6**: 451-455 [PMID: 25519526 DOI: 10.3920/BM2014.0118]

28 **Reider SJ**, Moosmang S, Tragust J, Trgovec-Greif L, Tragust S, Perschy L, Przysiecki N, Sturm S, Tilg H, Stuppner H, Rattei T, Moschen AR. Prebiotic Effects of Partially Hydrolyzed Guar Gum on the Composition and Function of the Human Microbiota-Results from the PAGODA Trial. *Nutrients* 2020; **12** [PMID: 32354152 DOI: 10.3390/nu12051257]

29 **Matsuzawa N**, Takamura T, Kurita S, Misu H, Ota T, Ando H, Yokoyama M, Honda M, Zen Y, Nakanuma Y, Miyamoto K, Kaneko S. Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. *Hepatology* 2007; **46**: 1392-1403 [PMID: 17929294 DOI: 10.1002/hep.21874]

30 **Grisham MB**, Hernandez LA, Granger DN. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. *Am J Physiol* 1986; **251**: G567-G574 [PMID: 3020994 DOI: 10.1152/ajpgi.1986.251.4.G567]

31 **Luther J**, Garber JJ, Khalili H, Dave M, Bale SS, Jindal R, Motola DL, Luther S, Bohr S, Jeoung SW, Deshpande V, Singh G, Turner JR, Yarmush ML, Chung RT, Patel SJ. Hepatic Injury in Nonalcoholic Steatohepatitis Contributes to Altered Intestinal Permeability. *Cell Mol Gastroenterol Hepatol* 2015; **1**: 222-232 [PMID: 26405687 DOI: 10.1016/j.jcmgh.2015.01.001]

32 **Nagahama S**, Korenaga D, Honda M, Inutsuka S, Sugimachi K. Assessment of the intestinal permeability after a gastrectomy and the oral administration of anticancer drugs in rats: nitric oxide release in response to gut injury. *Surgery* 2002; **131**: S92-S97 [PMID: 11821793]

33 **Kish L**, Hotte N, Kaplan GG, Vincent R, Tso R, Gänzle M, Rioux KP, Thiesen A, Barkema HW, Wine E, Madsen KL. Environmental particulate matter induces murine intestinal inflammatory responses and alters the gut microbiome. *PLoS One* 2013; **8**: e62220 [PMID: 23638009 DOI: 10.1371/journal.pone.0062220]

34 **Nagashima K**, Hisada T, Sato M, Mochizuki J. Application of new primer-enzyme combinations to terminal restriction fragment length polymorphism profiling of bacterial populations in human feces. *Appl Environ Microbiol* 2003; **69**: 1251-1262 [PMID: 12571054]

35 **Suau A**, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* 1999; **65**: 4799-4807 [PMID: 10543789]

36 **Nagahama K**, Mochizuki J, Suzuki S, Shimomura K. Phylogenetic Analysis of 16S ribosomal RNA gene sequences from human fecal microbiota and improved utility of terminal restriction fragment length polymorphism profiling. *Biosci Microflora* 2006; **25**: 99-107 [DOI: 10.12938/bifidus.25.99]

37 **Ushida K**, Sakata T. Effect of pH on oligosaccharide fermentation by porcine cecal digesta. *Animal Sci Technol* 1998; **69**: 100-107 [DOI: 10.2508/chikusan.69.100]

38 **Van Herck MA**, Vonghia L, Francque SM. Animal Models of Nonalcoholic Fatty Liver Disease-A Starter's Guide. *Nutrients* 2017; **9**: 1072 [PMID: 28953222 DOI: 10.3390/nu9101072]

39 **Henao-Mejia J**, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012; **482**: 179-185 [PMID: 22297845 DOI: 10.1038/nature10809]

40 **Mouries J**, Brescia P, Silvestri A, Spadoni I, Sorribas M, Wiest R, Mileti E, Galbiati M, Invernizzi P, Adorini L, Penna G, Rescigno M. Microbiota-driven gut vascular barrier disruption is a prerequisite for non-alcoholic steatohepatitis development. *J Hepatol* 2019; **71**: 1216-1228 [PMID: 31419514 DOI: 10.1016/j.jhep.2019.08.005]

41 **Pessentheiner AR**, Ducasa GM, Gordts PLSM. Proteoglycans in Obesity-Associated Metabolic Dysfunction and Meta-Inflammation. *Front Immunol* 2020; **11**: 769 [PMID: 32508807 DOI: 10.3389/fimmu.2020.00769]

42 **Power Guerra N**, Müller L, Pilz K, Glatzel A, Jenderny D, Janowitz D, Vollmar B, Kuhla A. Dietary-Induced Low-Grade Inflammation in the Liver. *Biomedicines* 2020; **8** [PMID: 33317065 DOI: 10.3390/biomedicines8120587]

43 **Carotti S**, Guarino MP, Vespasiani-Gentilucci U, Morini S. Starring role of toll-like receptor-4 activation in the gut-liver axis. *World J Gastrointest Pathophysiol* 2015; **6**: 99-109 [PMID: 26600967 DOI: 10.4291/wjgp.v6.i4.99]

44 **Pachikian BD**, Essaghir A, Demoulin JB, Catry E, Neyrinck AM, Dewulf EM, Sohet FM, Portois L, Clerbaux LA, Carpentier YA, Possemiers S, Bommer GT, Cani PD, Delzenne NM. Prebiotic approach alleviates hepatic steatosis: implication of fatty acid oxidative and cholesterol synthesis pathways. *Mol Nutr Food Res* 2013; **57**: 347-359 [PMID: 23203768 DOI: 10.1002/mnfr.201200364]

45 **Abdou RM**, Zhu L, Baker RD, Baker SS. Gut Microbiota of Nonalcoholic Fatty Liver Disease. *Dig Dis Sci* 2016; **61**: 1268-1281 [PMID: 26898658 DOI: 10.1007/s10620-016-4045-1]

46 **Le Roy T**, Llopis M, Lepage P, Bruneau A, Rabot S, Bevilacqua C, Martin P, Philippe C, Walker F, Bado A, Perlemuter G, Cassard-Doulcier AM, Gérard P. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. *Gut* 2013; **62**: 1787-1794 [PMID: 23197411 DOI: 10.1136/gutjnl-2012-303816]

47 **De Minicis S**, Rychlicki C, Agostinelli L, Saccomanno S, Candelaresi C, Trozzi L, Mingarelli E, Facinelli B, Magi G, Palmieri C, Marzioni M, Benedetti A, Svegliati-Baroni G. Dysbiosis contributes to fibrogenesis in the course of chronic liver injury in mice. *Hepatology* 2014; **59**: 1738-1749 [PMID: 23959503 DOI: 10.1002/hep.26695]

48 **Jin CJ**, Sellmann C, Engstler AJ, Ziegenhardt D, Bergheim I. Supplementation of sodium butyrate protects mice from the development of non-alcoholic steatohepatitis (NASH). *Br J Nutr* 2015; **114**: 1745-1755 [PMID: 26450277 DOI: 10.1017/S0007114515003621]

49 **Hiippala K**, Jouhten H, Ronkainen A, Hartikainen A, Kainulainen V, Jalanka J, Satokari R. The Potential of Gut Commensals in Reinforcing Intestinal Barrier Function and Alleviating Inflammation. *Nutrients* 2018; **10** [PMID: 30060606 DOI: 10.3390/nu10080988]

50 **Feng Y**, Wang Y, Wang P, Huang Y, Wang F. Short-Chain Fatty Acids Manifest Stimulative and Protective Effects on Intestinal Barrier Function Through the Inhibition of NLRP3 Inflammasome and Autophagy. *Cell Physiol Biochem* 2018; **49**: 190-205 [PMID: 30138914 DOI: 10.1159/000492853]

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ORIGINAL ARTICLE

Retrospective Cohort Study

Factors influencing the failure of interferon-free therapy for chronic hepatitis C: Data from the Polish EpiTer-2 cohort study

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Abstract

BACKGROUND

The introduction of direct-acting antiviral drugs into clinical practice has revolutionized the treatment of chronic hepatitis C, making it highly effective and safe for patients. However, few researchers have analyzed the factors causing therapy failure in some patients.

AIM

To analyze factors influencing the failure of direct antiviral drugs in the large, multicenter EpiTer-2 cohort in a real-world setting.

METHODS

The study cohort consisted of patients with chronic hepatitis C treated at 22 Polish centers from 2016-2020. Data collected from the online EpiTer-2 database included the following: hepatitis C virus (HCV) genotype, stage of fibrosis, hematology and liver function parameters, Child-Turcotte-Pugh and Model for End-stage Liver Disease scores, prior antiviral therapy, concomitant diseases, and drugs used in relation to hepatitis B virus (HBV) and/or human immunodeficiency virus (HIV) coinfections. Adverse events observed during the treatment and follow-up period were reported. Both standard and machine learning methods were used for statistical analysis.

Conflict-of-interest statement: Janczewska E has acted as a speaker and/or advisor for AbbVie, Gilead, MSD, Ipsen, and has received funding for clinical trials from AbbVie, Allergan, BMS, Celgene, Cymabay, Dr Falk Pharma, Exelixis, GSK, and MSD. Jakub Klapaczyński has acted as a speaker for Gilead and AbbVie. Mazur W has acted as a speaker and/or advisor for AbbVie, Gilead, Merck, and has received funding for clinical trials from AbbVie, Gilead, and Janssen. Zarębska-Michaluk D has acted as a speaker for AbbVie and Gilead. Dybowska D has received funding for participation in the conference: from AbbVie. Czauż-Andrzejuk A has received funding for clinical trials from AbbVie and Merck. Berak H has acted as a speaker and/or advisor for Gilead, Abbvie, and MSD. Krygier R has acted as a consultant for AbbVie and Gilead. Jaroszewicz J has acted as a speaker and/or advisor for AbbVie, Gilead, Merck, Roche, Alfasigma, MSD, Gilead and PRO.MED. CS. Piekarska A has acted as a speaker and/or advisor for AbbVie, Gilead, Merck, and Roche. Socha Ł has acted as a consultant for BMS. Brygida Adamek has acted as a speaker for AbbVie, Gilead, and MSD. Tomasiewicz K has acted as a speaker and/or advisor AbbVie, Alfa Wasserman, BMS, Gilead, Janssen, Merck, Roche, and has received funding for clinical trials from AbbVie, BMS, Gilead, Janssen, Merck, and Roche. Simon K has acted as a speaker and/or advisor: AbbVie, Gilead, Merck, Alfa-Wassermann, Novartis, Lilly, Bayer, and has received funding for clinical trials from: AbbVie, Allergan, Bayer, EISAI, Gilead, Intercept, Pfizer. Pawłowska M has acted as a speaker and/or advisor for AbbVie, Gilead, Merck, Roche and has received funding for clinical trials from AbbVie, Gilead, and Roche. Halota W has acted as a speaker and/or advisor for AbbVie, BMS, Gilead, Janssen, Merck, Roche, and has received funding for clinical trials from AbbVie, Gilead, and Roche. Flisiak

RESULTS

During analysis, 12614 patients with chronic hepatitis C were registered, of which 11938 (mean age: 52 years) had available sustained virologic response (SVR) data [11629 (97%) achieved SVR and 309 (3%) did not]. Most patients (78.1%) were infected with HCV genotype 1b. Liver cirrhosis was diagnosed in 2974 patients, while advanced fibrosis (F3) was diagnosed in 1717 patients. We included patients with features of hepatic failure at baseline [ascites in 142 (1.2%) and encephalopathy in 68 (0.6%) patients]. The most important host factors negatively influencing treatment efficacy were liver cirrhosis, clinical and laboratory features of liver failure, history of hepatocellular carcinoma, and higher body mass index. Among viral factors, genotype 3 and viral load also exerted an influence on treatment efficacy. Classical statistical analysis revealed that treatment ineffectiveness seemed to be influenced by the male sex, which was not confirmed by the multivariate analysis using the machine learning algorithm (random forest). Coinfection with HBV (including patients with on-treatment reactivation of HBV infection) or HIV, extrahepatic manifestations, and renal failure did not significantly affect the treatment efficacy.

CONCLUSION

In patients with advanced liver disease, individualized therapy (testing for resistance-associated variants and response-guided treatment) should be considered to maximize the chance of achieving SVR.

Key Words: Advanced liver disease; Chronic hepatitis C; Direct-acting antiviral drugs; Sustained virologic response; Interferon-free therapy; Antiviral therapy

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Core Tip: We analyzed factors influencing the failure of direct-acting antiviral drugs (DAAs) in a large, multicenter EpiTer-2 cohort of patients treated across 22 centers. Our findings demonstrate that failure of DAA treatment occurred mainly in patients with liver cirrhosis and deterioration of liver function. Our machine learning analysis further revealed that older age and creatinine and hemoglobin levels also influenced treatment failure, as did viral factors such as genotype 3 and viral load. Thus, in patients with advanced liver disease, individualized therapy (testing for resistance-associated variants, response-guided treatment) should be considered to maximize the chance of achieving a sustained virologic response.

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INTRODUCTION

The introduction of direct-acting antiviral drugs (DAAs) into clinical practice has revolutionized the treatment of chronic hepatitis C, making it highly effective and safe for patients. Interferon-free therapies with a short duration and the absence of significant adverse events (AEs) allow for a sustained virologic response (SVR) in over 90% of patients, both in randomized clinical trials[1-3] and in real-world settings[4,5]. Previous research has demonstrated that pangenotypic therapies significantly increase the effectiveness of treatment in patients infected with hepatitis C virus (HCV) genotype 3, which is considered more difficult to treat[6-9].

R has acted as a speaker and/or advisor, and has received funding for clinical trials from AbbVie, Gilead, Merck, and Roche. Lorenc B, Kołek MF, Tudrujek-Zdunek M, Sitko M, Buczyńska I, Citko J, Dobracka B, Deroń Z, Laurans Ł, Białkowska-Warzecha J and Tronina O have no conflict of interest to declare.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at ejanczewska@sum.edu.pl.

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Numerous studies have confirmed the high efficacy of treatment with DAAs in almost all groups of patients, including those with liver cirrhosis, renal failure, and organ transplants, regardless of age or concomitant diseases, including human immunodeficiency virus (HIV) or hepatitis B virus (HBV) coinfections[10,11].

Most of the publications evaluating interferon-free therapies are devoted to assessing their efficacy and safety. Few researchers, however, have analyzed the factors that cause the therapy to fail in some patients. Therefore, we aimed to analyze this issue in the large, multicenter EpiTer-2 cohort and to present the characteristics of patients in whom DAA treatment has failed in a real-world setting. Knowledge of these factors may aid in determining the qualifying criteria for antiviral therapy and the way it is conducted, which would further minimize the failure rate.

Achieving SVR does not completely exclude the development adverse consequences of chronic hepatitis C, but there are no better methods to prevent the progression of liver fibrosis, development of cirrhosis, liver failure, and hepatocellular carcinoma (HCC) than to eliminate the infection. Thus, all measures should be taken to maximize treatment efficacy.

MATERIALS AND METHODS

Study population

In 2016, on the initiative of investigators, a group of 22 Polish centers began collecting data on the efficacy and safety of drugs used in the treatment of patients with chronic hepatitis C. This study, called EpiTer-2, was supported by the Polish Association of Epidemiologists and Infectologists. The data were collected using a web-based questionnaire, in accordance with the National General Data Protection Regulation.

The therapies were financed by the National Health Fund under general health insurance. The parameters collected in the database were as follows: HCV genotype, stage of fibrosis, hematology and liver function parameters, Child-Turcotte-Pugh and Model for End-stage Liver Disease (MELD) scores, prior antiviral therapy, concomitant diseases and drugs used in relation to them, HBV and/or HIV coinfections.

Hepatic fibrosis was evaluated *via* a liver biopsy based on the METAVIR or Scheuer scoring system, transient elastography using the FibroScan (Echosens, Paris) device, or real-time shear wave elastography) using the Aixplorer (Supersonic, Aix-en-Provence) device.

HCV RNA was monitored prior to and after the treatment (end of treatment virologic response), and then after at least a 12-wk follow-up period (SVR). Two assays were used to measure HCV RNA, depending on local practices at the testing site: Roche COBAS TaqMan with a lower limit of quantification (LLOQ) of 15 IU/mL or Abbott RealTime with an LLOQ of 12 IU/mL.

Peripheral blood counts, liver function, and kidney function were evaluated to assess the safety of the therapy. The basic scope of tests and schedule of patients' visits were defined in the National Health Fund therapeutic program. For the safety of the patient, additional examinations were performed if necessary.

The drug used, the dosage and length of the treatment regimen, and the decision to add ribavirin were determined by treating physicians based on the applicable product characteristics and recommendations of the Polish Group of Experts for HCV[12].

AEs observed during the treatment and follow-up periods were reported as well, with particular attention to events related to liver disease.

Ethical considerations

This observational study was conducted in a real-world setting with approved drugs. Patients were not exposed to any experimental interventions, nor did the study intervene with the clinical management of the patient. The study only collected information from patient records. The analysis included routine examinations and tests performed in patients treated within the therapeutic program of the National Health Fund. The data were originally collected to assess treatment efficacy and safety in individual patients, not for scientific purposes. Hence, the treating physicians did not obtain approval from the ethics committee. According to local law (Pharmaceutical Law of 6th September 2001, art. 37al), non-interventional studies do not require ethics committee approval. Patients provided informed consent for treatment and the processing of personal data. Patient data were collected through an online system, and only physicians caring for patients had access to the patients' personal information. Planning, conduct, and reporting of the study were in line with the tenets outlined in

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the Declaration of Helsinki, as revised in 2013.

Statistical analysis

To identify the best predictors of HCV detectability by at least the 12th week of follow-up, machine learning (ML) techniques were used to develop a statistical model. First, variables with more than 10% of missing observations were removed from the dataset.

Because the dataset was unbalanced for the dependent variable, an oversampling technique was used. The data were split into learning (75%) and testing (25%) sets. Four ML models were built using the following algorithm types: k-nearest neighbor, support vector machine, classification and regression tree, and random forest. The algorithm with the best accuracy was used for further analysis. Then, the selected parameters were optimized to boost the algorithm performance. Twelve variables with the highest predictive value were plotted.

Standard statistical methods were used to compare data between patients positive and negative for HCV at least 12 wk after the end of treatment.

Data are presented as the mean [95% confidence interval (CI)] for continuous variables and as counts (%) for categorical variables. Groups were compared with nonparametric Mann-Whitney *U*-tests and Pearson chi-square tests.

The parametric tests were not used because of the unequal sample size (SVR n = 11629; non-SVR n = 309).

Analysis was performed using the R programming language in RStudio (R Core Team, 2020) and IBM SPSS Statistics 25 (IBM Corp., 2017). The level of statistical significance was set at $P < 0.05$.

RESULTS

Patients' disposition and treatment outcomes are presented in Figure 1. At the time of analysis, a total of 12614 patients with chronic hepatitis C were registered in the database, of which 11938 had available SVR data. Among them, 11629 (97%) achieved SVR, while 309 (3%) did not.

Characteristics of the study group

The baseline characteristics of patients with SVR and their relationships to treatment efficacy are presented in Tables 1 and 2. As there were missing data for some parameters, the number of patients for a given parameter is not always equal to the total number of patients.

The studied population consisted of 5762 men (48.3%) and 6176 women (51.7%), with a mean age of 52 years. Most patients (78.1%) were infected with HCV genotype 1b, which is typical for the population of Polish patients with chronic hepatitis C. Liver cirrhosis was diagnosed in 2974 patients, while advanced fibrosis (F3) was diagnosed in 1717 patients. The study group also included patients with features of hepatic failure at baseline: ascites in 142 (1.2%) patients and encephalopathy in 68 (0.6%) patients. Esophageal varices were diagnosed in 989 (10.5%) patients.

A history of HCC was documented in 179 patients (1.5%), and 146 (1.2%) patients underwent liver transplantation prior to the antiviral treatment. HIV coinfection was diagnosed in 587 (5%) patients, while HBV coinfection was diagnosed in 1570 (13.5%) patients, among whom 124 patients were HBsAg-positive.

Treatment regimens

Treatment regimens and the percentages of patients taking particular medications among patients who achieved or did not achieve SVR are presented in Figure 2. The most commonly used drugs during the study (2016-2020) were paritaprevir/ritonavir/ombitasvir +/- dasabuvir +/- ribavirin (RBV); ledipasvir/sofosbuvir (LDV/SOF) +/- RBV; grazoprevir/elbasvir +/- RBV, glecaprevir/pibrentasvir, and velpatasvir/SOF +/- RBV.

The remaining drugs, asunaprevir plus daclatasvir (ASV+DCV) or SOF + RBV, were used during the initial period of the EpiTer-2 study, and fewer patients were treated with these drugs.

The percentages of patients treated with VEL/SOF +/- RBV, ASV + DCV, and SOF + RBV were higher among patients without SVR than among those who achieved SVR.

The vast majority of patients received complete therapy as planned, although treatment was terminated prematurely in 86 (0.7%) and modified in 275 (2.2%). The modifications were mainly related to changes in the dose of RBV. The proportion of patients not completing full scheduled therapy was higher in the non-SVR group than

Table 1 Baseline characteristics of patients with and without sustained virologic response and their relationship to treatment efficacy

Variable	Total, *n* = 11938	SVR, *n* = 11629	Non-SVR, *n* = 309	*P* value
Sex, *n* (%)				
Male	5762 (48.3)	5553 (47.8)	209 (67.6)	< 0.001
Female	6176 (51.7)	6076 (52.2)	100 (32.4)	
Age	52.24 (51.77-52.70)	52.24 (51.77-52.71)	52.16 (50.74-53.58)	0.869
BMI (kg/m^2)	26.47 (26.29-26.64)	26.45 (26.26-26.62)	27.20 (26.68-27.72)	< 0.001
Fibrosis				
F0	277 (1.9)	225 (99.1)	2 (0.9)	
F1	4539 (38.8)	4469 (98.5)	70 (1.5)	
F2	2249 (19.2)	2204 (98.0)	45 (2.0)	< 0.001
F3	1717 (14.7)	1684 (98.1)	33 (1.9)	
F4	2974 (25.4)	2828 (95.1)	146 (4.9)	
Liver stiffness (kPa)	12.72 (12.45-12.99)	12.53 (12.29-12.76)	20.27 (14.78-25.76)	< 0.001
Child Pugh, *n* (%)				
A	11320 (96.9)	11050 (97.1)	270 (89.7)	
B	346 (3.0)	315 (2.8)	31 (10.3)	< 0.001
C	13 (0.1)	13 (0.1)	0 (0.0)	
MELD score	7.81 (7.76-7.85)	7.79 (7.74-7.84)	8.39 (8.11-8.67)	< 0.001
Esophageal varices, *n* (%)				
Yes	989 (10.5)	922 (10.1)	67 (27.1)	< 0.001
No	8413 (89.5)	8233 (89.9)	180 (72.9)	
Ascites at the start of the treatment, *n* (%)				
No	11742 (98.8)	11444 (98.9)	298 (96.4)	
Moderate	136 (1.2)	125 (1.1)	11 (3.6)	< 0.001
Tense	6 (0.0)	6 (0.0)	0 (0.0)	
Encephalopathy at the start of the treatment, *n* (%)				
No	11808 (99.4)	11507 (99.5)	301 (97.8)	
Grade 1-2	67 (0.6)	60 (0.5)	7 (2.2)	0.001
Grade 3-4	1 (0.0)	1 (0.0)	0 (0.0)	
History of hepatocellular carcinoma, *n* (%)				
Yes	179 (1.5)	167 (1.5)	12 (4.1)	< 0.001
No	11515 (98.5)	11231 (98.5)	284 (95.9)	
Extrahepatic manifestations, *n* (%)				
Yes	948 (8.3)	916 (8.2)	32 (10.8)	0.282
No	10513 (91.7)	10248 (91.8)	265 (89.2)	
HIV coinfection, *n* (%)				
Yes	587 (5.0)	564 (4.9)	23 (7.6)	0.098
No	11161 (95.0)	10881 (95.1)	280 (92.4)	
HBV coinfection, *n* (%)				
Yes	1570 (13.5)	1526 (13.4)	44 (14.6)	0.851
No	10098 (86.5)	9840 (86.6)	258 (85.4)	

HBsAg(+), *n* (%))	124 (8.0)	122 (8.1)	2 (4.5)	0.543
Reactivation of HBV infection, *n* (%)				
Yes	11 (0.1)	11 (0.1)	0 (0.0)	0.510
No	10707 (99.9)	10435 (99.9)	272 (100.0)	
History of liver transplantation, *n* (%)				
Yes	146 (1.2)	144 (1.3)	2 (0.7)	0.576
No	11555 (98.8)	11253 (98.7)	302 (99.3)	
Course of treatment, *n* (%)				
As planned	11516 (97.1)	11238 (97.3)	278 (90.2)	
Terminated early	86 (0.7)	71 (0.6)	15 (4.9)	< 0.001
Modified	257 (2.2)	242 (2.1)	15 (4.9)	
Ascites appearing while on treatment, *n* (%)				
Yes	64 (0.5)	57 (0.5)	7 (2.3)	< 0.001
No	11773 (99.5)	11473 (99.5)	300 (97.7)	
Encephalopathy appearing while on treatment, *n* (%)				
Yes	43 (0.4)	35 (0.3)	8 (2.6)	< 0.001
No	11773 (99.6)	11474 (99.7)	299 (97.4)	
Gastrointestinal bleeding while on treatment, *n* (%)				
Yes	16 (0.1)	14 (0.1)	2 (0.7)	0.044
No	11798 (99.9)	11494 (99.9)	304 (99.3)	

SVR: Sustained virologic response; BMI: Body mass index; MELD: Model for End-stage Liver Disease; HBV: Hepatitis B virus; INR: International normalized ratio; PLT: Platelets; ALT: Alanine aminotransferase; eGFR: Estimated glomerular filtration rate; HCV: Hepatitis C virus.

in the SVR group; however, overall, it was not high (4.9% for both terminated and modified treatments).

Factors influencing treatment efficacy assessed using "traditional" statistics

Host factors: In the group of patients who did not achieve SVR, men predominated (67.7%). No age-related differences between the SVR and non-SVR groups were observed in the standard statistical analysis. However, body mass index (BMI) and mean liver stiffness (20.27 *vs* 12.35 kPa) were higher in the non-SVR group than in the SVR group.

DAA therapy was more often ineffective in patients with liver cirrhosis (F4 4.9% *vs* F3 1.9%; F2 2.0%; F1 1.5%; F0 0.9%), and with symptoms of liver failure (ascites, encephalopathy, esophageal varices, or higher Child-Pugh or MELD scores) at baseline. In addition, the occurrence of symptoms of liver failure during therapy decreased the probability of achieving SVR. Significant differences in laboratory markers of liver injury and function [alanine aminotransferase, albumin, bilirubin, international normalized ratio (INR), and platelet count] were also observed between the SVR and non-SVR groups ($P < 0.001$). However, it is worth noting that all 13 patients classified into Child-Pugh class C at baseline achieved SVR.

The percentage of patients with a history of HCC was significantly higher in the non-SVR group than in the SVR group (4.2% *vs* 1.5%, $P < 0.001$). Coinfection with HBV (including patients with on-treatment reactivation of HBV infection) or HIV, extrahepatic manifestations, and renal failure did not significantly affect the efficacy of therapy.

Viral factors: Infection with the HCV genotype 3 was more common in the non-SVR group than in the SVR group (34.6% *vs* 10.5%). Viral load was also higher among those without SVR (6.36 $\log_{10}$ *vs* 6.39 $\log_{10}$).

Table 2 Baseline characteristics of patients with and without sustained virologic response and their relationship to treatment efficacy-laboratory parameters

Variable	Total, *n* = 11938	SVR, *n* = 11629	Non-SVR, *n* = 309	*P* value
Albumin (g/dL)	4.37 (4.2-4.45)	4.38 (4.29-4.46)	4.02 (3.76-4.28)	< 0.001
Bilirubin (mg/dL)	0.80 (0.79-0.81)	0.79 (0.78-0.80)	1.06 (0.98-1.15)	< 0.001
INR	1.10 (1.07-1.12)	1.10 (1.07-1.12)	1.11 (1.08-1.13)	< 0.001
PLT (K/μL)	191.47 (190.07-192.85)	192.62 (191.22-194.02)	147.63 (139.07-156.19)	< 0.001
ALT (U/L)	78.39 (77.19-79.59)	77.92 (76.71-79.13)	96.07 (87.16-104.98)	< 0.001
Creatinine (mg/dL)	0.92 (0.89-0.96)	0.93 (0.89-0.96)	0.81 (0.79-0.83)	0.749
eGFR, *n* (%)				
< 30 mL/min	137 (29.2)	137 (29.7)	0 (0.0)	
> 60 mL/min	160 (34.0)	157 (34.0)	3 (37.5)	0.252
30-60 mL/min	173 (36.8)	168 (36.3)	5 (62.5)	
Hemoglobin (g/dL)	14.40 (14.37-14.43)	14.40 (14.37-14.44)	14.35 (14.15-14.55)	0.812
HCV genotype, *n* (%)				
1A	434 (3.6)	426 (3.7)	8 (2.6)	
1B	9327 (78.1)	9147 (78.7)	180 (58.3)	
2	20 (0.2)	20 (0.2)	0 (0.0)	< 0.001
3	1328 (11.1)	1221 (10.5)	107 (34.6)	
4	575 (4.8)	565 (4.9)	10 (3.2)	
5	1 (0.0)	1 (0.0)	0 (0.0)	
6	2 (0.0)	2 (0.0)	0 (0.0)	
HCV RNA (log_{10})	6.37 (6.34-6.39)	6.36 (6.34-6.39)	6.39 (6.31-6.46)	0.004

SVR: Sustained virologic response; INR: International normalized ratio; PLT: Platelets; ALT: Alanine aminotransferase; eGFR: Estimated glomerular filtration rate; HCV: Hepatitis C virus.

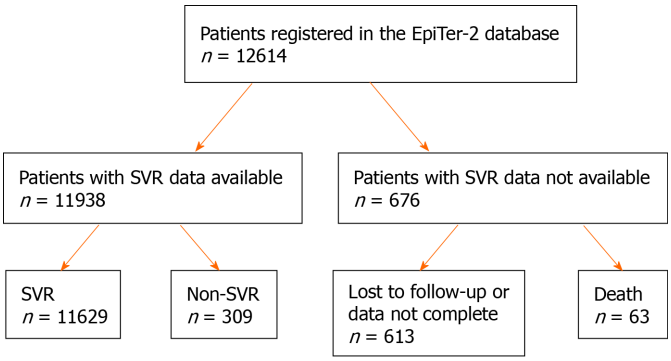


Figure 1 Patient disposition and treatment outcomes. SVR: Sustained virologic response.

Multivariate analysis using ML methods

ML techniques were used to develop a predictive model. The variables in which the missing data accounted for over 10% of all observations were extracted from the database and removed from the analysis. Although four models were constructed using ML algorithms, the random forest model was selected because it yielded the best prediction accuracy. The accuracy of the models is presented in Figure 3. The area under the receiver operating characteristic curve for the random forest model was 0.999 (Figure 4).

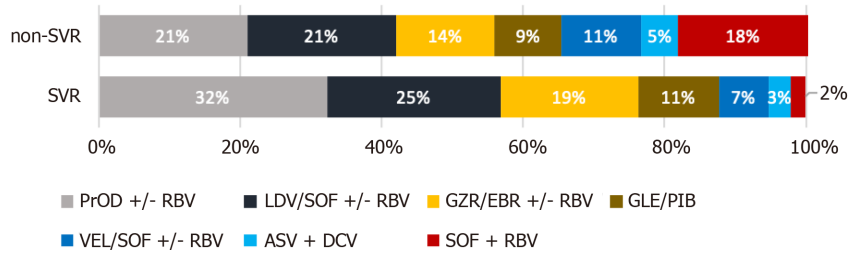


Figure 2 Distribution of treatment regimens among patients who did and did not achieve sustained virologic response. SVR: Sustained virologic response; RBV: Ribavirin; SOF: Sofosbuvir; PrOD: Paritaprevir/ritonavir +/- dasabuvir; LDV: Ledipasvir; GZR: Grazoprevir; EBR: Elbasvir; GLE: Glecaprevir; PIB: Pibrentasvir; VEL: Velpatasvir; ASV: Asunaprevir; DCV: Daclatasvir.

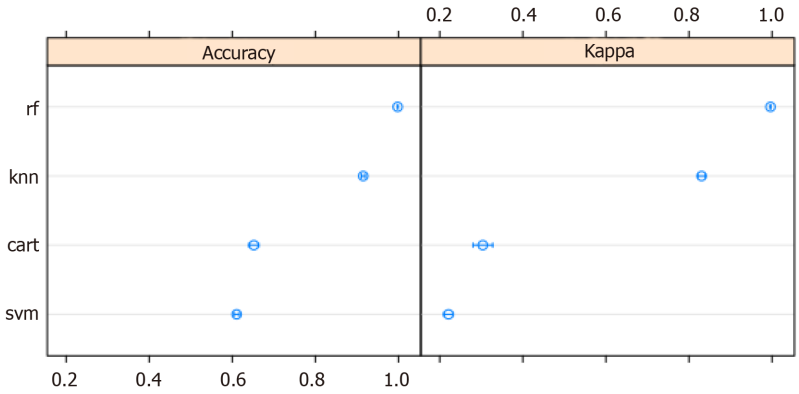


Figure 3 Prediction accuracy and the value of Cohen's κ statistic for individual machine learning algorithms. cart: Classification and regression tree; knn: K-nearest neighbors; svm: Support vector machine; rf: Random forest.

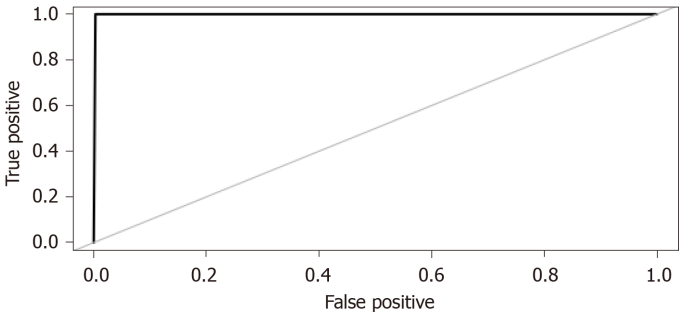


Figure 4 The receiver operating characteristic curve showing the accuracy of the final model for the test data.

The accuracy of the final model was checked using 10-fold cross-validation. The model was "taught" on the training data and then validated using the test data. The final model was built based on 14012 observations and 36 variables. Its accuracy was 0.9993 on the training set, and Cohen's κ statistic was 0.9985. When validating the model using the test set (n = 4670), an accuracy of 0.9985 (95%CI: 0.9969-0.9994) and a Cohen κ statistic of 0.9970 were obtained.

Based on the constructed model, the weights of variables influencing HCV RNA detectability at least 12 wk after the end of treatment (non-SVR) were determined. Two measures were used for this purpose: the average loss of accuracy, which is determined by how much the accuracy of the model has decreased after the removal of a specific variable (Figure 5), and the average loss of the Gini coefficient (Figure 6), on which the random forest algorithm is based. The greater the value of the loss of the Gini coefficient, the more important the variable is, because it leads to a reduction in the entropy of the output variable.

Figures 5 and 6 show the top 12 factors contributing to treatment failure.

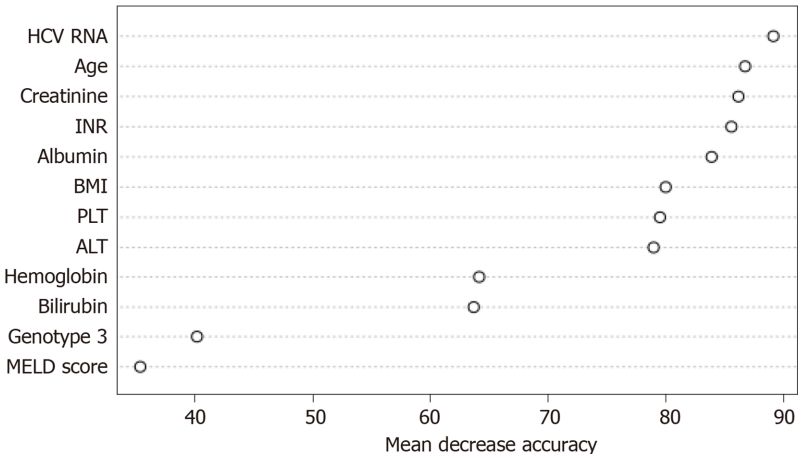


Figure 5 The most important factors influencing treatment failure: mean accuracy decrease. HCV: Hepatitis C virus; INR: International normalized ratio; BMI: Body mass index; PLT: Platelets; ALT: Alanine aminotransferase; MELD: Model for End-stage Liver Disease.

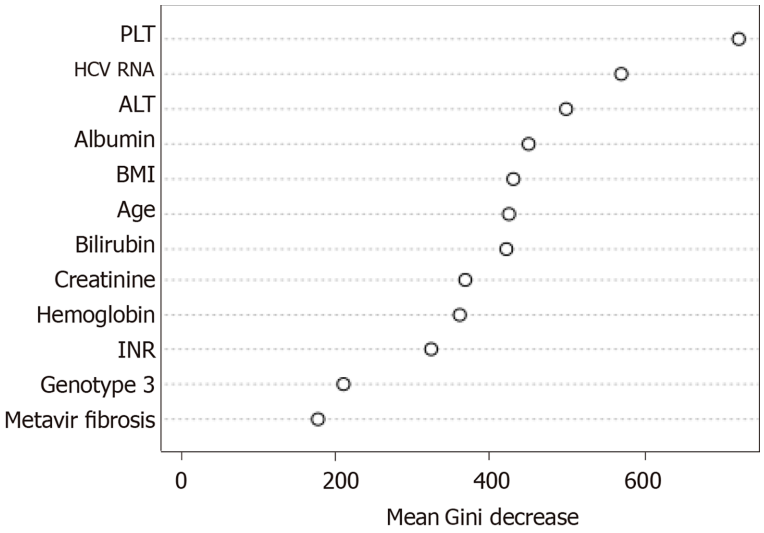


Figure 6 The most important factors influencing treatment failure: mean Gini coefficient decrease. HCV: Hepatitis C virus; INR: International normalized ratio; BMI: Body mass index; PLT: Platelets; ALT: Alanine aminotransferase; MELD: Model for End-stage Liver Disease.

In the case of the remaining variables, the degree of accuracy decrease indicated their minor importance in predicting therapy ineffectiveness.

Based on the statistical analysis of the factors influencing the failure of antiviral therapy performed with the use of the random forest algorithm, the following factors seem to be the most important: advancement of liver disease (platelets, albumin, INR, bilirubin, fibrosis), HCV characteristics (viral load, genotype 3), patient characteristics (age, BMI), hemoglobin levels, and creatinine levels. Conversely, the following factors seem to be of minor importance: HBV and HIV coinfections, extrahepatic manifestations of HCV infection, and coexistence of HCC.

Male sex was statistically significant in the conventional statistics. However, in the ML analysis, this parameter did not significantly affect treatment outcome (average accuracy decrease: 31st position, loss of the Gini coefficient: 14th position).

DISCUSSION

Treatment of chronic hepatitis C with DAAs rarely does not eliminate HCV infection. In our group, 97% of patients achieved SVR, which is consistent with the results of clinical trials[1-3,8,9] and other cohort studies[13-17]. In the analysis of our large, multicenter cohort of over 11000 patients, we assessed the factors that may have contributed to the ineffectiveness of DAA therapy in the remaining 3%.

Due to the specifics of the collected data (a very large study group, a large number of variables, a large disproportion between the number of patients who achieved and did not achieve SVR), we utilized ML techniques in addition to the traditional statistical analysis. Both types of statistical analysis revealed that the factors that have the greatest negative impact on the efficacy of DAA treatment are those related to the advancement of liver disease and impairment of its function. These observations are in line with the results of other cohort studies[18-22]. Because of the very low treatment failure rate, the numbers of patients failing to achieve SVR in these studies were significantly smaller than that in the present study. Gathering a group of 309 patients who did not achieve SVR makes our analysis more reliable than those conducted for groups with only several dozen non-SVR patients and can only be comparable to a few large study cohorts, such as the Veterans cohort[18].

In the classical statistical analysis, male sex seemed to be of importance, which is consistent with the findings of previous studies[17,18]. However, in the ML analysis, this factor was not among those with the greatest impact on treatment efficacy. Another difference between conventional statistics and the ML algorithm is the significance of age, creatinine, and hemoglobin in relation to treatment failure. These factors appeared to be irrelevant in the conventional analysis; however, they were among the important determinants of treatment failure in the random forest algorithm. Higher BMI was also an unfavorable prognostic factor in both types of statistical analysis. Among the virological factors examined, genotype 3 and viral load appeared to influence the efficacy of DAA treatment as well. Different results were obtained by Ioannou *et al*[18] in a large cohort of Veterans, in which, using conventional statistical methods, viral load and age had no effect on the treatment efficacy.

Neither HBV nor HIV coinfection influenced the results of therapy in our group.

Rial-Crestelo *et al*[23] in their publication, describes a cohort of 316 patients with HCV/HIV coinfection treated with DAAs between 2014 and 2018 (including 43.9% cirrhotics), in which the SVR rate was 90.9%. The factors with the greatest impact on the therapy ineffectiveness in this group were alcohol abuse and higher bilirubin levels. In our cohort, patients co-infected with HIV accounted for 5%, and only patients without active addictions were eligible for treatment. Higher bilirubin levels in our study were also associated with less effective therapy, as demonstrated by both traditional statistics and ML.

Part of the analyzed parameters showed a significant impact on the treatment effect in both "traditional" statistics and ML. For other parameters, we observed differences in statistical significance between these methods.

Direct comparison of these methods is difficult, because they involve different aspects of data collected. "Traditional" statistics cannot assess the interaction between many continuous variables and many factors simultaneously. We used this method for univariate analysis only.

ML algorithms are a multivariate way to analyze the data. It takes into account interaction between all variables, and this is a reason for inconsistency between "traditional" and ML sections. In our opinion, ML algorithms perform better because of the following reasons—the large data frame with more than 11000 observations and a significant disproportion between patients who achieved and did not achieve SVR (97% *vs* 3%); ML counts interactions between all variables; the oversampling technique allows us to have equal groups of HCV RNA detectability (in standard statistics, oversampling does not work because multiplication of data may result in biased outcomes).

The percentages of patients treated with VEL/SOF +/- RBV, ASV + DCV, and SOF + RBV were higher among patients without SVR than among those who achieved SVR.

The lower efficacy of treatment with SOF + RBV or ASV + DCV has been observed in previous studies and in clinical practice[24-27].

The relatively high percentage of patients treated with VEL/SOF +/- RBV among therapy failures is somewhat surprising. However, patients with hepatic failure were treated with this drug because treatment with a regimen containing protease inhibitors is contraindicated in this group. Thus, higher treatment failure rates may be associated with more severe liver disease. On the other hand, no such effect was observed in patients treated with LDV/SOF +/- RBV in the earlier period of the EpiTer-2 study, when relatively more patients with advanced liver disease were included[28].

Resistance-associated variants (RASs) were not analyzed in this study because they are not routinely used in clinical practice and are determined only in a few cases at select centers. Pre-treatment RAS data were not available for all patients, although some who qualified had them assessed as part of a separate study[29]. A study carried out on a population partially overlapping our study group reported an increased frequency of baseline NS5A RASs (particularly Y93H) in patients with advanced

fibrosis and cirrhosis. Data from the study by Parczewski *et al*[29], which included 265 patients, some of whom were subsequently treated in the EpiTer-2 study, suggest that the incidence of NS5A RASs increases in patients with advanced fibrosis and cirrhosis in comparison to levels observed in those with mild fibrosis, even in those with no history of antiviral therapy.

The frequent occurrence of RASs was also observed in the Italian cohort of 87 patients after failing DAAs therapy, 79.5% of whom were patients with cirrhosis[30].

Considering the lower efficacy of treatment in patients with advanced liver disease and a greater tendency to develop RASs in these groups[29-31], sequencing tests should be considered in patients with cirrhosis, especially those with the features of deteriorating liver function (both with signs of overt failure and those meeting the Child-Pugh A criteria, but with decreased platelet count and albumin and/or elevated bilirubin). This would allow for the selection of personalized therapy with the maximum chance of eliminating HCV infection, which is important in this particular group of patients at risk of complications of liver cirrhosis and/or the development of HCC.

Recently, there has been a tendency to shorten therapy in patients with cirrhosis, making its duration equal to that utilized in patients with less advanced liver disease[32,33]. Our data suggest that longer treatment durations should be considered in patients with cirrhosis, especially those with borderline or overt liver failure and high HCV viral load, with monitoring of early responses during the treatment period (response-guided therapy). Although the percentage of patients not achieving SVR was statistically low, this applies mainly to patients with cirrhosis, in whom the elimination of HCV sometimes determines their further health and even life. Therefore, a special approach to the treatment of these patients should be considered[33,34]. Due to the serious prognosis in these patients, it is important to maximize the effectiveness of the therapy in order to eliminate HCV infection as soon as possible, to prevent the development of additional RASs that can limit the efficacy of possible re-therapy, and to start the process of liver regeneration.

It would be advisable to conduct a further study to verify our findings and, if necessary, to develop guidelines for personalized therapy of such patients.

The strength of our study lies primarily in the inclusion of a large group of non-SVR patients treated in the setting of everyday clinical practice, which allowed us to assess the effects of treatment in patients with more advanced liver injury and concomitant diseases, in contrast to most clinical trials. There are, however, several limitations in our study. This was a cohort study in which various drugs were used depending on their availability over the 4-year data collection period. At that time, manufacturers also made changes to the characteristics of medicinal products used (*e.g.*, shortening the treatment period, recommended treatment with or without RBV). However, it is known that these changes were introduced after evidence from clinical trials showed that they did not significantly reduce the efficacy of treatment and may improve the patient's safety[32].

In addition, some data were not entered for all patients, which is the case in such large, multicenter, real-world projects. However, despite the omitted data, the size of the group was large enough to draw reliable conclusions.

CONCLUSION

In conclusion, our findings demonstrate that failure of treatment with DAAs occurs mainly in patients with liver cirrhosis and deterioration of liver function. Our ML analysis further revealed that older age, creatinine, and hemoglobin levels also influenced treatment failure, as did viral factors such as genotype 3 and viral load. In patients with advanced liver disease, individualization of therapy (RAS testing, response-guided treatment) should be considered to maximize the chance of achieving SVR.

ARTICLE HIGHLIGHTS

Research background

Treatment with direct-acting antiviral drugs (DAAs) is highly effective and safe. Interferon-free therapies allow for a sustained virologic response (SVR) in over 90% of patients, both in randomized clinical trials and in real-world settings.

Research motivation

Treatment of chronic hepatitis C with DAAs rarely does not eliminate hepatitis C virus (HCV) infection. Numerous studies have confirmed the high efficacy of treatment with direct-acting antivirals. Most of the publications evaluating interferon-free therapies are devoted to assessing their efficacy and safety. Few researchers, however, have analyzed the factors that cause the therapy to fail in some patients.

Research objectives

To analyze factors influencing the failure of direct antiviral drugs in the large, Polish multicenter EpiTer-2 cohort of 12614 patients in a real-world setting.

Research methods

The study cohort consisted of patients treated at 22 centers from 2016-2020. Both standard and machine learning methods were used for statistical analysis.

Research results

Among 11938 patients with SVR data available, 11629 (97%) achieved SVR and 309 (3%) did not. Most patients (78.1%) were infected with HCV genotype 1b. Liver cirrhosis was diagnosed in 2974 patients, advanced fibrosis (F3) in 1717 patients. The most important host factors negatively influencing treatment efficacy were liver cirrhosis, clinical and laboratory features of liver failure, history of hepatocellular carcinoma, and higher body mass index. Among viral factors, genotype 3 and viral load also exerted an influence on treatment efficacy.

Research conclusions

In patients with advanced liver disease, individualized therapy (testing for resistance-associated variants and response-guided treatment) should be considered to maximize the chance of achieving SVR.

Research perspectives

The EpiTer-2 is still an active study, and data on patients treated for HCV infection are still being collected. The obtained data will allow us to confirm the results of our research on a larger group of patients and to verify the validity of the hypothesis that individualization of therapy in patients with liver cirrhosis may improve the treatment efficacy.

ACKNOWLEDGEMENTS

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REFERENCES

1 **Ferenci P**, Bernstein D, Lalezari J, Cohen D, Luo Y, Cooper C, Tam E, Marinho RT, Tsai N, Nyberg A, Box TD, Younes Z, Enayati P, Green S, Baruch Y, Bhandari BR, Caruntu FA, Sepe T, Chulanov V, Janczewska E, Rizzardini G, Gervain J, Planas R, Moreno C, Hassanein T, Xie W, King M, Podsadecki T, Reddy KR; PEARL-III Study; PEARL-IV Study. ABT-450/r-ombitasvir and dasabuvir with or without ribavirin for HCV. *N Engl J Med* 2014; **370**: 1983-1992 [PMID: 24795200 DOI: 10.1056/NEJMoa1402338]

2 **Zeuzem S**, Ghalib R, Reddy KR, Pockros PJ, Ben Ari Z, Zhao Y, Brown DD, Wan S, DiNubile MJ, Nguyen BY, Robertson MN, Wahl J, Barr E, Butterton JR. Grazoprevir-Elbasvir Combination Therapy for Treatment-Naive Cirrhotic and Noncirrhotic Patients With Chronic Hepatitis C Virus Genotype 1, 4, or 6 Infection: A Randomized Trial. *Ann Intern Med* 2015; **163**: 1-13 [PMID: 25909356 DOI: 10.7326/M15-0785]

3 **Afdhal N**, Zeuzem S, Kwo P, Chojkier M, Gitlin N, Puoti M, Romero-Gomez M, Zarski JP, Agarwal K, Buggisch P, Foster GR, Bräu N, Buti M, Jacobson IM, Subramanian GM, Ding X, Mo H, Yang JC, Pang PS, Symonds WT, McHutchison JG, Muir AJ, Mangia A, Marcellin P; ION-1 Investigators. Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection. *N Engl J Med* 2014; **370**: 1889-1898 [PMID: 24725239 DOI: 10.1056/NEJMoa1402454]

4 **Mangia A**, Milligan S, Khalili M, Fagiuoli S, Shafran SD, Carrat F, Ouzan D, Papatheodoridis G, Ramji A, Borgia SM, Wedemeyer H, Losappio R, Pérez-Hernandez F, Wick N, Brown RS Jr, Lampertico P, Doucette K, Ntalla I, Ramroth H, Mertens M, Vanstraelen K, Turnes J. Global real-

world evidence of sofosbuvir/velpatasvir as simple, effective HCV treatment: Analysis of 5552 patients from 12 cohorts. *Liver Int* 2020; **40**: 1841-1852 [PMID: 32449966 DOI: 10.1111/liv.14537]

5 **Puenpatom A**, Cao Y, Yu X, Kanwal F, El-Serag HB, Kramer JR. Effectiveness of Elbasvir/Grazoprevir in US Veterans with Chronic Hepatitis C Virus Genotype 1b Infection. *Infect Dis Ther* 2020; **9**: 355-365 [PMID: 32297307 DOI: 10.1007/s40121-020-00293-7]

6 **Zuckerman E**, Gutierrez JA, Dylla DE, de Ledinghen V, Muir AJ, Gschwantler M, Puoti M, Caruntu F, Slim J, Nevens F, Sigal S, Cohen S, Fredrick LM, Pires Dos Santos AG, Rodrigues L Jr, Dillon JF. Eight Weeks of Treatment With Glecaprevir/Pibrentasvir Is Safe and Efficacious in an Integrated Analysis of Treatment-Naïve Patients With Hepatitis C Virus Infection. *Clin Gastroenterol Hepatol* 2020; **18**: 2544-2553. e6 [PMID: 32621971 DOI: 10.1016/j.cgh.2020.06.044]

7 **Boyle A**, Marra F, Peters E, Datta S, Ritchie T, Priest M, Heydtmann M, Barclay ST. Eight weeks of sofosbuvir/velpatasvir for genotype 3 hepatitis C in previously untreated patients with significant (F2/3) fibrosis. *J Viral Hepat* 2020; **27**: 371-375 [PMID: 31756019 DOI: 10.1111/jvh.13239]

8 **Zoratti MJ**, Siddiqua A, Morassut RE, Zeraatkar D, Chou R, van Holten J, Xie F, Druyts E. Pangenotypic direct acting antivirals for the treatment of chronic hepatitis C virus infection: A systematic literature review and meta-analysis. *EClinicalMedicine* 2020; **18**: 100237 [PMID: 31922124 DOI: 10.1016/j.eclinm.2019.12.007]

9 **Falade-Nwulia O**, Suarez-Cuervo C, Nelson DR, Fried MW, Segal JB, Sulkowski MS. Oral Direct-Acting Agent Therapy for Hepatitis C Virus Infection: A Systematic Review. *Ann Intern Med* 2017; **166**: 637-648 [PMID: 28319996 DOI: 10.7326/M16-2575]

10 **Li T**, Qu Y, Guo Y, Wang Y, Wang L. Efficacy and safety of direct-acting antivirals-based antiviral therapies for hepatitis C virus patients with stage 4-5 chronic kidney disease: a meta-analysis. *Liver Int* 2017; **37**: 974-981 [PMID: 27943605 DOI: 10.1111/liv.13336]

11 **Peters MG**, Kottilil S, Terrault N, Amara D, Husson J, Huprikar S, Florman S, Sulkowski MS, Durand CM, Luetkemeyer AF, Rogers R, Grab J, Haydel B, Blumberg E, Dove L, Emond J, Olthoff K, Smith C, Fishbein T, Masur H, Stock PG. Retrospective-prospective study of safety and efficacy of sofosbuvir-based direct-acting antivirals in HIV/HCV-coinfected participants with decompensated liver disease pre- or post-liver transplant. *Am J Transplant* 2020 [PMID: 33277801 DOI: 10.1111/ajt.16427]

12 **Polish Group of Experts for HCV**, Halota W, Flisiak R, Juszczyk J, Małkowski P, Pawłowska M, Simon K, Tomasiewicz K. Recommendations for the treatment of hepatitis C in 2017. *Clin Exp Hepatol* 2017; 3: 47-55 [PMID: 2885629 DOI: 10.5114/ceh.2017.67782]

13 **Calleja JL**, Crespo J, Rincón D, Ruiz-Antorán B, Fernandez I, Perelló C, Gea F, Lens S, García-Samaniego J, Sacristán B, García-Eliz M, Llerena S, Pascasio JM, Turnes J, Torras X, Morillas RM, Llaneras J, Serra MA, Diago M, Rodriguez CF, Ampuero J, Jorquera F, Simon MA, Arenas J, Navascues CA, Bañares R, Muñoz R, Albillos A, Mariño Z; Spanish Group for the Study of the Use of Direct-acting Drugs Hepatitis C Collaborating Group. Effectiveness, safety and clinical outcomes of direct-acting antiviral therapy in HCV genotype 1 infection: Results from a Spanish real-world cohort. *J Hepatol* 2017; **66**: 1138-1148 [PMID: 28189751 DOI: 10.1016/j.jhep.2017.01.028]

14 **Bischoff J**, Mauss S, Cordes C, Lutz T, Scholten S, Moll A, Jäger H, Cornberg M, Manns MP, Baumgarten A, Rockstroh JK. Rates of sustained virological response 12 weeks after the scheduled end of direct-acting antiviral (DAA)-based hepatitis C virus (HCV) therapy from the National German HCV registry: does HIV coinfection impair the response to DAA combination therapy? *HIV Med* 2018; **19**: 299-307 [PMID: 29368456 DOI: 10.1111/hiv.12579]

15 **Huang CF**, Iio E, Jun DW, Ogawa E, Toyoda H, Hsu YC, Haga H, Iwane S, Enomoto M, Lee DH, Wong G, Liu CH, Tada T, Chuang WL, Cheung R, Hayashi J, Tseng CH, Yasuda S, Tran S, Kam L, Henry L, Jeong JY, Nomura H, Park SH, Nakamuta M, Huang JF, Tai CM, Lo GH, Lee MH, Yang HI, Kao JH, Tamori A, Eguchi Y, Ueno Y, Furusyo N, Tanaka Y, Yu ML, Nguyen MH; REAL-C Investigators. Direct-acting antivirals in East Asian hepatitis C patients: real-world experience from the REAL-C Consortium. *Hepatol Int* 2019; **13**: 587-598 [PMID: 31463665 DOI: 10.1007/s12072-019-09974-z]

16 **Salmon D**, Trimoulet P, Gilbert C, Solas C, Lafourcade E, Chas J, Piroth L, Lacombe K, Katlama C, Peytavin G, Aumaitre H, Alric L, Boué F, Morlat P, Poizot-Martin I, Billaud E, Rosenthal E, Naqvi A, Miailhes P, Bani-Sadr F, Esterle L, Carrieri P, Dabis F, Sogni P, Wittkop L; ANRS CO13 Hepavih study Group. Factors associated with DAA virological treatment failure and resistance-associated substitutions description in HIV/HCV coinfected patients. *World J Hepatol* 2018; **10**: 856-866 [PMID: 30533186 DOI: 10.4254/wjh.v10.i11.856]

17 **Dalgard O**, Weiland O, Noraberg G, Karlsen L, Heggelund L, Färkkilâ M, Balslev U, Belard E, Øvrehus A, Skalshøi Kjær M, Krarup H, Thorup Røge B, Hallager S, Madsen LG, Lund Laursen A, Lagging M, Weis N. Sofosbuvir based treatment of chronic hepatitis C genotype 3 infections-A Scandinavian real-life study. *PLoS One* 2017; **12**: e0179764 [PMID: 28704381 DOI: 10.1371/journal.pone.0179764]

18 **Ioannou GN**, Beste LA, Chang MF, Green PK, Lowy E, Tsui JI, Su F, Berry K. Effectiveness of Sofosbuvir, Ledipasvir/Sofosbuvir, or Paritaprevir/Ritonavir/Ombitasvir and Dasabuvir Regimens for Treatment of Patients with Hepatitis C in the Veterans Affairs National Health Care System. *Gastroenterology* 2016; 151: 457-471. e5 [PMID: 2726705

19 **Chang CY**, Nguyen P, Le A, Zhao C, Ahmed A, Daugherty T, Garcia G, Lutchman G, Kumari R, Nguyen MH. Real-world experience with interferon-free, direct acting antiviral therapies in Asian Americans with chronic hepatitis C and advanced liver disease. *Medicine (Baltimore)* 2017; **96**: e6128

[PMID: 28178174 DOI: 10.1097/MD.0000000000006128]

20 **Ippolito AM**, Milella M, Messina V, Conti F, Cozzolongo R, Morisco F, Brancaccio G, Barone M, Santantonio T, Masetti C, Tundo P, Smedile A, Carretta V, Gatti P, Termite AP, Valvano MR, Bruno G, Fabrizio C, Andreone P, Zappimbulso M, Gaeta GB, Napoli N, Fontanella L, Lauletta G, Cuccorese G, Metrangolo A, Francavilla R, Ciracì E, Rizzo S, Andriulli A. HCV clearance after direct-acting antivirals in patients with cirrhosis by stages of liver impairment: The ITAL-C network study. *Dig Liver Dis* 2017; **49**: 1022-1028 [PMID: 28487083 DOI: 10.1016/j.dld.2017.03.025]

21 **Jiménez-Macías FM**, Cabanillas-Casafranca M, Maraver-Zamora M, Romero-Herrera G, García-García F, Correia-Varela-Almeida A, Cabello-Fernández A, Ramos-Lora M. Experience in real clinical practice with new direct acting antivirals in chronic hepatitis C. *Med Clin (Barc)* 2017; **149**: 375-382 [PMID: 28416232 DOI: 10.1016/j.medcli.2017.03.007]

22 **Terrault NA**, Zeuzem S, Di Bisceglie AM, Lim JK, Pockros PJ, Frazier LM, Kuo A, Lok AS, Shiffman ML, Ben Ari Z, Akushevich L, Vainorius M, Sulkowski MS, Fried MW, Nelson DR; HCV-TARGET Study Group. Effectiveness of Ledipasvir-Sofosbuvir Combination in Patients With Hepatitis C Virus Infection and Factors Associated With Sustained Virologic Response. *Gastroenterology* 2016; **151**: 1131-1140. e5 [PMID: 27565882 DOI: 10.1053/j.gastro.2016.08.004]

23 **Rial-Crestelo D**, Sepúlveda MA, González-Gasca FJ, Geijo-Martínez P, Martínez-Alfaro E, Barberá JR, Yzusqui M, Casallo S, García M, Muñoz Hornero C, Espinosa-Gimeno A, Torralba M; Grupo de Estudio de Castilla la Manche de enfermedades Infecciosas (GECMEI). Impact of interferon-free therapies in HIV/HCV co-infected patients on real clinical practice: results from a multicenter region-wide cohort study (2014-2018). *Eur J Gastroenterol Hepatol* 2021; **32**: 279-287 [PMID: 33252415 DOI: 10.1097/MEG.0000000000002012]

24 **Suzuki F**, Hatanaka N, Bando E, Nakamura K, Komoto A. Safety and effectiveness of daclatasvir and asunaprevir dual therapy in patients with genotype 1 chronic hepatitis C: results from postmarketing surveillance in Japan. *Hepatol Int* 2018; **12**: 244-253 [PMID: 29900486 DOI: 10.1007/s12072-018-9872-z]

25 **Kumada H**, Suzuki Y, Ikeda K, Toyota J, Karino Y, Chayama K, Kawakami Y, Ido A, Yamamoto K, Takaguchi K, Izumi N, Koike K, Takehara T, Kawada N, Sata M, Miyagoshi H, Eley T, McPhee F, Damokosh A, Ishikawa H, Hughes E. Daclatasvir plus asunaprevir for chronic HCV genotype 1b infection. *Hepatology* 2014; **59**: 2083-2091 [PMID: 24604476 DOI: 10.1002/hep.27113]

26 **Gane EJ**, Stedman CA, Hyland RH, Ding X, Svarovskaia E, Symonds WT, Hindes RG, Berrey MM. Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. *N Engl J Med* 2013; **368**: 34-44 [PMID: 23281974 DOI: 10.1056/NEJMoa1208953]

27 **Herbst DA Jr**, Reddy KR. Sofosbuvir, a nucleotide polymerase inhibitor, for the treatment of chronic hepatitis C virus infection. *Expert Opin Investig Drugs* 2013; **22**: 527-536 [PMID: 23448131 DOI: 10.1517/13543784.2013.775246]

28 **Flisiak R**, Zarębska-Michaluk D, Janczewska E, Staniaszek A, Gietka A, Mazur W, Tudrujek M, Tomasiewicz K, Belica-Wdowik T, Baka-Ćwierz B, Dybowska D, Halota W, Lorenc B, Sitko M, Garlicki A, Berak H, Horban A, Orłowska I, Simon K, Socha Ł, Wawrzynowicz-Syczewska M, Jaroszewicz J, Deroń Z, Czauż-Andrzejuk A, Citko J, Krygier R, Piekarska A, Laurans Ł, Dobracki W, Białkowska J, Tronina O, Pawłowska M. Treatment of HCV infection in Poland at the beginning of the interferon-free era-the EpiTer-2 study. *J Viral Hepat* 2018; **25**: 661-669 [PMID: 29316039 DOI: 10.1111/jvh.12861]

29 **Parczewski M**, Kordek J, Janczewska E, Pisula A, Łojewski W, Socha Ł, Wawrzynowicz-Syczewska M, Bociąga-Jasik M, Szymczak A, Cielniak I, Siwak E, Mularska E, Aksak-Wąs B, Urbańska A, Lübke N. Hepatitis C virus (HCV) genotype 1 NS5A resistance-associated variants are associated with advanced liver fibrosis independently of HCV-transmission clusters. *Clin Microbiol Infect* 2019; **25**: 513.e1-513. e6 [PMID: 29981869 DOI: 10.1016/j.cmi.2018.06.028]

30 **Starace M**, Minichini C, De Pascalis S, Macera M, Occhiello L, Messina V, Sangiovanni V, Adinolfi LE, Claar E, Precone D, Stornaiuolo G, Stanzione M, Ascione T, Caroprese M, Zampino R, Parrilli G, Gentile I, Brancaccio G, Iovinella V, Martini S, Masarone M, Fontanella L, Masiello A, Sagnelli E, Punzi R, Salomone Megna A, Santoro R, Gaeta GB, Coppola N. Virological patterns of HCV patients with failure to interferon-free regimens. *J Med Virol* 2018; **90**: 942-950 [PMID: 29315640 DOI: 10.1002/jmv.25022]

31 **Di Stefano M**, Faleo G, Farhan Mohamed AM, Morella S, Bruno SR, Tundo P, Fiore JR, Santantonio TA. Resistance Associated Mutations in HCV Patients Failing DAA Treatment. *New Microbiol* 2021; **44**: 12-18 [PMID: 33453702]

32 **Brown RS Jr**, Buti M, Rodrigues L, Chulanov V, Chuang WL, Aguilar H, Horváth G, Zuckerman E, Carrion BR, Rodriguez-Perez F, Urbánek P, Abergel A, Cohen E, Lovell SS, Schnell G, Lin CW, Zha J, Wang S, Trinh R, Mensa FJ, Burroughs M, Felizarta F. Glecaprevir/pibrentasvir for 8 wk in treatment-naïve patients with chronic HCV genotypes 1-6 and compensated cirrhosis: The EXPEDITION-8 trial. *J Hepatol* 2020; **72**: 441-449 [PMID: 31682879 DOI: 10.1016/j.jhep.2019.10.020]

33 **Lampertico P**, Mauss S, Persico M, Barclay ST, Marx S, Lohmann K, Bondin M, Zhang Z, Marra F, Belperio PS, Wedemeyer H, Flamm S Real-World Clinical Practice Use of 8-Week Glecaprevir/Pibrentasvir in Treatment-Naive Patients with Compensated Cirrhosis. Lampertico P, Mauss S, Persico M, Barclay ST, Marx S, Lohmann K, Bondin M, Zhang Z, Marra F, Belperio PS, Wedemeyer H, Flamm S Real-World Clinical Practice Use of 8-Week Glecaprevir/Pibrentasvir in Treatment-Naive Patients with Adv Ther 2020; 37(9): 4033-4042 [PMID: 3275482 DOI:

10.1007/s12325-020-01449-0]

34 **Peiffer KH**, Vermehren J, Kuhnhenn L, Susser S, Dietz J, Finkelmeier F, Weiler N, Welzel T, Grammatikos G, Zeuzem S, Sarrazin C. Interferon-free treatment choice according to baseline RASs leads to high SVR rates in HCV genotype 1 infected patients. *J Infect Chemother* 2018; **24**: 524-530 [PMID: 29628383 DOI: 10.1016/j.jiac.2018.02.008]

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ORIGINAL ARTICLE

Retrospective Study

Totally laparoscopic total gastrectomy using the modified overlap method and conventional open total gastrectomy: A comparative study

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Author contributions: Ko CS, Choi NR, Kim BS, Yook JH and Kim BS performed the literature search, conception and design, drafting of the article; Kim MJ performed the analysis and interpretation; all authors were involved in the critical revision and final approval of the article.

Institutional review board statement: This study was approved by the Institutional Review Board of the Asan Medical Center (approval No. 2019-0702).

Informed consent statement: Patients were not required to give informed consent for the study because the clinical data were obtained retrospectively after each patient agreed to treatment by written consent.

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Abstract

BACKGROUND

Although several methods of totally laparoscopic total gastrectomy (TLTG) have been reported. The best anastomosis technique for LTG has not been established.

AIM

To investigate the effectiveness and surgical outcomes of TLTG using the modified overlap method compared with open total gastrectomy (OTG) using the circular stapled method.

METHODS

We performed 151 and 131 surgeries using TLTG with the modified overlap method and OTG for gastric cancer between March 2012 and December 2018. Surgical and oncological outcomes were compared between groups using propensity score matching. In addition, we analyzed the risk factors associated with postoperative complications.

RESULTS

Patients who underwent TLTG were discharged earlier than those who underwent OTG [TLTG (9.62 ± 5.32) *vs* OTG (13.51 ± 10.67), $P < 0.05$]. Time to first flatus and soft diet were significantly shorter in TLTG group. The pain scores at all postoperative periods and administration of opioids were significantly lower in the TLTG group than in the OTG group. No significant difference in early, late and esophagojejunostomy (EJ)-related complications or 5-year recurrence free and overall survival between groups. Multivariate analysis demonstrated that body

additional data are available.

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mass index [odds ratio (OR), 1.824; 95% confidence interval (CI): 1.029-3.234, P = 0.040] and American Society of Anaesthesiologists (ASA) score (OR, 3.154; 95%CI: 1.084-9.174, P = 0.035) were independent risk factors of early complications. Additionally, age was associated with ≥ 3 Clavien-Dindo classification and EJ-related complications.

CONCLUSION
Although TLTG with the modified overlap method showed similar complication rate and oncological outcome with OTG, it yields lower pain score, earlier bowel recovery, and discharge. Surgeons should perform total gastrectomy cautiously and delicately in patients with obesity, high ASA scores, and older ages.

Key Words: Laparoscopic surgery; Gastrectomy; Anastomosis; Stomach neoplasms; Totally laparoscopic total gastrectomy

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Core Tip: The aim of the present study was to investigate the effectiveness and surgical outcomes of totally laparoscopic total gastrectomy (TLTG) using the modified overlap method compared with open total gastrectomy (OTG) using the circular stapled method. Although TLTG with the modified overlap method demonstrated similar complication rate and oncological outcome with OTG, it resulted in lower pain scores, and earlier bowel recovery and hospital discharge.

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INTRODUCTION

Laparoscopic total gastrectomy (LTG) is becoming increasingly used to treat upper or middle third gastric cancer because it shows earlier recovery and is considered less invasive[1-3]. Of the entire procedure of LTG, esophagojejunal reconstruction is the most crucial process. This is because the failure of esophagojejunostomy (EJ) such as leakage and stricture could induce the patients to suffer[4]. When performing EJ, EJ using linear stapler method is widely adopted due to its simplicity in comparison to the circular stapled method, such as overlap and functional method[5-9]. Nonetheless, the linear stapled method has a fundamental problem that it requires larger space to dissect around the distal esophagus than does the circular stapled method because the linear stapler needs to be inserted in the abdominal hiatus.

Recently, we developed a modified overlap method for totally LTG (TLTG) for overcoming these disadvantages of linear stapled method[10]. This method is performed with an intracorporeal side to side esophagojejunal anastomosis using a 45-mm linear stapler at 45° from the longitudinal direction of the esophagus (Figure 1). This procedure requires less dissection around abdominal esophagus; therefore, it can create a secure esophagojejunal anastomosis with reduced tension as circular stapled method.

Several studies have investigated the surgical outcomes of the TLTG compared with open total gastrectomy (OTG), including EJ-related complications[11,12]. However, to the best of our knowledge, there are few studies which compared TLTG with the overlap method and OTG with circular stapled method including oncological outcome.

The aim of the present study was to investigate the technical feasibility and oncological outcome of TLTG with the modified overlap method when compared with OTG with circular stapled method in the treatment of upper or middle third gastric cancer.

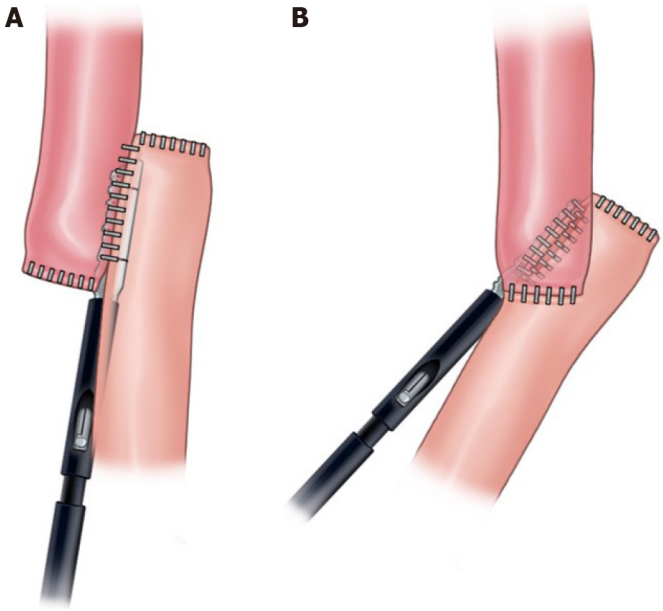


Figure 1 Two types of intracorporeal esophagojejunostomy methods. A: The conventional overlap method; B: The modified overlap method.

MATERIALS AND METHODS

Patients

This study was approved by the institutional review board of the Asan Medical Center. We reviewed the retrospectively collected and analyzed data of 462 patients who underwent curative TLTG (n = 178) and OTG (n = 284) as a treatment for upper or middle third gastric cancer between March 2012 and December 2018 at Asan Medical Center. We excluded all patients who received neoadjuvant chemotherapy, were diagnosed with esophagogastric junction cancer, underwent resection additional organs except for gall bladder, and did not have a gastric cancer diagnosis. Finally, 151 and 131 patients who underwent TLTG and OTG, respectively, were enrolled. All TLTG procedures were performed as TLTG with the modified overlap method. We evaluated TNM (tumor-node-metastasis) stage using the American Joint Committee on Cancer (AJCC), 7th edition[13]. Clinical characteristics and pathologic data were compared between TLTG and OTG groups. Additionally, we evaluated surgical outcomes, including EJ-related complications, and oncologic outcomes, including recurrence free survival (RFS) and overall survival (OS). Early and late complications were defined as events occurring within or after 30 d postoperatively, respectively. EJ complications, including bleeding, leakage and stricture, were diagnosed *via* upper gastrointestinal series, esophagogastroduodenoscopy, computed tomography, and clinical signs. These complications were reviewed and classified based on the Clavien-Dindo classification system (CDC)[14]. Patients were matched using propensity score matching (PSM) analysis and surgical and oncological outcomes were evaluated.

Surgical technique of anastomosis

We performed TLTG with modified overlap method and OTG with circular stapled method, which was similar to our previous studies[3,10]. Both procedures were performed by a single experienced surgeon who conducts approximately 300 cases of gastrectomy annually.

Statistical analysis

In the un-matched group, numerical variables were presented as the mean ± SD using the Student's t-test or Kruskal-Wallis test. Categorical variables was performed using the Chi-square test. Univariate and multivariate analyses were performed for the entire patient cohort (un-matched group) using logistic regression. Variables were included in the multivariate analysis if their univariate significance was < 0.1.

To reduce the impact of treatment selection bias and potential confounding factors in this observational study, we performed rigorous adjustments for significant differences in the baseline characteristics of patients using the logistic regression models with generalized estimating equations (GEE) with a propensity score matched

set. When that technique was used, the propensity scores were estimated without considering the outcomes using multiple logistic regression analysis. A full non-parsimonious model was developed that included all the variables shown in Table 1. Model discrimination was assessed using the C statistic and model calibration was evaluated using Hosmer-Lemeshow statistics. Overall, the model was well calibrated (Hosmer-Lemeshow test; P = 0.368) with reasonable discrimination (C statistic = 0.858). We matched the two groups (1:1 ratio) using a 'greedy nearest-neighbour' algorithm method. The Matching balance was measured based on the standardized mean differences. A > 10% difference in the absolute value was considered significantly imbalanced.

In the matched group, numerical variables were reported as the means ± SD using the paired *t*-test. Categorical variables were performed using McNemar's test or Marginal homogeniety test. To evaluate the association between type of surgery, and complication and survival (and recurrence), the propensity score adjusted model was applied. Finally, the logistic regression model with GEE was applied using propensity score-based matching. The Cox proportional hazards model was applied using propensity score-based matching with robust standard errors. All reported P values are two-sided; values < 0.05 were considered statistically significant. Data manipulation and statistical analyses were performed using SAS® Version 9.4 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Clinicopathological characteristics

The clinical variables are summarized in Table 1. There was a significant difference in body mass index (BMI), tumor size, and pathologic tumor stage before PSM between groups (all P < 0.05); however, these differences disappeared after PSM. There were no statistically significant differences in all baseline variables included in the model between groups.

Surgical outcomes and postoperative complications in PSM

All surgical outcomes and postoperative complications are shown in Table 2. There was no significant difference in operation time between groups (P = 0.351). Patients who underwent TLTG had significantly lower pain scores on all postoperative days than patients who underwent OTG. Moreover, patients in the TLTG group required significantly less analgesic and opioid administration than in the OTG group. The TLTG group reported earlier time to first flatus (3.62 ± 0.84 d *vs* 4.15 ± 0.87 d, P = 0.002) and soft diet (4.62 ± 2.67 d *vs* 7.47 ± 7.92 d, P = 0.001). Furthermore, patients who underwent TLTG stayed statistically significantly fewer days at the hospital after surgery than patients who underwent OTG (9.62 ± 5.32 d *vs* 13.51 ± 10.67 d; P < 0.001). No significant differences in postoperative complications were noted between the two groups (P = 0.161).

Postoperative complications, including EJ-related complications, are summarized in Table 3. There were no significant differences in the early and late postoperative overall complications between groups (P = 0.317 and P = 0.257, respectively). In addition, there was no difference in the incidence of patients with ≥ 3 CDC complications in the early and late postoperative periods between groups (P = 0.428 and P > 0.999, respectively). There was no significant differences in EJ-related complications. Table 4 shows the details of EJ-related complications. Five cases of EJ leakage were observed, and two cases of EJ bleeding were found. Four patients with CDC 3 complications required interventions such as endoscopic management and pigtail drainage, whereas 2 patients with CDC 2 complications fully recovered by conservative treatment. One postoperative mortality occurred due to EJ bleeding.

Oncologic outcomes of PSM

There were no significant differences in the number of retrieved lymph nodes between groups (P = 0.713). The 5-year RFS and OS are shown in Figure 2. There were no significant differences in pathologic tumor stage between groups after PSM. The 5-year RFS rates of patients who underwent TLTG and OTG were 87.7% and 92.3%, respectively; however, these differences were no significant (P = 0.653). The 5-year OS rates of patients who underwent TLTG and OTG were 74.6% and 80.4%, respectively (P = 0.476).

Table 1 Patient clinical characteristics

Variable	Total set (*n* = 282)		*P* value	SD	PSM set (1:1) (*n* = 122)		*P* value	SD
	TLTG (*n* = 151)	OTG (*n* = 131)			TLTG (*n* = 61)	OTG (*n* = 61)		
Age (yr)	60.74 ± 11.55	59.11 ± 11.02	0.231	0.144	58.30 ± 11.26	58.70 ± 10.65	0.841	0.037
Gender			0.257	0.136			0.847	0.035
Male	94 (62.25)	90 (68.70)			40 (65.57)	41 (67.21)		
Female	57 (37.75)	41 (31.30)			21 (34.43)	20 (32.79)		
BMI (kg/m²)	24.57 ± 3.25	23.69 ± 3.21	0.023	0.273	24.01 ± 2.98	23.98 ± 2.73	0.957	0.010
ASA score			0.859	0.044			0.885	0.073
I	30 (19.87)	26 (19.85)			13 (21.31)	14 (22.95)		
II	114 (75.50)	97 (74.05)			46 (75.41)	44 (72.13)		
III	7 (4.64)	8 (6.11)			2 (3.28)	3 (4.92)		
Number of comorbidities			0.068	0.222			0.655	0.083
0-2	137 (90.73)	126 (96.18)			59 (75.41)	44 (72.13)		
> 2	14 (9.27)	5 (3.82)			2 (3.28)	3 (4.92)		
Combined operation			0.176	0.161			> 0.999	0
No	136 (90.07)	111 (84.73)			55 (90.16)	55 (90.16)		
Yes	15 (9.93)	20 (15.27)			6 (9.84)	6 (9.84)		
History of abdominal surgery			0.061	0.254			0.846	0.103
No	120 (79.47)	102 (77.86)			50 (81.97)	48 (78.69)		
Minor surgery	24 (15.89)	14 (10.69)			6 (9.84)	6 (9.84)		
Major surgery	7 (4.64)	15 (11.45)			5 (8.20)	7 (11.48)		
Tumor size (mm, median)	31 (22, 46)	64 (36, 85)	< 0.001	0.841	39 (27, 62)	50 (28, 68)	0.865	0.028
Pathologic tumor stage			< 0.001	1.012			0.824	0.085
I	109 (72.19)	38 (29.01)			31 (50.82)	33 (54.10)		
II	28 (18.54)	41 (31.30)			19 (31.15)	19 (31.15)		
III	14 (9.27)	52 (39.69)			11 (18.03)	9 (14.75)		

Values are expressed as mean ± SD or *n* (%). PSM: Propensity score matching; BMI: Body mass index; ASA score: American Society of Anesthesiologists Physical Status Classification; TLTG: Totally laparoscopic gastrectomy; OTG: Open total gastrectomy.

Risk factors for postoperative complications

Tables 5 and 6 demonstrate the risk factors for postoperative complications after TLTG and OTG. BMI and American Society of Anaesthesiologists (ASA) scores were significantly associated with the occurrence of early complications in the univariate analysis. In addition, ASA score and age were significantly associated with the incidence of ≥ 3 CDC and EJ-related complications, respectively. Multivariate analysis demonstrated that BMI [odds ratio (OR), 1.824; 95% confidence interval (CI): 1.029-3.234, P = 0.040] and ASA score (OR, 3.154; 95%CI: 1.084-9.174, P = 0.035) were independent risk factors of early complications. Furthermore, multivariate analysis revealed that age was associated with ≥ 3 CDC and EJ-related complications.

DISCUSSION

To the best of our knowledge, this is the first study to compare feasibility and oncological outcomes between patients who underwent TLTG with the modified overlap method and OTG. This study demonstrated that TLTG with the modified overlap method is a technically safe procedure based on acceptable postoperative

Table 2 Early surgical outcomes and pathologic data in patients undergoing the totally laparoscopic gastrectomy with the modified overlap method and open total gastrectomy

Variable	Total set (*n* = 282)		*P* value	PSM set (1:1) (*n* = 122)		*P* value
	TLTG (*n* = 151)	OTG (*n* = 131)		TLTG (*n* = 61)	OTG (*n* = 61)	
Operative time (min)	147.68 ± 29.64	145.24 ± 35.48	0.282	147.11 ± 25.48	143.46 ± 38.09	0.351
Time to first flatus (d)	3.73 ± 0.90	4.14 ± 0.81	< 0.001	3.62 ± 0.84	4.15 ± 0.87	**0.002**
Time to soft diet (d)	4.99 ± 3.78	7.24 ± 6.29	< 0.001	4.62 ± 2.67	7.47 ± 7.92	**0.001**
Perioperative transfusion (*n*)			< 0.001			**0.035**
No	145 (96.03)	107 (81.68)		59 (96.72)	52 (85.25)	
Yes	6 (3.97)	24 (18.32)		2 (3.28)	9 (14.75)	
Hospital day after surgery (d)	9.96 ± 6.36	13.06 ± 11.09	< 0.001	9.62 ± 5.32	13.51 ± 10.67	**< 0.001**
Pick of pain score (VAS)	4 (3.92)	5 (2.51)	0.494	3 (3.16)	1 (1.05)	0.317
Pain score at postoperative day	3.19 ± 1.04	3.83 ± 1.14	< 0.001	3.23 ± 1.09	4.07 ± 1.35	**< 0.001**
Pain score at postoperative day 1	2.98 ± 1.07	3.76 ± 1.13	< 0.001	2.97 ± 0.87	3.77 ± 1.07	**< 0.001**
Pain score at postoperative day 3	2.68 ± 1.17	3.10 ± 1.28	< 0.001	2.75 ± 1.31	3.16 ± 1.27	**< 0.001**
Pain score at postoperative day 5	1.93 ± 1.13	2.61 ± 1.49	< 0.001	1.82 ± 1.13	2.64 ± 1.21	**< 0.001**
Administration of analgesics (*n*)	9.74 ± 8.92	16.22 ± 18.06	< 0.001	10.61 ± 11.07	16.92 ± 13.72	**< 0.001**
Administration of opioid (*n*)	2.89 ± 5.49	5.43 ± 11.51	< 0.001	3.21 ± 6.98	4.48 ± 5.15	**0.031**
Retrieved LN	39.53 ± 15.59	41.03 ± 15.31	0.265	38.67 ± 13.82	38.13 ± 14.52	0.713

Values are expressed as mean ± SD or *n* (%) or median (range). PSM: Propensity score matching; LN: Lymph node; PRM: Proximal resection margin; TLTG: Totally laparoscopic gastrectomy; OTG: Open total gastrectomy.

complications, including EJ-related complications.

The overlap method is a widely used EJ reconstruction method in TLTG, it which can lessen the tension in the anastomosis and reduce mesentery division. This secures additional jejunum length for anastomosis[15,16]. This method involves a linear stapler for anastomosis; therefore, the area around the abdominal esophagus requires sufficient dissection. Furthermore, and space in the hiatus and length of the esophagus in which the stapler will be placed should be secured. This may lead to tension in the esophagus after anastomosis and hiatal hernia caused by excessive hiatus dissection. We have devised a novel method to minimize these risks, named the modified overlap method. We use a linear stapler; however, compared with the existing side-to-side anastomosis, less esophageal dissection is required. Further, anastomosis is completed obliquely at 45°; therefore, the resulting anastomosis is similar to when a circular stapler is used because end to side anastomosis is possible. This study proved the TLTG with this modified overlap method showed no significant difference in EJ complications when compared with OTG.

A previous comparative study of LTG and OTG reported similar EJ anastomotic complications; however, a previous large multicenter cohort study in Japan has shown that open surgery is safer for EJ reconstruction[11,12,17]. This indicates that controversy remains regarding the superior method for EJ anastomosis between OTG and LTG. Furthermore, international treatment guidelines, including the Korean gastric cancer treatment and Japanese gastric cancer treatment guidelines, do not yet recognize LTG as a standard treatment[18,19]. Nonetheless, our data indicate that a randomized clinical trial assessing the surgical and oncological outcomes using the modified overlap method should be conducted to confirm its safety and efficacy.

In this study, we overcame operative time and lymphadenectomy issues using the TLTG with the modified overlap method. First, laparoscopic gastrectomy surgery is longer than open gastrectomy[2,20,21]. However, the institution in which this study was conducted is a high-volume center where more than a thousand laparoscopic gastrectomies are performed annually. The lead surgeon in this study performs > 300 gastric cancer operations per year. All surgical team members in this institution are skilled and experienced; therefore, we predicted a reduced operative time while maintaining acceptable surgical and oncological outcomes. However, it may be

Table 3 Postoperative complications

Variable	Total set (*n* = 282)		*P* value	PSM set (1:1) (*n* = 122)		*P* value
	TLTG (*n* = 151)	OTG (*n* = 131)		TLTG (*n* = 61)	OTG (*n* = 61)	
Early complications						
No	119 (78.81)	99 (75.57)	0.518	48 (78.69)	43 (70.49)	0.317
Yes	32 (21.19)	32 (24.43)		13 (21.31)	18 (29.51)	
Late complications						
No	142 (94.04)	125 (95.42)	0.607	56 (91.80)	59 (96.72)	0.257
Yes	9 (5.96)	6 (4.58)		5 (8.20)	2 (3.28)	
CDC			0.426			0.564
0-2	138 (91.39)	116 (88.55)		56 (91.80)	54 (88.52)	
≥ 3	13 (8.61)	15 (11.45)		5 (8.20)	7 (11.48)	
EJ related complications			0.090			0.270
No	148 (98.01)	123 (93.89)		59 (96.72)	56 (91.80)	
Yes	3 (1.99)	8 (6.11)		2 (3.28)	5 (8.20)	

Values are expressed as mean ± SD or *n* (%). PSM: Propensity score matching; TLTG: Totally laparoscopic gastrectomy; OTG: Open total gastrectomy; CDC: Clavien-Dindo classification; EJ: Esophagojejunostomy.

Table 4 Characteristics of the patients with esophagojejunostomy-related complications

Case	Sex	Age	Primary operation	TNMstage	Early or late	Type of complication	CDC	Treatment	Hospital day
1	F	79	TLTG	III	Early	Bleeding	5	Operation	8
2	M	65	TLTG	II	Early	Leakage	3A	Intervention	20
3	M	74	OTG	I	Early	Leakage	2	Conservative	14
4	M	74	OTG	I	Early	Bleeding	3A	Intervention	72
5	M	60	OTG	I	Early	Leakage	3A	Intervention	48
6	M	66	OTG	I	Early	Leakage	2	Conservative	25
7	M	63	OTG	I	Early	Leakage	3A	Intervention	32

TNM: Tumor-node-metastasis; CDC: Clavien-Dindo classification; F: Female; M: Male; TLTG: Totally laparoscopic gastrectomy; OTG: Open total gastrectomy.

difficult to apply the results of this study to low-volume centers or inexperienced surgeons.

Second, lymph node dissection is an important procedure in gastric cancer surgery because the oncologic outcome is dependent on a proper lymphadenectomy[22]. The AJCC recommends that ≥ 30 lymph nodes be removed for lymphadenectomy in gastric cancer[23]. In this study, there was no statistically significant difference in the number of retrieved lymph nodes between groups; ≥ 30 lymph nodes were removed in both groups. In addition, this study showed that the 5-year overall and RFS after PSM analysis did not differ between groups.

In general, the risk factors associated with surgical complications in laparoscopic gastrectomy are comorbidity, surgeon experience, age, malnutrition, gender, and chronic liver disease[24-26]. Most studies have included and analyzed patients who underwent total and distal gastrectomies. Fewer studies have analyzed total gastrectomy alone. Kosuga *et al*[25] and Martin *et al*[26] have classified total gastrectomy as a risk factor for complications (OR 1.63 and 3.13, respectively). This indicates that it is important to evaluate risk factors relative to limited total gastrectomy. Li *et al*[27] have shown that old age combined with splenectomy is a risk factor for overall complications after total gastrectomy. In this study, preoperative BMI and ASA scores were risk factors associated with early complications. Further, old age

Table 5 Univariate analysis of risk factors for overall early, Clavien-Dindo classification ≥ 3, and esophagojejunostomy-related complications

Variables	Early complications		CDC ≥ 3 complications		EJ-related complications	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Type of surgery		0.518		0.428		0.090
TLTG	1		1		1	
OTG	1.202 (0.688-2.100)		1.373 (0.628-3.002)		3.209 (0.833–12.356)	
Age		0.289		0.051		**0.035**
< 60	1		1		1	
≥ 60	1.358 (0.772-2.390)		2.347 (0.997-5.525)		9.220 (1.164-73.022)	
BMI		**0.045**		0.380		0.402
< 25	1		1		1	
≥ 25	1.773 (1.012-3.109)		1.419 (0.649-3.101)		1.678 (0.500-5.633)	
ASA score		**0.030**		**0.036**		
1-2	1		1			
3	3.224 (1.122-9.265)		3.682 (1.088-12.456)			
Number of comorbidities		0.342				0.752
0-2	1				1	
> 2	1.631 (0.594-4.480)				1.406 (0.170-11.599)	
Combined operation		0.685		0.381		0.735
No	1		1		1	
Yes	0.833 (0.346-2.008)		0.515 (0.117-2.272)		0.697 (0.086-5.618)	
History of abdominal surgery		0.130		0.323		
No	1		1		1	
Yes	1.640 (0.864-3.111)		1.554 (0.648-3.726)		1.408 (0.362-5.478)	0.622
Tumor size		0.521		0.442		0.053
< 5 cm	1		1		1	
≥ 5 cm	0.830 (0.470-1.466)	0.521	0.727 (0.323-1.638)		3.786 (0.983-14.585)	
Operation time		0.225		0.605		0.805
< 150 min	1		1		1	
≥ 150 min	1.414 (0.808-2.477)		1.230 (0.562-2.692)		1.165 (0.347-3.912)	
Retrieved lymph node		0.714		0.662		0.246
< 30	1		1		1	
≥ 30	0.888 (0.471-1.676)		1.235 (0.480-3.181)		3.416 (0.429-27.170)	
Pathologic tumor stage		0.418		0.864		0.875
I	1		1		1	
II	0.666 (0.328-1.351)		1.246 (0.497-3.126)		0.701 (0.138-3.568)	
III	0.704 (0.346-1.431)		0.950 (0.348-2.592)		1.119 (0.27-4.617)	

Values are expressed as mean ± SD or *n* (%). TLTG: Totally laparoscopic total gastrectomy; OTG: Open total gastrectomy; CDC: Clavien-Dindo classification; EJ: Esophagojejunostomy; CI: Confidence interval; OR: Odds ratio.

was a risk factor associated with EJ complications. Patients over 60-year-old with a BMI over 25 and ASA scores of ≥ 3 were more likely to have surgical complications; therefore, caution is required during surgery and careful perioperative management is necessary.

Table 6 Multivariate analysis of risk factors for early, Clavien-Dindo classification ≥ 3, and esophagojejunostomy-related complications

Variables	Early complications		CDC ≥ 3 complications		EJ related complications	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Type of surgery						
TLTG	1		1		1	
OTG	1.275 (0.719-2.262)	0.405	1.431 (0.650-3.153)	0.373	3.546 (0.908-13.854)	0.069
Age						
< 60			1		1	
≥ 60			2.391 (1.013-5.641)	0.047	9.925 (1.245-79.107)	0.030
BMI						
< 25	1					
≥ 25	1.824 (1.029-3.234)	0.040				
ASA score						
1-2	1					
3	3.154 (1.084-9.174)	0.035				

Values are expressed as mean ± SD or *n* (%). TLTG: Totally laparoscopic total gastrectomy; OTG: Open total gastrectomy; CDC: Clavien-Dindo classification; EJ: Esophagojejunostom; CI: Confidence interval; OR: Odds ratio; BMI: Body mass index; ASA score: American Society of Anesthesiologists Physical Status Classification.

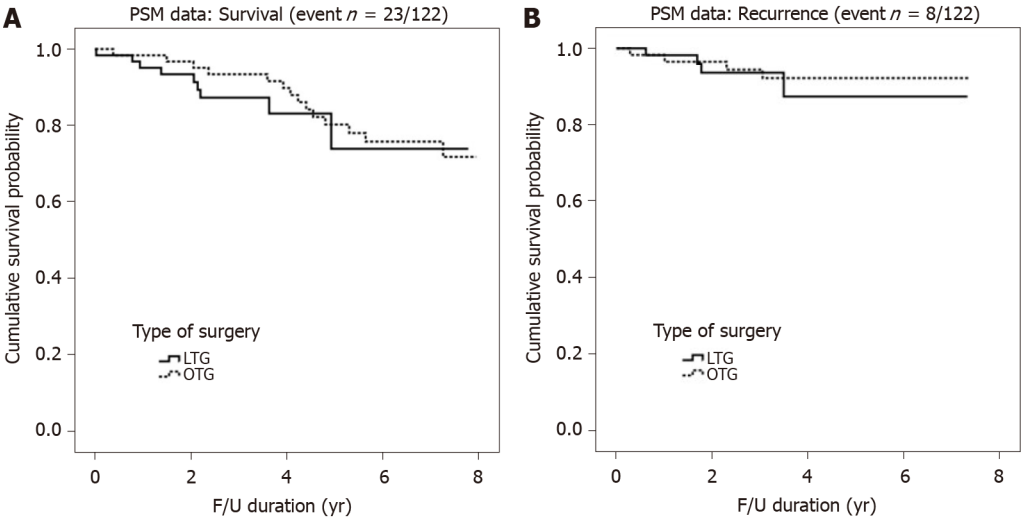


Figure 2 Survival curves for matched patients. A: Overall survival; B: Recurrence free survival. PSM: Propensity score matching; LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy; F/U: Follow up.

This study has some limitations. First, it is a retrospective study performed by a single experienced surgeon at a high-volume center. Therefore, our method might not be appropriate for relatively inexperienced surgeons or small-volume institutes. Second, the number of enrolled patients is relatively small; therefore, subgroup analysis, such as distinguishing between early gastric cancer and advanced gastric cancer or grouping by stage, was not possible.

CONCLUSION

In conclusion, we confirmed that TLTG with the modified overlap method had several advantages over OTG. These included a lower pain score, earlier bowel recovery, and discharge based on acceptable postoperative complications and oncological outcomes.

ARTICLE HIGHLIGHTS

Research background

Although several methods of totally laparoscopic total gastrectomy (TLTG) have been reported, the best anastomosis technique of TLTG has not been conclusively established. Recently, we developed a modified overlap method for TLTG for overcoming these disadvantages of linear stapled method. This procedure requires less dissection around abdominal esophagus; therefore, it can create a secure esophagojejunal anastomosis with reduced tension as circular stapled method.

Research motivation

Whether is a more optimal anastomotic method of esophagojejunostomy in TLTG and open total gastrectomy (OTG) remains unclear. Especially, there was no report about comparing between TLTG with overlap method and OTG.

Research objectives

The aim of this study was to investigate the effectiveness and surgical outcomes including recurrence and survival of TLTG using the modified overlap method compared with OTG using the circular stapled method.

Research methods

We performed 151 TLTG with modified overlap method and 131 OTG for gastric cancer between March 2012 and December 2018 at Asan Medical Center. We evaluated surgical and oncological outcomes between the two groups using propensity score matching. In addition, we analyzed risk factors associated with postoperative complications for improvement of postoperative management of gastric cancer surgery.

Research results

The patients who underwent TLTG were discharged earlier than those who underwent OTG. Time to first flatus and soft diet were significantly shorter in TLTG group. Pain score at all postoperative period and administration of opioid were significantly lower after the TLTG. No statistically significant difference was found between the two groups in terms of early, late and esophagojejunostomy-related complications. Significant differences were observed not with respect to 5-year recurrence free survival and overall survival.

Research conclusions

TLTG with modified overlap method have favorable surgical and oncological outcomes compared with OTG. Furthermore, the surgeon should perform total gastrectomy cautiously and delicately especially with the patients with obese, high American Society of Anaesthesiologists score and old age.

Research perspectives

Based on our results, we confirmed that the TLTG with modified overlap method has several advantages over OTG. However, this study has certain limitations. It is a retrospective study performed by a single experienced surgeon at a high-volume center, and the number of enrolled patients is relatively small.

REFERENCES

1 **Chen K**, Pan Y, Zhai ST, Yu WH, Pan JH, Zhu YP, Chen QL, Wang XF. Totally laparoscopic *vs* open total gastrectomy for gastric cancer: A case-matched study about short-term outcomes. *Medicine (Baltimore)* 2017; **96**: e8061 [PMID: 28930841 DOI: 10.1097/MD.0000000000008061]

2 **Haverkamp L**, Weijs TJ, van der Sluis PC, van der Tweel I, Ruurda JP, van Hillegersberg R. Laparoscopic total gastrectomy *vs* open total gastrectomy for cancer: a systematic review and meta-analysis. *Surg Endosc* 2013; **27**: 1509-1520 [PMID: 23263644 DOI: 10.1007/s00464-012-2661-1]

3 **Kim HS**, Kim BS, Lee IS, Lee S, Yook JH. Comparison of totally laparoscopic total gastrectomy and open total gastrectomy for gastric cancer. *J Laparoendosc Adv Surg Tech A* 2013; **23**: 323-331 [PMID: 23379920 DOI: 10.1089/lap.2012.0389]

4 **Gong W**, Li J. Combat with esophagojejunal anastomotic leakage after total gastrectomy for gastric cancer: A critical review of the literature. *Int J Surg* 2017; **47**: 18-24 [PMID: 28935529 DOI: 10.1016/j.ijsu.2017.09.019]

5 **Ebihara Y**, Okushiba S, Kawarada Y, Kitashiro S, Katoh H. Outcome of functional end-to-end

esophagojejunostomy in totally laparoscopic total gastrectomy. *Langenbecks Arch Surg* 2013; **398**: 475-479 [PMID: 23354359 DOI: 10.1007/s00423-013-1051-z]

6 **Ito H**, Inoue H, Odaka N, Satodate H, Onimaru M, Ikeda H, Takayanagi D, Nakahara K, Kudo SE. Evaluation of the safety and efficacy of esophagojejunostomy after totally laparoscopic total gastrectomy using a trans-orally inserted anvil: a single-center comparative study. *Surg Endosc* 2014; **28**: 1929-1935 [PMID: 24488351 DOI: 10.1007/s00464-014-3417-x]

7 **Jeong O**, Jung MR, Kim GY, Kim HS, Ryu SY, Park YK. Comparison of short-term surgical outcomes between laparoscopic and open total gastrectomy for gastric carcinoma: case-control study using propensity score matching method. *J Am Coll Surg* 2013; **216**: 184-191 [PMID: 23211117 DOI: 10.1016/j.jamcollsurg.2012.10.014]

8 **Okabe H**, Obama K, Tanaka E, Nomura A, Kawamura J, Nagayama S, Itami A, Watanabe G, Kanaya S, Sakai Y. Intracorporeal esophagojejunal anastomosis after laparoscopic total gastrectomy for patients with gastric cancer. *Surg Endosc* 2009; **23**: 2167-2171 [PMID: 18553203 DOI: 10.1007/s00464-008-9987-8]

9 **Shim JH**, Yoo HM, Oh SI, Nam MJ, Jeon HM, Park CH, Song KY. Various types of intracorporeal esophagojejunostomy after laparoscopic total gastrectomy for gastric cancer. *Gastric Cancer* 2013; **16**: 420-427 [PMID: 23097123 DOI: 10.1007/s10120-012-0207-9]

10 **Choi M**, Ko CS, Yook JH, Kim BS. Comparative outcomes between totally laparoscopic total gastrectomy with the modified overlap method for early gastric cancer and advanced gastric cancer: review of 149 consecutive cases. *Wideochir Inne Tech Maloinwazyjne* 2020; **15**: 437-445 [PMID: 32904610 DOI: 10.5114/wiitm.2020.96098]

11 **Inokuchi M**, Otsuki S, Fujimori Y, Sato Y, Nakagawa M, Kojima K. Systematic review of anastomotic complications of esophagojejunostomy after laparoscopic total gastrectomy. *World J Gastroenterol* 2015; **21**: 9656-9665 [PMID: 26327774 DOI: 10.3748/wjg.v21.i32.9656]

12 **Lee JH**, Nam BH, Ryu KW, Ryu SY, Park YK, Kim S, Kim YW. Comparison of outcomes after laparoscopy-assisted and open total gastrectomy for early gastric cancer. *Br J Surg* 2015; **102**: 1500-1505 [PMID: 26398912 DOI: 10.1002/bjs.9902]

13 **Union for International Cancer Control**. What is TNM cancer staging system? [cited 17 March 2021]. In: Union for International Cancer Control (UICC) homepage [Internet]. Available from: https://www.uicc.org/resources/tnm

14 **Dindo D**, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205-213 [PMID: 15273542 DOI: 10.1097/01.sla.0000133083.54934.ae]

15 **Kawamura H**, Ohno Y, Ichikawa N, Yoshida T, Homma S, Takahashi M, Taketomi A. Anastomotic complications after laparoscopic total gastrectomy with esophagojejunostomy constructed by circular stapler (OrVil™) *vs* linear stapler (overlap method). *Surg Endosc* 2017; **31**: 5175-5182 [PMID: 28488177 DOI: 10.1007/s00464-017-5584-z]

16 **Son SY**, Cui LH, Shin HJ, Byun C, Hur H, Han SU, Cho YK. Modified overlap method using knotless barbed sutures (MOBS) for intracorporeal esophagojejunostomy after totally laparoscopic gastrectomy. *Surg Endosc* 2017; **31**: 2697-2704 [PMID: 27699517 DOI: 10.1007/s00464-016-5269-z]

17 **Kodera Y**, Yoshida K, Kumamaru H, Kakeji Y, Hiki N, Etoh T, Honda M, Miyata H, Yamashita Y, Seto Y, Kitano S, Konno H. Introducing laparoscopic total gastrectomy for gastric cancer in general practice: a retrospective cohort study based on a nationwide registry database in Japan. *Gastric Cancer* 2019; **22**: 202-213 [PMID: 29427039 DOI: 10.1007/s10120-018-0795-0]

18 **Guideline Committee of the Korean Gastric Cancer Association (KGCA)**; Development Working Group & Review Panel. Erratum: Korean Practice Guideline for Gastric Cancer 2018: an Evidence-based, Multi-disciplinary Approach. *J Gastric Cancer* 2019; **19**: 372-373 [PMID: 31598379 DOI: 10.5230/jgc.2019.19.e32]

19 **Japanese Gastric Cancer Association**. Japanese gastric cancer treatment guidelines 2018 (5th edition). *Gastric Cancer* 2021; **24**: 1-21 [PMID: 32060757 DOI: 10.1007/s10120-020-01042-y]

20 **Deng Y**, Zhang Y, Guo TK. Laparoscopy-assisted *vs* open distal gastrectomy for early gastric cancer: A meta-analysis based on seven randomized controlled trials. *Surg Oncol* 2015; **24**: 71-77 [PMID: 25791201 DOI: 10.1016/j.suronc.2015.02.003]

21 **Zeng YK**, Yang ZL, Peng JS, Lin HS, Cai L. Laparoscopy-assisted *vs* open distal gastrectomy for early gastric cancer: evidence from randomized and nonrandomized clinical trials. *Ann Surg* 2012; **256**: 39-52 [PMID: 22664559 DOI: 10.1097/SLA.0b013e3182583e2e]

22 **Schwarz RE**, Smith DD. Clinical impact of lymphadenectomy extent in resectable gastric cancer of advanced stage. *Ann Surg Oncol* 2007; **14**: 317-328 [PMID: 17094022 DOI: 10.1245/s10434-006-9218-2]

23 **Edge SB**, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti AI; American Joint Committee of Cancer. AJCC cancer staging manual. 7th ed. New York: Springer, 2010

24 **Kim MC**, Kim W, Kim HH, Ryu SW, Ryu SY, Song KY, Lee HJ, Cho GS, Han SU, Hyung WJ; Korean Laparoscopic Gastrointestinal Surgery Study (KLASS) Group. Risk factors associated with complication following laparoscopy-assisted gastrectomy for gastric cancer: a large-scale korean multicenter study. *Ann Surg Oncol* 2008; **15**: 2692-2700 [PMID: 18663532 DOI: 10.1245/s10434-008-0075-z]

25 **Kosuga T**, Ichikawa D, Komatsu S, Kubota T, Okamoto K, Konishi H, Shiozaki A, Fujiwara H, Otsuji E. Clinical and surgical factors associated with organ/space surgical site infection after laparoscopic gastrectomy for gastric cancer. *Surg Endosc* 2017; **31**: 1667-1674 [PMID: 27506433

DOI: 10.1007/s00464-016-5156-7]

26 **Martin AN**, Das D, Turrentine FE, Bauer TW, Adams RB, Zaydfudim VM. Morbidity and Mortality After Gastrectomy: Identification of Modifiable Risk Factors. *J Gastrointest Surg* 2016; **20**: 1554-1564 [PMID: 27364726 DOI: 10.1007/s11605-016-3195-y]

27 **Li Z**, Liu Y, Bai B, Yu D, Lian B, Zhao Q. Surgical and Long-Term Survival Outcomes After Laparoscopic and Open Total Gastrectomy for Locally Advanced Gastric Cancer: A Propensity Score-Matched Analysis. *World J Surg* 2019; **43**: 594-603 [PMID: 30229383 DOI: 10.1007/s00268-018-4799-z]

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ORIGINAL ARTICLE

Retrospective Study

Radiofrequency ablation *vs* surgical resection in elderly patients with hepatocellular carcinoma in Milan criteria

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Abstract

BACKGROUND

Surgical resection and radiofrequency ablation (RFA) represent two possible strategy in treatment of hepatocellular carcinoma (HCC) in Milan criteria.

AIM

To evaluate short- and long-term outcome in elderly patients (> 70 years) with HCC in Milan criteria, which underwent liver resection (LR) or RFA.

METHODS

The study included 594 patients with HCC in Milan criteria (429 in LR group and 165 in RFA group) managed in 10 European centers. Statistical analysis was performed using the Kaplan-Meier method before and after propensity score matching (PSM) and Cox regression.

RESULTS

After PSM, we compared 136 patients in the LR group with 136 patients in the RFA group. Overall survival at 1, 3, and 5 years was 91%, 80%, and 76% in the LR group and 97%, 67%, and 41% in the RFA group respectively (P = 0.001). Disease-free survival at 1, 3, and 5 years was 84%, 60% and 44% for the LR group, and 63%, 36%, and 25% for the RFA group (P = 0.001).Postoperative Clavien-Dindo III-IV complications were lower in the RFA group (1% *vs* 11%, P = 0.001) in association with a shorter length of stay (2 d *vs* 7 d, P = 0.001).In multivariate analysis, Model for End-stage Liver Disease (MELD) score (> 10) [odds ratio (OR) = 1.89], increased value of international normalized ratio (> 1.3) (OR = 1.60), treatment with radiofrequency (OR = 1.46) ,and multiple nodules (OR = 1.19) were independent predictors of a poor overall survival while a high MELD score (> 10) (OR = 1.51) and radiofrequency (OR = 1.37) were independent factors associated with a higher recurrence rate.

CONCLUSION

Despite a longer length of stay and a higher rate of severe postoperative complications, surgery provided better results in long-term oncological outcomes as compared to ablation in elderly patients (> 70 years) with HCC in Milan criteria.

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Core Tip: Surgical resection and radiofrequency ablation represent two possible strategy in treatment of hepatocellular carcinoma in Milan criteria. In order to evaluate which of the two therapeutic options can provide better short-term and oncological outcomes, we compared data from 10 European centers before and after propensity score matching. Despite a longer length of stay and a higher rate of severe postoperative complications, surgery provided better results in long-term oncological outcomes as compared to ablation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third global cause of cancer-related death[1]. The therapeutic strategy for patients with HCC varies considerably, according to the Barcelona clinic liver cancer (BCLC) algorithm[2], from liver transplantation to resection or ablation, passing through a series of options (chemoembolization, systemic supportive therapy, and systemic chemotherapy), based on the stage of neoplasia and patient's general condition. The best radical treatment is still debated. For patients within Milan criteria, liver transplantation represents the treatment of choice, but unfortunately it is not suitable for all patients due to the scarcity of donors or due to an age limit[3-5].

For patients with very early and early-stage HCC (BCLC 0-A), liver resection (LR) represented the treatment of choice when liver function was well-preserved and when the remnant liver was sufficient. In elderly patients, these conditions were sometimes more precarious and required a more careful evaluation of the risk-benefit ratio in terms of treatment. In fact, the prognostic role of advanced age was not defined in patients with HCC subjected to resection nor was there any mention in official guidelines[6,7].

The aim of our work was to evaluate short-term and long-term outcomes in elderly HCC patients (> 70 years) within Milan criteria, undergoing LR or radiofrequency ablation (RFA).

MATERIALS AND METHODS

Patients

A multicentric retrospective study included 594 patients who were managed from January 2009 to January 2019 in the following centers: Centre Hépato-Biliaire Paul Brousse, Villejuif, France; Hôpital Henry Mondor, Créteil, France; Hospital Universitario Reina Sofia, Cordoba, Spain; Hôpitaux Universitaires Genève, Switzerland; Ospedale Niguarda, Milan, Italy; Nouvel Hôpital Civil, Strasbourg, France; Ospedale San Raffaele, Milan, Italy; Ospedale Miulli,Bari, Italy; Policlinico di Modena, Italy; Centre Hospitalier Universitaire, Reims, France).

We included patients who underwent laparoscopic and open LR or RFA in the study. Inclusion criteria: ≥ 70 years old patients, with Child A-B disease, in BCLC 0/A stage, with tumor within Milan criteria (solitary HCC < 5 cm in diameter, or multiple HCC < 3 lesions, each < 3 cm in diameter). Exclusion criteria: patients with tumor

beyond Milan criteria, with radiological evidence of major portal/hepatic vein branch invasion, with evidence of extrahepatic disease.

The diagnosis of HCC was based on non-invasive findings [ultrasonography, computed tomography (CT) scan, magnetic resonance imaging] or histopathology (with biopsy), according to the European Association for Study of Liver (EASL) consensus criteria[2]. The type of treatment was planned in multidisciplinary team discussions including surgeons, hepatologists, oncologists, interventional radiologists, and pathologists.

RFA procedure

RFA was performed using an internally cooled electrode. Depending on tumor size and position, either a single or clustered electrode was used for ablation under ultrasound guidance percutaneously or using a laparoscopic or open approach. The procedure was performed under local anesthesia and intravenous sedation for percutaneous ablation, and under general anesthesia for laparoscopic and open ablations. A control liver ultrasound was performed on the first postoperative day to assess the quality of the ablation in term of necrotic area.

LR procedure

The surgical strategy was tailored based on tumor size, position, and liver function. The type of LR was defined according to the Brisbane classification[8]. Anatomical resection, wedge resection, minor and major resections were performed under general anesthesia, with an open or laparoscopic approach. Minor resection was defined as the resection of two or fewer Couinaud's liver segments, and major resection was defined as the resection of three or more liver segments. Intraoperative ultrasonography was used routinely. A Pringle's maneuver was used during hepatectomy to control intraoperative bleeding.

Follow-up

Short-term outcomes included operative time, blood transfusion, complications based on the Clavien-Dindo classification[9], length of hospital stay and mortality within 90 d. Long-term outcomes evaluated rates of overall survival (OS) and disease free survival. Liver function (complete blood count, liver test, and coagulation profile) were assessed on postoperative days 1, 3, and 5. Follow-up was performed with CT-scan and blood tests (including liver function and oncological markers) once every 3 mo during the first year and every 4 mo thereafter. For patients undergoing to RFA, a CT-scan at 1 mo after ablation was performed, in order to assess results of treatment according mRECIST (modified Response Evaluation Criteria in Solid Tumors) criteria[10]. Recurrence treatment included repeat resection, ablation, trans-arterial chemo-embolization, liver transplantation, percutaneous ethanol injection, sorafenib chemotherapy, or supportive care according to the EASL-EORTC (European Organisation for Research and Treatment of Cancer) clinical practice guidelines[2].

Statistical analysis

Statistical analysis was performed using the IBM SPSS 20 software. Continuous variables were compared using an independent sample t-test and Mann-Whitney U test. Categorical variables were compared using the chi-square test and Kruskal-Wallis test respectively. Recurrence-free survival (RFS) and OS curves were constructed using the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazards model was used in a stepwise manner (entry criterion $P = 0.05$ and removal criterion $P = 0.1$) to explore independent prognostic RFS and OS factors. We performed a propensity score matching (PSM) analysis to decrease selection bias by building a matched group of patients to compare perioperative characteristics, short-term and long-term outcomes in resection and ablation groups. Variables entered in our propensity model were co-morbidities, American Society of Anaesthesiologists (ASA) score, Child and MELD scores, number of lesions, and tumor size. We calculated propensity scores by applying these variables to a logistic regression model and calculated C-statistics to evaluate the goodness of fit. One-to-one PSM was performed with a caliper width ranging from the < 0.2 pooled standard deviation of estimated propensity scores. A total of 136 patients out of 429 in the resection group and a total of 136 patients out of 165 in the ablation group were matched for further analyses. The relative prognostic significance of the variables in predicting OS and overall recurrence was established using univariate and multivariate Cox proportional hazards regression models. All variables with a P value < 0.05 in the univariate analysis were subjected to the multivariate comparison. Results of the multivariate

analysis were presented as relative risk with a corresponding 95% confidence interval.

RESULTS

Before PSM

We identified 594 patients within the Milan criteria. A total of 429 patients underwent LR and 165 RFA. Perioperative data are described in Table 1. The RFA group presented more co-morbidities than the LR group (64% *vs* 33%, $P = 0.001$). The RFA group also had a higher percentage of patients with ASA score III-IV than the LR group (73% *vs* 60%, $P = 0.001$), and a greater MELD score value (8 *vs* 6, $P = 0.001$). The LR group was associated with a larger tumor size than the RFA group (30 mm *vs* 24 mm, $P = 0.001$). Perioperative and postoperative data are described in Table 2. Operative time was significantly increased in the resection group as compared to the RFA group (205 min *vs* 25 min, $P = 0.0001$). Additionally, the perioperative blood transfusion rate was markedly higher in the LR group than in the RFA group (15% *vs* 8%, $P = 0.001$).

The RFA postoperative course was burdened by a lower rate of serious complications (Clavien-Dindo III-IV) than the LR group (1% *vs* 9%, $P = 0.001$). The RFA group had also significantly shorter postoperative hospital stays than the LR group (2 d *vs* 6 d, $P = 0.001$).

The estimated 1-, 3-, and 5-year OS rates were 91.9%, 84%, and 75.5% for the LR group and 92%, 66.4%, and 37.8% for the RFA group ($P = 0.001$, Figure 1). The estimated 1-, 3-, and 5-year disease-free survival rates were 85.7%, 63.7%, and 50.3% for the LR group, and 66.7%, 37.8%, and 27.7% for the RFA group ($P = 0.001$, Figure 2).

After PSM

After matching, we obtained a comparable population for both groups (Table 1). The variables included in the PSM are comorbidities, ASA and MELD score, tumor size and number of lesions. The use of these parameters for the PSM allowed us to obtain two samples to be compared more homogeneous, and therefore to have short- and long-term results between the two groups less burdened by other variables, although the number of patients in the two groups are smaller after pairing Perioperative and postoperative results are described in Table 2. The postoperative course of the RFA group was burdened by a lower rate of serious complications (Clavien-Dindo III-IV) as compared to the LR group (1% *vs* 11%, $P = 0.001$). The RFA group had also significantly shorter postoperative hospital stays than the LR group (2 d *vs* 7 d, $P = 0.001$). Operative time was significantly increased in the LR group as compared to the RFA group (median range: 190 min *vs* 25 min, $P = 0.001$). In addition, the perioperative blood transfusion rate was markedly higher in the LR group than in the RFA group (17% *vs* 8%, $P = 0.001$).

The estimated 1-, 3-, and 5-year OS rates were 91%, 80.2%, and 76.6% for the LR group and 97.7%, 68.9%, and 40.8% for the RFA group ($P = 0.001$, Figure 3) respectively. The estimated 1-, 3-, and 5-year disease-free survival rates were 84.5%, 60.6%, and 44.4% for the LR group, and 63.2%, 35.7%, and 25.1% for the RFA group ($P = 0.001$, Figure 4) respectively.

Multivariate analysis

We evaluated factors influencing overall and disease-free survival using univariate and multivariate analyses. Multivariate analyses (Table 3) showed that the therapeutic choice of radiofrequency [hazard ratio: 1.46; (1.1-1.79), $P = 0.001$], international normalized ratio > 1.3 mg/dL [hazard ratio 1.60, (1.03-2.49), $P = 0$ 03], and MELD score > 10 [1.89, (1.21-2.92), $P = 0.005$] were independent risk factors for OS. Concerning the rate of recurrence (Table 4), radiofrequency [1.37 (1.17-1.60), $P = 0.0001$] and MELD score > 10 [1.51, (1.04-2.17) $P = 0.0001$] were both considered poor prognostic factors.

DISCUSSION

This study suggested that surgical treatment provided better results in terms of long-term oncological outcomes (OS and disease-free survival) as compared to ablative treatment (RFA) in elderly HCC patients (> 70 years) within the Milan criteria, despite a longer and more complicated postoperative course.

Table 1 Preoperative and clinical characteristics of patients with hepatocellular carcinoma in Milan criteria who underwent surgical resection and radiofrequency ablation

	Before PSM			After PSM		
	RFA (*n* = 165)	Surgery (*n* = 429)	*P* value	RFA (*n* = 136)	Surgery (*n* = 136)	*P* value
Male, *n* (%)	116 (70)	319 (74)	0.35	98 (72)	104 (76)	0.48
Age (yr) median (range)	75 (70-89)	74.9 (70-90)	0.71	75 (70-88)	74.7 (70-86.1)	0.56
BMI (kg/cm²) median (range)	26.7 (19-51)	26.7 (19-52)	0.37	26.7 (19-51)	26 (21-41)	0.85
Co-morbidities > 2, *n* (%)	107 (64)	142 (33)	0.001	83 (61)	81 (60)	0.90
Cause of Cirrhosis *n* (%)			0.002			0.11
Hepatitis C virus	89 (54)	217 (50)		73 (54)	68 (50)	
Hepatitis B virus	10 (6)	80 (19)		10 (7)	22 (16)	
Alcohol	37 (22)	60 (14)		31(23)	23 (17)	
Others	29 (18)	72 (17)		22 (16)	23 (17)	
ASA score, *n* (%)			0.004			0.59
I-II	45 (27)	172 (40)		41 (30)	36 (26)	
III-IV	120 (73)	257 (60)		95 (70)	100 (74)	
Blood tests median (range)						
Bilirubin (mg/dL)	1 (0.2-2.8)	0.9 (0.18-4.5)	0.41	1 (0.2-2.8)	0.8 (0.2-4.5)	0.02
Creatinine (mg/dL)	0.9 (0.5-2.5)	1 (0.2-2.5)	0.03	0.9 (0.5-2.3)	0.9 (0.4-2.5)	0.02
Platelet count × 10^9/L	131 (10-856)	178 (45-900)	0.00	131.5 (10-856)	155 (47-573)	0.57
INR	1.1 (0.9-2.4)	1.2 (0.6-2.5)	0.00	1.1 (0.9-2.4)	1.1 (0.8-2.5)	0.00
Child Pugh, *n* (%)			0.52			0.87
A	139 (84)	370 (86)		114 (84)	116 (85)	
B	26 (16)	59 (14)		22 (16)	20 (15)	
MELD median (range)	8 (6-18)	6 (6-17)	0.00	8 (6-18)	8 (6-17)	0.05
Tumors number, *n* (%)			0.07			0.71
Single nodule	142 (86)	392 (91)		117 (86)	120 (88)	
Multi nodules	23 (14)	37 (9)		19 (14)	16 (12)	
Tumor size (mm) *n* (%)	24 (10-50)	30 (7-50)	0.00	25 (10-50)	24.5 (7-50)	0.9
< 20	49 (30)	33 (8)	0.00	36 (26)	28 (21)	0.31
20-50	116 (70)	396 (92)		100 (74)	108 (79)	
Bilobar tumor, *n* (%)	8 (5)	8 (2)	0.08	6 (4)	2 (1)	0.28
Tumor location, *n* (%)			0.28			0.17
1	2 (1)	8 (2)		1 (0.7)	1 (0.7)	
2	14 (8)	41 (10)		13 (9)	14 (10)	
3	12 (7)	42 (10)		10 (7)	20 (15)	
4	20 (12)	53 (12)		15 (11)	13 (9)	
5	28 (17)	63 (15)		21 (15)	27 (19)	
6	27 (16)	87 (20)		23 (17)	29 (21)	
7	18 (11)	60 (14)		16 (12)	11 (8)	
8	44 (28)	75 (17)		37 (27)	21 (15)	
Histologically proven, *n* (%)	42 (25)	115 (27)	0.75	36 (26)	42 (31)	0.50

Previous treatment, *n* (%)	53 (32)	53 (12)	0.00	42 (31)	21 (15)	0.004

Continuous variables were compared using an independent sample *t*-test and Mann-Whitney *U* test. Categorical variables were compared using the chi-square test and Kruskal-Wallis test respectively. PSM: Propensity score matching; BMI: Body mass index; ASA: American Society of Anesthesiologists; MELD: Model for End-Stage Liver Disease; RFA: Radiofrequency ablation; INR: International normalized ratio.

Table 2 Clinical and perioperative characteristics of patients with hepatocellular carcinoma in Milan criteria who underwent surgical resection and radiofrequency ablation

	Before PSM			After PSM		
	RFA (*n* = 165)	Surgery (*n* = 429)	*P* value	RFA (*n* = 136)	Surgery (*n* = 136)	*P* value
Operative time (min) median (range)	25 (5-250)	205 (55-600)	0.002	25 (5-250)	190 (55-600)	0.001
Blood transfusion, *n* (%)	13 (8)	66 (15)	0.01	11(8)	23 (17)	0.04
Dindo-Clavien Classification, *n* (%)			0.001			0.002
I-II	163 (99)	378 (91)		134 (98)	121 (89)	
III-IV	2 (1)	38 (9)		2 (1)	15 (11)	
Postoperative complication, *n* (%)			0.001			0.002
Yes	31 (19)	188 (44)		28 (21)	75 (55)	
No	134 (81)	241 (56)		108 (79)	61 (45)	
Type of complication, *n* (%)						
Liver failure	1 (1)	35 (8)	0.002	1 (0.7)	14 (10)	0.001
Ascites	3 (2)	60 (14)	0.003	3 (2)	17 (12)	0.002
Biliary leakage	0 (0)	9 (2)	0.064	0 (0)	3 (2)	0.25
Hemorrhage	4 (2)	19 (4)	0.340	3 (2)	11 (8)	0.51
Systemic Infection	4 (2)	30 (7)	0.03	4 (3)	14 (10)	0.03
Intra-abdominal abscess	0 (0)	23 (5)	0.00	0 (0)	8 (6)	0.007
Wound infection	2 (1)	12 (3)	0.37	2 (1)	7 (5)	0.17
Portal thrombosis	1 (1)	3 (1)	1.007	1 (0.7)	2 (1)	1
Pulmonary	7 (4)	33 (8)	0.15	6 (4)	15 (11)	0.07
Cardiac	1 (1)	18 (4)	0.03	1 (0.7)	8 (6)	0.03
Renal	1 (1)	18 (4)	0.03	1 (0.7)	6 (4)	0.12
Reoperation, *n* (%)	0 (0)	7 (2)	0.19	0 (0)	2 (1)	0.5
Postoperative treatment, *n* (%)	3 (2)	19 (4)	0.15	3 (2)	10 (7)	0.08
Length of hospital stay median (range)	2 (1-23)	6 (1-203)	0.00	2 (1-23)	7 (1-203)	0.00
Mortality 90 d, *n* (%)	3 (2)	13 (3)	0.001	3 (2)	4 (3)	1

Continuous variables were compared using an independent sample *t*-test and Mann-Whitney *U* test. Categorical variables were compared using the chi-square test and Kruskal-Wallis test respectively. PSM: Propensity score matching; RFA: Radiofrequency ablation.

Increased life expectancy, ageing, and the accumulation of chronic pathologies, such as obesity, diabetes, and some inadequate living habits (excess alcohol and smoking) led to the establishment of conditions of oxidative stress and inflammation which seemed to be the substratum favouring the onset of HCC, despite the reduction in the incidence rate of HBV and HCV-related liver disease, especially in Western countries[11].

Table 3 Univariate and multivariate models for survival

	Univariate analysis	Multivariate analysis	
	P value	RR (95%CI)	*P* value
RFA	0.001	1.46 (1.1-1.79)	0.001
Age ≤ 75 yr	0.63		
Male	0.001		
Co-morbidity ≥ 2	0.001		
BMI < 24	0.001		
ASA score III-IV	0.07		
TBil (mg/dL > 2)	0.001		
Crea (mg/dL > 1)	0.57		
PLT (U/μL > 150 × 10^3)	0.001		
INR (> 1.3)	0.001	1.60 (1.03-2.49)	0.03
Tumor size < 3 cm	0.001		
Multiple nodule	0.001	1.19 (1.08-4.17)	0.03
Child Pugh A	0.001		
MELD > 10	0.007	1.89 (1.21-2.92)	0.005

The relative prognostic significance of the variables in predicting overall survival and overall recurrence was established using univariate and multivariate Cox proportional hazards regression models. Radiofrequency ablation, international normalized ratio > 1.3 mg/dL, and Model for End-Stage Liver Disease score >10 were independent risk factors for overall survival. RFA: Radiofrequency ablation; INR: International normalized ratio; MELD: Model for End-Stage Liver Disease; RR: Relative risk; CI: Confidence interval; BMI: Body mass index; ASA: American Society of Anesthesiologists; PLT: Platelets; TBil: Total bilirubin; Crea: Creatinine.

For this reason, our study aimed to examine a part of the population still growing today, that of the elderly, (≥ 70 years), and namely patients within Milan criteria unsuitable for liver transplantation due to a reached limit of age and who should be managed either with RFA or LR. We analyzed short-term outcomes, namely perioperative characteristics (operative time, postoperative complications, length of hospital stay, and mortality within 90 d), as well as long-term outcomes, namely oncological results (OS and disease-free survival). LR was the treatment of choice in patients with very early and early-stage HCC, with a well-preserved liver function and sufficient residual liver volume. Radiofrequency was indicated in patients who were not candidates to surgery, and who presented with higher rates of local disease control and OS than other local ablative therapies[2,12,13] inducing a tumor necrosis, which guarantees a valid control of margin[14]. Microwave ablation is an alternative procedure, equally based on induction tumor destruction with heat generation, but with a different mechanism, and which seems to show promising performances although in treatment of HCC of 3-5 cm size, adjacent to vessels or gallbladder[15].

The most effective therapeutic strategy in the very early and early stages of HCC was still a matter for debate.

Radiofrequency made use of less invasiveness, thereby presenting a shorter hospital stay, fewer costs, a lower rate of major complications, as shown in multiple randomized clinical trials and meta-analyses[16-20]. On the other hand, LR provided better oncological outcomes in terms of local disease control[17,19,21] and in long-term OS[17,22,23], as reported in randomized controlled trials and meta-analyses, also and above all, considering the characteristics of HCC which presented a tendency to micro-dissemination in portal and hepatic veins and to the generation of micro-metastases around the lesion[24,25].

Recent studies have shown that this therapeutic algorithm can also be extended to elderly patients[26-29]. Old age represented an important risk factor concerning postoperative morbidity and mortality, especially in association with major surgical procedures, but thanks to the evolution of surgical techniques and an increasingly careful postoperative management, it was possible to extend the resective treatment even to the most advanced age groups. According to these findings, our data also showed a shorter postoperative course in patients undergoing RFA, in terms of major

Table 4 Univariate and multivariate models for recurrence

	Univariate analysis	Multivariate analysis	
	***P* value**	**RR (95%CI)**	***P* value**
RFA	0.001	1.37 (1.17-1.60)	0.0001
Age ≤ 75 yr	0.28		
Male	0.91		
Co-morbidity ≥ 2	0.01		
BMI < 24	0.61		
ASA score III-IV	0.61		
Bilirubin (mg/dL > 2)	0.37		
Creatinine (mg/dL > 1)	0.62		
PLT (U/μL > 150 × 10^3)	0.001		
INR (> 1.3)	0.02		
Tumor size < 3 cm	0.001		
Multiple nodule	0.07		
Child Pugh A	0.26		
MELD > 10	0.01	1.51 (1.04-2.17)	0.03

The relative prognostic significance of the variables in predicting overall survival and overall recurrence was established using univariate and multivariate Cox proportional hazards regression models. Radiofrequency ablation and Model for End-Stage Liver Disease score > 10 were considered poor prognostic factors. RFA: Radiofrequency ablation; INR: International normalized ratio; MELD: Model for End-Stage Liver Disease; RR: Relative risk; CI: Confidence interval; BMI: Body mass index; ASA: American Society of Anesthesiologists; PLT: Platelets.

postoperative complications (Clavien-Dindo III-IV) (1% *vs* 9%, $P = 0.001$), length of hospital stay (median range: 2 d *vs* 6 d, $P = 0.001$), and also mortality rate within 90 d (0% *vs* 3%, $P = 0.02$).

Directly related to the type of procedure, data regarding operative time (median range: 25 min *vs* 205 min, $P = 0.001$) and percentage of perioperative blood transfusions (8% *vs* 15%, $P = 0.001$) highlighted the lower invasiveness of the ablative strategy. Although there were studies which showed overlapping[30] or even more satisfactory[31] results in the long-term outcomes of RFA managed patients, our work resulted in a clear superiority in patients managed with LR with a 1-, 3-, 5-year OS of 91.9%, 84%, and 75.5% as compared to 92%, 66.4%, and 37.8% for the RFA group ($P = 0.001$), and a 1-, 3-, and 5-year disease-free survival rate of 85.7%, 63.7%, and 50.3% for the LR group, and 66.7%, 37.8%, and 27.7% for the RFA group ($P = 0.001$).

To prevent any potential selection bias, we applied a PSM. Once co-morbidities, ASA and MELD score, tumor number, and tumor size of both groups had become balanced, we confirmed that LR provided better OS and disease-free survival than RFA treatment in elderly HCC patients with Milan criteria. On the other hand, the percentage of postoperative complications and blood transfusions, operative time, and length of hospital stay were also higher in the resection group even after PSM while the mortality value within 90 d was no longer significant. These data put emphasis on how important it was not only for the preoperative assessment of patients, which allowed us to choose a targeted and tailored therapeutic strategy, but also for the preoperative preparation of patients, which was related to the chosen procedure and made them less susceptible to any events secondary to the treatment itself, especially in elderly patients.

Elderly patients were often considered a high risk group for major surgery regarding the higher incidence of co-morbidities. In the past, this assumption seemed to have been a limit in the evaluation of the therapeutic choice. As a result, this category of patients was often undertreated, and this attitude may have distorted the previous results in terms of overall and recurrent survival.

This study had some limitations. First of all, because of its retrospective nature, there was a possibility of unavoidable selection bias. In addition, only 25.4% of patients undergoing RFA had a biopsy, although they had received a radiological diagnosis according to guidelines. Third, the surgical procedures adopted were very

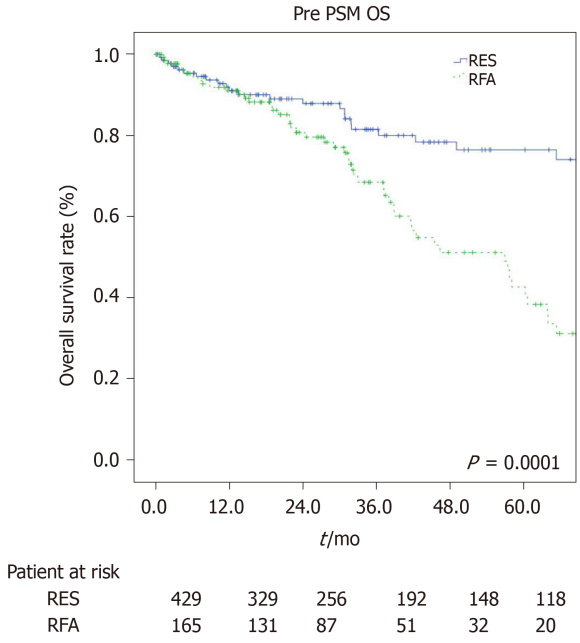


Figure 1 Survival curves (Kaplan-Meier method) of patients with hepatocellular carcinoma in Milan criteria who underwent surgical resection and radiofrequency ablation before propensity score matching. Overall survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. Overall survival significantly differs between the two groups. PSM: Propensity score matching; OS: Overall survival; RES: Resection; RFA: Radiofrequency ablation.

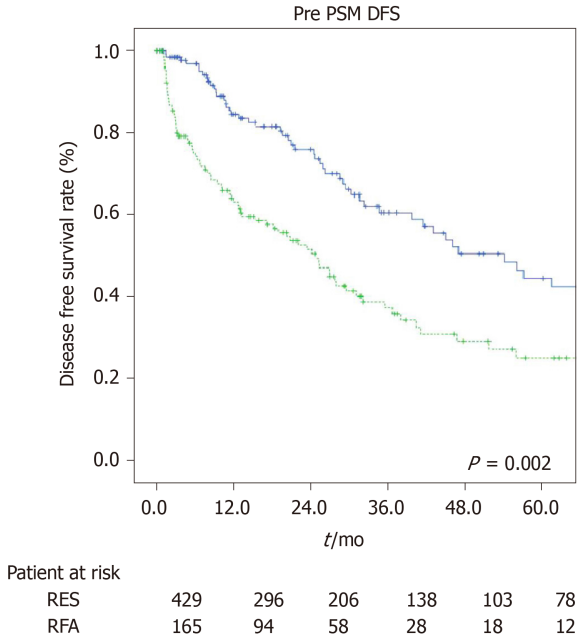


Figure 2 Tumor recurrence curves (Kaplan-Meier method) of patients with hepatocellular carcinoma in Milan criteria who underwent surgical resection and radiofrequency ablation before propensity score matching. Recurrence-free survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. hepatocellular carcinoma recurrence significantly differs between the two groups. PSM: Propensity score matching; DFS: Disease-free survival; RES: Resection; RFA: Radiofrequency ablation.

different, from anatomical to non-anatomical ones. In addition to this, there was the multicentric nature of the study which could be a determining factor in further bias in selection and evaluation criteria.

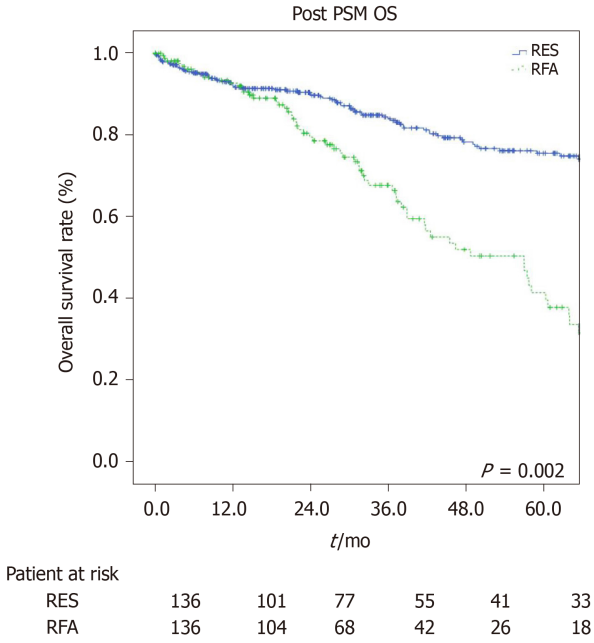


Figure 3 Survival curves (Kaplan-Meier method) of patients with hepatocellular carcinoma in Milan criteria who underwent surgical resection and radiofrequency ablation after propensity score matching. Overall survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. After propensity score matching, survival remained significantly different. PSM: Propensity score matching; OS: Overall survival; RES: Resection; RFA: Radiofrequency ablation.

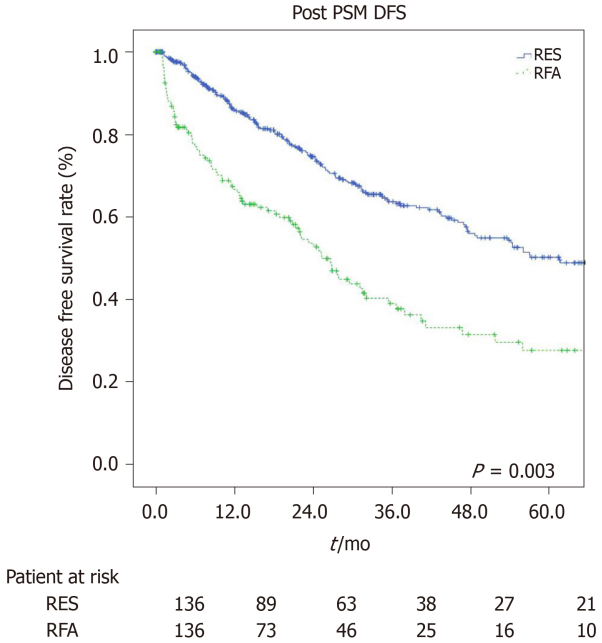


Figure 4 Tumor recurrence curves (Kaplan-Meier method) of patients with hepatocellular carcinoma in Milan criteria who underwent surgical resection and radiofrequency ablation after propensity score matching. Recurrence-free survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. After propensity score matching, recurrence remained significantly different. PSM: Propensity score matching; DFS: Disease-free survival; RES: Resection; RFA: Radiofrequency ablation.

CONCLUSION

In conclusion, our work reached similar results to the ones of several recent studies which had it that LR guaranteed better outcomes in terms of overall and disease-free survival than RFA in elderly HCC patients within Milan criteria, and so it was mandatory to outline the best therapeutic strategy without foreclosures, rather

respecting the parameters of patient selection and tailored treatment. LR should be considered for patients with a better liver function and a longer life expectancy, in order to balance the postoperative risk of treatment with benefits in long-term overall and disease-free survival.

ARTICLE HIGHLIGHTS

Research background

Surgical resection and radiofrequency ablation represent two alternative treatment for hepatocellular carcinoma.

Research motivation

Evaluation between surgery and radiofrequency ablation in elderly patients with hepatocellular carcinoma within Milan criteria.

Research objectives

To evaluate short- and long-term outcome in elderly patients (> 70 years) with HCC in Milan criteria, which underwent liver resection (LR) or radiofrequency ablation.

Research methods

Analysis of results from multicentric data about overall and disease-free survival linked to the two strategy.

Research results

Our data before and after propensity score matching show a better overall survival and disease-free survival at 1, 3, and 5 years in patient who underwent LR compared to ablation group.

Research conclusions

This retrospective multicenter study shows that LR provides better overall and disease-free survival despite a higher rate of post-operative complications and longer hospital stay when compared with ablation in elderly patients.

Research perspectives

With data from this retrospective multicenter study, a purpose of multicenter randomized controlled trials should be considered.

REFERENCES

1 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

2 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

3 **Cherqui D**, Laurent A, Mocellin N, Tayar C, Luciani A, Van Nhieu JT, Decaens T, Hurtova M, Memeo R, Mallat A, Duvoux C. Liver resection for transplantable hepatocellular carcinoma: long-term survival and role of secondary liver transplantation. *Ann Surg* 2009; **250**: 738-746 [PMID: 19801927 DOI: 10.1097/SLA.0b013e3181bd582b]

4 **Sogawa H**, Shrager B, Jibara G, Tabrizian P, Roayaie S, Schwartz M. Resection or transplant-listing for solitary hepatitis C-associated hepatocellular carcinoma: an intention-to-treat analysis. *HPB (Oxford)* 2013; **15**: 134-141 [PMID: 23036070 DOI: 10.1111/j.1477-2574.2012.00548.x]

5 **Spolverato G**, Vitale A, Ejaz A, Kim Y, Maithel SK, Cosgrove DP, Pawlik TM. The relative net health benefit of liver resection, ablation, and transplantation for early hepatocellular carcinoma. *World J Surg* 2015; **39**: 1474-1484 [PMID: 25665675 DOI: 10.1007/s00268-015-2987-7]

6 **Tandon P**, Garcia-Tsao G. Prognostic indicators in hepatocellular carcinoma: a systematic review of 72 studies. *Liver Int* 2009; **29**: 502-510 [PMID: 19141028 DOI: 10.1111/j.1478-3231.2008.01957.x]

7 **Zhao LY**, Huo RR, Xiang X, Torzilli G, Zheng MH, Yang T, Liang XM, Huang X, Tang PL, Xiang BD, Li LQ, You XM, Zhong JH. Hepatic resection for elderly patients with hepatocellular carcinoma: a systematic review of more than 17,000 patients. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 1059-1068 [PMID: 30145919 DOI: 10.1080/17474124.2018.1517045]

8 **Terminology Committee of the International Hepato-Pancreato-Biliary Association**; Strasberg SM, Belghiti J, Clavien P-A, Gadzijev E, Garden JO, Lau WY, Makuuchi M, Strong RW. The

Brisbane 2000 Terminology of Liver Anatomy and Resections. *HPB* 2000; **2**: 333-339
9 **Dindo D**, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205-213 [PMID: 15273542 DOI: 10.1097/01.sla.0000133083.54934.ae]
10 **Llovet JM**, Lencioni R. mRECIST for HCC: Performance and novel refinements. *J Hepatol* 2020; **72**: 288-306 [PMID: 31954493 DOI: 10.1016/j.jhep.2019.09.026]
11 **Petrick JL**, Florio AA, Znaor A, Ruggieri D, Laversanne M, Alvarez CS, Ferlay J, Valery PC, Bray F, McGlynn KA. International trends in hepatocellular carcinoma incidence, 1978-2012. *Int J Cancer* 2020; **147**: 317-330 [PMID: 31597196 DOI: 10.1002/ijc.32723]
12 **Lau WY**, Leung TW, Yu SC, Ho SK. Percutaneous local ablative therapy for hepatocellular carcinoma: a review and look into the future. *Ann Surg* 2003; **237**: 171-179 [PMID: 12560774 DOI: 10.1097/01.SLA.0000048443.71734.BF]
13 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]
14 **Ng KK**, Lam CM, Poon RT, Ai V, Tso WK, Fan ST. Thermal ablative therapy for malignant liver tumors: a critical appraisal. *J Gastroenterol Hepatol* 2003; **18**: 616-629 [PMID: 12753142 DOI: 10.1046/j.1440-1746.2003.02991.x]
15 **Yu J**, Yu XL, Han ZY, Cheng ZG, Liu FY, Zhai HY, Mu MJ, Liu YM, Liang P. Percutaneous cooled-probe microwave *vs* radiofrequency ablation in early-stage hepatocellular carcinoma: a phase III randomised controlled trial. *Gut* 2017; **66**: 1172-1173 [PMID: 27884919 DOI: 10.1136/gutjnl-2016-312629]
16 **Livraghi T**, Meloni F, Di Stasi M, Rolle E, Solbiati L, Tinelli C, Rossi S. Sustained complete response and complications rates after radiofrequency ablation of very early hepatocellular carcinoma in cirrhosis: Is resection still the treatment of choice? *Hepatology* 2008; **47**: 82-89 [PMID: 18008357 DOI: 10.1002/hep.21933]
17 **Huang J**, Yan L, Cheng Z, Wu H, Du L, Wang J, Xu Y, Zeng Y. A randomized trial comparing radiofrequency ablation and surgical resection for HCC conforming to the Milan criteria. *Ann Surg* 2010; **252**: 903-912 [PMID: 21107100 DOI: 10.1097/SLA.0b013e3181efc656]
18 **Feng K**, Yan J, Li X, Xia F, Ma K, Wang S, Bie P, Dong J. A randomized controlled trial of radiofrequency ablation and surgical resection in the treatment of small hepatocellular carcinoma. *J Hepatol* 2012; **57**: 794-802 [PMID: 22634125 DOI: 10.1016/j.jhep.2012.05.007]
19 **Wang Y**, Luo Q, Li Y, Deng S, Wei S, Li X. Radiofrequency ablation *vs* hepatic resection for small hepatocellular carcinomas: a meta-analysis of randomized and nonrandomized controlled trials. *PLoS One* 2014; **9**: e84484 [PMID: 24404166 DOI: 10.1371/journal.pone.0084484]
20 **Fang Y**, Chen W, Liang X, Li D, Lou H, Chen R, Wang K, Pan H. Comparison of long-term effectiveness and complications of radiofrequency ablation with hepatectomy for small hepatocellular carcinoma. *J Gastroenterol Hepatol* 2014; **29**: 193-200 [PMID: 24224779 DOI: 10.1111/jgh.12441]
21 **Xu XL**, Liu XD, Liang M, Luo BM. Radiofrequency Ablation *vs* Hepatic Resection for Small Hepatocellular Carcinoma: Systematic Review of Randomized Controlled Trials with Meta-Analysis and Trial Sequential Analysis. *Radiology* 2018; **287**: 461-472 [PMID: 29135366 DOI: 10.1148/radiol.2017162756]
22 **Ueno S**, Sakoda M, Kubo F, Hiwatashi K, Tateno T, Baba Y, Hasegawa S, Tsubouchi H; Kagoshima Liver Cancer Study Group. Surgical resection *vs* radiofrequency ablation for small hepatocellular carcinomas within the Milan criteria. *J Hepatobiliary Pancreat Surg* 2009; **16**: 359-366 [PMID: 19300896 DOI: 10.1007/s00534-009-0069-7]
23 **Yun WK**, Choi MS, Choi D, Rhim HC, Joh JW, Kim KH, Jang TH, Lee JH, Koh KC, Paik SW, Yoo BC. Superior long-term outcomes after surgery in child-pugh class a patients with single small hepatocellular carcinoma compared to radiofrequency ablation. *Hepatol Int* 2011; **5**: 722-729 [PMID: 21484104 DOI: 10.1007/s12072-010-9237-8]
24 **Shi M**, Zhang CQ, Zhang YQ, Liang XM, Li JQ. Micrometastases of solitary hepatocellular carcinoma and appropriate resection margin. *World J Surg* 2004; **28**: 376-381 [PMID: 15022021 DOI: 10.1007/s00268-003-7308-x]
25 **Sasaki A**, Kai S, Iwashita Y, Hirano S, Ohta M, Kitano S. Microsatellite distribution and indication for locoregional therapy in small hepatocellular carcinoma. *Cancer* 2005; **103**: 299-306 [PMID: 15578688 DOI: 10.1002/cncr.20798]
26 **Oweira H**, Petrausch U, Helbling D, Schmidt J, Mannhart M, Mehrabi A, Schöb O, Giryes A, Abdel-Rahman O. Early stage hepatocellular carcinoma in the elderly: A SEER database analysis. *J Geriatr Oncol* 2017; **8**: 277-283 [PMID: 28389117 DOI: 10.1016/j.jgo.2017.03.002]
27 **Lim KC**, Chow PK, Allen JC, Siddiqui FJ, Chan ES, Tan SB. Systematic review of outcomes of liver resection for early hepatocellular carcinoma within the Milan criteria. *Br J Surg* 2012; **99**: 1622-1629 [PMID: 23023956 DOI: 10.1002/bjs.8915]
28 **Kishida N**, Hibi T, Itano O, Okabayashi K, Shinoda M, Kitago M, Abe Y, Yagi H, Kitagawa Y. Validation of hepatectomy for elderly patients with hepatocellular carcinoma. *Ann Surg Oncol* 2015; **22**: 3094-3101 [PMID: 25582743 DOI: 10.1245/s10434-014-4350-x]
29 **Pompili M**, Saviano A, de Matthaeis N, Cucchetti A, Ardito F, Federico B, Brunello F, Pinna AD, Giorgio A, Giulini SM, De Sio I, Torzilli G, Fornari F, Capussotti L, Guglielmi A, Piscaglia F, Aldrighetti L, Caturelli E, Calise F, Nuzzo G, Rapaccini GL, Giuliante F. Long-term effectiveness of resection and radiofrequency ablation for single hepatocellular carcinoma ≤3 cm. Results of a

multicenter Italian survey. *J Hepatol* 2013; **59**: 89-97 [PMID: 23523578 DOI: 10.1016/j.jhep.2013.03.009]

30 **Chen MS**, Li JQ, Zheng Y, Guo RP, Liang HH, Zhang YQ, Lin XJ, Lau WY. A prospective randomized trial comparing percutaneous local ablative therapy and partial hepatectomy for small hepatocellular carcinoma. *Ann Surg* 2006; **243**: 321-328 [PMID: 16495695 DOI: 10.1097/01.sla.0000201480.65519.b8]

31 **Peng ZW**, Lin XJ, Zhang YJ, Liang HH, Guo RP, Shi M, Chen MS. Radiofrequency ablation *vs* hepatic resection for the treatment of hepatocellular carcinomas 2 cm or smaller: a retrospective comparative study. *Radiology* 2012; **262**: 1022-1033 [PMID: 22357902 DOI: 10.1148/radiol.11110817]

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ORIGINAL ARTICLE

Clinical Trials Study

Responses to faecal microbiota transplantation in female and male patients with irritable bowel syndrome

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Author contributions: El-Salhy M designed the study, obtained the funding, administered the study, recruited the patients, performed faecal microbiota transplantation, collected, analyzed and interpreted the data, and drafted the manuscript; Casen C contributed to the design of the study, analyzed the faecal bacteria and critically revised the manuscript for important intellectual content; Valeur J contributed to the design of the study, analyzed the short-chain fatty acids and critically revised the manuscript for important intellectual content; Hausken T and Hatlebakk JG contributed to the design of the study and to the analysis and interpretation of the data, and critically revised the manuscript for important intellectual content.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the University of Bergen.

Clinical trial registration statement:

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Abstract

BACKGROUND

Faecal microbiota transplantation (FMT) seems to be a promising treatment for irritable bowel syndrome (IBS) patients. In Western countries (United States and Europe), there is a female predominance in IBS. A sex difference in the response to FMT has been reported recently in IBS patients.

AIM

To investigate whether there was a sex difference in the response to FMT in the IBS patients who were included in our previous randomized controlled trial of the efficacy of FMT.

METHODS

The study included 164 IBS patients who participated in our previous randomized controlled trial. These patients had moderate-to-severe IBS symptoms belonging to the IBS-D (diarrhoea-predominant), IBS-C (constipation-predominant) and IBS-M (mixed) subtypes, and had not responded to the National Institute for Health and Care Excellence (NICE)-modified diet. They belonged in three groups: placebo (own faeces), and active treated group (30-g or 60-g superdonor faeces). The patients completed the IBS severity scoring system (IBS-SSS), Fatigue Assessment Scale (FAS) and the IBS quality of life scale (IBS-QoL) questionnaires

This study is registered at www.clinicaltrials.gov (NCT03822299) and www.cristin.no (ID657402).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrolment.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The authors have read the CONSORT 2010 statement, and the manuscript was prepared and revised according to the CONSORT 2010 statement.

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Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

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at the baseline and 2 wk, 1 mo and 3 mo after FMT. They also provided faecal samples at the baseline and 1 mo after FMT. The faecal bacteria profile and dysbiosis were determined using the 16S rRNA gene polymerase chain reaction DNA amplification covering V3-V9; probe labelling by single nucleotide extension and signal detection. The levels of short-chain fatty acids (SCFAs) were determined by gas chromatography and flame ionization.

RESULTS

There was no sex difference in the response to FMT either in the placebo group or active treated group. There was no difference between females and males in either the placebo group or actively treated groups in the total score on the IBS-SSS, FAS or IBS-QoL, in dysbiosis, or in the faecal bacteria or SCFA level. However, the response rate was significantly higher in females with diarrhoea-predominant (IBS-D) than that of males at 1 mo, and 3 mo after FMT. Moreover, IBS-SSS total score was significantly lower in female patients with IBS-D than that of male patients both 1 mo and 3 mo after FMT.

CONCLUSION

There was no sex difference in the response to FMT among IBS patients with moderate-to-severe symptoms who had previously not responded to NICE-modified diet. However, female patients with IBS-D respond better and have higher reduction of symptoms than males after FMT.

Key Words: Dysbiosis; Fatigue; Microbiome; Quality of life; Short-chain fatty acids; Superdonor

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Core Tip: A sex difference in the response to faecal microbiota transplantation (FMT) was previous reported for a subgroup of refractory irritable bowel syndrome (IBS) patients with severe bloating who had not responded to at least three conventional therapies for IBS. This subgroup only contained patients with diarrhoea-predominant (IBS-D) or mixed (IBS-M) IBS. The present study found no sex difference in the response to FMT among IBS patients with moderate-to-severe symptoms of IBS-D, constipation-predominant (IBS-C) and IBS-M. However, female patients with IBS-D respond better and have higher reduction of symptoms than males after FMT.

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INTRODUCTION

The gut microbiota plays an important role in the pathophysiology of irritable bowel syndrome (IBS)[1,2]. The composition of the gut bacteria in IBS patients differs from that of healthy subjects[2-6]. IBS patients have lower abundances of the butyrate-producing bacteria, *Erysipelotrichaceae* and *Ruminococcaceae* compared with healthy controls[7,8]. Methane-producing bacteria, *Methanobacteriales* were found to be more abundant in IBS patients with constipation as a predominant symptom (IBS-C) and less abundant in IBS patients with diarrhoea as a predominant symptom (IBS-D) compared with healthy individuals[7,8]. Moreover, IBS patients have been found to have increased abundances of *Veillonella*, *Lactobacillus* and *Ruminococcus* bacteria and decreased abundances of *Bifidobacterium*, *Faecalibacterium* and *Erysipelotrichaceae* methanogens[7,8]. IBS patients also have a lower diversity of gut bacteria (dysbiosis) than healthy subjects[4-6,9].

Faecal microbiota transplantation (FMT) has previously been performed in IBS patients in seven randomized controlled trials (RCTs)[10-16]. Four of these RCTs

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showed that FMT had good effects on symptoms and the quality of life[10,12,15,16]. while the other three RCTs found no effects[11,13,14]. It soon became clear that carefully selecting the donor based on clinical and microbial criteria as well as the dose of the transplant are important for a successful outcome of FMT[17].

In Western countries (United States and Europe) there is a sex difference in IBS, with a female:male ratio of 2:1[18-20]. However, in Asia there is no such female predominance[21-24]. A recently published RCT on FMT in IBS found that females responded better to FMT than did males[16]. A recent RCT of IBS patients performed by our group found that FMT led to marked reductions in IBS symptoms and fatigue and an improvement in the quality of life[12]. These improvements were accompanied by marked changes in the faecal bacteria profile and the profile of short-chain fatty acids (SCFAs) of the patients[12,25].

The present study investigated whether there is a sex difference in the response to FMT in terms of symptoms, dysbiosis, and bacteria and SCFA profiles in the same cohort of patients that we had investigated in our previous study[12].

MATERIALS AND METHODS

Study design and randomization of patients

The design of this study has been described in detail previously[12]. In brief, patients completed three questionnaires to assess their symptoms and quality of life at the baseline and 2 wk, 1 mo and 3 mo after FMT. They also provided faecal samples at the baseline and 1 mo after FMT. Polyethylene glycol and loperamide were allowed as rescue medication during the study. The patients were randomized 1:1:1 to placebo (own faeces), 30-g (superdonor faeces) or 60-g (superdonor faeces) FMT[12]. The 30- and 60-g superdonor-faeces groups were pooled together and called the active treated group in order to increase the sample size and reduce the probability of type-II statistical errors.

Patients

This study included 164 patients who had participated in our previous study[12]. The characteristics of these patients are given in Table 1. The patients enrolled in this study have been described in detail previously[12]. In brief, patients attending the outpatient clinic at Stord Hospital who fulfilled the Rome IV criteria for a diagnosis of IBS were recruited. All of the recruited patients had previously not responded to consuming the National Institute for Health and Care Excellence (NICE)-modified diet for at least 3 mo[12]. They also received a course of IBS treatment that slightly improved their symptoms.

The inclusion criteria were being aged between 18 and 75 years and having moderate-to-severe IBS symptoms, as indicated by a score of 175 on the IBS severity scoring system (IBS-SSS). The exclusion criteria were being pregnant or planning pregnancy, lactating, the presence of systemic disease, having immune deficiency or being treated by immune-modulating medication, or having a psychiatric illness, excessive alcohol consumption or drug abuse. Patients who took probiotics, antibiotics or IBS medications within 8 wk prior to study inclusion were also excluded[12].

Donor

The single superdonor used in this study has been described in detail previously[12]. Briefly, he was screened according to the European guidelines for FMT donors[26]. He was a healthy 36-year-old male, non-smoker, not taking any medication regularly and had a normal body mass index. He had been born *via* a vaginal delivery, breastfed and had taken only a few courses of antibiotics during his life. He exercised regularly and took sport-specific dietary supplements, which made his diet richer than average in protein, fibre, minerals and vitamins. He was normobiotic, but his faecal bacteria profile deviated from the healthy subjects abundance in 14 of the 39 bacteria markers[12].

Collection, preparation and administration of faecal samples

Faecal samples were frozen immediately and kept at -20 °C until they were delivered frozen to the laboratory, where they were kept at -80 °C. The process of FMT has been described in detail previously[12]. In brief, the patients randomized to the placebo FMT group received 30 g of their own faeces (autologous), while those in the 30-g and 60-g FMT groups received 30 g and 60 g of the superdonor's faeces (allogenic),

Table 1 Characteristics of the patients in the placebo and active treated groups

	Placebo				Active treated			
	Total	Females	Males	*P* value	Total	Females	Males	*P* value
n	**55**	**47**	**8**		**109**	**85**	**24**	
Age, yr (median, range)	38.5 (18-75)	38.0 (18-73)	47.0 (20-75)	0.3	39.0 (18-73)	40.0 (18-73)	32.0 (21-65)	0.07
IBS-D	21	19	2	0.7	42	30	12	0.4
IBS-C	22	18	4		40	32	8	
IBS-M	12	10	2		27	23	4	
IBS duration, yr	15.5 ± 7.9	16.2 ± 8.0	15.0 ± 9.0	0.9	17.3 ± 8.9	16.8 ± 8.2	18.0 ± 9.2	0.9
Age at IBS onset, yr (median, range)	20.0 (15-35)	20.5 (16-35)	19.0 (15-30)	0.4	20.0 (15-36)	20.0 (16-35)	20 (15-33)	0.6
IBS-SSS total score	315.2 ± 77.1	320.1 ± 77.8	286.9 ± 69.3	0.5	312.9 ± 82.0	319.1 ± 77.3	297.7 ± 82.0	0.4
Moderate symptoms[1] (%)	23 (42)	17 (36)	6 (75)	0.06	45 (41)	30 (35)	13 (54)	0.1
Severe symptoms[2] (%)	32 (58)	30 (64)	2 (25)		64 (59)	55 (65)	11 (46)	

Data are *n*, *n* (%) or mean ± SD values.

[1]Irritable bowel syndrome severity scoring system total score between 175 and 300.

[2]Irritable bowel syndrome severity scoring system total score of ≥ 300. IBS: Irritable bowel syndrome; IBS-D: Irritable bowel syndrome with diarrhoea-predominant; IBS-C: Irritable bowel syndrome with constipation-predominant; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; IBS-SSS: Irritable bowel syndrome severity scoring system.

respectively. The transplant was administered to the distal duodenum *via* a gastroscope[12].

Symptom and quality-of-life assessments

Symptoms were assessed using the IBS-SSS and the Fatigue Assessment Scale (FAS)[27-31]. Quality of life was measured using the IBS quality of life scale (IBS-QoL)[32-34]. Response was defined as a decrease of ≥ 50 points in the IBS-SSS total score after FMT.

Microbiome analysis and dysbiosis index

The faecal bacteria profile and dysbiosis were determined by the GA-map Dysbiosis Test (Genetic Analysis, Oslo, Norway) using the 16S rRNA gene polymerase chain reaction DNA amplification covering V3-V9; probe labelling by single nucleotide extension and signal detection by BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, United States)[6]. The bacterial markers used detected bacteria within 5 phyla (Firmicutes, Proteobacteria, Bacteroidetes, Tenericutes and Verrucomicrobia) that cover 10 bacterial classes, 36 genera and 32 species[6]. This test assesses > 300 bacteria at different taxonomic levels[9]. The dysbiosis index (DI) was measured on a 5-point scale from 1 to 5, where DI values 1-2 indicates normobiosis, 3-5 indicates dysbiosis[6].

Determination of faecal SCFA levels

The method used to determine faecal SCFA levels has been described in detail previously[25]. Briefly, the faecal samples were homogenized with a solution containing 3 mmol/L 2-ethylbutyric acid and 0.5 mmol/L H_2SO_4. The homogenate was vacuum distilled, and the SCFA levels were determined by gas chromatography (Agilent 7890 A, Agilent, CA, United States) using a capillary column (serial no. USE400345H, Agilent J&W GC columns, Agilent) and flame ionization[35,36] levels of total SCFAs, acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric acid, isocapronic and n-capronic acids, were determined and were expressed in units of mmol/kg wet weight.

Statistical analysis

The sample size required in each arm of the previously published trial was calculated by assuming that a placebo effect was 40% and an effect response was 80%. The total

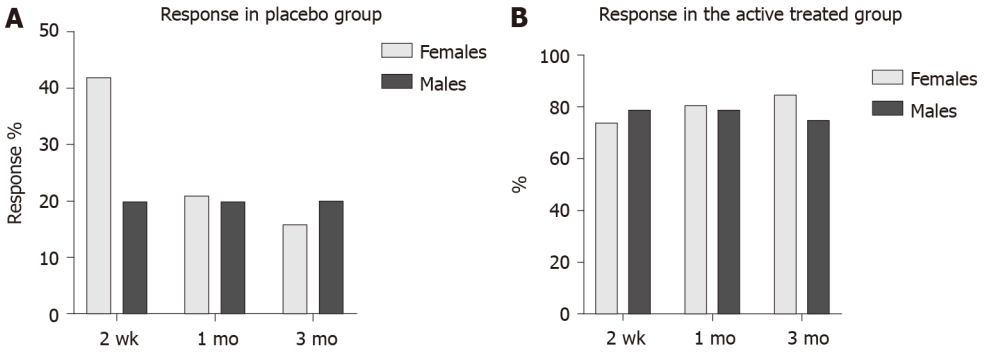


Figure 1 Response to faecal microbiota transplantation in females and males at different intervals after faecal microbiota transplantation in the two groups. A: Placebo group; B: Active treated group.

sample size was estimated to be 60 patients, with 20 in each arm ($\alpha = 0.05$, $1\text{-}\beta = 0.80$)[12]. In the present study a new calculation for the sample size was done based on the response rates obtained from our previous RCT[12]. Thus, assuming that the females' response is 90% and males' response is 60%, The total sample size was estimated to be 22 with 11 females and 11 males ($\alpha = 0.05$, $1\text{-}\beta = 0.80$). The 30- and 60-g superdonor-faeces groups were pooled together and called the active treated group in order to increase the sample size and reduce the probability of type-II statistical errors. Differences in response and dysbiosis between females and males in the placebo and the active treated group were analyzed using the χ^2 test. Differences between females and males in the total scores on the IBS-SSS, FAS and IBS-QoL, and in faecal bacteria and SCFA levels were analyzed using the Mann-Whitney test. These analyses were performed using GraphPad Prism (version 8, La Jolla, CA, United States).

Ethics

The Regional Committee for Medical and Health Research Ethics West, Bergen, Norway approved the study (approval No. 2017/1197/REK vest). All subjects provided both oral and written consents to participate. The study was registered at www.clinicaltrials.gov (NCT03822299) and www.cristin.no (ID657402).

RESULTS

Symptom and quality-of-life assessments

In the placebo group, the response did not differ between females and males at 2 wk, 1 mo and 3 mo after FMT ($P = 0.4$, 0.9 and 0.8, respectively). The responses in the active treated group did not differ between females and males after 2 wk, 1 mo and 3 mo ($P = 0.6$, 0.8 and 0.3, respectively) (Figure 1). The response rate was significantly higher in females with IBS-D than that of males at 1 mo, and 3 mo after FMT (Table 2 and Figure 2). There was no significant difference of response rates between female and male patients with either moderate or severe IBS symptoms (Table 3 and Figure 3).

The IBS-SSS total score did not differ significantly between female and male IBS patients in either the placebo or the active treated group (Table 4 and Figure 4). However, IBS-SSS total score was significantly lower in female patients with IBS-D than that of male patients both 1 mo and 3 mo after FMT (Table 5 and Figure 5). The IBS-SSS total score did not differ significantly between females and males in patients with moderate or severe IBS symptoms (Table 6 and Figure 3).

The FAS total score also did not differ significantly between female and male IBS patients in the active treated group (Table 7 and Figure 6), but it was lower in males than females in the placebo group at 3 mo after FMT. This could have been due to a type-I statistical error. There was no significant difference between female and male IBS patients belonging to different IBS-subtypes IBS symptoms (Table 8 and Figure 7). However, the FAS total score was lower in males IBS patients with IBS-D than that of females 2 wk after FMT.

The IBS-QoL total score did differ between females and males in both the placebo and active treated groups (Table 9 and Figure 8), being higher in males than in females at the baseline. IBS-QoL total scores did not differ significantly between female and male patients belonging to different IBS-subtypes (Table 10 and Figure 9).

Table 2 The response rates of females and males in different irritable bowel syndrome-subtypes at different intervals after faecal microbiota transplantation

Time after FMT	IBS-D			IBS-C			IBS-M		
	Females	Males	*P* value	Females	Males	*P* value	Females	Males	*P* value
2 wk (%)	73	58	0.3	65	50	0.7	72	55	0.3
1 mo (%)	90	42	**0.0003**	69	75	0.7	65	60	0.9
3 mo (%)	90	42	**0.0003**	70	75	0.3	63	80	0.6

IBS-D: Irritable bowel syndrome with diarrhoea-predominant symptom; IBS-C: Irritable bowel syndrome with constipation-predominant symptom; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; FMT: Faecal microbiota transplantation.

Table 3 The response rates in females and males with either moderate or severe irritable bowel syndrome symptoms

Time after FMT	Moderate symptoms[1]			Severe symptoms[2]		
	Females	Males	*P* value	Females	Males	*P* value
2 wk (%)	58	61	0.999	78	91	0.4
1 mo (%)	61	61	0.999	78	91	0.4
3 mo (%)	63	56	0.778	77	82	0.999

[1]Irritable bowel syndrome severity scoring system total score between 175 and 300.
[2]Irritable bowel syndrome severity scoring system total score of ≥ 300. FMT: Faecal microbiota transplantation.

Table 4 Irritable bowel syndrome severity scoring system total scores of females and males in the two study groups at different times after faecal microbiota transplantation

Time	Placebo		*P* value	Active treated		*P* value
	Females	Males		Females	Males	
0	320 ± 78	287 ± 69	0.2	319 ± 77	297 ± 82	0.3
2 wk	254 ± 106	256 ± 90	0.9	199 ± 102	205 ± 95	0.6
1 mo	277 ± 98	272 ± 89	0.8	196 ± 108	193 ± 94	0.9
3 mo	288 ± 90	266 ± 100	0.6	173 ± 116	183 ± 105	0.5

Data are mean ± SD values.

Microbiome analysis

The faecal bacteria levels in the placebo group did not differ between female and male IBS patients at the baseline and 1 mo after FMT (Table 11 and Figure 10). Similarly, there were no differences in the faecal bacteria levels between female and male IBS patients in the active treated group (Table 12 and Figure 11).

In the placebo group, 26 females (55%) and 4 males (50%) had dysbiosis ($P = 0.8$) at the baseline, while 25 females (53%) and 4 males (50%) had dysbiosis ($P = 0.9$) at 1 mo after FMT. In the active treated group, 52 females (61%) and 13 males (54%) had dysbiosis ($P = 0.3$) at the baseline, while 41 females (48%) and 9 males (38%) had dysbiosis ($P = 0.2$) at 1 mo after FMT.

Faecal SCFA levels

The faecal levels of total SCFAs and acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocapronic and capronic acids did not differ between female and males IBS patients in both the placebo and active treated groups at the baseline and 1 mo after FMT (Table 13 and Figure 12).

Table 5 The irritable bowel syndrome severity scoring system total scores in females and males belonging to different irritable bowel syndrome-sub-types

Time after FMT	IBS-D			IBS-C			IBS-M		
	Females	Males	*P* value	Females	Males	*P* value	Females	Males	*P* value
2 wk	190.5 ± 191.4	204.0 ± 92.2	0.6	228.1 ± 116.2	239.2 ± 113.8	0.5	202.8 ± 121.3	225.0 ± 65.8	0.5
1 mo	177.8 ± 94.9	226.9 ± 73.3	**0.02**	228.8 ± 118.1	215.8 ± 115.7	0.6	219.9 ± 136.6	197.0 ± 65.2	0.8
3 mo	157.8 ± 102.9	212.3 ± 96.9	**0.03**	212.8 ± 124.0	234.6 ± 131.8	0.5	219.2 ± 146.3	149.0 ± 36.0	0.5

Data are mean ± SD values. IBS-D: Irritable bowel syndrome with diarrhoea-predominant symptom; IBS-C: Irritable bowel syndrome with constipation-predominant symptom; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; FMT: Faecal microbiota transplantation.

Table 6 Irritable bowel syndrome severity scoring system total scores in females and males with moderate or severe irritable bowel syndrome symptoms

Time after FMT	Moderate symptoms[1]			Severe symptoms[2]		
	Females	Males	*P* value	Females	Males	*P* value
2 wk	166.5 ± 70.0	167.2 ± 55.8	0.8	225.1 ± 114.0	259.5 ± 102.3	0.3
1 mo	162.1 ± 73.1	179.4 ± 63.8	0.5	229.2 ± 120.2	214.1 ± 118.1	0.7
3 mo	155.3 ± 76.9	175.3 ± 100.2	0.5	214.6 ± 131.8	202.3 ± 120.6	0.8

Data are mean ± SD values.

[1]Irritable bowel syndrome severity scoring system total score between 175 and 300.

[2]Irritable bowel syndrome severity scoring system total score of ≥ 300. FMT: Faecal microbiota transplantation.

Table 7 Fatigue Assessment Scale total scores of females and males in the two study groups at different times after faecal microbiota transplantation

Time	Placebo			Active treated		
	Females	Males	*P* value	Females	Males	*P* value
0	31 ± 5	29 ± 4	0.3	32 ± 5	30 ± 5	0.09
2 wk	31 ± 6	29 ± 6	0.2	28 ± 6	28 ± 5	0.5
1 mo	31 ± 6	27 ± 7	0.1	27 ± 7	29 ± 5	0.5
3 mo	30 ± 4	26 ± 4	**0.01**	29 ± 6	27 ± 5	0.7

Data mean ± SD values.

DISCUSSION

The present study found that the response to FMT did not differ between females and males. Furthermore, the total scores on the IBS-SSS, FAS and IBS-QoL did not differ between females and males in the active treated groups before FMT and at different times after FMT. In the placebo group, the total score of IBS-QoL was higher in males than males and the FAS total score was lower in males than females 3 mo after FMT. This indicates that the effects of active treated FMT did not differ between males and females regarding IBS symptoms, fatigue and quality of life. Moreover, there was no difference between females and males regarding dysbiosis or the faecal bacteria.

SCFA profiles following FMT did not differ between females and males in both the placebo and the active treated groups. SCFAs regulate intestinal motility and the secretion and absorption of water and electrolytes[37,38]. Moreover, SCFAs increase also the secretion and up-regulate the gene expression of peptide YY (PYY)[39,40]. PYY is a mediator of the ileal brake and stimulates the absorption of water and electrolytes in the colon[37]. The faecal level of total SCFAs increased significantly in IBS patients after 1 mo and remained elevated at 1 year after FMT (unpublished

Table 8 Fatigue Assessment Scale total scores of females and males irritable bowel syndrome patients belonging to different irritable bowel syndrome-subtypes

Time after FMT	IBS-D			IBS-C			IBS-M		
	Females	Males	*P* value	Females	Males	*P* value	Females	Males	*P* value
2 wk	30.1 ± 3.6	27.0 ± 3.6	**0.04**	28.0 ± 6.3	29.3 ± 7.4	0.5	26.7 ± 5.4	26.3 ± 2.1	0.9
1 mo	27.1 ± 5.2	26.6 ± 4.6	0.6	27.1 ± 6.7	31.3 ± 6.1	0.1	28.3 ± 8.3	28.3 ± 4.0	0.9
3 mo	27.5 ± 5.7	27.8 ± 5.0	0.4	26.0 ± 6.2	29.2 ± 5.0	0.2	26.8 ± 7.6	24.8 ± 2.2	0.7

Data are mean ± SD values. IBS-D: Irritable bowel syndrome with diarrhoea-predominant symptom; IBS-C: Irritable bowel syndrome with constipation-predominant symptom; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; FMT: Faecal microbiota transplantation.

Table 9 Irritable bowel syndrome quality of life scale total scores of females and males in the two study groups at different times after faecal microbiota transplantation

Time	Placebo			Active treated		
	Females	Males	*P* value	Females	Males	*P* value
0	116 ± 20	130 ± 11	**0.03**	111 ± 23	114 ± 21	0.9
2 wk	123 ± 29	120 ± 23	0.7	122 ± 24	118 ± 27	0.6
1 mo	123 ± 26	121 ± 26	0.8	126 ± 24	119 ± 29	0.4
3 mo	112 ± 24	118 ± 26	0.2	132 ± 23	131 ± 25	0.9

Data are mean ± SD values.

Table 10 Irritable bowel syndrome quality of life scale total scores of females and males irritable bowel syndrome patients belonging to different irritable bowel syndrome-subtypes

Time after FMT	IBS-D			IBS-C			IBS-M		
	Females	Males	*P* value	Females	Males	*P* value	Females	Males	*P* value
2 wk	123.3 ± 98	123.7 ± 25.5	0.8	120.5 ± 23.1	102.4 ± 28.0	0.06	123.1 ± 24.6	131.5 ± 13.0	0.4
1 mo	131.3 ± 20.8	129.6 ± 28.2	0.9	121.9 ± 24.4	111.4 ± 29.1	01	125.4 ± 25.2	130.5 ± 11.3	0.8
3 mo	136.4 ± 16.6	134.5 ± 22.7	0.9	128.7 ± 24.4	129.1 ± 31.3	0.6	129.5 ± 28.6	124.8 ± 22.0	0.5

Data are mean ± SD values. IBS-D: Irritable bowel syndrome with diarrhoea-predominant symptom; IBS-C: Irritable bowel syndrome with constipation-predominant symptom; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; FMT: Faecal microbiota transplantation.

data)[25]. Similarly, the faecal level of butyric acid increased in IBS patients after 1 mo and remained elevated at 1 year after FMT (unpublished data)[25]. Butyrate is a major energy source for colonic epithelial cells, interacts with the immune response, modulates the oxidative stress, and decreases both intestinal-cell permeability and intestinal motility[41]. Butyrate modulates also colonic hypersensitivity[42-44]. At 1 year after FMT levels of isobutyric and isovaleric acids were increased in IBS patients, indicating a shift in microbial fermentation from a saccharolytic to a proteolytic pattern[45]. It is worthy of note that in IBS patients, who adhered to a low-FODMAPs (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) diet an increase in the levels of isobutyric and isovaleric acids have been found[46]. Moreover, the level of acetic acid which induces visceral hypersensitivity decreased significantly at 1 year after FMT[47].These changes in SCFAs after FMT appear to be one of the mechanisms underlying the effects seen in IBS patients after FMT. That is why the difference between females and males regarding the changes in SCFAs was assessed.

Holvoet *et al*[16] reported that females responded better to FMT than did males. That RCT differed from the present study in terms of the characteristics of the included patients, the size of the patient cohort and the dose of the faecal transplants[16]. The trial of Holvoet *et al*[16] included a subgroup of refractory IBS

Table 11 Faecal bacteria levels in the female and male irritable bowel syndrome patients in the placebo group at the baseline and 1 mo after faecal microbiota transplantation

Bacteria	Baseline		1 mo after FMT	
	Females	Males	Females	Males
Actinobacteria	-0.235 ± 0.763	-0.365 ± 0.768	-0.250 ± 0.954	-0.375 ± 0.838
Actinomycetales	0.118 ± 0.382	-0.212 ± 0.536	0.175 ± 0.594	0.100 ± 0.496
Bifidobacterium spp.	-0.020 ± 0.607	-0.154 ± 0.539	0.025 ± 0.660	-0.075 ± 0.572
Alistipes	-0.863 ± 0.895	-0.885 ± 0.900	-0.875 ± 0.853	-0.800 ± 0.709
Alistipes onderdonkii	-0.667 ± 0.792	-0.615 ± 0.718	-0.650 ± 0.834	-0.550 ± 0.783
Bacteroides fragilis	-0.255 ± 0.689	-0.212 ± 0.637	0.175 ± 0.501	0.050 ± 0.221
Bacteroides spp. and *Prevotella spp.*	-0.980 ± 1.157	-0.885 ± 1.182	-0.750 ± 1.032	-0.900 ± 0.687
Bacteroides stercoris	-0.137 ± 0.448	-0.154 ± 0.415	0.025 ± 0.158	0.100 ± 0.304
Bacteroides zoogleoformans	0.078 ± 0.272	0.038 ± 0.194	0.025 ± 0.158	0 ± 0
Parabacteroides johnsonii	0.039 ± 0.196	0.077 ± 0.269	0.050 ± 0.221	0.100 ± 0.304
Parabacteroides spp.	-0.451 ± 0.642	-0.327 ± 0.706	-0.425 ± 0.747	-0.225 ± 0.480
Firmicutes	-0.431 ± 0.575	-0.385 ± 0.566	-0.325 ± 0.526	-0.400 ± 0.591
Bacilli	0.235 ± 1.124	0.192 ± 0.991	0.271 ± 1.132	0.150 ± 1.001
Catenibacterium mitsuokai	0.000 ± 0.400	0.135 ± 0.525	0.050 ± 0.289	0.100 ± 0.441
Clostridia	-0.020 ± 0.244	-0.077 ± 0.269	-0.025 ± 0.276	0.0 ± 0.036
Clostridium spp.	0.039 ± 0.196	0.038 ± 0.194	0.0 ± 0.0	0.050 ± 0.316
Dialister invisus	0.118 ± 0.381	-0.173 ± 0.474	0.200 ± 0.405	0.225 ± 0.158
Dialister invisus and *Megasphaera micronuciformis*	0.059 ± 0.238	0.173 ± 0.474	0.125 ± 0.335	0.025 ± 0.158
Dorea spp.	0.569 ± 0.700	0.500 ± 0.700	0.625 ± 0.628	0.667 ± 0.806
Eubacterium biforme	0.412 ± 0.753	0.269 ± 0.598	0.275 ± 0.640	0.400 ± 0.633
Eubacterium hallii	0.804 ± 0.939	0.673 ± 0.879	0.655 ± 0.730	0.650 ± 0.597
Eubacterium rectale	0.078 ± 0.337	0.058 ± 0.235	0.050 ± 0.221	0.025 ± 0.158
Eubacterium siraeum	-1.412 ± 0.963	-1.288 ± 0.161	-1.475 ± 1.086	-1.200 ± 1.265
Faecalibacterium prausnitzii	-0.431 ± 0.671	-0.500 ± 0.804	-0.550 ± 0.745	-0.500 ± 0.599
Lachnospiraceae	0.196 ± 0.566	0.269 ± 0.630	0.325 ± 0.730	0.275 ± 0.640
Lactobacillus ruminis and *Pediococcus acidilactici*	0.059 ± 0.311	0.077 ± 0.334	0.0 ± 0.0	0.025 ± 0.158
Lactobacillus spp.	0.353 ± 0.594	0.269 ± 0.528	0.325 ± 0.616	0.475 ± 0.680
Phascolarctobacterium spp.	0.078 ± 0337	0.077 ± 0.337	0.125 ± 0.404	0.075 ± 0.350
Ruminococcus albus and *Ruminococcus bromii*	0.353 ± 0.658	0.404 ± 0.721	0.325 ± 0.616	0.450 ± 0.749
Ruminococcus gnavus	0.431 ± 0.878	0.577 ± 0.878	0.450 ± 0.815	0.325 ± 0.764
Streptococcus agalactiae & Eubacterium rectale	0.157 ± 0.367	0.250 ± 0.480	0.110 ± 0.304	0.125 ± 0.345
Streptococcus salivarius ssp. Thermophilus and *Streptococcus sanguinis*	0.412 ± 0.606	0.346 ± 0.556	0.675 ± 0.888	0.475 ± 0.751
Streptococcus salivarius ssp. thermophilus	0.628 ± 0.871	0.577 ± 0.915	0.500 ± 0.934	0.600 ± 0.928
Streptococcus spp.	0.471 ± 0.833	0.423 ± 0.696	0.400 ± 0.709	0.450 ± 0.815
Veillonella spp.	-0.177 ± 0.518	-0.173 ± 0.648	-0.175 ± 0.385	-0.150 ± 0.534
Proteobacteria	0.294 ± 0.576	0.289 ± 0.499	0.275 ± 0.599	0.325 ± 0.616
Shigella spp. and *Escherichia spp.*	-0.275 ± 0.940	-0.212 ± 0.893	-0.200 ± 0.853	-0.335 ± 0.920
Mycoplasma hominis	-0.451 ± 0.503	-0.404 ± 0.496	-0.450 ± 0.504	-0.450 ± 0.503

Akkermansia muciniphila	0.471 ± 0.644	0.365 ± 0.627	0.450 ± 0.714	0.650 ± 0.802

The bacterial levels are mean ± SD relative values to a normobiotic microbiota profile of 165 healthy subjects. FMT: Faecal microbiota transplantation.

patients with severe bloating who had not responded to at least three conventional therapies for IBS. This subgroup contained only patients with the IBS-D or mixed (IBS-M) subtypes. The patients included in the present study had moderate-to-severe IBS symptoms belonging to the IBS-D, IBS-C and IBS-M subtypes, and had not responded to the NICE-modified diet. The patient cohort investigated by Holvoet *et al*[16] included 62 patients: 19 in the placebo group and 43 in the active treated group. The present study investigated a cohort of 164 patients: 55 in the placebo group and 109 in the active treated group. It is worthy of note that in the trial of Holvoet *et al*[16] included 30 females and 13 males in the active treated group and 8 females and 11 males in the placebo group. The present study included 85 females and 24 males in the active treated group and included 47 females and 8 males in the placebo group. Thus, this makes the present study less constrained than the RCT of Holvoet *et al*[16] regarding power and sample sizes. Moreover, the dose of the faecal transplant from the donor was not reported for the RCT by Holvoet *et al*[16], while in the present study the active treated group received either 30 g or 60 g of a superdonor transplant. In our previously published RCT we showed that the response rates increased with increased dose[12]. These differences make it difficult to compare the outcomes of the present study with those of Holvoet *et al*[16]. However, in the present study, female patients with IBS-D had a significant higher response rate to FMT and lower IBS-SSS score after FMT than males. These observations could explain the discrepancy between the findings of Holvoet *et al*[16] and the present study as in Holvoet *et al*[16] study the cohort of patients included were only IBS-D and IBS-M IBS-subtypes.

CONCLUSION

In conclusion, there is no sex difference in the response to FMT in IBS patients with moderate-to-severe IBS symptoms belonging to the three of IBS subtypes of IBS-C and IBS-M in patients who did not responded to NICE-modified diet. Female patients with IBS-D had a significant higher response rate to FMT and lower IBS-SSS score after FMT than males.

Table 12 Gut bacteria levels in female and male irritable bowel syndrome patients in the active treated group at the baseline and 1 mo after faecal microbiota transplantation

Bacteria	Baseline		1 mo after FMT	
	Females	Males	Females	Males
Actinobacteria	-0.250 ± 0.954	-0.375 ± 0.838	-0.250 ± 0.719	-0.2350 ± 0.636
Actinomycetales	0.175 ± 0.594	0.100 ± 0.496	0.068 ± 0255	0.145 ± 0.412
Bifidobacterium spp.	0.025 ± 0.660	-0.075 ± 0.572	-0.045 ± 0.526	-0.063 ± 0.433
Alistipes	-0.875 ± 0.853	-0.800 ± 0.709	-0.886 ± 0.869	-0.783 ± 0.821
Alistipes onderdonkii	-0.650 ± 0.834	-0.550 ± 0.783	-0.523 ± 0.699	-0.354 ± 0.565
Bacteroides fragilis	0.175 ± 0.501	0.050 ± 0.221	0.159 ± 0.480	0.104 ± 0.371
Bacteroides spp. and *Prevotella spp.*	-0.750 ± 1.032	-0.800 ± 0.687	-1.091 ± 1.996	-0.708 ± 0.967
Bacteroides stercoris	0.025 ± 0.158	0.100 ± 0.304	0.023 ± 0.151	0.146 ± 0.357
Bacteroides zoogleoformans	0.025 ± 0.158	0 ± 0	0.091 ± 0.291	0.083 ± 0.347
Parabacteroides johnsonii	0.050 ± 0.221	0.100 ± 0.304	0.045 ± 0.302	0.021 ± 0.144
Parabacteroides spp.	-0.425 ± 0.747	-0.225 ± 0.480	-0.455 ± 0.504	-0.313 ± 0.468
Firmicutes	-0.325 ± 0.526	-0.400 ± 0.591	-0.546 ± 0.627	-0.454 ± 0.483
Bacilli	0.271 ± 1.132	0.150 ± 1.001	0.205 ± 1.047	0.042 ± 0.824
Catenibacterium mitsuokai	0.050 ± 0.289	0.100 ± 0.441	0.023 ± 0.151	0.104 ± 0.515
Clostridia	-0.025 ± 0.276	0.0 ± 0.036	0.068 ± 0.255	0.021 ± 252
Clostridium spp.	0.0 ± 0.0	0.050 ± 0.316	0.223 ± 0.151	0.063 ± 0.245
Dialister invisus	0.200 ± 0.405	0.225 ± 0.158	0.091 ± 0.362	0.146 ± 0.505
Dialister invisus and *Megasphaera micronuciformis*	0.125 ± 0.335	0.025 ± 0.158	0.068 ± 0.034	0.104 ± 0.308
Dorea spp.	0.625 ± 0.628	0.667 ± 0.806	0.727 ± 0.758	0.663 ± 0.796
Eubacterium biforme	0.275 ± 0.640	0.400 ± 0.633	0.477 ± 0.791	0.563 ± 0.769
Eubacterium hallii	0.655 ± 0.730	0.550 ± 0.597	0.886 ± 0.993	0.979 ± 1.021
Eubacterium rectale	0.050 ± 0.221	0.025 ± 0.158	0-068 ± 0.255	0.042 ± 0.202
Eubacterium siraeum	-1.475 ± 1.086	-1.200 ± 1.265	-1.295 ± 0.930	-1.208 ± 0.988
Faecalibacterium prausnitzii	-0.550 ± 0.745	-0.500 ± 0.599	-0.568 ± 0.759	-0.521 ± 0.825
Lachnospiraceae	0.325 ± 0.730	0.275 ± 0.640	0.205 ± 0.553	0.125 ± 0.489
Lactobacillus ruminis and *Pediococcus acidilactici*	0.0 ± 0.0	0.025 ± 0.158	0.021 ± 0.146	0.188 ± 0.571
Lactobacillus spp.	0.325 ± 0.616	0.475 ± 0.680	0.500 ± 0.731	0.583 ± 0.679
Phascolarctobacterium spp.	0.125 ± 0.404	0.075 ± 0.350	0.091 ± 0.362	0.083 ± 0.347
Ruminococcus albus and *Ruminococcus bromii*	0.325 ± 0.616	0.450 ± 0.749	0.205 ± 0.553	0.271 ± 0.574
Ruminococcus gnavus	0.450 ± 0.815	0.325 ± 0.764	0.364 ± 0.810	0.250 ± 0.636
Streptococcus agalactiae & Eubacterium rectale	0.110 ± 0.304	0.125 ± 0.345	0.267 ± 0.495	0.083 ± 0.279
Streptococcus salivarius ssp. thermophilus and *Streptococcus sanguinis*	0.675 ± 0.888	0.475 ± 0.751	0.455 ± 0.504	0.292 ± 0.459
Streptococcus salivarius ssp. thermophilus	0.500 ± 0.934	0.600 ± 0.928	0.523 ± 0.821	0.604 ± 0.844
Streptococcus spp.	0.400 ± 709	0.450 ± 0.815	0.444 ± 0.841	0.396 ± 0.610
Veillonella spp.	-0.175 ± 0.385	-0.150 ± 0.534	-0.273 ± 0.544	-0.208 ± 0.504
Proteobacteria	0.275 ± 0.599	0.325 ± 0.616	0.717 ± 0.750	0.583 ± 0.498
Shigella spp. and *Escherichia spp.*	-0.200 ± 0.853	-0.335 ± 0.920	-0.151 ± 1.077	-0.188 ± 0.790
Mycoplasma hominis	-0.450 ± 0.504	-0.450 ± 0.503	-0.500 ± 0.506	-0.479 ± 0.505

Akkermansia muciniphila	0.450 ± 0.714	0.650 ± 0.802	0.741 ± 0.713	0.813 ± 0.915

The bacterial levels are relative values to a normobiotic microbiota profile of 165 healthy subjects (mean ± SD). FMT: Faecal microbiota transplantation.

Table 13 The short-chain fatty acids concentration in the faeces of the placebo group and the patients that received donor's faecs (faecal microbiota transplantation) at the baseline and 1 mo after faecal microbiota transplantation

Acids	Placebo				Active treated			
	Baseline		1 mo after FMT		Baseline		1 mo after FMT	
	Females	Males	Females	Males	Females	Males	Females	Males
Total SCFAs	72 ± 37	69 ± 23.	73 ± 37	69 ± 23	77 ± 40	72 ± 40	87 ± 42	89 ± 26
Acetic acid	42 ± 18	40 ± 15	41 ± 17	40 ± 14	44 ± 21	44 ± 20	46 ± 13	40.2 ± 15.0
Propionic acid	12 ± 8	11 ± 5	12 ± 8	11 ± 5	13 ± 10	13 ± 8	14 ± 4	11 ± 7
Iso-butyric acid	2 ± 2	1 ± 1	1 ± 2	1 ± 1	1 ± 1	1 ± 1	2 ± 2	1 ± 1
Butyric acid	14 ± 9	12 ± 6	13 ± 8	12 ± 6	11 ± 8	12 ± 9	18 ± 14	16 ± 10
Iso-valeric acid	2 ± 2	2 ± 1	2 ± 2	2 ± 1	2 ± 1	2 ± 2	2 ± 2	2 ± 1
Valeric acid	2 ± 2	1 ± 1	2 ± 2	1 ± 1	2 ± 2	2 ± 2	2 ± 1	2 ± 1
Iso-capronic acid	0.1 ± 0.04	0.01 ± 0.07	0.5 ± 0.7	0.4 ± 0.7	0.0 ± 0.0	0.02 ± 0.08	0.01 ± 0.04	0.0 ± 0.0
Capronic acid	0.5 ± 0.7	0.5 ± 0.7	0.01 ± 0.04	0.1 ± 0.08	0.7 ± 1.3	0.6 ± 0.8	0.6 ± 0.9	0.5 ± 0.9

The values were expressed as mmol/kg wet weight (mean ± SD). FMT: Faecal microbiota transplantation; SCFAs: Short-chain fatty acids.

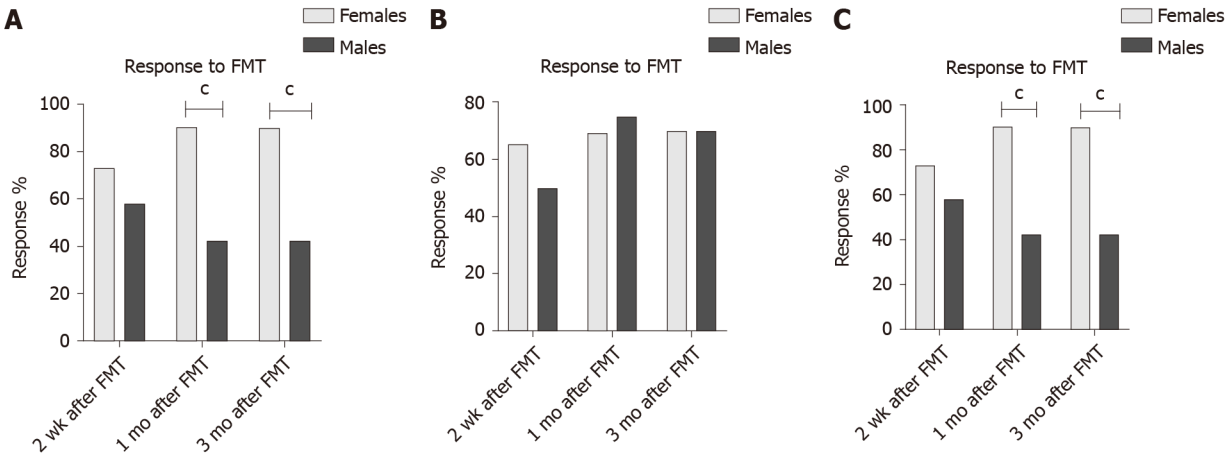


Figure 2 Response rates to faecal microbiota transplantation of female and male irritable bowel syndrome patients. A: Irritable bowel syndrome with diarrhoea-predominant; B: Irritable bowel syndrome with constipation-predominant; C: Irritable bowel syndrome with mixed diarrhoea and constipation. $^{c}P < 0.001$. FMT: Faecal microbiota transplantation.

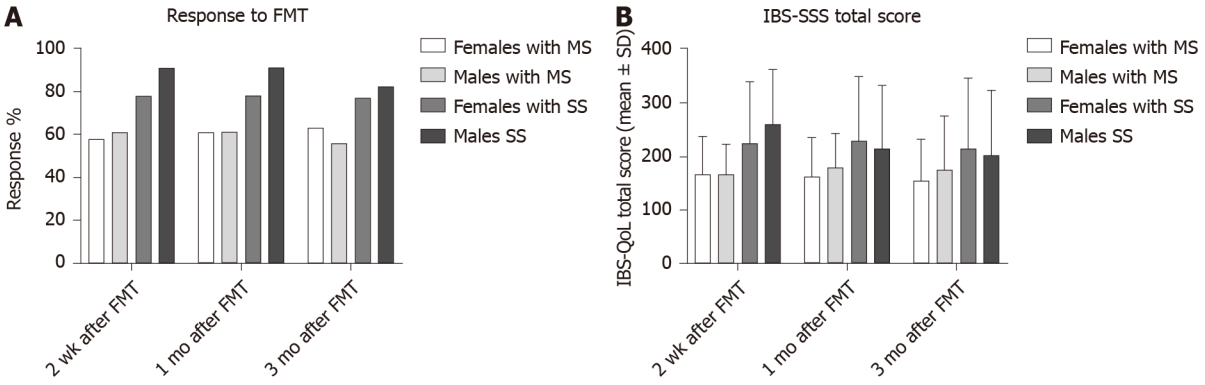


Figure 3 Response rates to faecal microbiota transplantation and the total irritable bowel syndrome-severity scoring system scores of irritable bowel syndrome patients with moderate irritable bowel syndrome symptoms (irritable bowel syndrome-severity scoring system total score between 175 and 300) and with severe irritable bowel syndrome symptoms (irritable bowel syndrome-severity scoring system total score of ≥ 300). A: Faecal microbiota transplantation; B: Irritable bowel syndrome severity scoring system total score. MS: Moderate irritable bowel syndrome symptoms; SS: Severe irritable bowel syndrome symptoms; FMT: Faecal microbiota transplantation.

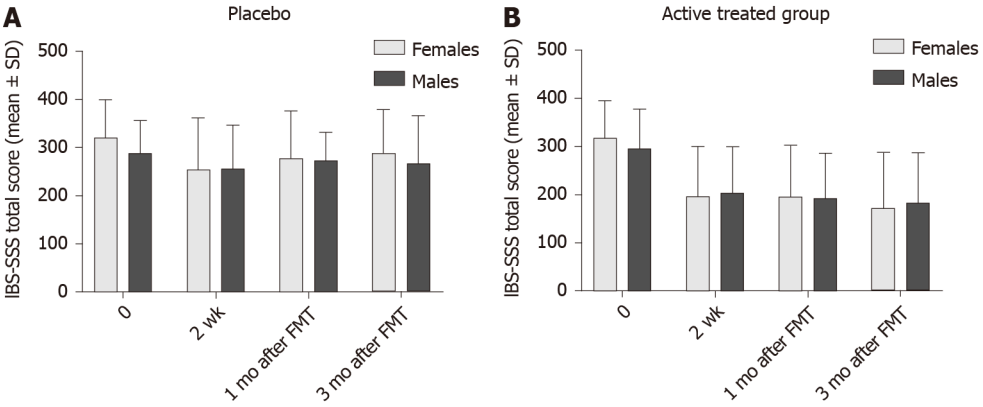


Figure 4 The total irritable bowel syndrome-severity scoring system scores in females and males. A: Placebo group; B: Active treated group. FMT: Faecal microbiota transplantation.

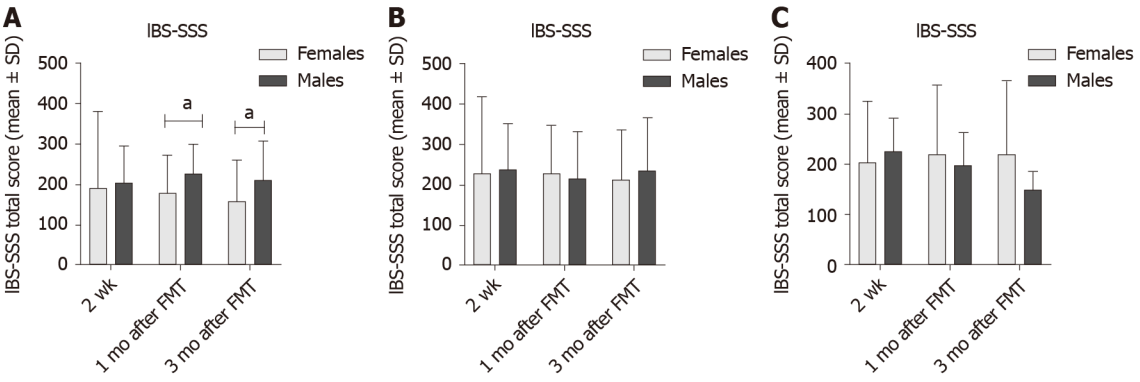


Figure 5 The total irritable bowel syndrome-severity scoring system scores in females and males. A: Irritable bowel syndrome with diarrhoea-predominant; B: Irritable bowel syndrome with constipation-predominant; C: Irritable bowel syndrome with mixed diarrhoea and constipation. $^{a}P < 0.05$. IBD-SSS: Irritable bowel syndrome-severity scoring system; FMT: Faecal microbiota transplantation.

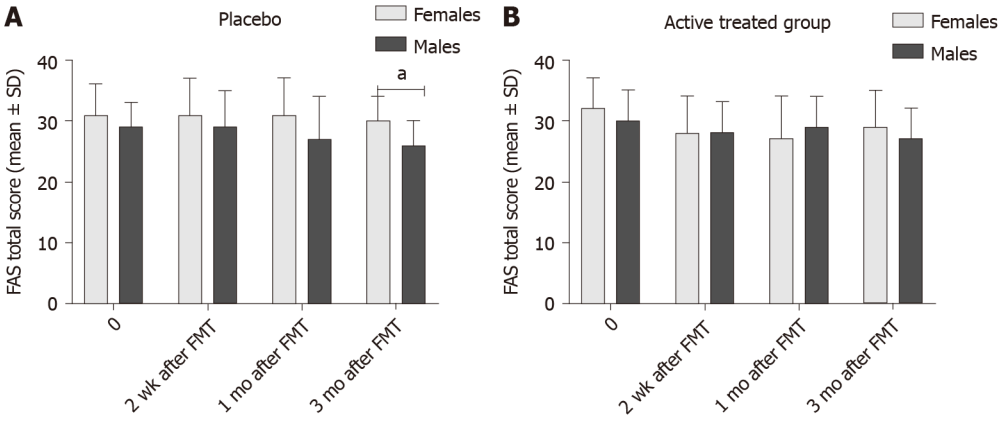


Figure 6 The total Fatigue Assessment Scale scores in female and male irritable bowel syndrome patients. A: Placebo group; B: Active treated group. [a]*P* < 0.05. FAS: Fatigue Assessment Scale; FMT: Faecal microbiota transplantation.

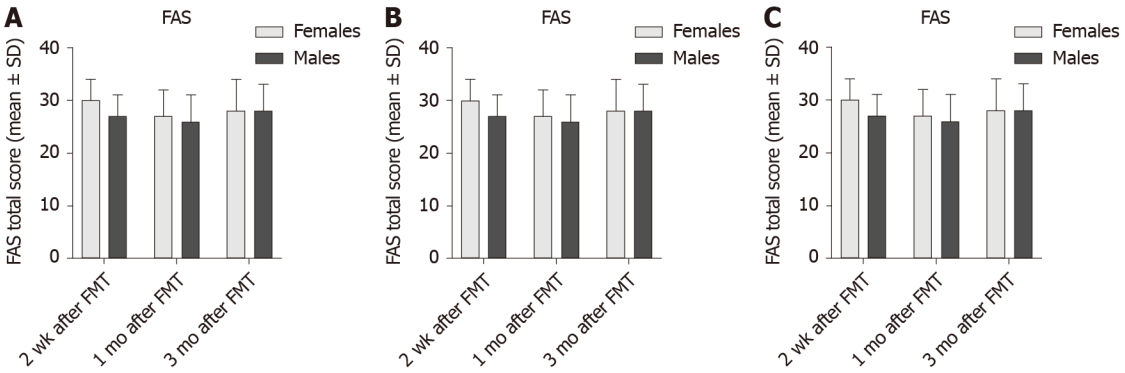


Figure 7 Total Fatigue Assessment Scale scores in female and male patients. A: Irritable bowel syndrome with diarrhoea-predominant; B: Irritable bowel syndrome with constipation-predominant; C: Irritable bowel syndrome with mixed diarrhoea and constipation. FAS: Fatigue Assessment Scale; FMT: Faecal microbiota transplantation.

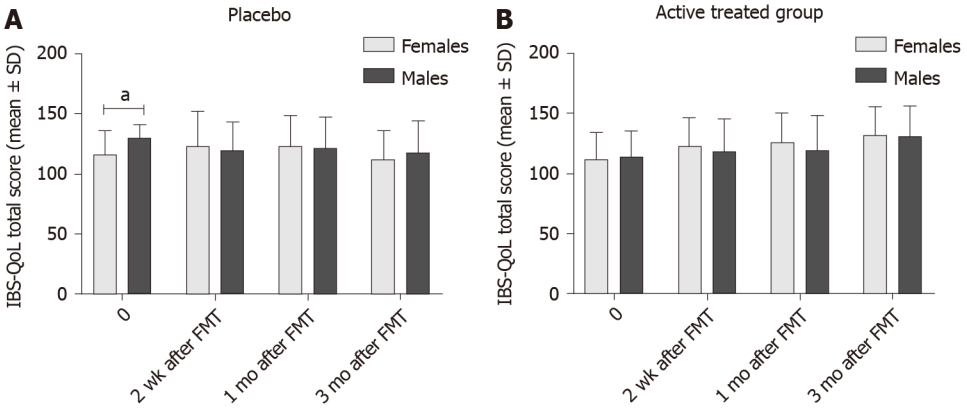


Figure 8 Total irritable bowel syndrome quality of life scale scores in female and male patients. A: Placebo group; B: Active treated group. [a]*P* < 0.05. IBS-QoL: Irritable bowel syndrome quality of life scale; FMT: Faecal microbiota transplantation.

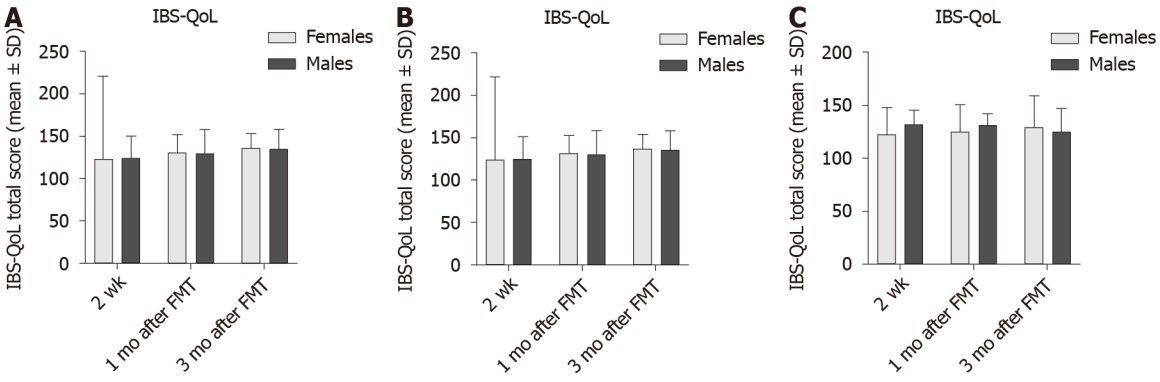


Figure 9 Total irritable bowel syndrome quality of life scale scores in females and males. A: Irritable bowel syndrome with diarrhoea-predominant; B: Irritable bowel syndrome with constipation-predominant; C: Irritable bowel syndrome with mixed diarrhoea and constipation. IBS-QoL: Irritable bowel syndrome quality of life scale; FMT: Faecal microbiota transplantation.

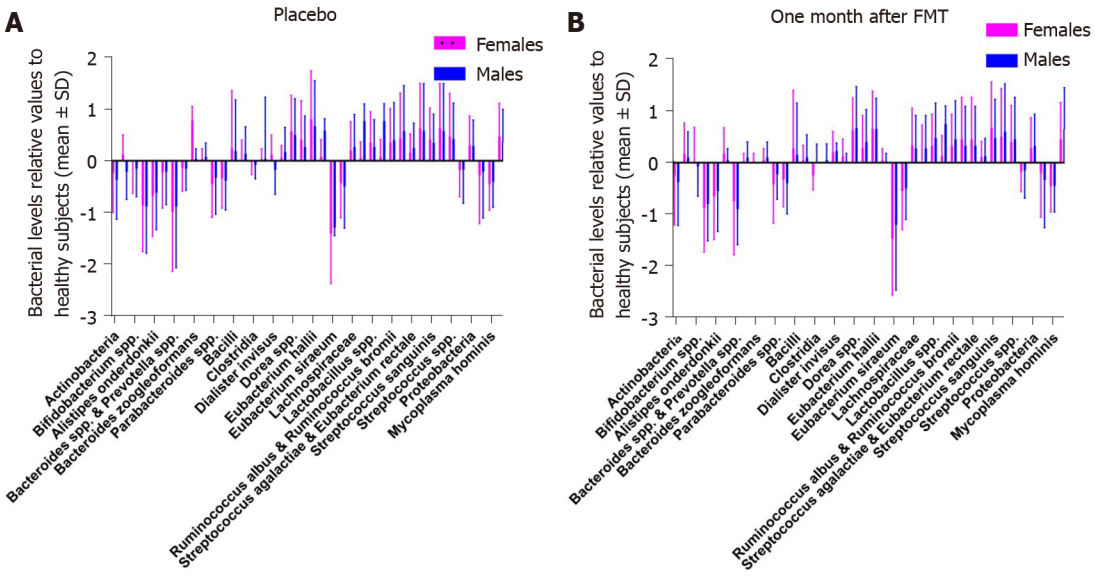


Figure 10 Bacteria levels in the faeces of female and male irritable bowel syndrome patients in the placebo group at the baseline and 1 mo after faecal microbiota transplantation. A: Baseline; B: 1 mo after faecal microbiota transplantation. The bacterial levels are relative values to a normobiotic microbiota profile of 165 healthy subjects. FMT: Faecal microbiota transplantation.

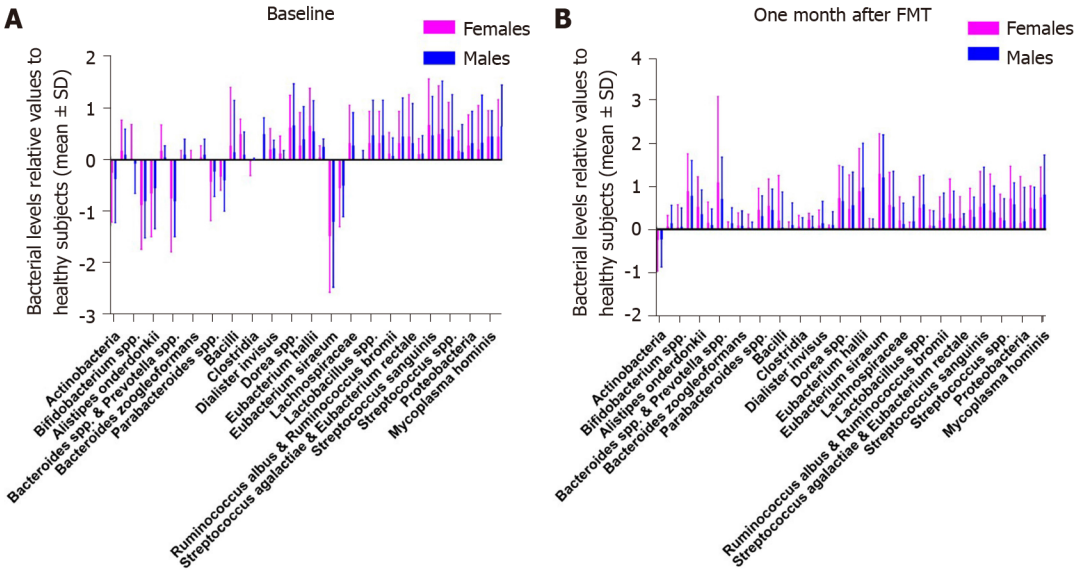


Figure 11 Faecal bacteria levels in the female and male irritable bowel syndrome patients in the active treated group at the baseline and 1 mo after faecal microbiota transplantation. A: Baseline; B: 1 mo after faecal microbiota transplantation. The bacterial levels are expressed as in Figure 10. FMT: Faecal microbiota transplantation.

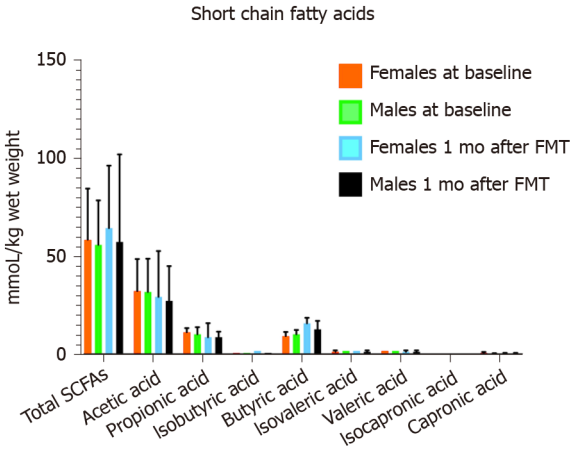


Figure 12 The faecal short chain fatty acids concentration of female and male patients in both the placebo and active treated groups at the baseline and 1 mo after faecal microbiota transplantation. The values were expressed as mmol/kg wet weight (mean ± SD).

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is a common chronic disorder, where intestinal microbiota plays a pivotal role in its pathophysiology. Faecal microbiota transplantation for IBS appears to be a promising treatment of IBS.

Research motivation

In Western countries, there is a female predominance in IBS with female:male ratio of 2:1. In a recent randomized double-blind placebo-controlled trial on faecal microbiota transplantation (FMT) in IBS females responded better to FMT than did males.

Research objectives

We aimed to investigate whether there is a sex difference in the response to FMT in terms of symptoms, dysbiosis, and bacteria and short-chain fatty acids (SCFAs) profiles in the same cohort of patients that we had investigated in our previous randomized controlled trial.

Research methods

This study included 164 patients who fulfilled the Rome IV criteria for the diagnosis of IBS. These patient's cohort included IBS diarrhoea-predominant (IBS-D), IBS-constipation predominant (IBS-C) and mixed diarrhoea and constipation (IBS-M) subtypes. They were randomized to placebo (own faeces), 30 g or 60 g donor's faeces at a ratio of 1:1:1. The faecal transplant was administered *via* gastroscope to the duodenum. Patients completed IBS severity scoring system (IBS-SSS), the Fatigue Assessment Scale (FAS) and the IBS quality of life scale (IBS-QoL) questionnaires at the baseline and 2 wk, 1 mo and 3 mo after FMT. They also provided faecal samples at the baseline and 1 mo after FMT. Response was defined as a decrease of ≥ 50 points in the IBS-SSS total score after FMT. The faecal bacteria profile and dysbiosis were determined by the GA-map Dysbiosis Test (Genetic Analysis, Oslo, Norway) using the 16S rRNA gene. The levels of faecal SCFAs were determined by gas chromatography.

Research results

There was no sex difference in the response to FMT either in the placebo group or active treated group. There was no difference between females and males in either the placebo group or actively treated groups in the total score on the IBS-SSS, FAS or IBS-QoL, in dysbiosis, or in the faecal bacteria or SCFA level. However, the response rate was significantly higher in females with IBS-D than that of males at 1 mo, and 3 mo after FMT. Moreover, IBS-SSS total score was significantly lower in female patients with IBS-D than that of male patients both 1 mo and 3 mo after FMT.

Research conclusions

There is no sex difference in the response to FMT in IBS patients with moderate-to-severe IBS symptoms belonging to the three of IBS subtypes of IBS-C and IBS-M in patients who did not responded to National Institute for Health and Care Excellence-modified diet. However, female patients with IBS-D had a significant higher response rate to FMT and lower IBS-SSS score after FMT than males.

Research perspectives

The present observation that female patients with IBS-D respond better to FMT than males raise several questions as to the cause of this difference. Further studies are needed to explore the difference in diet and life style between females and males as possible causes for this difference.

REFERENCES

1 **El-Salhy M**. Recent developments in the pathophysiology of irritable bowel syndrome. *World J Gastroenterol* 2015; **21**: 7621-7636 [PMID: 26167065 DOI: 10.3748/wjg.v21.i25.7621]

2 **Ohman L**, Simrén M. Intestinal microbiota and its role in irritable bowel syndrome (IBS). *Curr Gastroenterol Rep* 2013; **15**: 323 [PMID: 23580243 DOI: 10.1007/s11894-013-0323-7]

3 **Öhman L**, Törnblom H, Simrén M. Crosstalk at the mucosal border: importance of the gut microenvironment in IBS. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 36-49 [PMID: 25446728 DOI: 10.1038/nrgastro.2014.200]

4 **Wilson BC**, Vatanen T, Cutfield WS, O'Sullivan JM. The Super-Donor Phenomenon in Fecal Microbiota Transplantation. *Front Cell Infect Microbiol* 2019; **9**: 2 [PMID: 30719428 DOI: 10.3389/fcimb.2019.00002]

5 **Maier L**, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, Brochado AR, Fernandez KC, Dose H, Mori H, Patil KR, Bork P, Typas A. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* 2018; **555**: 623-628 [PMID: 29555994 DOI: 10.1038/nature25979]

6 **Casén C**, Vebø HC, Sekelja M, Hegge FT, Karlsson MK, Ciemniejewska E, Dzankovic S, Frøyland C, Nestestog R, Engstrand L, Munkholm P, Nielsen OH, Rogler G, Simrén M, Öhman L, Vatn MH, Rudi K. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment Pharmacol Ther* 2015; **42**: 71-83 [PMID: 25973666 DOI: 10.1111/apt.13236]

7 **Pozuelo M**, Panda S, Santiago A, Mendez S, Accarino A, Santos J, Guarner F, Azpiroz F, Manichanh C. Reduction of butyrate- and methane-producing microorganisms in patients with Irritable Bowel Syndrome. *Sci Rep* 2015; **5**: 12693 [PMID: 26239401 DOI: 10.1038/srep12693]

8 **Chong PP**, Chin VK, Looi CY, Wong WF, Madhavan P, Yong VC. The Microbiome and Irritable Bowel Syndrome - A Review on the Pathophysiology, Current Research and Future Therapy. *Front Microbiol* 2019; **10**: 1136 [PMID: 31244784 DOI: 10.3389/fmicb.2019.01136]

9 **Enck P**, Mazurak N. Dysbiosis in Functional Bowel Disorders. *Ann Nutr Metab* 2018; **72**: 296-306 [PMID: 29694952 DOI: 10.1159/000488773]

10 **Johnsen PH**, Hilpüsch F, Cavanagh JP, Leikanger IS, Kolstad C, Valle PC, Goll R. Faecal microbiota

transplantation *vs* placebo for moderate-to-severe irritable bowel syndrome: a double-blind, randomised, placebo-controlled, parallel-group, single-centre trial. *Lancet Gastroenterol Hepatol* 2018; **3**: 17-24 [PMID: 29100842 DOI: 10.1016/S2468-1253(17)30338-2]

11 **Halkjær SI**, Christensen AH, Lo BZS, Browne PD, Günther S, Hansen LH, Petersen AM. Faecal microbiota transplantation alters gut microbiota in patients with irritable bowel syndrome: results from a randomised, double-blind placebo-controlled study. *Gut* 2018; **67**: 2107-2115 [PMID: 29980607 DOI: 10.1136/gutjnl-2018-316434]

12 **El-Salhy M**, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; **69**: 859-867 [PMID: 31852769 DOI: 10.1136/gutjnl-2019-319630]

13 **Aroniadis OC**, Brandt LJ, Oneto C, Feuerstadt P, Sherman A, Wolkoff AW, Kassam Z, Sadovsky RG, Elliott RJ, Budree S, Kim M, Keller MJ. Faecal microbiota transplantation for diarrhoea-predominant irritable bowel syndrome: a double-blind, randomised, placebo-controlled trial. *Lancet Gastroenterol Hepatol* 2019; **4**: 675-685 [PMID: 31326345 DOI: 10.1016/S2468-1253(19)30198-0]

14 **Holster S**, Lindqvist CM, Repsilber D, Salonen A, de Vos WM, König J, Brummer RJ. The Effect of Allogenic Versus Autologous Fecal Microbiota Transfer on Symptoms, Visceral Perception and Fecal and Mucosal Microbiota in Irritable Bowel Syndrome: A Randomized Controlled Study. *Clin Transl Gastroenterol* 2019; **10**: e00034 [PMID: 31009405 DOI: 10.14309/ctg.0000000000000034]

15 **Lahtinen P**, Jalanka J, Hartikainen A, Mattila E, Hillilä M, Punkkinen J, Koskenpato J, Anttila VJ, Tillonen J, Satokari R, Arkkila P. Randomised clinical trial: faecal microbiota transplantation *vs* autologous placebo administered *via* colonoscopy in irritable bowel syndrome. *Aliment Pharmacol Ther* 2020; **51**: 1321-1331 [PMID: 32343000 DOI: 10.1111/apt.15740]

16 **Holvoet T**, Joossens M, Vázquez-Castellanos JF, Christiaens E, Heyerick L, Boelens J, Verhasselt B, van Vlierberghe H, De Vos M, Raes J, De Looze D. Fecal Microbiota Transplantation Reduces Symptoms in Some Patients With Irritable Bowel Syndrome With Predominant Abdominal Bloating: Short- and Long-term Results From a Placebo-Controlled Randomized Trial. *Gastroenterology* 2021; **160**: 145-157. e8 [PMID: 32681922 DOI: 10.1053/j.gastro.2020.07.013]

17 **Benech N**, Sokol H. Fecal microbiota transplantation in gastrointestinal disorders: time for precision medicine. *Genome Med* 2020; **12**: 58 [PMID: 32605650 DOI: 10.1186/s13073-020-00757-y]

18 **Grundmann O**, Yoon SL. Irritable bowel syndrome: epidemiology, diagnosis and treatment: an update for health-care practitioners. *J Gastroenterol Hepatol* 2010; **25**: 691-699 [PMID: 20074154 DOI: 10.1111/j.1440-1746.2009.06120.x]

19 **Camilleri M**. Diagnosis and Treatment of Irritable Bowel Syndrome: A Review. *JAMA* 2021; **325**: 865-877 [PMID: 33651094 DOI: 10.1001/jama.2020.22532]

20 **Canavan C**, West J, Card T. The epidemiology of irritable bowel syndrome. *Clin Epidemiol* 2014; **6**: 71-80 [PMID: 24523597 DOI: 10.2147/CLEP.S40245]

21 **Kwan AC**, Hu WH, Chan YK, Yeung YW, Lai TS, Yuen H. Prevalence of irritable bowel syndrome in Hong Kong. *J Gastroenterol Hepatol* 2002; **17**: 1180-1186 [PMID: 12453277 DOI: 10.1046/j.1440-1746.2002.02871.x]

22 **Husain N**, Chaudhry IB, Jafri F, Niaz SK, Tomenson B, Creed F. A population-based study of irritable bowel syndrome in a non-Western population. *Neurogastroenterol Motil* 2008; **20**: 1022-1029 [PMID: 18492027 DOI: 10.1111/j.1365-2982.2008.01143.x]

23 **Xiong LS**, Chen MH, Chen HX, Xu AG, Wang WA, Hu PJ. A population-based epidemiologic study of irritable bowel syndrome in South China: stratified randomized study by cluster sampling. *Aliment Pharmacol Ther* 2004; **19**: 1217-1224 [PMID: 15153175 DOI: 10.1111/j.1365-2036.2004.01939.x]

24 **Chang FY**, Lu CL, Chen TS. The current prevalence of irritable bowel syndrome in Asia. *J Neurogastroenterol Motil* 2010; **16**: 389-400 [PMID: 21103420 DOI: 10.5056/jnm.2010.16.4.389]

25 **El-Salhy M**, Valeur J, Hausken T, Gunnar Hatlebakk J. Changes in fecal short-chain fatty acids following fecal microbiota transplantation in patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2021; **33**: e13983 [PMID: 32945066 DOI: 10.1111/nmo.13983]

26 **Cammarota G**, Ianiro G, Tilg H, Rajilić-Stojanović M, Kump P, Satokari R, Sokol H, Arkkila P, Pintus C, Hart A, Segal J, Aloi M, Masucci L, Molinaro A, Scaldaferri F, Gasbarrini G, Lopez-Sanroman A, Link A, de Groot P, de Vos WM, Högenauer C, Malfertheiner P, Mattila E, Milosavljević T, Nieuwdorp M, Sanguinetti M, Simren M, Gasbarrini A; European FMT Working Group. European consensus conference on faecal microbiota transplantation in clinical practice. *Gut* 2017; **66**: 569-580 [PMID: 28087657 DOI: 10.1136/gutjnl-2016-313017]

27 **Francis CY**, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. *Aliment Pharmacol Ther* 1997; **11**: 395-402 [PMID: 9146781 DOI: 10.1046/j.1365-2036.1997.142318000.x]

28 **Roalfe AK**, Roberts LM, Wilson S. Evaluation of the Birmingham IBS symptom questionnaire. *BMC Gastroenterol* 2008; **8**: 30 [PMID: 18651941 DOI: 10.1186/1471-230X-8-30]

29 **Hendriks C**, Drent M, Elfferich M, De Vries J. The Fatigue Assessment Scale: quality and availability in sarcoidosis and other diseases. *Curr Opin Pulm Med* 2018; **24**: 495-503 [PMID: 29889115 DOI: 10.1097/MCP.0000000000000496]

30 **Drent M**, Lower EE, De Vries J. Sarcoidosis-associated fatigue. *Eur Respir J* 2012; **40**: 255-263 [PMID: 22441750 DOI: 10.1183/09031936.00002512]

31 **Atkins C**, Fordham R, Clark AB, Stockl A, Jones AP, Wilson AM. Feasibility study of a randomised controlled trial to investigate the treatment of sarcoidosis-associated fatigue with methylphenidate

(FaST-MP): a study protocol. *BMJ Open* 2017; **7**: e018532 [PMID: 29208618 DOI: 10.1136/bmjopen-2017-018532]

32 **Drossman DA**, Patrick DL, Whitehead WE, Toner BB, Diamant NE, Hu Y, Jia H, Bangdiwala SI. Further validation of the IBS-QOL: a disease-specific quality-of-life questionnaire. *Am J Gastroenterol* 2000; **95**: 999-1007 [PMID: 10763950 DOI: 10.1111/j.1572-0241.2000.01941.x]

33 **Wong RK**, Drossman DA. Quality of life measures in irritable bowel syndrome. *Expert Rev Gastroenterol Hepatol* 2010; **4**: 277-284 [PMID: 20528115 DOI: 10.1586/egh.10.19]

34 **Arslan G**, Lind R, Olafsson S, Florvaag E, Berstad A. Quality of life in patients with subjective food hypersensitivity: applicability of the 10-item short form of the Nepean Dyspepsia Index. *Dig Dis Sci* 2004; **49**: 680-687 [PMID: 15185878 DOI: 10.1023/b:ddas.0000026318.81635.3b]

35 **Zijlstra JB**, Beukema J, Wolthers BG, Byrne BM, Groen A, Dankert J. Pretreatment methods prior to gaschromatographic analysis of volatile fatty acids from faecal samples. *Clin Chim Acta* 1977; **78**: 243-250 [PMID: 884859 DOI: 10.1016/0009-8981(77)90312-6]

36 **Høverstad T**, Fausa O, Bjørneklett A, Bøhmer T. Short-chain fatty acids in the normal human feces. *Scand J Gastroenterol* 1984; **19**: 375-381 [PMID: 6740214]

37 **Soret R**, Chevalier J, De Coppet P, Poupeau G, Derkinderen P, Segain JP, Neunlist M. Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. *Gastroenterology* 2010; **138**: 1772-1782 [PMID: 20152836 DOI: 10.1053/j.gastro.2010.01.053]

38 **Hamer HM**, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* 2008; **27**: 104-119 [PMID: 17973645 DOI: 10.1111/j.1365-2036.2007.03562.x]

39 **Zhou J**, Martin RJ, Tulley RT, Raggio AM, McCutcheon KL, Shen L, Danna SC, Tripathy S, Hegsted M, Keenan MJ. Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. *Am J Physiol Endocrinol Metab* 2008; **295**: E1160-E1166 [PMID: 18796545 DOI: 10.1152/ajpendo.90637.2008]

40 **Karaki S**, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, Furness JB, Kuwahara A. Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. *Cell Tissue Res* 2006; **324**: 353-360 [PMID: 16453106 DOI: 10.1007/s00441-005-0140-x]

41 **Zhou J**, Martin RJ, Raggio AM, Shen L, McCutcheon K, Keenan MJ. The importance of GLP-1 and PYY in resistant starch's effect on body fat in mice. *Mol Nutr Food Res* 2015; **59**: 1000-1003 [PMID: 25631638 DOI: 10.1002/mnfr.201400904]

42 **Zhang J**, Song L, Wang Y, Liu C, Zhang L, Zhu S, Liu S, Duan L. Beneficial effect of butyrate-producing Lachnospiraceae on stress-induced visceral hypersensitivity in rats. *J Gastroenterol Hepatol* 2019; **34**: 1368-1376 [PMID: 30402954 DOI: 10.1111/jgh.14536]

43 **Long X**, Li M, Li LX, Sun YY, Zhang WX, Zhao DY, Li YQ. Butyrate promotes visceral hypersensitivity in an IBS-like model *via* enteric glial cell-derived nerve growth factor. *Neurogastroenterol Motil* 2018; **30**: e13227 [PMID: 29052293 DOI: 10.1111/nmo.13227]

44 **Banasiewicz T**, Krokowicz Ł, Stojcev Z, Kaczmarek BF, Kaczmarek E, Maik J, Marciniak R, Krokowicz P, Walkowiak J, Drews M. Microencapsulated sodium butyrate reduces the frequency of abdominal pain in patients with irritable bowel syndrome. *Colorectal Dis* 2013; **15**: 204-209 [PMID: 22738315 DOI: 10.1111/j.1463-1318.2012.03152.x]

45 **Tana C**, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. *Neurogastroenterol Motil* 2010; **22**: 512-519, e114 [PMID: 19903265 DOI: 10.1111/j.1365-2982.2009.01427.x]

46 **Valeur J**, Røseth AG, Knudsen T, Malmstrøm GH, Fiennes JT, Midtvedt T, Berstad A. Fecal Fermentation in Irritable Bowel Syndrome: Influence of Dietary Restriction of Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols. *Digestion* 2016; **94**: 50-56 [PMID: 27487397 DOI: 10.1159/000448280]

47 **Winston J**, Shenoy M, Medley D, Naniwadekar A, Pasricha PJ. The vanilloid receptor initiates and maintains colonic hypersensitivity induced by neonatal colon irritation in rats. *Gastroenterology* 2007; **132**: 615-627 [PMID: 17258716 DOI: 10.1053/j.gastro.2006.11.014]

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ORIGINAL ARTICLE

Observational Study

Standard *vs* magnifying narrow-band imaging endoscopy for diagnosis of *Helicobacter pylori* infection and gastric precancerous conditions

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Abstract

BACKGROUND

Advances in endoscopic imaging enable the identification of patients at high risk of gastric cancer. However, there are no comparative data on the utility of standard and magnifying narrow-band imaging (M-NBI) endoscopy for diagnosing *Helicobacter pylori* (*H. pylori*) infection, gastric atrophy, and intestinal metaplasia.

AIM

To compare the diagnostic performance of standard and M-NBI endoscopy for *H. pylori* gastritis and precancerous conditions.

METHODS

In 254 patients, standard endoscopy findings were classified into mosaic-like appearance (type A), diffuse homogenous redness (type B), and irregular redness with groove (type C). Gastric mucosal patterns visualized by M-NBI were classified as regular round pits with polygonal sulci (type Z-1), more dilated and linear pits without sulci (type Z-2), and loss of gastric pits with coiled vessels (type Z-3).

RESULTS

The diagnostic accuracy of standard and M-NBI endoscopy for *H. pylori* gastritis was 93.3% and 96.1%, respectively. Regarding gastric precancerous conditions,

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the accuracy of standard and M-NBI endoscopy was 72.0% *vs* 72.6% for moderate to severe atrophy, and 61.7% *vs*. 61.1% for intestinal metaplasia in the corpus, respectively. Compared to type A and Z-1, types B+C and Z-2+Z-3 were significantly associated with moderate to severe atrophy [odds ratio (OR) = 5.56 and 8.67] and serum pepsinogen I/II ratio of ≤ 3 (OR = 4.48 and 5.69).

CONCLUSION
Close observation of the gastric mucosa by standard and M-NBI endoscopy is useful for the diagnosis of *H. pylori* gastritis and precancerous conditions.

Key Words: Endoscopy; Magnifying narrow-band imaging; *Helicobacter pylori*; Gastric atrophy; Intestinal metaplasia; Pepsinogen

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Core Tip: In Correa's model of gastric carcinogenesis, *Helicobacter pylori* infection, gastric atrophy and intestinal metaplasia are linked to gastric cancer development. The low level of serum pepsinogens was known to be highly associated with extensive atrophic gastritis. High-resolution and magnifying narrow-band imaging (M-NBI) facilitate the detailed examination of gastrointestinal mucosa. However, there was no comparative data regarding the usefulness of standard and M-NBI endoscopy for H. pylori infection and gastric precancerous conditions. We found the significant relationship between endoscopic mucosal patterns and degree of gastric precancerous conditions (moderate to severe gastric atrophy and serum pepsinogen I/II ratio of ≤ 3). These results seem to be valuable for identifying a group at risk of gastric cancer using high quality endoscopy.

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INTRODUCTION

The global prevalence of *Helicobacter pylori* (*H. pylori*) infection is reportedly > 50%[1]. In the *H. pylori*–infected stomach, chronic active inflammation of the mucosa becomes persistent, leading to gastric atrophy and intestinal metaplasia (IM)[2]. According to Correa's model of gastric carcinogenesis, gastric atrophy and IM are linked to progression to gastric cancer[3]. Advanced gastric atrophy and IM are considered to be precancerous conditions because they correlate with gastric carcinogenesis[4-6]. Accurate diagnosis of gastric precancerous conditions is essential for identifying patients at risk of gastric cancer[7].

Narrow-band imaging (NBI) is an innovative optical method that facilitates detailed examination of the gastric mucosa[8]. Furthermore, magnifying NBI (M-NBI) endoscopy with 80-fold magnification can visualize the fine mucosal structure and microvessels[9]. M-NBI endoscopy can be used to diagnose *H. pylori* infection and classify gastritis by histological severity[10]. Recent improvements in the resolution (> 1 million pixels) of gastrointestinal endoscopy have enhanced image quality, facilitating characterization of the gastric mucosal pattern[11]. Close observation of the gastric corpus mucosa by standard endoscopy without magnification enables prediction of *H. pylori* gastritis[12]. Moreover, the severity of gastric atrophy and IM differ according to the endoscopic mucosal pattern. In a systematic review, standard endoscopy was effective as an alternative method for diagnosing *H. pylori* infection[13]. However, there are no comparative data on the utility of standard and M-NBI endoscopy for diagnosing *H. pylori* infection, gastric atrophy, and IM.

In this study, we evaluated the diagnostic performance of standard and M-NBI endoscopy for *H. pylori* infection and advanced gastritis, and investigated the association between the endoscopic mucosal pattern and gastric precancerous

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conditions.

MATERIALS AND METHODS

Patients and study design

From June 2016 to April 2020, we prospectively enrolled patients who underwent gastroscopy for epigastric symptoms, diagnostic work-up for gastric neoplasia, and gastric cancer screening. Before endoscopic examination, all patients had the informed consents about the evaluation of *H. pylori* infection status and gastric precancerous conditions. We performed a complete blood cell count, blood chemistry assays, coagulation test, chest X-ray, and electrocardiogram. The exclusion criteria were: Age < 20 or > 80 years, anemia, severe systemic disease, current use of proton pump inhibitors, history of *H. pylori* eradication, and history of gastric surgery. The study protocol was approved by the Institutional Review Board of our hospital (SCHUH 2016-05-001) and was registered at ClinicalTrials.gov (NCT04489030).

Endoscopic equipment and procedure

Endoscopic procedures were performed using a high-resolution endoscope (GIF-H260Z, H290Z; Olympus, Tokyo, Japan). The whole stomach was examined in a routine manner by a single experienced endoscopist (Cho JH). First, we performed close-up observation of the mucosal patterns at the greater curvature of the middle or lower corpus *via* non-magnified white-light imaging in structural enhancement mode A5. We captured endoscopic images while maintaining a distance of ≤ 10 mm between the endoscope tip and mucosal surface. We previously classified abnormal mucosal patterns using a simple standard endoscopy technique (Figure 1)[12]. When a regular arrangement of numerous minute red dots (normal pattern) was absent, abnormal patterns were categorized as mosaic-like appearance (type A), diffuse homogenous redness (type B), or irregular redness with groove (type C). After completing the mucosal examination by standard endoscopy, the greater curvature side of the gastric corpus was observed by M-NBI. Before the procedure, a soft black hood was attached to the endoscope tip to fix the distance between the endoscope tip and mucosal surface to about 2 mm. When white-light endoscopic image was magnified 80-fold (Supplementary Figure 1), we pressed the NBI button on the handle for the enhanced visualization of mucosal structures and microvessels[14]. NBI was set in enhancement mode A7 and color mode 2. Next, we classified the M-NBI still images as normal or one of three abnormal patterns according to Yagi's classification (Figure 2)[15]. A regular arrangement of collecting venules (RAC) and honeycomb-like subepithelial capillary network was considered a normal pattern. If RAC was not visualized by M-NBI endoscopy, abnormal mucosal patterns were classified as regular round pits with polygonal sulci (type Z-1), more dilated and linear pits without sulci (type Z-2), or loss of gastric pits with coiled vessels (type Z-3). In cases of mixed mucosal patterns, the most prominent was reported after discussion with another expert endoscopist (Jeon SR).

Evaluation of H. pylori infection and gastric precancerous conditions

To increase the *H. pylori* detection rate, a biopsy was taken from the greater curvature of the gastric corpus. *H. pylori* infection status was confirmed by rapid urease test (Pronto Dry; Gastrex Sarl, Gilly les Citeaux, France) or molecular test (Seeplex® *H. pylori*-ClaR ACE Detection; Seegene Inc., Seoul, South Korea). For the histological assessment of glandular atrophy and IM, biopsy specimens were obtained from the lesser curvature of the gastric antrum and corpus. The specimens were fixed in 10% formalin and embedded in paraffin wax, and 5-μm sections were stained with hematoxylin and eosin. The gastric atrophy was defined as loss of glandular tissue and scored on a four-point scale in accordance with the updated Sydney System[16]. The degree of atrophy was classified as mild (1-2), moderate (3-4), or severe (5-6) by summing the scores of the antrum (score 0-3) and corpus (score 0-3). IM was diagnosed by the presence of goblet cells in foveolar epithelium[17]. The pathological examination was performed by an expert pathologist (Jin SY), who was blinded to the patients' data and endoscopic findings.

Before endoscopy, blood samples were collected during a 12-h fasting period. Blood samples were immediately centrifuged at 4 °C and stored at -70 °C until required. Serum pepsinogen (PG) I and PG II levels were measured by latex turbidimetric immunoassay (HiSens; HBI, Anyang, South Korea), and the PG I/II ratio was calculated[18]. A serum PG I level of ≤ 70 ng/mL and PG I/II ratio of ≤ 3 are highly

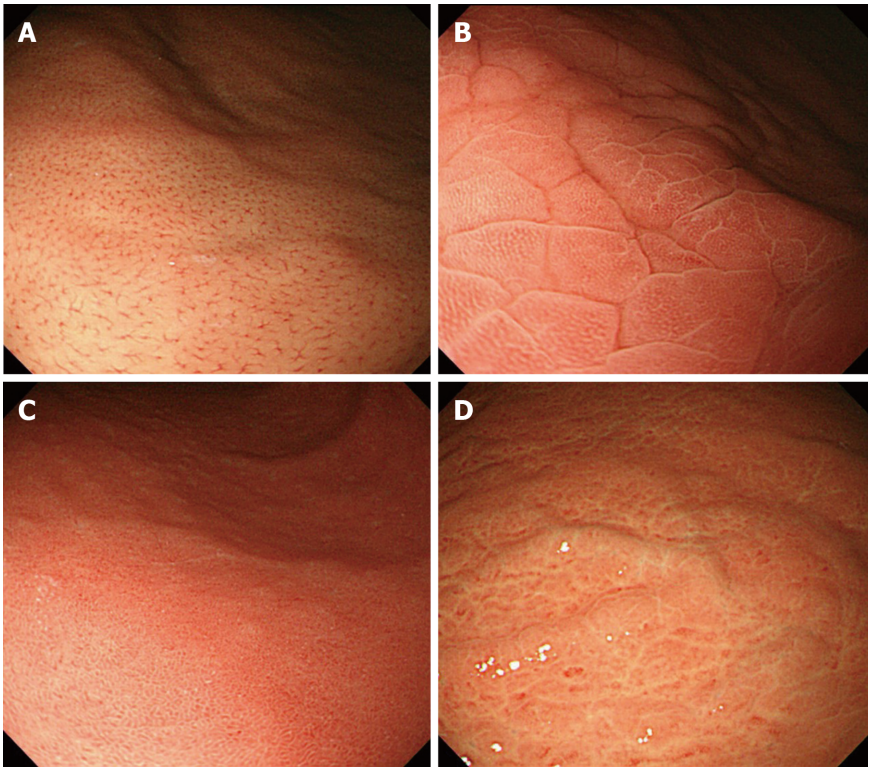


Figure 1 Normal and three abnormal mucosal patterns in the gastric corpus by standard endoscopy. A: Normal pattern, numerous minute red dots; B: Type A, mosaic-like appearance; C: Type B, diffuse homogenous redness; D: Type C, irregular redness with grooves.

associated with extensive atrophic gastritis[19]. A cutoff PG I/II ratio of ≤ 3 was used to assess the risk of gastric precancerous conditions.

Statistical analysis

Continuous data are presented as means and standard deviations and categorical data as numbers and percentages. The Pearson chi-squared test or linear-by-linear association was used to analyze categorical data. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the two endoscopic classifications were compared by McNemar test. Diagnostic performance was assessed by computing area under the curve (AUC) values. The adjusted odds ratios (ORs) of endoscopic mucosal patterns for predicting gastric precancerous conditions were calculated by logistic regression analysis. Statistical analysis was conducted using SPSS software (version 19.0; IBM Corp., Armonk, NY, United States). A value of $P < 0.05$ was considered indicative of statistical significance.

RESULTS

Characteristics of the study population

A total of 254 patients was eligible for the study (Table 1). The mean age was 45.9 (± 14.6) years and the proportion of male patients was 46.9%. The most frequent endoscopic findings were chronic active gastritis (53.1%), peptic ulcer (4.3%), gastric neoplasia (9.8%), and non-neoplastic polyp (7.5%). Current *H. pylori* infection was confirmed in 64.2% of the patients. According to the degree of gastric atrophy, 117 patients (66.9%) were classified into the none/mild atrophy group, 37 (21.1%) into the moderate atrophy group, and 21 (12.0%) into the severe atrophy group. Regarding the extent of IM, 115 patients (65.7%) had no IM in the antrum or corpus, and 22 (12.6%) and 38 (21.7%) patients had IM in the antrum only and in both the antrum and the corpus, respectively. The mean serum PG I and PG II concentrations and PG I/II ratio were 64.4 ± 32.4 ng/mL, 20.5 ± 14.7 ng/mL, and 4.11 ± 2.2, respectively. The mean serum gastrin concentration was 123.1 ± 149.9 pg/mL.

Table 1 Baseline characteristics of the study population

Characteristic	*n* = 254
Age (yr, mean ± SD)	45.9 ± 14.6
Sex, male, *n* (%)	119 (46.9)
Indication for endoscopy, *n* (%)	
Screening	132 (52.0)
Dyspepsia	42 (16.5)
Abdominal pain	47 (18.5)
Other	33 (13.0)
Endoscopic diagnosis, *n* (%)	
Peptic ulcer	11 (4.3)
Gastric neoplasia	25 (9.8)
Non-neoplastic polyp	19 (7.5)
Chronic active gastritis	135 (53.1)
Helicobacter pylori infection, *n* (%)	163 (64.2)
Gastric atrophy, *n* (%)	
None/mild	117 (66.9)
Moderate	37 (21.1)
Severe	21 (12.0)
Intestinal metaplasia, *n* (%)	
None	115 (65.7)
Antrum only	22 (12.6)
Corpus	38 (21.7)
Serum PG (ng/mL, mean ± SD)	
PG I	64.4 ± 32.4
PG II	20.5 ± 14.7
PG I/II ratio	4.11 ± 2.2
Serum gastrin (pg/mL, mean ± SD)	123.1 ± 149.9

SD: Standard deviation; PG: Pepsinogen.

Diagnostic performance of standard and M-NBI endoscopy

By standard endoscopy, the *H. pylori* infection rate was 91.5% (n = 43/47) for type A, 100% (n = 84/84) for type B, and 100% (n = 23/23) for type C (Table 2). By M-NBI endoscopy, the proportion of *H. pylori* infection was 97.6% (n = 124/127) for type Z-1, 96.3% (n = 26/27) for type Z2, and 100% (n = 7/7) for type Z-3. The rate of advanced gastritis was calculated according to the endoscopic mucosal pattern among 175 patients whose samples were subjected to pathologic examination. Moderate to severe gastric atrophy and the presence of IM in the corpus were considered to be advanced gastritis with a high risk of gastric cancer. By standard endoscopy, the proportions of patients with moderate to severe atrophy were 27.3% (n = 9/33) for type A, 74.0% (n = 37/50) for type B, and 50.0% (n = 10/20) for type C. By M-NBI endoscopy, the proportions of patients with moderate to severe atrophy were 45.9% (n = 39/85) for type Z-1, 88.2% (n = 15/17) for type Z-2, and 100% (n = 4/4) for type Z-3. If the gastric mucosal pattern was normal, however, the rate of moderate to severe atrophy was 2.8% (n = 2/72) by standard endoscopy and 0% (n = 0/69) by M-NBI endoscopy. Similarly, the rate of IM in the corpus differed significantly between normal and abnormal mucosal patterns by both standard (types A+B+C, $P < 0.001$) and M-NBI endoscopy (types Z-1+Z-2 +Z-3, $P < 0.001$).

Table 2 *Helicobacter pylori* gastritis, gastric atrophy, and intestinal metaplasia observed by standard and magnifying narrow-band imaging endoscopy

Endoscopy	*H. pylori* gastritis		Gastric atrophy		Intestinal metaplasia	
	Negative	Positive	None/mild	Moderate/severe	None/antrum	Corpus
	(*n* = 91)	(*n* = 163)	(*n* = 117)	(*n* = 58)	(*n* = 137)	(*n* = 38)
Standard						
Normal	87/100 (87.0)	13/100 (13.0)	70/72 (97.2)	2/72 (2.8)	71/72 (98.6)	1/72 (1.4)
Type A	4/47 (8.5)	43/47 (91.5)	24/33 (72.7)	9/33 (27.3)	25/33 (75.8)	8/33 (24.2)
Type B	0/84 (0)	84/84 (100)	13/50 (26.0)	37/50 (74.0)	24/50 (48.0)	26/50 (52.0)
Type C	0/23 (0)	23/23 (100)	10/20 (50.0)	10/20 (50.0)	17/20 (85.0)	3/20 (15.0)
M-NBI						
Normal	87/93 (93.5)	6/93 (6.5)	69/69 (100)	0/69 (0)	69/69 (100)	0/69 (0)
Type Z-1	3/127 (2.4)	124/127 (97.6)	46/85 (54.1)	39/85 (45.9)	59/85 (69.4)	26/85 (30.6)
Type Z-2	1/27 (3.7)	26/27 (96.3)	2/17 (11.8)	15/17 (88.2)	7/17 (41.2)	10/17 (58.8)
Type Z-3	0/7 (0)	7/7 (100)	0/4 (0)	4/4 (100)	2/4 (50.0)	2/4 (50.0)

M-NBI: Magnifying narrow-band imaging; *H. pylori*: *Helicobacter pylori*.

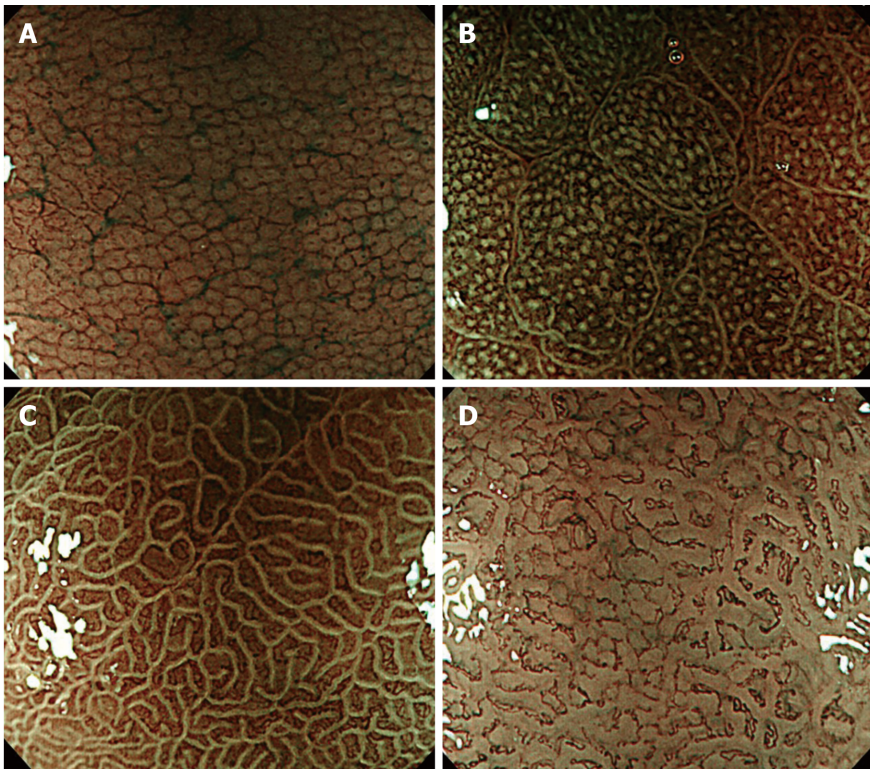


Figure 2 Micromucosal patterns in the gastric corpus observed by magnifying narrow-band imaging endoscopy. A: Normal pattern characterized by regular arrangement of collecting venules and honeycomb-like subepithelial capillary network; B: Type Z-1, regular round pits with polygonal sulci; C: Type Z-2, more dilated and linear pits without sulci; D: Type Z-3, loss of gastric pits with coiled microvessels.

For the diagnosis of *H. pylori* gastritis, the sensitivity of standard endoscopy was 92.0%, while the specificity was 95.6%, the PPV was 97.4%, the NPV was 87.0%, and the accuracy was 93.3%. The sensitivity of M-NBI endoscopy was 96.3%, while the specificity was 95.6%, the PPV was 97.5%, the NPV was 93.5%, and the accuracy was 96.1%. The sensitivity of M-NBI endoscopy was significantly greater than that of standard endoscopy (P = 0.016; Table 3). For the diagnosis of moderate to severe atrophy, the sensitivity of standard endoscopy was 96.6%, while the specificity was

Table 3 Diagnostic performance of standard and magnifying narrow-band imaging endoscopy for *Helicobacter pylori* gastritis, gastric atrophy, and intestinal metaplasia

Endoscopy	Diagnosis	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	AUC
Standard	*H. pylori* gastritis	92.0[a]	95.6	97.4	87.0	93.3	0.93
	Moderate to severe atrophy	96.6	59.8	54.4	97.2	72.0	0.78
	Intestinal metaplasia, corpus	97.4	51.8	35.9	98.6	61.7	0.74
M-NBI	*H. pylori* gastritis	96.3[a]	95.6	97.5	93.5	96.1	0.96
	Moderate to severe atrophy	100	59.0	54.7	100	72.6	0.79
	Intestinal metaplasia, corpus	100	50.4	35.8	100	61.1	0.75

[a]$P = 0.016$, McNemar test indicated a significant difference in sensitivity for diagnosis of *Helicobacter pylori* infection between standard and magnifying narrow-band imaging endoscopy. M-NBI: Magnifying narrow-band imaging; PPV: Positive predictive value; NPV: Negative predictive value; AUC: area under the curve; *H. pylori*: *Helicobacter pylori*.

59.8%, the PPV was 54.4%, the NPV was 97.2%, and the accuracy was 72.0%. The sensitivity of M-NBI endoscopy was 100%, while the specificity was 59.0%, the PPV was 54.7%, the NPV was 100%, and the accuracy was 72.6%. For diagnosis of IM in the corpus, the sensitivity of standard endoscopy was 97.4%, while the specificity was 51.8%, the PPV was 35.9%, the NPV was 98.6%, and the accuracy was 61.7%. The sensitivity of M-NBI endoscopy was 100%, while the specificity was 50.4%, the PPV was 35.8%, the NPV was 100%, and the accuracy was 61.1%. The diagnostic performance of standard and M-NBI endoscopy for moderate to severe atrophy and IM in the corpus was not significantly different. The AUC values of standard and M-NBI endoscopy were 0.93 and 0.96 for *H. pylori* gastritis, 0.78 and 0.79 for moderate to severe atrophy, and 0.74 and 0.75 for IM in the corpus, respectively.

Association of endoscopic patterns with gastric precancerous conditions

Table 4 shows the relationships between endoscopic mucosal patterns and a serum PG I/II ratio of ≤ 3. Among 127 patients who underwent measurement of serum PG concentration, 44.1% (n = 56) had a PG I/II ratio of ≤ 3. Of patients with a normal mucosal pattern, the rate of a PG I/II ratio of ≤ 3 was 2.6% (n = 1/39) by standard endoscopy and 0% (n = 0/36) by M-NBI endoscopy. By standard endoscopy, the rate of a PG I/II ratio of ≤ 3 was 36.0% (n = 9/25) for type A, 71.7% (n = 33/46) for type B, and 76.5% (n = 13/17) for type C. By M-NBI endoscopy, the rate of a PG I/II ratio of ≤ 3 was 54.2% (n = 39/72) for type Z-1, 86.7% (n = 13/15) for type Z-2, and 100% (n = 4/4) for type Z-3. The rate of a PG I/II ratio of ≤ 3 differed between normal and abnormal mucosal patterns according to two endoscopic classifications ($P < 0.001$).

The age- and sex-adjusted ORs of abnormal mucosal patterns for gastric precancerous conditions were calculated by logistic regression analyses (Table 5). Compared to type A, the OR for moderate to severe atrophy was 5.56 [95% confidence interval (CI): 2.07-14.92, $P = 0.001$] for types B+C. Compared to type Z-1, the OR for moderate to severe atrophy was 8.67 (95%CI: 1.82–41.30, $P = 0.007$) for types Z-2+Z-3. Compared to type A, the OR for a serum PG I/II ratio of ≤ 3 was 4.48 (95%CI: 1.60-12.54, $P = 0.004$) for types B+C. Compared to type Z-1, the OR for a serum PG I/II ratio of ≤ 3 was 5.69 (95%CI: 1.19-27.18, $P = 0.029$) for types Z-2+Z-3. For IM in the corpus, there was no significant difference in abnormal mucosal patterns according to two endoscopic classifications (type A *vs* types B+C, $P = 0.189$; and type Z-1 *vs* types Z2+Z-3, $P = 0.162$, respectively).

DISCUSSION

Pathological examination plays an important role in the diagnosis of gastrointestinal diseases by endoscopy[20]. Endoscopists tend to focus on detecting abnormal lesions, and macroscopic examination by conventional endoscopy has limitations for characterizing mucosal lesions. Therefore, the final diagnosis of detected lesions is dependent

Table 4 Serum pepsinogen I/II ratio according to gastric mucosal pattern observed by standard and magnifying narrow-band imaging endoscopy

PG I/II ratio	Standard (%)				M-NBI (%)			
	Normal	Type A	Type B	Type C	Normal	Type Z-1	Type Z-2	Type Z-3
> 3 (n = 71)	38/39 (97.4)	16/25 (64.0)	13/46 (28.3)	4/17 (23.5)	36/36 (100)	33/72 (45.8)	2/15 (13.3)	0/4 (0)
≤ 3 (n = 56)	1/39 (2.6)	9/25 (36.0)	33/46 (71.7)	13/17 (76.5)	0/36 (0)	39/72 (54.2)	13/15 (86.7)	4/4 (100)

PG: Pepsinogen; M-NBI: Magnifying narrow-band imaging.

Table 5 Logistic regression analysis of the associations of endoscopic mucosal patterns with gastric precancerous conditions

Mucosal pattern	Moderate to severe atrophy		Intestinal metaplasia, corpus		PG I/II ratio ≤ 3	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Standard		0.001		0.189		0.004
Type A	1 (ref.)		1 (ref.)		1 (ref.)	
Type B + C	5.56 (2.07-14.92)		1.96 (0.72-5.33)		4.48 (1.60-12.54)	
M-NBI		0.007		0.162		0.029
Type Z-1	1 (ref.)		1 (ref.)		1 (ref.)	
Type Z-2 + Z-3	8.67 (1.82-41.30)		2.12 (0.74-6.07)		5.69 (1.19-27.18)	

M-NBI: Magnifying narrow-band imaging; PG: Pepsinogen; OR: Odds ratio; CI: Confidence interval.

on the pathological report. To identify those at risk of gastric cancer, the presence of precancerous conditions is evaluated by non-targeted protocol-guided biopsies in different areas[21]. However, multiple mucosal biopsies increase medical costs and the procedure time.

High-resolution and high-magnification endoscopy facilitate detailed examination of the gastrointestinal mucosa[22]. In 2002, Yagi *et al*[23] used magnifying endoscopy to show that RAC was a characteristic finding in the normal stomach without *H. pylori* infection. Abnormal mucosal patterns without RAC were classified as Z-1 to Z-3 in accordance with the degree of mucosal damage in the *H. pylori*–infected stomach[24]. Anagnostopoulos *et al*[25] demonstrated that magnifying endoscopic examination could identify normal gastric mucosa, *H. pylori*–related gastritis, and gastric atrophy in a Western population. The severity of chronic gastritis has been investigated based on the micro-mucosal patterns observed by M-NBI[26]. Kanzaki *et al*[27] reported that groove-type mucosa had a higher grade of atrophy and IM compared to foveolar-type mucosa. However, M-NBI endoscopy is not available in all endoscopy units and training program is required prior to its clinical application[28].

In a previous study, we determined *H. pylori* infection status by close observation of the gastric corpus mucosa by high-resolution endoscopy without magnification[12]. The sensitivity and specificity of all abnormal patterns for predicting *H. pylori* infection were 93.3% and 89.1%, respectively, and the overall diagnostic accuracy was 91.6%. The inter- and intra-observer agreement for the endoscopic mucosal patterns was 91.7% and 90.0%, respectively. This simplified endoscopic technique has enabled reliable prediction of gastric *H. pylori* infection in other countries[29,30]. Recent advances in endoscopic imaging technology have increased the diagnostic accuracy for *H. pylori* infection[13,31]. To our knowledge, this is the first study comparing diagnostic performance between standard and M-NBI endoscopy for *H. pylori* infection. We also analyzed the degree of gastric atrophy, IM, and serum PG levels according to two endoscopic classifications. A decrease in the serum PG I/II ratio is reportedly a non-invasive marker for advanced corpus gastritis and gastric cancer[32,33]. Wang *et al*[34] reported that the serum PG level was strongly correlated with the Operative Link for Gastritis Assessment (OLGA) and Operative Link for Gastric Intestinal Metaplasia (OLGIM) stage. Therefore, a serum PG I/II ratio of ≤ 3 is a serologic marker of gastric precancerous conditions.

Except for sensitivity, the diagnostic performance for *H. pylori* infection was similar between standard and M-NBI endoscopy. Seven patients with a normal pattern by standard endoscopy had *H. pylori* infection. In contrast, these patients were classified as type Z-1 (n = 4), type Z-2 (n = 2), and type Z-3 (n = 1) by M-NBI endoscopy. This is likely because of the ability of M-NBI to examine the superficial microanatomy of the areae gastricae and the pit pattern, which is not possible with standard endoscopy. Nevertheless, standard endoscopy may enable detection of *H. pylori* infection in routine clinical practice (diagnostic accuracy, 93.3%).

For diagnosis of gastric atrophy and IM, standard and M-NBI endoscopy had excellent sensitivity, NPV (> 95%), and AUC (> 0.7) values; however, the specificity and PPV values were not acceptable. According to the Kimura–Takemoto classification, gastric atrophy progresses more frequently along the lesser curvature than the greater curvature of the corpus[35]. When atrophic change extends into the anterior and posterior mucosa of the corpus, the topographic pattern of IM becomes more diffuse throughout the stomach[36]. In this study, we focused on the mucosal pattern in the greater curvature of the gastric corpus. Atrophy of the gastric mucosa and transformation to IM occur last at the greater curvature of the corpus, hampering evaluation of gastric precancerous conditions. A systematic screening protocol for the stomach has been developed to ensure high-quality endoscopic evaluation[37], and enables detection of gastric atrophy and IM in various areas of the stomach[38]. Using NBI without magnification, Pimentel-Nunes *et al*[39] created an endoscopic classification to grade gastric IM; a tubulovillous mucosal pattern was highly concordant with histological IM. However, image-enhanced endoscopic findings for diagnosing and grading gastric atrophy remain to be established[40].

The *H. pylori*–infected stomach exhibits redness and swelling of the corpus mucosa[41,42]. Histologically, *H. pylori* infection causes infiltration of neutrophils and mononuclear cells in the gastric mucosa[43]. In biopsy specimens, lymphoid hyperplasia is a specific immunological reaction to *H. pylori* infection[44]. During long-term *H. pylori*–induced inflammation, the presence of lymphoid hyperplasia decreased with increasing severity of gastric atrophy and IM. In young women, nodular gastritis is induced by *H. pylori* infection and lymphoid follicles in nodular lesions can be detected histologically[45]. Miyamoto *et al*[46] reported that nodular gastritis showed a lower atrophic score than other forms of *H. pylori*–related gastritis. Similarly, we postulated that a swollen areae gastricae would be present in early stage *H. pylori* infection. Therefore, type A was used as a reference for evaluating the risk of gastric cancer according to abnormal mucosal pattern by standard endoscopy. By M-NBI endoscopy, type Z-1 was reported to correlate with less severe gastric atrophy and IM compared to other types[27]. In this study, types A and B+C showed significantly different serum PG I/II ratios (3.82 *vs* 2.74, P = 0.007), as did types Z-1 and Z-2+Z-3 (3.24 *vs* 2.28, P = 0.005). However, there was no significant difference among abnormal mucosal patterns for IM in the corpus. Based on the multifocal patterns of IM, an endoscopic mucosal examination of the entire stomach is recommended[47]. Marcos *et al*[48] reported that NBI endoscopic grading of IM was useful for risk assessment of early gastric neoplasia.

This study had several limitations. First, we did not evaluate the severity of gastric atrophy and IM using the OLGA and OLGIM staging systems. Second, only *H. pylori* treatment-naïve patients were enrolled. Therefore, the results may be not be applicable to endoscopic surveillance after *H. pylori* eradication. Finally, this study was conducted in a single center. A multicenter trial involving endoscopists with varying levels of experience is required to confirm the reliability of the results.

CONCLUSION

In conclusion, close observation of the gastric corpus mucosa by standard and M-NBI endoscopy enables diagnosis of *H. pylori* infection and gastric precancerous conditions. Furthermore, our results suggest an association of endoscopic mucosal patterns with moderate to severe atrophy and a serum PG I/II ratio of ≤ 3.

ARTICLE HIGHLIGHTS

Research background

In Correa's model of gastric carcinogenesis, *Helicobacter pylori* (*H. pylori*) infection,

gastric atrophy and intestinal metaplasia (IM) are linked to gastric cancer development. The low level of serum pepsinogens (PG) was known to be highly associated with extensive atrophic gastritis.

Research motivation

High-resolution and magnifying narrow-band imaging (M-NBI) facilitate the detailed examination of gastrointestinal mucosa. M-NBI endoscopy can be used to diagnose *H. pylori* infection and classify gastritis by histological severity. Moreover, recent improvements in the resolution (> 1 million pixels) of gastrointestinal endoscopy have enhanced image quality, facilitating characterization of the gastric mucosal pattern. Close observation of the gastric corpus mucosa by standard endoscopy without magnification enables prediction of *H. pylori* gastritis and precancerous lesions.

Research objectives

To date, there was no comparative data regarding the usefulness of standard and M-NBI endoscopy for *H. pylori* infection and gastric precancerous conditions. We compared the diagnostic performance of standard and M-NBI endoscopy for *H. pylori* gastritis and precancerous conditions.

Research methods

In total, 254 patients who underwent gastroscopy were prospectively enrolled. Standard endoscopy findings of the gastric mucosal surface were classified into mosaic-like appearance (type A), diffuse homogenous redness (type B), and irregular redness with groove (type C). Gastric mucosal patterns visualized by M-NBI endoscopy were classified as regular round pits with polygonal sulci (type Z-1), more dilated and linear pits without sulci (type Z-2), and loss of gastric pits with coiled vessels (type Z-3). We evaluated the utility of the two endoscopic classifications for the diagnosis of *H. pylori* gastritis, gastric atrophy, IM, and a serum PG I/II ratio of ≤ 3.

Research results

The diagnostic accuracy of standard and M-NBI endoscopy for *H. pylori* gastritis was 93.3% and 96.1%, respectively. Regarding gastric precancerous conditions, the diagnostic accuracy of standard and M-NBI endoscopy was 72.0% *vs* 72.6% for moderate to severe atrophy, and 61.7% *vs* 61.1% for IM in the corpus, respectively. Compared to type A and Z1, types B+C and Z-2+Z-3 were significantly associated with moderate to severe atrophy [odds ratio (OR) = 5.56, P = 0.001; OR = 8.67, P = 0.007] and a serum PG I/II ratio of ≤ 3 (OR = 4.48, P = 0.004; OR = 5.69, P = 0.029).

Research conclusions

Close observation of the gastric corpus mucosa by standard and M-NBI endoscopy enables diagnosis of *H. pylori* infection and gastric precancerous conditions. Furthermore, our results suggest an association of endoscopic mucosal patterns with moderate to severe atrophy and a serum PG I/II ratio of ≤ 3.

Research perspectives

By gastric mucosal observation in detail, optical diagnosis of *H. pylori*–related gastritis may be achieved in real time. In the future, a multicenter trial is required to confirm the reliability of our results.

REFERENCES

1 **Hooi JKY**, Lai WY, Ng WK, Suen MMY, Underwood FE, Tanyingoh D, Malfertheiner P, Graham DY, Wong VWS, Wu JCY, Chan FKL, Sung JJY, Kaplan GG, Ng SC. Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis. *Gastroenterology* 2017; **153**: 420-429 [PMID: 28456631 DOI: 10.1053/j.gastro.2017.04.022]

2 **Kuipers EJ**, Uyterlinde AM, Peña AS, Roosendaal R, Pals G, Nelis GF, Festen HP, Meuwissen SG. Long-term sequelae of Helicobacter pylori gastritis. *Lancet* 1995; **345**: 1525-1528 [PMID: 7791437 DOI: 10.1016/s0140-6736(95)91084-0]

3 **Correa P**, Piazuelo MB. The gastric precancerous cascade. *J Dig Dis* 2012; **13**: 2-9 [PMID: 22188910 DOI: 10.1111/j.1751-2980.2011.00550.x]

4 **Kaji K**, Hashiba A, Uotani C, Yamaguchi Y, Ueno T, Ohno K, Takabatake I, Wakabayashi T, Doyama H, Ninomiya I, Kiriyama M, Ohyama S, Yoneshima M, Koyama N, Takeda Y, Yasuda K. Grading of Atrophic Gastritis is Useful for Risk Stratification in Endoscopic Screening for Gastric Cancer. *Am J Gastroenterol* 2019; **114**: 71-79 [PMID: 30315306 DOI: 10.1038/s41395-018-0259-5]

5 **Shichijo S**, Hirata Y, Sakitani K, Yamamoto S, Serizawa T, Niikura R, Watabe H, Yoshida S, Yamada A, Yamaji Y, Ushiku T, Fukayama M, Koike K. Distribution of intestinal metaplasia as a predictor of gastric cancer development. *J Gastroenterol Hepatol* 2015; **30**: 1260-1264 [PMID: 25777777 DOI: 10.1111/jgh.12946]

6 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789 [PMID: 11556297 DOI: 10.1056/NEJMoa001999]

7 **Pimentel-Nunes P**, Libânio D, Marcos-Pinto R, Areia M, Leja M, Esposito G, Garrido M, Kikuste I, Megraud F, Matysiak-Budnik T, Annibale B, Dumonceau JM, Barros R, Fléjou JF, Carneiro F, van Hooft JE, Kuipers EJ, Dinis-Ribeiro M. Management of epithelial precancerous conditions and lesions in the stomach (MAPS II): European Society of Gastrointestinal Endoscopy (ESGE), European Helicobacter and Microbiota Study Group (EHMSG), European Society of Pathology (ESP), and Sociedade Portuguesa de Endoscopia Digestiva (SPED) guideline update 2019. *Endoscopy* 2019; **51**: 365-388 [PMID: 30841008 DOI: 10.1055/a-0859-1883]

8 **Gono K**, Obi T, Yamaguchi M, Ohyama N, Machida H, Sano Y, Yoshida S, Hamamoto Y, Endo T. Appearance of enhanced tissue features in narrow-band endoscopic imaging. *J Biomed Opt* 2004; **9**: 568-577 [PMID: 15189095 DOI: 10.1117/1.1695563]

9 **Muto M**, Horimatsu T, Ezoe Y, Morita S, Miyamoto S. Improving visualization techniques by narrow band imaging and magnification endoscopy. *J Gastroenterol Hepatol* 2009; **24**: 1333-1346 [PMID: 19702901 DOI: 10.1111/j.1440-1746.2009.05925.x]

10 **Tahara T**, Shibata T, Nakamura M, Yoshioka D, Okubo M, Arisawa T, Hirata I. Gastric mucosal pattern by using magnifying narrow-band imaging endoscopy clearly distinguishes histological and serological severity of chronic gastritis. *Gastrointest Endosc* 2009; **70**: 246-253 [PMID: 19386303 DOI: 10.1016/j.gie.2008.11.046]

11 **ASGE Technology Committee**. . High-definition and high-magnification endoscopes. *Gastrointest Endosc* 2014; **80**: 919-927 [PMID: 25442091 DOI: 10.1016/j.gie.2014.06.019]

12 **Cho JH**, Chang YW, Jang JY, Shim JJ, Lee CK, Dong SH, Kim HJ, Kim BH, Lee TH, Cho JY. Close observation of gastric mucosal pattern by standard endoscopy can predict Helicobacter pylori infection status. *J Gastroenterol Hepatol* 2013; **28**: 279-284 [PMID: 23189930 DOI: 10.1111/jgh.12046]

13 **Glover B**, Teare J, Patel N. A systematic review of the role of non-magnified endoscopy for the assessment of *H. pylori* infection. *Endosc Int Open* 2020; **8**: E105-E114 [PMID: 32010741 DOI: 10.1055/a-0999-5252]

14 **Cho JH**, Jeon SR, Jin SY. Clinical applicability of gastroscopy with narrow-band imaging for the diagnosis of *Helicobacter pylori* gastritis, precancerous gastric lesion, and neoplasia. *World J Clin Cases* 2020; **8**: 2902-2916 [PMID: 32775373 DOI: 10.12998/wjcc.v8.i14.2902]

15 **Yagi K**, Nakamura A, Sekine A. Comparison between magnifying endoscopy and histological, culture and urease test findings from the gastric mucosa of the corpus. *Endoscopy* 2002; **34**: 376-381 [PMID: 11972268 DOI: 10.1055/s-2002-25281]

16 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181 [PMID: 8827022 DOI: 10.1097/00000478-199610000-00001]

17 **Correa P**, Piazuelo MB, Wilson KT. Pathology of gastric intestinal metaplasia: clinical implications. *Am J Gastroenterol* 2010; **105**: 493-498 [PMID: 20203636 DOI: 10.1038/ajg.2009.728]

18 **Cho JH**, Jeon SR, Kim HG, Jin SY, Park S. The serum pepsinogen levels for risk assessment of gastric neoplasms: New proposal from a case-control study in Korea. *Medicine (Baltimore)* 2017; **96**: e7603 [PMID: 28723806 DOI: 10.1097/MD.0000000000007603]

19 **Kodoi A**, Yoshihara M, Sumii K, Haruma K, Kajiyama G. Serum pepsinogen in screening for gastric cancer. *J Gastroenterol* 1995; **30**: 452-460 [PMID: 7550854 DOI: 10.1007/BF02347560]

20 **Carpenter HA**, Talley NJ. Gastroscopy is incomplete without biopsy: clinical relevance of distinguishing gastropathy from gastritis. *Gastroenterology* 1995; **108**: 917-924 [PMID: 7875496 DOI: 10.1016/0016-5085(95)90468-9]

21 **El-Zimaity HM**, Graham DY. Evaluation of gastric mucosal biopsy site and number for identification of Helicobacter pylori or intestinal metaplasia: role of the Sydney System. *Hum Pathol* 1999; **30**: 72-77 [PMID: 9923930 DOI: 10.1016/s0046-8177(99)90303-9]

22 **Kiesslich R**, Goetz M, Hoffman A, Galle PR. New imaging techniques and opportunities in endoscopy. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 547-553 [PMID: 21894196 DOI: 10.1038/nrgastro.2011.152]

23 **Yagi K**, Nakamura A, Sekine A. Characteristic endoscopic and magnified endoscopic findings in the normal stomach without Helicobacter pylori infection. *J Gastroenterol Hepatol* 2002; **17**: 39-45 [PMID: 11895551 DOI: 10.1046/j.1440-1746.2002.02665.x]

24 **Yagi K**, Honda H, Yang JM, Nakagawa S. Magnifying endoscopy in gastritis of the corpus. *Endoscopy* 2005; **37**: 660-666 [PMID: 16010611 DOI: 10.1055/s-2005-861423]

25 **Anagnostopoulos GK**, Yao K, Kaye P, Fogden E, Fortun P, Shonde A, Foley S, Sunil S, Atherton JJ, Hawkey C, Ragunath K. High-resolution magnification endoscopy can reliably identify normal gastric mucosa, Helicobacter pylori-associated gastritis, and gastric atrophy. *Endoscopy* 2007; **39**: 202-207 [PMID: 17273960 DOI: 10.1055/s-2006-945056]

26 **Kawamura M**, Abe S, Oikawa K, Terai S, Saito M, Shibuya D, Kato K, Shimada T, Uedo N, Masuda T. Topographic differences in gastric micromucosal patterns observed by magnifying endoscopy with

narrow band imaging. *J Gastroenterol Hepatol* 2011; **26**: 477-483 [PMID: 21155881 DOI: 10.1111/j.1440-1746.2010.06527.x]

27 **Kanzaki H**, Uedo N, Ishihara R, Nagai K, Matsui F, Ohta T, Hanafusa M, Hanaoka N, Takeuchi Y, Higashino K, Iishi H, Tomita Y, Tatsuta M, Yamamoto K. Comprehensive investigation of areae gastricae pattern in gastric corpus using magnifying narrow band imaging endoscopy in patients with chronic atrophic fundic gastritis. *Helicobacter* 2012; **17**: 224-231 [PMID: 22515361 DOI: 10.1111/j.1523-5378.2012.00938.x]

28 **Nakanishi H**, Doyama H, Ishikawa H, Uedo N, Gotoda T, Kato M, Nagao S, Nagami Y, Aoyagi H, Imagawa A, Kodaira J, Mitsui S, Kobayashi N, Muto M, Takatori H, Abe T, Tsujii M, Watari J, Ishiyama S, Oda I, Ono H, Kaneko K, Yokoi C, Ueo T, Uchita K, Matsumoto K, Kanesaka T, Morita Y, Katsuki S, Nishikawa J, Inamura K, Kinjo T, Yamamoto K, Yoshimura D, Araki H, Kashida H, Hosokawa A, Mori H, Yamashita H, Motohashi O, Kobayashi K, Hirayama M, Kobayashi H, Endo M, Yamano H, Murakami K, Koike T, Hirasawa K, Miyaoka Y, Hamamoto H, Hikichi T, Hanabata N, Shimoda R, Hori S, Sato T, Kodashima S, Okada H, Mannami T, Yamamoto S, Niwa Y, Yashima K, Tanabe S, Satoh H, Sasaki F, Yamazato T, Ikeda Y, Nishisaki H, Nakagawa M, Matsuda A, Tamura F, Nishiyama H, Arita K, Kawasaki K, Hoppo K, Oka M, Ishihara S, Mukasa M, Minamino H, Yao K. Evaluation of an e-learning system for diagnosis of gastric lesions using magnifying narrow-band imaging: a multicenter randomized controlled study. *Endoscopy* 2017; **49**: 957-967 [PMID: 28637065 DOI: 10.1055/s-0043-111888]

29 **Yumang ZL**, Pangilinan JA. Close observation endoscopy of gastric mucosal pattern in predicting Helicobacter pylori infection a single center cross-sectional study (a preliminary report). *Gut Liver* 2018; **12**: S26

30 **Garcés-Durán R**, García-Rodríguez A, Córdova H, Cuatrecasas M, Ginès À, González-Suárez B, Araujo I, Llach J, Fernández-Esparrach G. Association between a regular arrangement of collecting venules and absence of Helicobacter pylori infection in a European population. *Gastrointest Endosc* 2019; **90**: 461-466 [PMID: 31108089 DOI: 10.1016/j.gie.2019.05.027]

31 **Qi Q**, Guo C, Ji R, Li Z, Zuo X, Li Y. Diagnostic Performance of Magnifying Endoscopy for Helicobacter pylori Infection: A Meta-Analysis. *PLoS One* 2016; **11**: e0168201 [PMID: 27992489 DOI: 10.1371/journal.pone.0168201]

32 **Gantuya B**, Oyuntsetseg K, Bolor D, Erdene-Ochir Y, Sanduijav R, Davaadorj D, Tserentogtokh T, Uchida T, Yamaoka Y. Evaluation of serum markers for gastric cancer and its precursor diseases among high incidence and mortality rate of gastric cancer area. *Gastric Cancer* 2019; **22**: 104-112 [PMID: 29934751 DOI: 10.1007/s10120-018-0844-8]

33 **Kang JM**, Kim N, Yoo JY, Park YS, Lee DH, Kim HY, Lee HS, Choe G, Kim JS, Jung HC, Song IS. The role of serum pepsinogen and gastrin test for the detection of gastric cancer in Korea. *Helicobacter* 2008; **13**: 146-156 [PMID: 18321304 DOI: 10.1111/j.1523-5378.2008.00592.x]

34 **Wang X**, Lu B, Meng L, Fan Y, Zhang S, Li M. The correlation between histological gastritis staging- 'OLGA/OLGIM' and serum pepsinogen test in assessment of gastric atrophy/intestinal metaplasia in China. *Scand J Gastroenterol* 2017; **52**: 822-827 [PMID: 28436254 DOI: 10.1080/00365521.2017.1315739]

35 **Kimura K**. Chronological transition of the fundic-pyloric border determined by stepwise biopsy of the lesser and greater curvatures of the stomach. *Gastroenterology* 1972; **63**: 584-592 [PMID: 5077145 DOI: 10.1016/S0016-5085(19)33241-X]

36 **Cassaro M**, Rugge M, Gutierrez O, Leandro G, Graham DY, Genta RM. Topographic patterns of intestinal metaplasia and gastric cancer. *Am J Gastroenterol* 2000; **95**: 1431-1438 [PMID: 10894575 DOI: 10.1111/j.1572-0241.2000.02074.x]

37 **Yao K**. The endoscopic diagnosis of early gastric cancer. *Ann Gastroenterol* 2013; **26**: 11-22 [PMID: 24714327 DOI: 10.1016/S0016-5107(79)73384-0]

38 **Banks M**, Graham D, Jansen M, Gotoda T, Coda S, di Pietro M, Uedo N, Bhandari P, Pritchard DM, Kuipers EJ, Rodriguez-Justo M, Novelli MR, Ragunath K, Shepherd N, Dinis-Ribeiro M. British Society of Gastroenterology guidelines on the diagnosis and management of patients at risk of gastric adenocarcinoma. *Gut* 2019; **68**: 1545-1575 [PMID: 31278206 DOI: 10.1136/gutjnl-2018-318126]

39 **Pimentel-Nunes P**, Libânio D, Lage J, Abrantes D, Coimbra M, Esposito G, Hormozdi D, Pepper M, Drasovean S, White JR, Dobru D, Buxbaum J, Ragunath K, Annibale B, Dinis-Ribeiro M. A multicenter prospective study of the real-time use of narrow-band imaging in the diagnosis of premalignant gastric conditions and lesions. *Endoscopy* 2016; **48**: 723-730 [PMID: 27280384 DOI: 10.1055/s-0042-108435]

40 **Rodríguez-Carrasco M**, Esposito G, Libânio D, Pimentel-Nunes P, Dinis-Ribeiro M. Image-enhanced endoscopy for gastric preneoplastic conditions and neoplastic lesions: a systematic review and meta-analysis. *Endoscopy* 2020; **52**: 1048-1065 [PMID: 32663879 DOI: 10.1055/a-1205-0570]

41 **Uchiyama K**, Ida K, Okuda J, Asai Y, Ohyama Y, Kuroda M, Matsumoto N, Takami T, Ogawa T, Takaori K. Correlations of hemoglobin index (IHb) of gastric mucosa with Helicobacter pylori (H. pylori) infection and inflammation of gastric mucosa. *Scand J Gastroenterol* 2004; **39**: 1054-1060 [PMID: 15545161 DOI: 10.1080/00365520410009645]

42 **Kato T**, Yagi N, Kamada T, Shimbo T, Watanabe H, Ida K; Study Group for Establishing Endoscopic Diagnosis of Chronic Gastritis. Diagnosis of Helicobacter pylori infection in gastric mucosa by endoscopic features: a multicenter prospective study. *Dig Endosc* 2013; **25**: 508-518 [PMID: 23369058 DOI: 10.1111/den.12031]

43 **Wyatt JI**. Histopathology of gastroduodenal inflammation: the impact of Helicobacter pylori.

Histopathology 1995; **26**: 1-15 [PMID: 7713479 DOI: 10.1111/j.1365-2559.1995.tb00614.x]

44 **Chen XY**, Liu WZ, Shi Y, Zhang DZ, Xiao SD, Tytgat GN. Helicobacter pylori associated gastric diseases and lymphoid tissue hyperplasia in gastric antral mucosa. *J Clin Pathol* 2002; **55**: 133-137 [PMID: 11865009 DOI: 10.1136/jcp.55.2.133]

45 **Shiotani A**, Kamada T, Kumamoto M, Nakae Y, Nakamura Y, Kakudo K, Haruma K. Nodular gastritis in Japanese young adults: endoscopic and histological observations. *J Gastroenterol* 2007; **42**: 610-615 [PMID: 17701123 DOI: 10.1007/s00535-007-2073-5]

46 **Miyamoto M**, Haruma K, Yoshihara M, Hiyama T, Sumioka M, Nishisaka T, Tanaka S, Chayama K. Nodular gastritis in adults is caused by Helicobacter pylori infection. *Dig Dis Sci* 2003; **48**: 968-975 [PMID: 12772798 DOI: 10.1023/a:1023016000096]

47 **Correa P**, Haenszel W, Cuello C, Zavala D, Fontham E, Zarama G, Tannenbaum S, Collazos T, Ruiz B. Gastric precancerous process in a high risk population: cohort follow-up. *Cancer Res* 1990; **50**: 4737-4740 [PMID: 2369748 DOI: 10.1097/00002820-199008000-00007]

48 **Marcos P**, Brito-Gonçalves G, Libânio D, Pita I, Castro R, Sá I, Dinis-Ribeiro M, Pimentel-Nunes P. Endoscopic grading of gastric intestinal metaplasia on risk assessment for early gastric neoplasia: can we replace histology assessment also in the West? *Gut* 2020; **69**: 1762-1768 [PMID: 32051208 DOI: 10.1136/gutjnl-2019-320091]

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EDITORIAL

Celiac Disease in Asia beyond the Middle East and Indian subcontinent: Epidemiological burden and diagnostic barriers

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Abstract

Celiac Disease (CD) had been considered uncommon in Asia for a long time. However, several studies suggested that, in the Indian subcontinent and Middle East countries, CD is present and as prevalent as in Western countries. Outside these Asian regions, the information about the epidemiology of CD is still lacking or largely incomplete for different and variable reasons. Here, we discuss the epidemiological aspects and the diagnostic barriers in several Asian regions including China, Japan, Southeast Asia and Russia/Central Asia. In some of those regions, especially Russia and Central Asia, the prevalence of CD is very likely to be underestimated. Several factors may, to a different extent, contribute to CD underdiagnosis (and, thus, underestimation of its epidemiological burden), including the poor disease awareness among physicians and/or patients, limited access to diagnostic resources, inappropriate use or interpretation of the serological tests, absence of standardized diagnostic and endoscopic protocols, and insufficient expertise in histopathological interpretation.

Key Words: Celiac disease; Epidemiology; Prevalence; Asia; China; Japan; Russia; Central Asia; HLA-DQB1; Diagnostic barriers

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Core Tip: This editorial discusses the main epidemiological characteristics of Celiac Disease in Asia outside the Indian subcontinent and Middle East countries. Indeed, information about the epidemiology of Celiac Disease is still lacking or largely incomplete in those Asian regions (China, Japan, South-East Asia, and Russia/Centra

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Asia), where the disease prevalence is likely be underestimated. Factors contributing to diagnostic difficulties in these Asian regions are discussed.

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INTRODUCTION

The global prevalence of Celiac Disease (CD) is estimated to be around 1% in the general population. However, this estimation is based on epidemiological studies that mainly come from the European and South/North American continents[1,2]. CD had been considered to be uncommon in Asia for a long time, but several studies published in the previous two decades demonstrated that CD is present and is as prevalent in the Indian subcontinent and in the Middle East, as it is in Western countries[3-5].

A few years ago, a meta-analysis by Singh *et al*[6] reported that the pooled prevalence of CD in Asia was around 0.5% without any significant difference between children and adults. Except for one study from Malaysia, all the clinical studies were from the Middle East (Turkey, n = 5; Iran, n = 4; Israel, n = 2; Saudi Arabia, n = 2; Jordan, n = 1) and India (n = 3). A recent study estimated that the prevalence of CD might be as high as 1% even in those Asian countries, based on an assessment of the HLA-DQB1*02 allelic frequencies and wheat consumption in the general population[7]. Indeed, the HLA-DQB1*02 allelic variant is the major HLA-related CD predisposing allele, and the appropriate HLA genetic background is as necessary (but not sufficient) as the dietary gluten intake to trigger of the immunopathological process underlying CD[8,9]. In India, this estimate was confirmed by a community-based study by Makharia *et al*[10], who found a CD prevalence of 1.04% in the north of India, where both the dietary habits and *HLA-DQ* genetic background favor the development of CD. A study from Iran including 1,500 healthy school children serologically screened for CD, led to a biopsy-confirmed diagnosis of CD in 0.6% of this pediatric cohort. As most of the cases appeared to be silent, it is reasonable to expect a higher prevalence in the general population[11]. The situation may be the same in Turkey, where Tatar *et al*[12] screened 2,000 healthy blood donors for CD markers and found that 1.3% (n = 26) were positive, and 12 donors (0.6%) were diagnosed with CD after biopsy. Considering that most of the serologically positive donors (n = 14) could not be contacted or refused endoscopy and all the diagnosed cases had been silent, the overall CD prevalence is probably greater.

For various reasons, epidemiological information about CD in other Asian regions is incomplete. The available data from China, Japan, Southeast Asia, and Russia/Central Asia are discussed below.

CHINA

In the last decade, more studies investigating the epidemiology of CD have been published from China than from the other Asian regions discussed here. In 2011, Wang *et al*[13] published a national multicenter study describing 14 Chinese children histologically diagnosed with CD after serological screening because of chronic diarrhea. Yuan *et al*[14] reported that the presence of DQB1*0201 allele was not negligible in the Chinese population, with an overall frequency of 10.5%. The highest frequency of HLA-DQB1*02 was > 20%-25% in the northwestern region, where non-Chinese ethnic minorities (*e.g.*, Kazakh and Uygur ethnicities) are present in the population. Recently, the same authors reported a CD seropositivity rate of 2.19% in a cross-sectional study including 19,778 Chinese adolescents and young adults[15].

Dietary exposure to gluten has increased over the past 50 years in the Chinese population. Wheat has become the second most consumed staple food, after rice. China is one of the world's largest producers and consumers of wheat, especially in

the northern regions. Therefore, CD is an emerging disease in this country; and, despite the lack of epidemiological studies with a complete diagnostic definition, the awareness of this disease and its potential impact on health should be increased in such a large population[14]. In 2019, Chen and Li[16] reported that lack of awareness among the population and health professionals have contributed to the hidden epidemiological burden of CD in China. They also reported that local lack of resources could limit the access to standardized tissue acquisition and histological evaluation. Consequently, in most studies, seropositive individuals could not undergo the duodenal biopsy needed for the confirmation of a CD diagnosis.

JAPAN

In Japan, the epidemiological burden of CD is extremely low. In 2018, Fukunaga *et al*[17] described only two biopsy-based CD diagnoses in a group of 2,055 people including 2,008 asymptomatic individuals and 47 adults complaining of chronic abdominal symptoms, which corresponds to < 0.1% prevalence. That finding is consistent with the very low frequency of the HLA-DQ2/DQ8 immunotype. Indeed, in a study including 371 unrelated healthy apheresis blood donors, the HLA-DQB1*02 allele frequency was reported to be < 1% even though the DQB1*03:02 allele is relatively common (10.8%)[18]. Although the dietary exposure to gluten has been increasing in Japan, the consumption of wheat is still relatively low (it is estimated to be approximately one-third of that consumed in Western countries)[17].

The same research group reported a CD seropositivity rate (based on anti-tissue transglutaminase (tTG) immunoglobulin (Ig) A antibody) of 0.19% in 2,005 Japanese adults tested in 2008-2013. That result is consistent with another study that reported a positivity rate of 0.2% (based on a 10 U/mL titer cutoff) in 2014-2016[17,19]. Because of the presumed low prevalence, in Japanese clinical practice, CD may be rarely considered by physicians who manage patients with chronic abdominal symptoms[17].

It must be emphasized that the available studies were mostly, if not completely, focused on asymptomatic/healthy people. The actual prevalence of CD seropositivity and diagnosis may thus be higher than that reported so far. Nevertheless, Hokari and Higashiyama[20] observed that Japanese physicians are unlikely to overlook CD, as the endoscopic assessment is well-established in Japan for patients complaining of chronic abdominal symptoms. This procedure is performed by well-trained endoscopists and the histopathological appearance of CD mucosa is well-known. Therefore, the low epidemiological burden of CD in Japan is considered to be real: it is consistent with the immunogenetic background and dietary habits, and there are no major diagnostic barriers. However, this situation may change in the near future if wheat consumption increases.

SOUTHEAST ASIA

Few clinical studies assessed the epidemiological burden of CD in this Asian region[21]. In most countries of Southeast Asia, the allele frequency of HLA-DQB1*02 is estimated to be < 10%-15%[6]. The low dietary intake of gluten also contributes to the low prevalence of CD. In Vietnam, CD autoimmunity was assessed in 1,961 children and around 1% were found positive for anti-tTG IgA[22]. In Thailand, CD serology was assessed in 46 children with type 1 diabetes mellitus and only one child was positive for anti-tTG IgA. Considering an expected prevalence of > 5% (and up to 10%) in such a risk group, the prevalence of CD is assumed to be low in Thai children and in the general population[23,24].

In Malaysia, Yap *et al*[25] reported a relatively high CD seroprevalence of 1.25% in healthy young adults. This finding might be explained by the fact that the Malaysian population includes three main ethnicities (*i.e.* Malay, Chinese, and Indian), but HLA genotyping was not performed in that study. These authors noted that CD is underdiagnosed in Malaysia and discussed the diagnostic barriers in this country. Lack of medical awareness of CD (resulting in a low rate of request for CD serology tests), the use of less sensitive CD serological markers, inappropriate application of endoscopic protocols, and under-recognition of mild CD histopathological patterns, were all considered to be contributing factors.

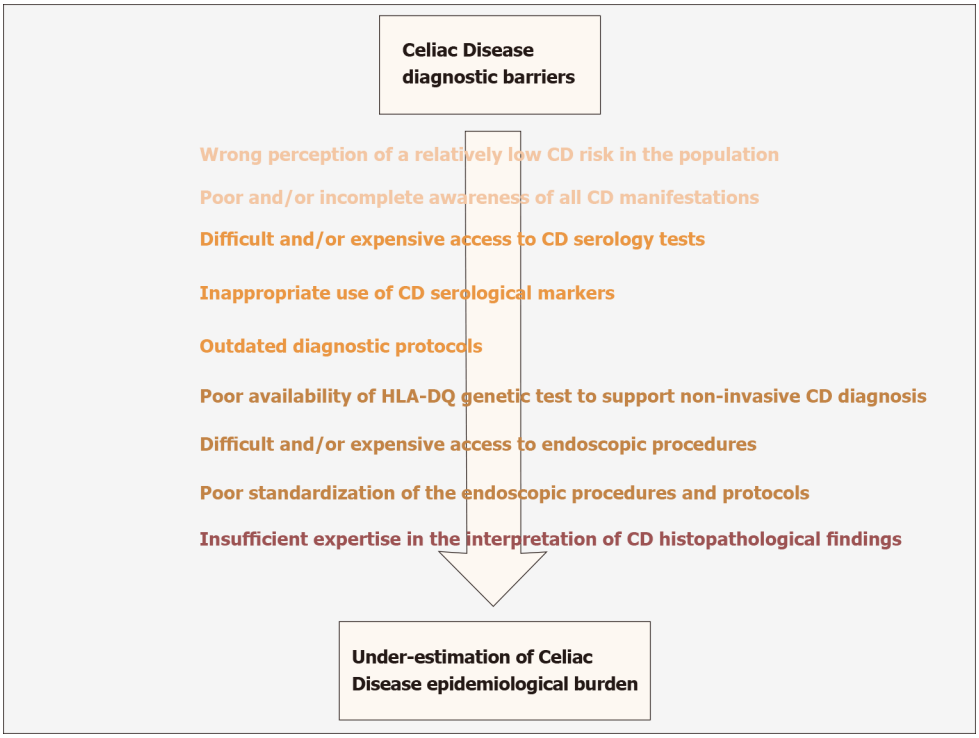


Figure 1 Schematic overview of CD diagnostic barriers in Kazakhstan (CD: Celiac Disease).

RUSSIA AND CENTRAL ASIA

A review by Savvateeva *et al*[26] is the primary English-language publication of the overall and indirect epidemiological evidence of CD in Russia. Most studies considered in this review were conducted from 2000 to 2014 and were published in Russian language. The authors concluded that the prevalence of CD in children has increased in the last few decades and is at least 0.6%, although significant inter-regional variations should be considered, because of the geographical extent of this region. The carrier frequency of HLA-DQ2/DQ8 haplotypes in the Russian population, especially in the western region, seems to be comparable to that in Europe. Savvateeva *et al*[26] reported epidemiological trends and prevalence rates similar to those in Europe, but do not discuss potential barriers hampering CD diagnosis in their country. However, they do declare well-established therapeutical support and follow-up protocols for CD patients in Russia.

Epidemiological data from Central Asia and, more precisely, from Kazakhstan are also mentioned in the aforementioned article by Savvateeva *et al*[26]. They reported a CD prevalence of < 0.4% or one case in every 262 children. However, the diagnostic approach based on anti-gliadin antibodies and the study design were prone to a significant underestimation of the actual prevalence[21,26]. Moreover, our group recently showed that the carrier frequencies of HLA-DQB1*02 and HLA-DQB1*03:02 in Kazakhstani healthy blood donors are 38% and 12.5%, respectively, and these numbers are comparable to those described in Caucasian populations. Considering the high consumption of wheat foods in Kazakhstan, we concluded that it is reasonable to expect that the CD prevalence in this country may be comparable to that reported in Europe[27].

Large well-designed clinical studies are needed to provide a reliable estimate of the CD epidemiological burden in Central Asia. It is likely that CD is underdiagnosed in Kazakhstan. Several barriers currently contribute to the underdiagnosis of CD in this country, including the inappropriate use of serological tests, limited access to diagnostic tools for economic reasons, the absence of standard protocols for endoscopic procedures, and difficulties in histopathological interpretation[27,28]. The combined effects of all those obstacles lead to the underestimation of the diagnostic and epidemiological burden in this country, as summarized in Figure 1. These considerations might also apply in other regions of Central Asia that have even fewer economic resources.

CONCLUSION

Outside the Indian subcontinent and Middle East countries, the epidemiological burden of CD in Asia is very likely to be underestimated, especially in Russia and Central Asia, where wheat is a staple food and the genetic predisposition to CD is comparable to Europe.

In agreement with other authors[29,30], several factors (that vary by country and regions) can contribute to hamper CD diagnosis and, thus, the estimation of its epidemiological burden. Overall, these factors include poor disease awareness among physicians and/or patients, limited access to diagnostic resources (because of economic and/or organizational and/or geographical reasons), inappropriate use or interpretation of the available serological tests, absence of standardized diagnostic and endoscopic protocols, and insufficient expertise in histopathological interpretation.

REFERENCES

1 **Lindfors K**, Ciacci C, Kurppa K, Lundin KEA, Makharia GK, Mearin ML, Murray JA, Verdu EF, Kaukinen K. Coeliac disease. *Nat Rev Dis Primers* 2019; **5**: 3 [PMID: 30631077 DOI: 10.1038/s41572-018-0054-z]

2 **Lebwohl B**, Sanders DS, Green PHR. Coeliac disease. *Lancet* 2018; **391**: 70-81 [PMID: 28760445 DOI: 10.1016/S0140-6736(17)31796-8]

3 **Ashtari S**, Pourhoseingholi MA, Rostami K, Aghdaei HA, Rostami-Nejad M, Busani L, Tavirani MR, Zali MR. Prevalence of gluten-related disorders in Asia-Pacific region: a systematic review. *J Gastrointestin Liver Dis* 2019; **28**: 95-105 [PMID: 30851178 DOI: 10.15403/jgld.2014.1121.281.sys]

4 **Cummins AG**, Roberts-Thomson IC. Prevalence of celiac disease in the Asia-Pacific region. *J Gastroenterol Hepatol* 2009; **24**: 1347-1351 [PMID: 19702902 DOI: 10.1111/j.1440-1746.2009.05932.x]

5 **Comba A**, Eren NB, Demir E. Prevalence of celiac disease among school-age children in Çorum, Turkey. *Turk J Gastroenterol* 2018; **29**: 595-600 [PMID: 30260783 DOI: 10.5152/tjg.2018.18020]

6 **Singh P**, Arora S, Singh A, Strand TA, Makharia GK. Prevalence of celiac disease in Asia: A systematic review and meta-analysis. *J Gastroenterol Hepatol* 2016; **31**: 1095-1101 [PMID: 26678020 DOI: 10.1111/jgh.13270]

7 **Makharia GK**, Catassi C. Celiac Disease in Asia. *Gastroenterol Clin North Am* 2019; **48**: 101-113 [PMID: 30711203 DOI: 10.1016/j.gtc.2018.09.007]

8 **Poddighe D**, Rebuffi C, De Silvestri A, Capittini C. Carrier frequency of HLA-DQB1*02 allele in patients affected with celiac disease: A systematic review assessing the potential rationale of a targeted allelic genotyping as a first-line screening. *World J Gastroenterol* 2020; **26**: 1365-1381 [PMID: 32256023 DOI: 10.3748/wjg.v26.i12.1365]

9 **Megiorni F**, Mora B, Bonamico M, Barbato M, Nenna R, Maiella G, Lulli P, Mazzilli MC. HLA-DQ and risk gradient for celiac disease. *Hum Immunol* 2009; **70**: 55-59 [PMID: 19027045 DOI: 10.1016/j.humimm.2008.10.018]

10 **Makharia GK**, Verma AK, Amarchand R, Bhatnagar S, Das P, Goswami A, Bhatia V, Ahuja V, Datta Gupta S, Anand K. Prevalence of celiac disease in the northern part of India: a community based study. *J Gastroenterol Hepatol* 2011; **26**: 894-900 [PMID: 21182543 DOI: 10.1111/j.1440-1746.2010.06606.x]

11 **Dehghani SM**, Haghighat M, Mobayen A, Rezaianzadeh A, Geramizadeh B. Prevalence of celiac disease in healthy Iranian school children. *Ann Saudi Med* 2013; **33**: 159-161 [PMID: 23563005 DOI: 10.5144/0256-4947.2013.159]

12 **Tatar G**, Elsurer R, Simsek H, Balaban YH, Hascelik G, Ozcebe OI, Buyukasik Y, Sokmensuer C. Screening of tissue transglutaminase antibody in healthy blood donors for celiac disease screening in the Turkish population. *Dig Dis Sci* 2004; **49**: 1479-1484 [PMID: 15481323 DOI: 10.1023/b:ddas.0000042250.59327.91]

13 **Wang XQ**, Liu W, Xu CD, Mei H, Gao Y, Peng HM, Yuan L, Xu JJ. Celiac disease in children with diarrhea in 4 cities in China. *J Pediatr Gastroenterol Nutr* 2011; **53**: 368-370 [PMID: 21701402 DOI: 10.1097/MPG.0b013e31822a0128]

14 **Yuan J**, Gao J, Li X, Liu F, Wijmenga C, Chen H, Gilissen LJ. The tip of the "celiac iceberg" in China: a systematic review and meta-analysis. *PLoS One* 2013; **8**: e81151 [PMID: 24324669 DOI: 10.1371/journal.pone.0081151]

15 **Yuan J**, Zhou C, Gao J, Li J, Yu F, Lu J, Li X, Wang X, Tong P, Wu Z, Yang A, Yao Y, Nadif S, Shu H, Jiang X, Wu Y, Gilissen L, Chen H. Prevalence of Celiac Disease Autoimmunity Among Adolescents and Young Adults in China. *Clin Gastroenterol Hepatol* 2017; **15**: 1572-1579. e1 [PMID: 28433781 DOI: 10.1016/j.cgh.2017.04.025]

16 **Chen CY**, Li JN. Insufficient awareness of celiac disease in China: population-based screening is needed. *Chin Med J (Engl)* 2019; **132**: 1513-1515 [PMID: 31188159 DOI: 10.1097/CM9.0000000000000305]

17 **Fukunaga M**, Ishimura N, Fukuyama C, Izumi D, Ishikawa N, Araki A, Oka A, Mishiro T, Ishihara S, Maruyama R, Adachi K, Kinoshita Y. Celiac disease in non-clinical populations of Japan. *J*

Gastroenterol 2018; **53**: 208-214 [PMID: 28389733 DOI: 10.1007/s00535-017-1339-9]
18 **Saito S**, Ota S, Yamada E, Inoko H, Ota M. Allele frequencies and haplotypic associations defined by allelic DNA typing at HLA class I and class II loci in the Japanese population. *Tissue Antigens* 2000; **56**: 522-529 [PMID: 11169242 DOI: 10.1034/j.1399-0039.2000.560606.x]
19 **Fukunaga M**, Ishimura N, Abe T, Takeda M, Isomura M, Kinoshita Y, Ishihara S. Serological screening for celiac disease in adults in Japan: Shimane CoHRE study. *JGH Open* 2020; **4**: 558-560 [PMID: 32782937 DOI: 10.1002/jgh3.12334]
20 **Hokari R**, Higashiyama M. Extremely low prevalence of Celiac disease in Japan: Eternal silence or just the calm before the storm? *JGH Open* 2020; **4**: 554-555 [PMID: 32782935 DOI: 10.1002/jgh3.12352]
21 **Poddighe D**, Rakhimzhanova M, Marchenko Y, Catassi C. Pediatric Celiac Disease in Central and East Asia: Current Knowledge and Prevalence. *Medicina (Kaunas)* 2019; **55** [PMID: 30642036 DOI: 10.3390/medicina55010011]
22 **Zanella S**, De Leo L, Nguyen-Ngoc-Quynh L, Nguyen-Duy B, Not T, Tran-Thi-Chi M, Phung-Duc S, Le-Thanh H, Malaventura C, Vatta S, Ziberna F, Mazzocco M, Volpato S, Phung-Tuyet L, Le-Thi-Minh H, Borgna-Pignatti C. Cross-sectional study of coeliac autoimmunity in a population of Vietnamese children. *BMJ Open* 2016; **6**: e011173 [PMID: 27329441 DOI: 10.1136/bmjopen-2016-011173]
23 **Thammarakcharoen T**, Hirankarn N, Sahakitrungruang T, Thongmee T, Kuptawintu P, Kanoonthong S, Chongsrisawat V. Frequency of HLA-DQB1*0201/02 and DQB1*0302 alleles and tissue transglutaminase antibody seropositivity in children with type 1 diabetes mellitus. *Asian Pac J Allergy Immunol* 2017; **35**: 82-85 [PMID: 27543737 DOI: 10.12932/AP0751]
24 **Pham-Short A**, Donaghue KC, Ambler G, Phelan H, Twigg S, Craig ME. Screening for Celiac Disease in Type 1 Diabetes: A Systematic Review. *Pediatrics* 2015; **136**: e170-e176 [PMID: 26077482 DOI: 10.1542/peds.2014-2883]
25 **Yap TW**, Chan WK, Leow AH, Azmi AN, Loke MF, Vadivelu J, Goh KL. Prevalence of serum celiac antibodies in a multiracial Asian population--a first study in the young Asian adult population of Malaysia. *PLoS One* 2015; **10**: e0121908 [PMID: 25799401 DOI: 10.1371/journal.pone.0121908]
26 **Savvateeva LV**, Erdes SI, Antishin AS, Zamyatnin AA Jr. Overview of Celiac Disease in Russia: Regional Data and Estimated Prevalence. *J Immunol Res* 2017; **2017**: 2314813 [PMID: 28316996 DOI: 10.1155/2017/2314813]
27 **Poddighe D**, Turganbekova A, Baymukasheva D, Saduakas Z, Zhanzakova Z, Abdrakhmanova S. Genetic predisposition to celiac disease in Kazakhstan: Potential impact on the clinical practice in Central Asia. *PLoS One* 2020; **15**: e0226546 [PMID: 31895924 DOI: 10.1371/journal.pone.0226546]
28 **Penny HA**, Raju SA, Lau MS, Marks LJ, Baggus EM, Bai JC, Bassotti G, Bontkes HJ, Carroccio A, Danciu M, Derakhshan MH, Ensari A, Ganji A, Green PHR, Johnson MW, Ishaq S, Lebwohl B, Levene A, Maxim R, Mohaghegh Shalmani H, Rostami-Nejad M, Rowlands D, Spiridon IA, Srivastava A, Volta U, Villanacci V, Wild G, Cross SS, Rostami K, Sanders DS. Accuracy of a no-biopsy approach for the diagnosis of coeliac disease across different adult cohorts. *Gut* 2020 [PMID: 33139268 DOI: 10.1136/gutjnl-2020-320913]
29 **Agarwal A**, Chauhan A, Ahuja V, Makharia GK. Opportunities and challenges in the management of celiac disease in Asia. *JGH Open* 2020; **4**: 795-799 [PMID: 33102747 DOI: 10.1002/jgh3.12381]
30 **Dhawan A**, Agarwal A, Mulder CJ, Makharia GK. Celiac disease in the East and the West: Bridging the gaps between the guidelines and their implementation in daily practice is mandatory. *Indian J Gastroenterol* 2019; **38**: 185-189 [PMID: 31313236 DOI: 10.1007/s12664-019-00970-7]

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FRONTIER

Biomarkers in autoimmune pancreatitis and immunoglobulin G4-related disease

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Abstract

Solitary organ autoimmune disorders, formerly known as autoimmune pancreatitis (AIP), autoimmune sialadenitis, and autoimmune sclerosing cholangitis, are now considered organ-specific manifestations of systemic immunoglobulin G4-related disease (IgG4-RD). AIP and IgG4-RD are characterized by elevated serum concentration of IgG4 antibody (Ab), accumulation of IgG4-expressing plasmacytes in the affected organs, and involvement of multiple organs. It is well established that enhanced IgG4 Ab responses are a hallmark of AIP and IgG4-RD for diagnosis and monitoring disease activity. However, a significant fraction of patients with AIP and IgG4-RD who develop chronic fibroinflammatory responses have normal serum concentrations of this IgG subtype. In addition, disease flare-up is sometimes seen even in the presence of normalized serum concentrations of IgG4 Ab after successful induction of remission by prednisolone. Therefore, it is necessary to identify new biomarkers based on the understanding of the pathophysiology of AIP and IgG4-RD. Recently, we found that activation of plasmacytoid dendritic cells producing both interferon-α (IFN-α) and interleukin-33 (IL-33) mediate murine AIP and human IgG4-RD. More importantly, we provided evidence that serum concentrations of IFN-α and IL-33 could be useful biomarkers for the diagnosis and monitoring of AIP and IgG4-RD activity after induction of remission in these autoimmune disorders. In this Frontier article, we have summarized and discussed biomarkers of AIP and IgG4-RD, including Igs, autoAbs, and cytokines to provide useful information not only for clinicians but also for researchers.

Key Words: Biomarker; Autoimmune pancreatitis; Immunoglobulin G4-related disease; Plasmacytoid dendritic cells; Cytokine; Chemokine

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Core Tip: Autoimmune pancreatitis (AIP) and immunoglobulin G4-related disease (IgG4-RD) are new disease entities characterized by enhanced IgG4 antibody responses. Serum concentration of IgG4 antibody is widely used as a useful biomarker for diagnosis and disease activity monitoring in AIP and IgG4-RD. Recent studies have highlighted the importance of cytokine responses in the immunopathogenesis of these disorders. In this Frontier article, we have summarized our knowledge regarding cytokine responses in AIP and IgG4-RD and then discussed the utility of serum concentrations of cytokines as possible biomarkers.

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a unique form of the chronic fibroinflammatory disorder of the pancreas, which is driven by autoimmune responses[1]. AIP is classified into type 1 and type 2, and more than 95% of AIP cases represent the former, which is a pancreatic manifestation of systemic immunoglobulin G4-related disease (IgG4-RD)[2-4]. In this article, type 1 AIP is hereafter referred to as AIP. AIP and IgG4-RD are recently established disease entities proposed by rheumatologists and gastroenterologists[2-4]. As awareness and recognition of these disorders by physicians increase, the number of patients diagnosed with AIP and IgG4-RD is growing. Thus, the clinical manifestations and immunopathogenesis of AIP and IgG4-RD are attracting much attention from physicians and researchers.

IgG4-RD occurs most commonly in elderly men; it is characterized by a marked elevation of serum IgG4 antibody (Ab) and accumulation of plasma cells secreting IgG4 Ab into injured organs[2-4]. Another important feature of IgG4-RD is multiple organ involvement: this disorder preferentially affects the pancreas, bile duct, lung, salivary glands, and kidney. AIP is a pancreatic manifestation of IgG4-RD. The elevated concentration of serum IgG4 Ab is widely used as a diagnostic marker for AIP and IgG4-RD[5,6]. In addition, patients with IgG4-RD exhibiting multiple organ involvement display higher concentrations of serum IgG4 Ab[7,8], suggesting that measurement of serum IgG4 concentration is useful not only for the diagnosis but also for the evaluation of disease activity. It should be noted, however, that the concentration of this IgG subtype is not always regarded as a perfect biomarker for the diagnosis or evaluation of disease activity in AIP and IgG4-RD. In fact, serum concentration of IgG4 Ab is elevated in a significant fraction of patients with pancreatic cancer[9] and about 20% of patients with AIP display normal serum concentration of IgG4 Ab[10]. Furthermore, patients with AIP sometimes relapse even if they have normal serum concentration of IgG4 Ab[11]. Therefore, it is necessary to identify other biomarkers that could be useful for the diagnosis and evaluation of disease activity in AIP and IgG4-RD.

Remarkable progress has been made in understanding the immunopathogenesis of AIP and IgG4-RD. Elucidation of immune networks associated with the development of these autoimmune disorders has led us to identify candidate biomarkers other than IgG4 Ab. In this Frontier article, we summarize recent progress in the biomarkers of AIP and IgG4-RD based on the knowledge of abnormal immune microenvironments.

IMMUNOPATHOGENESIS OF AUTOIMMUNE PANCREATITIS AND IGG4-RD

Adaptive immunity

AIP and IgG4-RD are characterized by enhanced IgG4 Ab responses; thus, immune microenvironments leading to IgG4 Ab production are likely to be involved in the development of these disorders[2-4]. Various types of differentiated T cell subpopu-

lations are involved in the enhanced IgG4 Ab response (Figure 1). These effector T cells include T helper type 2 (Th2) cells, regulatory T cells (Tregs), follicular helper T (Tfh) cells, and cytotoxic $CD4^+$ T cells ($CD4^+$ CTLs)[4]. Cytokines produced by effector T cell subpopulations promote IgG4 Ab production by B cells.

Interleukin-4 (IL-4), IL-10, and IL-13 secreted by Th2 cells and/or Tregs promoted IgG4 Ab production by healthy control B cells *in vitro*[12]. In fact, expression of IL-10, IL-13, and transforming growth factor-β1 (TGF-β1), also produced by Th2 and/or Tregs, was found to be higher in the livers of patients with IgG4-RD than in the livers of patients with other autoimmune biliary diseases[13]. Moreover, the cytokine responses seen in IgG4-RD were accompanied by the enhanced expression of forkhead box P3 (FOXP3), a critical transcription factor for Tregs[13]. Koyabu *et al*[14] reported that the number of Tregs correlated with that of $IgG4^+$ cells in the livers of IgG4-RD patients. In line with the enhanced expression of Th2 and Treg-associated cytokines in the liver, patients with IgG4-RD displayed higher expression of Th2 cytokines and chemokines such as IL-4, IL-5, IL-10, C-C motif chemokine ligand 17 (CCL17), and CCL22 in the salivary glands as compared with the levels of these molecules in healthy controls and individuals with the Sjogren syndrome[15]. Such enhanced Th2 responses in the salivary glands were accompanied by TGF-β1 production and accumulation of $FOXP3^+$ Tregs[15]. These data support the idea that Th2 cells and Tregs are involved in the development of AIP and IgG4-RD. Given that Tregs are potent negative regulators of autoimmune reactions, it might be possible that activation of Tregs is an epiphenomenon of persistent strong inflammation rather than a component of inflammation in AIP and IgG4-RD.

Ectopic germinal center formation is observed in the salivary glands of IgG4-RD patients[15]. Tfh cells, which express B cell lymphoma 6 (BCL6) and C-X-C chemokine receptor type 5 (CXCR5), and produce IL-21, play critical roles in germinal center reactions[4]. Expression levels of BCL6, CXCR5, and IL-21 in the ectopic germinal centers in the salivary glands were significantly higher in patients with IgG4-RD than in those with Sjogren syndrome[16]. Tfh cells isolated from the salivary glands and peripheral blood of patients with IgG4-RD had a greater capacity to stimulate IgG4 Ab production by B cells than tonsillar Tfh cells[17,18]. The percentage of circulating Tfh2 cells, defined as $CXCR5^+CXCR3^-$ C-C chemokine receptor type 6^- cells, positively correlated with serum IgG4 concentration in patients with IgG4-RD[19]. Thus, these data support the idea that Tfh cells are involved in the immunopathogenesis of IgG4-RD through the induction of germinal center reaction. However, the roles played by Tfh cells in AIP have not been clarified.

$CD4^+$ CTLs are a unique population of effector T cells that are often seen in patients with chronic viral infections. $CD4^+$ CTLs are localized in the salivary glands of IgG4-RD patients[20,21]. These cells express T-box-expressed-in-T-cells (T-bet) and produce interferon-g (IFN-γ)[4]. Although both Th1 cells and $CD4^+$ CTLs express T-bet and IFN-γ, they differ in expression levels of myeloid cell markers (CD11b) and in the ability to produce CCL4 and IL-1β[20,21]. In addition, these cells produce several cytotoxic proteins, including perforin and granzymes[20,21]. More importantly, these cells secrete TGF-β1, one of the prototypical pro-fibrogenic factors. Thus, $CD4^+$ CTLs are involved in chronic fibroinflammatory responses associated with IgG4-RD. However, accumulation of $CD4^+$ CTLs has not been verified in the pancreas of patients with AIP.

Innate immunity

Innate immunity is one of the major host defense mechanisms against microbial infections[22,23]. Recognition of microbial components by Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) induces pro-inflammatory cytokine responses to eradicate microbial infections[22,23]. It is well established that excessive innate immune responses underlie various types of autoimmune disorders[4,24]. Recent studies have highlighted the importance of innate immunity in AIP and IgG4-RD (Figure 1)[4].

We were the first to address the role of innate immunity in the development of AIP and IgG4-RD. We initially examined whether peripheral blood mononuclear cells (PBMCs) isolated from IgG4-RD patients produced pro-inflammatory cytokines upon exposure to TLR ligands and found that they secreted more IgG4 Ab and Th2 cytokines than PBMCs from healthy controls[25]. In the subsequent studies, we utilized a co-culture system composed of peripheral blood $CD14^+$ monocytes, $CD19^+$ B cells, and $CD3^+$ T cells isolated from patients with AIP and IgG4-RD. This co-culture system allowed us to show that B cells produced a large amount of IgG4 Ab in the presence of NLR and TLR ligands upon co-culture with monocytes isolated from patients with AIP and IgG4-RD, but not with monocytes from healthy controls[26].

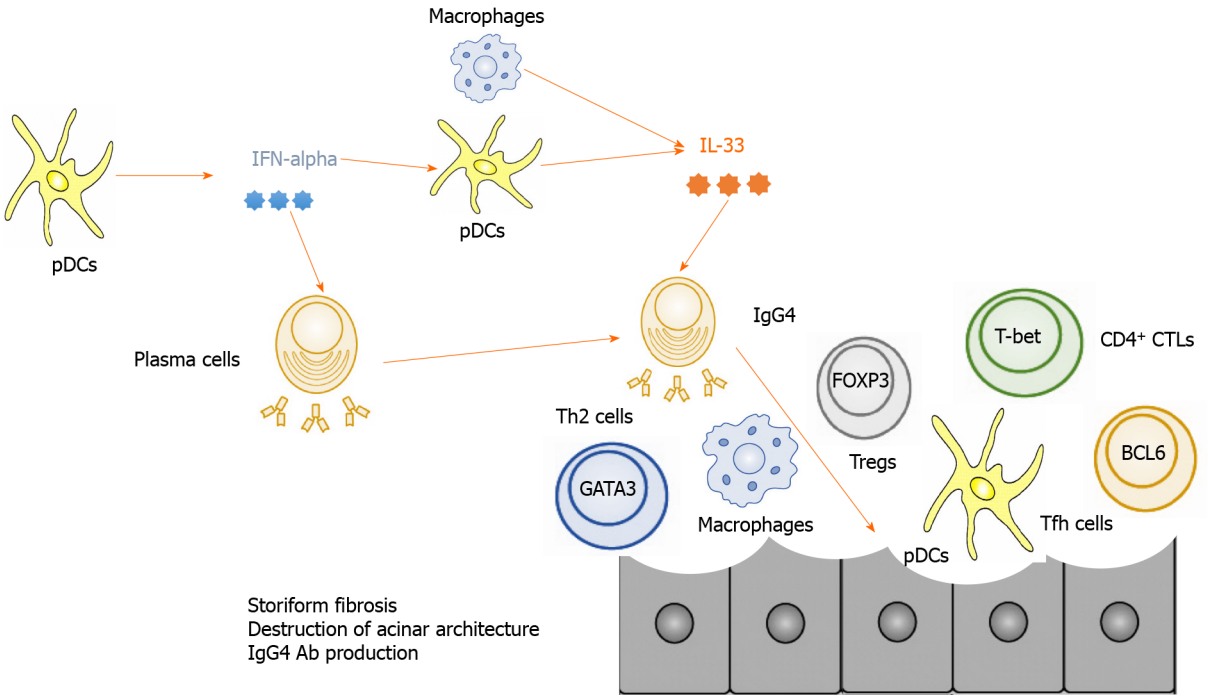


Figure 1 Immunopathogenesis of autoimmune pancreatitis and immunoglobulin G4-related disease. Plasmacytoid dendritic cells produce interferon-α and interleukin-33 and thereby mediate chronic fibroinflammatory responses in the pancreas. T helper type 2 cells expressing GATA-binding protein 3, regulatory T cells expressing forkhead box P3, follicular helper T cells expressing B cell lymphoma 6, and CD4$^+$ cytotoxic T cells expressing T-box-expressed-in-T-cells are involved in the development of autoimmune pancreatitis and immunoglobulin G4-related disease. IgG4: Immunoglobulin G4; pDCs: Plasmacytoid dendritic cells; IFN: Interferon; IL: Interleukin; Th2: T helper type 2 cells; Tregs: Regulatory T cells; GATA3: GATA-binding protein 3; FOXP3: Forkhead box P3; Tfh: Follicular helper T; BCL6: B cell lymphoma 6; T-bet: T-box-expressed-in-T-cells.

Interestingly, peripheral blood monocytes isolated from patients with AIP and IgG4-RD efficiently induced IgG4 Ab production by B cells from healthy controls in a T cell-independent manner. Stimulation of TLRs and NLRs led to the production of B cell-activating factor (BAFF), which induced IgG4 Ab responses[26]. In addition to monocytes, peripheral blood basophils isolated from patients with AIP and IgG4-RD also promoted IgG4 Ab production by B cells from healthy controls in a T cell-independent and BAFF-dependent manner[27]. These pioneering studies fully support the concept that excessive innate immune responses are involved in the development of AIP and IgG4-RD. Indeed, the expression of TLRs was verified in the salivary glands and pancreas of patients with AIP and IgG4-RD[28-30].

It remains uncertain whether innate immune responses are shared by peripheral blood and affected organs in AIP and IgG4-RD. To identify innate immune cells responsible for the development of AIP and IgG4-RD, we utilized a murine experimental model of AIP and IgG4-RD. Repeated intraperitoneal injections of polyinosinic-polycytidylic acid [poly (I:C)] into MRL/MpJ mice led to the development of AIP characterized by the destruction of the pancreatic acinar architecture, immune cell infiltration, and fibrosis[31,32]. Thus, this murine experimental AIP model recapitulated pathological findings observed in human AIP. Extensive flow cytometry analyses revealed massive accumulation of plasmacytoid dendritic cells (pDCs), defined as PDCA-1$^+$B220^{low}, in the pancreas[31]. pDCs are a specialized DC population that produce type I IFNs (IFN-α) upon recognition of TLR7 and TLR9 ligands[33]. Indeed, activation and accumulation of pDCs in the pancreas mediated experimental AIP through the production of IFN-α, because the depletion of pDCs or neutralization of type I IFN by Abs efficiently prevented the development of AIP[31]. Furthermore, pDCs expressing IFN-α and BAFF were found in the pancreas of patients with AIP and IgG4-RD, and peripheral blood pDCs isolated from these patients promoted IgG4 Ab production by healthy control B cells in a type I IFN dependent manner[31]. Thus, these results strongly suggest that activation of pDCs and type I IFN production are prominent features of murine experimental and human AIP.

Although a unique form of fibrosis, called storiform fibrosis, is one of the characteristic findings in human AIP[2-4], molecular mechanisms accounting for the induction and generation of this fibrogenic response have been poorly understood. We recently discovered that the type I IFN-IL-33 axis plays a pro-inflammatory and pro-

fibrogenic role in chronic alcoholic pancreatitis[34]. Type I IFN production by pancreatic acinar cells acts in concert with TNF-a produced by pancreatic macrophages to induce a robust production of IL-33 by the former cells[34]. Given that type I IFN produced by pDCs mediates experimental AIP, we hypothesized that IL-33 is involved in the generation of chronic fibroinflammatory responses in the pancreas. pDCs, which accumulate in the pancreas after repeated injections of poly (I:C), produced IL-33 in a type I IFN-dependent manner[32]. Importantly, the blockade of IL-33-mediated signaling pathways by an Ab against the IL-33 receptor attenuated chronic fibroinflammatory responses of the pancreas, which was accompanied by a marked reduction in pro-fibrogenic cytokines such as IL-13 and TGF-β1[32]. Immunofluorescence studies of pancreatic specimens from patients with AIP and IgG4-RD confirmed pancreatic localization of pDCs expressing IL-33[32]. Taken together, these results support the idea that activation of pDCs followed by the production of IFN-α and IL-33 mediates both experimental and human AIP. However, it should be noted that pDCs are not the only cellular source of IL-33. For example, M2 macrophages have been shown to co-localize with IL-33 in the salivary glands of IgG4-RD patients [30,35].

IMMUNOGLOBULINS AS BIOMARKERS IN AUTOIMMUNE PANCREATITIS AND IGG4-RD

The diagnosis of AIP and IgG4-RD relies on the detection of elevated serum concentration of IgG4 Ab as well as on the characteristic pathological findings, including abundant infiltration of IgG4-expressing plasma cells, storiform fibrosis, and obliterative phlebitis[2-4]. Thus, serum level of IgG4 Ab is widely used as an established biomarker for the diagnosis of AIP and IgG4-RD. Moreover, serum concentration of IgG4 declines rapidly after the induction of remission by prednisolone[11]. Indeed, serum concentration of IgG4 Ab was much higher in patients with AIP and IgG4-RD than in individuals with chronic alcoholic pancreatitis and in healthy controls in our previous study[36]. Therefore, there is no doubt that measurement of serum IgG4 Ab concentration in clinical practice is necessary not only for the diagnosis of AIP and IgG4-RD but also for the assessment of disease activity. However, IgG4 Ab level is not always informative for the diagnosis or assessment of disease activity in such patients. Around 20% of patients with AIP have normal serum concentration of IgG4 Ab[10]. Furthermore, a significant fraction of patients with pancreatic cancer also exhibit elevated IgG4 Ab concentration[9]. Moreover, disease flare-up is sometimes seen in patients with AIP and IgG4-RD, even on the background of normalized serum concentrations of IgG4 Ab[11].

IgG4 Ab is unique in that it has a limited ability to activate Fcγ receptors and complements[37]. Thus, IgG4 Ab is considered to play non-pathogenic rather than pathogenic roles in the development of AIP and IgG4-RD. Shiokawa *et al*[38] directly addressed this issue by utilizing a passive transfer of patient IgG subtypes into neonatal mice. They found that pancreatic injury was successfully induced by a passive transfer of total IgG isolated from patients with AIP, but not by total IgG from healthy controls. The degree of pancreatic injury was much greater in neonatal mice treated with the IgG1 Ab from AIP patients than in those that received the IgG4 Ab from the same patients. Moreover, pancreatic injury induced by a passive transfer of IgG1 Ab was efficiently inhibited by a co-transfer of IgG4 Ab[38]. These studies conducted by Shiokawa *et al*[38] strongly suggest that IgG1 Ab rather than IgG4 Ab contributes to the immunopathogenesis of AIP and IgG4-RD. Consistent with this idea, serum concentrations of both IgG1 and IgG4 Abs were significantly higher in patients with AIP and IgG4-RD than in individuals with chronic alcoholic pancreatitis or in healthy controls (Table 1)[36].

As for the other IgG subtypes, no significant differences were observed in serum concentrations of IgG3 Ab in patients with AIP and IgG4-RD in comparison with those in patients with chronic alcoholic pancreatitis or in healthy controls[36]. Serum concentration of IgG2 Ab was significantly lower in patients with AIP and IgG4-RD than in patients with chronic alcoholic pancreatitis (Table 1)[36]. In contrast, another report showed that serum concentration of IgG2 Ab was elevated in patients suffering from IgG4-RD[39]. This discrepancy can be explained by the difference in the organ distribution of IgG4-RD. Serum concentration of this IgG subtype is preferentially elevated in patients with orbital IgG4-RD, but not in those with pancreatic IgG4-RD[36,39].

Table 1 Possible biomarkers in autoimmune pancreatitis and immunoglobulin G4-related disease

Biomarkers		Ref.
Immunoglobulins	IgG1	Minaga *et al*[36]
	IgG2	Chan *et al*[39]
	IgE	Minaga *et al*[36], Culver *et al*[41]
	IgM	Taguchi *et al*[40]
Cytokines	IFN-α	Arai *et al*[31], Minaga *et al*[36], Minaga *et al*[53]
	IL-5	Yamamoto *et al*[49]
	IL-6	Tsukuda *et al*[60]
	IL-33	Furukawa *et al*[35], Minaga *et al*[36], Minaga *et al*[53]
	BAFF	Arai *et al*[31], Kiyama *et al*[58]
Chemokines	CCL17	Umeda *et al*[63]
Autoantibodies	Laminin 511	Shiokawa *et al*[42]
	Annexin A11	Hubers *et al*[43]
	Galectin-3	Perugino *et al*[44]

BAFF: B cell activating factor; CCL17: C-C motif chemokine ligand 17.

Serum total IgG concentration is also elevated in patients with AIP and IgG4-RD[2-4]. Thus, elevations in serum concentrations of both total IgG and IgG4 are prominent features of patients with AIP and IgG4-RD. In contrast to augmented IgG and IgG4 levels, serum concentrations of IgM and IgA are decreased in patients with AIP and IgG4-RD[40]. Furthermore, serum concentration of IgM inversely correlated with those of IgG and IgG4 (Table 1)[40]. The diagnostic value of reduced serum concentrations of IgA and IgM needs to be determined in future studies.

Co-occurrence of AIP/IgG4-RD and allergic disorders is often observed[2-4]. In fact, serum concentration of IgE is significantly higher in patients with AIP and IgG4-RD than in those with chronic alcoholic pancreatitis and in healthy controls[36]. This raises the possibility that serum concentration of IgE can be used as a biomarker for AIP and IgG4-RD. In line with this idea, approximately 50% of patients with AIP and IgG4-RD exhibit elevated serum concentration of IgE[41]. Moreover, changes in serum concentration of IgE are associated with the relapse of these disorders[41]. Therefore, serum concentration of IgE can be used as a biomarker for the diagnosis and prediction of relapse in AIP and IgG4-RD[41].

AUTOANTIBODIES AS BIOMARKERS IN AUTOIMMUNE PANCREATITIS AND IGG4-RD

Although AIP and IgG4-RD are considered to be caused by autoimmune reactions, autoAbs responsible for the development of autoimmunity have not been identified. Recently, three different types of autoAbs have been identified[42-44]. These autoAbs recognize laminin 511, annexin A11, and galectin-3 (Table 1). Fifty-one percent of AIP patients were positive for autoAb against laminin 511-E8, a truncated variant of the extracellular matrix protein laminin 511. Furthermore, serum IgG1 purified from AIP patients co-localized with laminin 511 in the pancreas of neonatal mice upon passive transfer[42]. Huber *et al*[43] identified annexin A11, a calcium-dependent phospholipid-binding protein, as a candidate autoantigen in AIP. Interestingly, annexin A11-specific IgG4 and IgG1 Abs purified from patients with AIP shared antigenic epitopes and IgG4 autoAbs inhibited pathogenic binding of IgG1 Ab to the shared epitopes[43]. These data suggest that IgG1 autoAbs rather than IgG4 autoAbs play pathogenic roles in the development of AIP and IgG4-RD. Confirmation of these results awaits future studies that should address the diagnostic utility of these autoAbs in a large number of patients with AIP and IgG4-RD. However, identification of autoAbs associated with AIP and IgG4-RD strongly supports the idea that these disorders arise from autoimmune reactions.

ADAPTIVE IMMUNITY CYTOKINES AS BIOMARKERS IN AUTOIMMUNE PANCREATITIS AND IGG4-RD

Effector $CD4^+$ T cell subpopulations, including Th2 cells, Tregs, Tfh cells, and $CD4^+$ CTLs, are involved in the immunopathogenesis of AIP and IgG4-RD, as shown by the localization of these T cells in the affected organs. Th2 cells, Tregs, Tfh cells, and $CD4^+$ CTLs cells were detected in the peripheral blood of patients with AIP or IgG4-RD[17,19,20,45-48] and found to be markedly decreased after induction of remission[20,46], raising the possibility that serum concentrations of cytokines derived from effector T cells can be useful biomarkers (Table 1 and Figure 2). Yamamoto *et al*[49] found that serum concentration of IL-5 was elevated in patients with IgG4-RD, whereas serum concentrations of IL-10, IL-13, IL-21, and TGF-β1 were comparable in patients and healthy controls. Given that the major cellular source of IL-5 is Th2 cells, these data support the idea that the activation status of Th2 cells might be a surrogate marker for IgG4-RD and AIP. In line with this notion, bile concentrations of Th2 cytokines such as IL-4, IL-5, and IL-13 were significantly higher in the patients with IgG4-related sclerosing cholangitis than in those with primary sclerosing cholangitis[50]. Mechanistically, these Th2 cytokines induce bile leakage due to the impairment of the tight junction-associated biliary epithelial cell barrier, thereby causing chronic biliary inflammation[50]. Moreover, two cases of IgG4-RD successfully treated with dupilumab that neutralizes IL-4 receptor α have been reported[51,52]. Thus, Th2 responses may underlie the immunopathogenesis of AIP and IgG4-RD. Therefore, Th2 cytokines, especially IL-5, can be used as biomarkers of AIP and IgG4-RD.

A positive correlation between serum concentration of IgG4 Ab and circulating numbers of Tfh cells has been demonstrated in patients with IgG4-RD[17,19,46]. However, the utility of serum concentration of IL-21, a prototypical cytokine produced by Tfh cells, for IgG4-RD diagnosis has not been verified. Similarly, the usefulness of serum concentrations of IFN-γ and TGF-β1 produced by $CD4^+$ CTLs as biomarkers of AIP and IgG4-RD has not been reported either.

Therefore, at present, the utility of adaptive immunity cytokines as biomarkers for AIP and IgG4-RD is limited. The reason why previous studies did not successfully identify adaptive immunity cytokines as biomarkers might be partially explained by a broad range of affected organs in AIP and IgG4-RD or by complex effector T cell responses. Thus, the classification of IgG4-RD into subtypes by affected organ distribution might lead to the identification of biomarkers specific for each such subtype.

INNATE IMMUNITY CYTOKINES AS BIOMARKERS IN AUTOIMMUNE PANCREATITIS AND IGG4-RD

Activation of pDCs and the subsequent robust production of IFN-α and IL-33 are characteristic pathogenic immune responses in experimental AIP and human IgG4-RD[31,32]. These findings led us to examine whether serum concentrations of IFN-α and IL-33 could be useful biomarkers for AIP and IgG4-RD (Table 1 and Figure 2). For this purpose, we measured serum concentrations of these cytokines in patients with AIP and IgG4-RD who met the well-established diagnostic criteria[5,6,36]. In comparison with the patients with chronic alcoholic pancreatitis and healthy controls, the patients with AIP and IgG4-RD displayed markedly elevated serum concentrations of IFN-α and IL-33[36]. In contrast, serum levels of the prototypical proinflammatory cytokines IL-1β and IL-6 were comparable in the patients with AIP/IgG4-RD and those with chronic alcoholic pancreatitis[36]. Serum concentrations of IFN-α and IL-33 positively correlated with those of IgG4 Ab[36]. Thus, measurements of serum concentrations of IFN-α and IL-33 may be very useful for the diagnosis of AIP and IgG4-RD[36].

We then evaluated the diagnostic performance of serum IFN-α and IL-33 concentrations for AIP and IgG4-RD. Surprisingly, the diagnostic performance of serum IFN-α and IL-33 concentrations as diagnostic markers for AIP and IgG4-RD was comparable to that of serum IgG4 Ab, as calculated by the receiver operating characteristic curve analysis[36]. Moreover, the induction of remission by prednisolone markedly reduced serum concentrations of IFN-α and IL-33. Thus, serum IFN-α and IL-33 concentrations can be biomarkers that are useful not only for the diagnosis but also for the assessment of disease activity. Taken together, our data strongly suggested that serum concentrations of IFN-α and IL-33 might serve as novel biomarkers in AIP

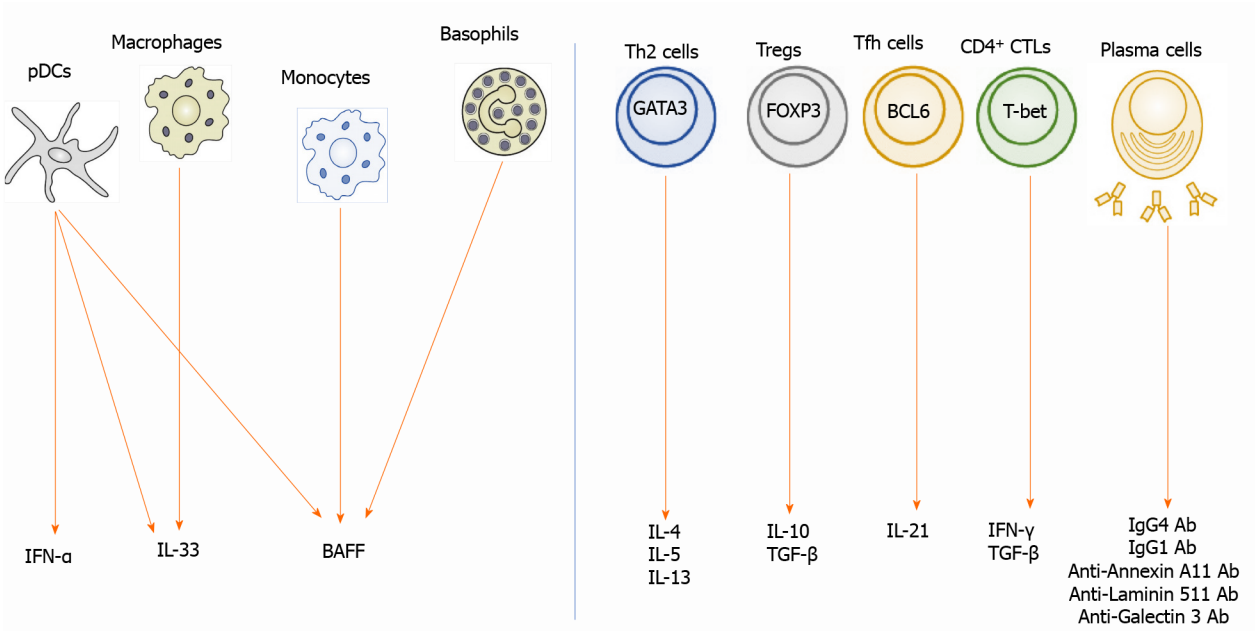


Figure 2 Biomarkers in autoimmune pancreatitis and immunoglobulin G4-related disease. Left panel: Interferon-a (IFN-α) is produced by plasmacytoid dendritic cells (pDCs). Interleukin-33 (IL-33) is produced by pDCs and macrophages. B cell-activating factor (BAFF) is produced by pDCs, monocytes, and basophils; Right panel: IL-4, IL-5, and IL-13 are produced by T helper type 2 cells expressing GATA-binding protein 3 (GATA3). IL-10 and transforming growth factor-β1 (TGF-β) are produced by regulatory T cells (Tregs) expressing forkhead box P3. IL-21 is produced by follicular helper T cells expressing B cell lymphoma 6. TGF-β is produced by Tregs and CD4$^+$ cytotoxic T cells (CTLs) expressing T-box-expressed-in-T-cells. IFN-γ is produced by CD4$^+$ CTLs. Plasma cells produce immunoglobulin G1 (IgG1) and IgG4 Ab. These cytokines are possible biomarkers for autoimmune pancreatitis and IgG4-related disease. IgG: Immunoglobulin G; pDCs: Plasmacytoid dendritic cells; IFN: Interferon; IL: Interleukin; BAFF: B cell-activating factor; Th2: T helper type 2 cells; Tregs: Regulatory T cells; GATA3: GATA-binding protein 3; FOXP3: Forkhead box P3; Tfh: Follicular helper T; BCL6: B cell lymphoma 6; T-bet: T-box-expressed-in-T-cells; CTLs: CD4$^+$ cytotoxic T cells.

and IgG4-RD. This idea is fully supported by an observation of an AIP/IgG4-RD case in which serum concentrations of IFN-α and IL-33 were markedly reduced soon after the induction of remission, whereas those of IgG4 remained unchanged even after the successful induction of remission[53]. Identification of the IFN-α-IL-33 axis as a crucial pathogenic pathway as well as a biomarker leads us to speculate that patients with AIP and IgG4-RD can be treated with biologics targeting IFN-α as in the case of systemic lupus erythematosus[54,55].

BAFF and a proliferation-inducing ligand (APRIL) are cytokines produced by antigen-presenting cells[56]. BAFF and APRIL are crucial factors for B cell survival and thus, they promote Ig production[56]. Given that AIP and IgG4-RD are characterized by elevated concentrations of serum total IgG and IgG4, in particular, it is likely that BAFF and APRIL are involved in the immunopathogenesis of these disorders. Indeed, pDCs producing BAFF have been demonstrated in the pancreas of patients with AIP and IgG4-RD[31]. Moreover, T cell-independent class switch recombination of IgG4 Ab requires BAFF production by monocytes[26]. The involvement of B cell survival factors in the development of AIP and IgG4-RD is further supported by the high probability of successful remission induction by rituximab in patients with IgG4-RD[57]. As for the utility of BAFF and APRIL as biomarkers for AIP and IgG4-RD, serum concentrations of BAFF and APRIL were significantly higher in patients with these disorders than in healthy controls[31,58]. In addition, induction of remission by prednisolone markedly reduced serum concentrations of BAFF[58]. Therefore, measurements of serum concentrations of BAFF and APRIL might be very useful not only for the diagnosis but also for monitoring disease activity.

IL-6 is a pleiotropic cytokine associated with autoimmune responses[59]. Although serum concentrations of this cytokine were comparable in patients with chronic pancreatitis, patients with AIP/IgG4-RD, and healthy controls in our study, elevated serum IL-6 level might help to discriminate a specific type of patients with AIP and IgG4-RD. Tsukuda *et al*[60] compared clinical manifestations of patients with AIP and IgG4-RD in relation to serum concentration of IL-6. They found that hepatosplenomegaly and biliary tract involvement tended to be more prevalent in patients with high IL-6 serum level than in those with low IL-6 concentration[60]. However, we need to be cautious regarding the interpretation of these data, because hepatosplenomegaly is often seen in patients with multicentric Castleman disease, an IL-6-driven systemic

autoimmune disorder[61]. Therefore, some patients with AIP and IgG4-RD might exhibit clinical manifestations similar to those of Castleman disease.

These previous studies on innate immunity cytokines have opened up new research vistas that can facilitate identification of novel biomarkers in AIP and IgG4-RD. In particular, serum concentrations of IFN-α and IL-33, which faithfully reflect disease activity, may be informative diagnostic examinations in AIP and IgG4-RD[36].

CHEMOKINES AS BIOMARKERS IN AUTOIMMUNE PANCREATITIS AND IGG4-RD

AIP and IgG4-RD are characterized by Th2 responses[2-4]. The prototypical Th2 chemokine, thymus and activation-regulated chemokine, also known as CCL17, is a well-established biomarker for atopic dermatitis[62]. Given that atopic dermatitis and IgG4-RD share Th2 responses, it is likely that serum concentration of CCL17 could be a useful biomarker for IgG4-RD and AIP. Umeda *et al*[63] explored the utility of serum concentration of CCL17 as a possible biomarker for IgG4-RD (Table 1). They found that serum concentration of CCL17 was significantly higher in patients with IgG4-RD than in those with Sjogren syndrome or in healthy controls[63]. Although no association between serum concentrations of CCL17 and IgG4 Ab have been observed, those of CCL17 positively correlated with the number of affected organs. However, the utility of this chemokine as a biomarker for AIP has not been examined.

As mentioned above, production of IFN-α by pDCs is a prominent feature of AIP and IgG4-RD. Excessive IFN-α responses result in the robust production of chemokines such as C-X-C motif chemokine ligand 9 (CXCL9) and CXCL10[31]. In fact, the development of experimental AIP is accompanied by the enhanced expression of CXCL9 and CXCL10 in the pancreas[31]. The utility of CXCL9 and CXCL10 as biomarkers for AIP and IgG4-RD awaits future studies with the use of samples from patients with AIP and IgG4-RD.

CONCLUSION

AIP and IgG4-RD are newly established disease entities[2-4]. Both disorders are characterized by elevated serum concentration of IgG4 Ab and accumulation of IgG4-expressing plasma cells in the affected organs[2-4]. Moreover, the induction of remission by prednisolone is accompanied by a marked decrease in serum concentration of IgG4 Ab in patients with AIP and IgG4-RD[2-4]. Therefore, serum concentration of this IgG subtype is undoubtedly a useful biomarker for both the diagnosis and assessment of disease activity. However, a significant fraction of patients with AIP display active disease even at normal serum IgG4 Ab concentration. Recent elegant studies have shown that IgG1 rather than IgG4 plays the main pathogenic role in the development of AIP and IgG4-RD[38]. Thus, elevated IgG4 Ab responses seen in AIP and IgG4-RD are an epiphenomenon associated with chronic inflammatory reactions. Therefore, novel biomarkers based on the understanding of immunopathogenesis need to be established. We have recently found that pDCs producing IFN-α and IL-33 mediate experimental AIP and human IgG4-RD[31,32]. Interestingly, serum concentrations of IFN-α and IL-33 have been identified as potent biomarkers for the diagnosis and assessment of disease activity in AIP and IgG4-RD[36,53]. Based on recently identified biomarkers of these disorders, we propose diagnostic algorithm for patients with AIP and IgG4-RD exhibiting normal or slightly elevated concentrations of serum IgG4 Ab (Figure 3). As shown in Figure 3, measurement of serum concentrations of cytokines and autoAbs in combination with serum IgG4 Ab might be useful for the diagnosis of AIP and IgG4-RD affluent in diversity.

As our knowledge of the immunopathogenesis of AIP and IgG4-RD increases, many candidate biomarkers will likely be identified in the future. The discovery of such biomarkers will contribute to the clinical practice and advance further our understanding of AIP and IgG4-RD immunopathogenesis.

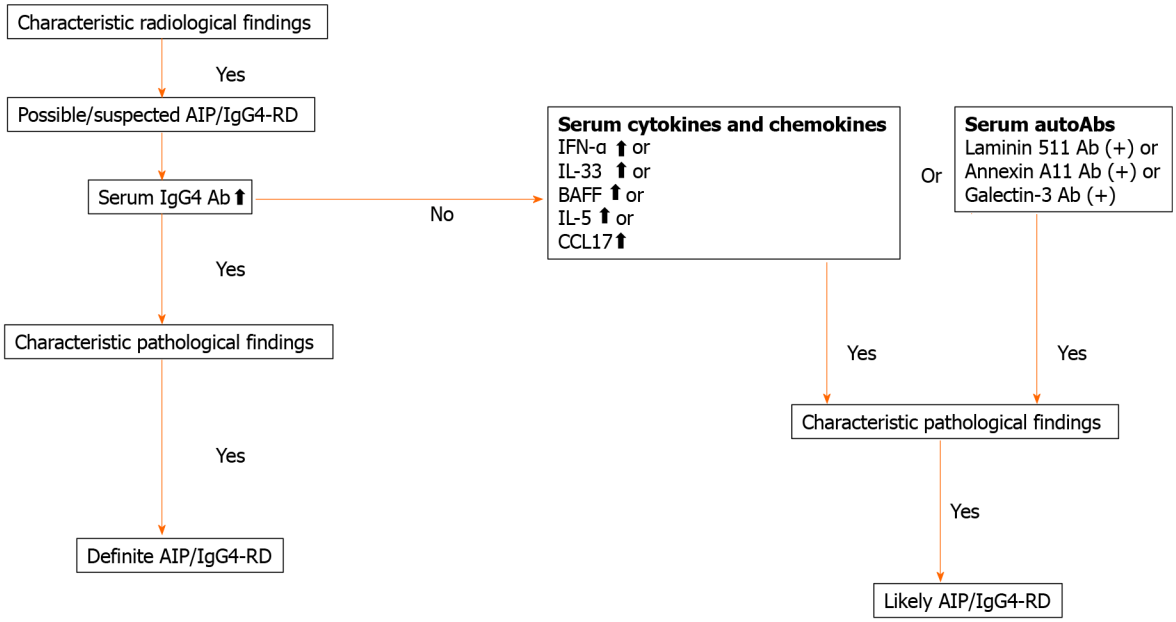


Figure 3 Diagnostic algorithm for autoimmune pancreatitis and immunoglobulin G4-related disease. Serum concentration of cytokines and chemokines or the presence of serum auto-antibodies may be useful for diagnosis of autoimmune pancreatitis and immunoglobulin G4 (IgG4)-related disease displaying normal or slightly elevated concentrations of IgG4. AIP: Autoimmune pancreatitis; IgG4-RD: Immunoglobulin G4-related disease; IFN: Interferon; IL: Interleukin; BAFF: B cell-activating factor; CCL17: C-C motif chemokine ligand 17.

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REFERENCES

1 **Kamisawa T**, Chari ST, Lerch MM, Kim MH, Gress TM, Shimosegawa T. Recent advances in autoimmune pancreatitis: type 1 and type 2. *Gut* 2013; **62**: 1373-1380 [PMID: 23749606 DOI: 10.1136/gutjnl-2012-304224]
2 **Stone JH**, Zen Y, Deshpande V. IgG4-related disease. *N Engl J Med* 2012; **366**: 539-551 [PMID: 22316447 DOI: 10.1056/NEJMra1104650]
3 **Kamisawa T**, Zen Y, Pillai S, Stone JH. IgG4-related disease. *Lancet* 2015; **385**: 1460-1471 [PMID: 25481618 DOI: 10.1016/S0140-6736(14)60720-0]
4 **Watanabe T**, Minaga K, Kamata K, Kudo M, Strober W. Mechanistic Insights into Autoimmune Pancreatitis and IgG4-Related Disease. *Trends Immunol* 2018; **39**: 874-889 [PMID: 30401468 DOI: 10.1016/j.it.2018.09.005]
5 **Kawa S**, Kamisawa T, Notohara K, Fujinaga Y, Inoue D, Koyama T, Okazaki K. Japanese Clinical Diagnostic Criteria for Autoimmune Pancreatitis, 2018: Revision of Japanese Clinical Diagnostic Criteria for Autoimmune Pancreatitis, 2011. *Pancreas* 2020; **49**: e13-e14 [PMID: 31856100 DOI: 10.1097/MPA.0000000000001443]
6 **Umehara H**, Okazaki K, Nakamura T, Satoh-Nakamura T, Nakajima A, Kawano M, Mimori T, Chiba T. Current approach to the diagnosis of IgG4-related disease - Combination of comprehensive diagnostic and organ-specific criteria. *Mod Rheumatol* 2017; **27**: 381-391 [PMID: 28165852 DOI: 10.1080/14397595.2017.1290911]
7 **Tang J**, Cai S, Ye C, Dong L. Biomarkers in IgG4-related disease: A systematic review. *Semin Arthritis Rheum* 2020; **50**: 354-359 [PMID: 31280934 DOI: 10.1016/j.semarthrit.2019.06.018]
8 **Culver EL**, Sadler R, Simpson D, Cargill T, Makuch M, Bateman AC, Ellis AJ, Collier J, Chapman RW, Klenerman P, Barnes E, Ferry B. Elevated Serum IgG4 Levels in Diagnosis, Treatment Response, Organ Involvement, and Relapse in a Prospective IgG4-Related Disease UK Cohort. *Am J Gastroenterol* 2016; **111**: 733-743 [PMID: 27091321 DOI: 10.1038/ajg.2016.40]
9 **Ghazale A**, Chari ST, Smyrk TC, Levy MJ, Topazian MD, Takahashi N, Clain JE, Pearson RK, Pelaez-Luna M, Petersen BT, Vege SS, Farnell MB. Value of serum IgG4 in the diagnosis of autoimmune pancreatitis and in distinguishing it from pancreatic cancer. *Am J Gastroenterol* 2007; **102**: 1646-1653 [PMID: 17555461 DOI: 10.1111/j.1572-0241.2007.01264.x]
10 **Kamisawa T**, Takuma K, Tabata T, Inaba Y, Egawa N, Tsuruta K, Hishima T, Sasaki T, Itoi T. Serum IgG4-negative autoimmune pancreatitis. *J Gastroenterol* 2011; **46**: 108-116 [PMID: 20824290 DOI: 10.1007/s00535-010-0317-2]

11 **Kamisawa T**, Shimosegawa T, Okazaki K, Nishino T, Watanabe H, Kanno A, Okumura F, Nishikawa T, Kobayashi K, Ichiya T, Takatori H, Yamakita K, Kubota K, Hamano H, Okamura K, Hirano K, Ito T, Ko SB, Omata M. Standard steroid treatment for autoimmune pancreatitis. *Gut* 2009; **58**: 1504-1507 [PMID: 19398440 DOI: 10.1136/gut.2008.172908]

12 **Jeannin P**, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol* 1998; **160**: 3555-3561 [PMID: 9531318]

13 **Zen Y**, Fujii T, Harada K, Kawano M, Yamada K, Takahira M, Nakanuma Y. Th2 and regulatory immune reactions are increased in immunoglobin G4-related sclerosing pancreatitis and cholangitis. *Hepatology* 2007; **45**: 1538-1546 [PMID: 17518371 DOI: 10.1002/hep.21697]

14 **Koyabu M**, Uchida K, Miyoshi H, Sakaguchi Y, Fukui T, Ikeda H, Takaoka M, Hirohara J, Nishio A, Uemura Y, Uemoto S, Okazaki K. Analysis of regulatory T cells and IgG4-positive plasma cells among patients of IgG4-related sclerosing cholangitis and autoimmune liver diseases. *J Gastroenterol* 2010; **45**: 732-741 [PMID: 20087609 DOI: 10.1007/s00535-010-0199-3]

15 **Tanaka A**, Moriyama M, Nakashima H, Miyake K, Hayashida JN, Maehara T, Shinozaki S, Kubo Y, Nakamura S. Th2 and regulatory immune reactions contribute to IgG4 production and the initiation of Mikulicz disease. *Arthritis Rheum* 2012; **64**: 254-263 [PMID: 21898360 DOI: 10.1002/art.33320]

16 **Maehara T**, Moriyama M, Nakashima H, Miyake K, Hayashida JN, Tanaka A, Shinozaki S, Kubo Y, Nakamura S. Interleukin-21 contributes to germinal centre formation and immunoglobulin G4 production in IgG4-related dacryoadenitis and sialoadenitis, so-called Mikulicz's disease. *Ann Rheum Dis* 2012; **71**: 2011-2019 [PMID: 22753386 DOI: 10.1136/annrheumdis-2012-201477]

17 **Chen Y**, Lin W, Yang H, Wang M, Zhang P, Feng R, Chen H, Peng L, Zhang X, Zhao Y, Zeng X, Zhang F, Zhang W, Lipsky PE. Aberrant Expansion and Function of Follicular Helper T Cell Subsets in IgG4-Related Disease. *Arthritis Rheumatol* 2018; **70**: 1853-1865 [PMID: 29781221 DOI: 10.1002/art.40556]

18 **Kamekura R**, Takano K, Yamamoto M, Kawata K, Shigehara K, Jitsukawa S, Nagaya T, Ito F, Sato A, Ogasawara N, Tsubomatsu C, Takahashi H, Nakase H, Himi T, Ichimiya S. Cutting Edge: A Critical Role of Lesional T Follicular Helper Cells in the Pathogenesis of IgG4-Related Disease. *J Immunol* 2017; **199**: 2624-2629 [PMID: 28916523 DOI: 10.4049/jimmunol.1601507]

19 **Akiyama M**, Suzuki K, Yamaoka K, Yasuoka H, Takeshita M, Kaneko Y, Kondo H, Kassai Y, Miyazaki T, Morita R, Yoshimura A, Takeuchi T. Number of Circulating Follicular Helper 2 T Cells Correlates With IgG4 and Interleukin-4 Levels and Plasmablast Numbers in IgG4-Related Disease. *Arthritis Rheumatol* 2015; **67**: 2476-2481 [PMID: 25989153 DOI: 10.1002/art.39209]

20 **Mattoo H**, Mahajan VS, Maehara T, Deshpande V, Della-Torre E, Wallace ZS, Kulikova M, Drijvers JM, Daccache J, Carruthers MN, Castelino FV, Stone JR, Stone JH, Pillai S. Clonal expansion of CD4(+) cytotoxic T lymphocytes in patients with IgG4-related disease. *J Allergy Clin Immunol* 2016; **138**: 825-838 [PMID: 26971690 DOI: 10.1016/j.jaci.2015.12.1330]

21 **Maehara T**, Mattoo H, Ohta M, Mahajan VS, Moriyama M, Yamauchi M, Drijvers J, Nakamura S, Stone JH, Pillai SS. Lesional CD4+ IFN-γ+ cytotoxic T lymphocytes in IgG4-related dacryoadenitis and sialoadenitis. *Ann Rheum Dis* 2017; **76**: 377-385 [PMID: 27358392 DOI: 10.1136/annrheumdis-2016-209139]

22 **Takeda K**, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005; **17**: 1-14 [PMID: 15585605 DOI: 10.1093/intimm/dxh186]

23 **Philpott DJ**, Sorbara MT, Robertson SJ, Croitoru K, Girardin SE. NOD proteins: regulators of inflammation in health and disease. *Nat Rev Immunol* 2014; **14**: 9-23 [PMID: 24336102 DOI: 10.1038/nri3565]

24 **Ganguly D**. Do Type I Interferons Link Systemic Autoimmunities and Metabolic Syndrome in a Pathogenetic Continuum? *Trends Immunol* 2018; **39**: 28-43 [PMID: 28826817 DOI: 10.1016/j.it.2017.07.001]

25 **Akitake R**, Watanabe T, Zaima C, Uza N, Ida H, Tada S, Nishida N, Chiba T. Possible involvement of T helper type 2 responses to Toll-like receptor ligands in IgG4-related sclerosing disease. *Gut* 2010; **59**: 542-545 [PMID: 20332525 DOI: 10.1136/gut.2009.200972]

26 **Watanabe T**, Yamashita K, Fujikawa S, Sakurai T, Kudo M, Shiokawa M, Kodama Y, Uchida K, Okazaki K, Chiba T. Involvement of activation of toll-like receptors and nucleotide-binding oligomerization domain-like receptors in enhanced IgG4 responses in autoimmune pancreatitis. *Arthritis Rheum* 2012; **64**: 914-924 [PMID: 21971969 DOI: 10.1002/art.33386]

27 **Watanabe T**, Yamashita K, Sakurai T, Kudo M, Shiokawa M, Uza N, Kodama Y, Uchida K, Okazaki K, Chiba T. Toll-like receptor activation in basophils contributes to the development of IgG4-related disease. *J Gastroenterol* 2013; **48**: 247-253 [PMID: 22744834 DOI: 10.1007/s00535-012-0626-8]

28 **Fukui Y**, Uchida K, Sakaguchi Y, Fukui T, Nishio A, Shikata N, Sakaida N, Uemura Y, Satoi S, Okazaki K. Possible involvement of Toll-like receptor 7 in the development of type 1 autoimmune pancreatitis. *J Gastroenterol* 2015; **50**: 435-444 [PMID: 25005350 DOI: 10.1007/s00535-014-0977-4]

29 **Yanagawa M**, Uchida K, Ando Y, Tomiyama T, Yamaguchi T, Ikeura T, Fukui T, Nishio A, Uemura Y, Miyara T, Okamoto H, Satoi S, Okazaki K. Basophils activated *via* TLR signaling may contribute to pathophysiology of type 1 autoimmune pancreatitis. *J Gastroenterol* 2018; **53**: 449-460 [PMID: 28921377 DOI: 10.1007/s00535-017-1390-6]

30 **Ishiguro N**, Moriyama M, Furusho K, Furukawa S, Shibata T, Murakami Y, Chinju A, Haque ASMR, Gion Y, Ohta M, Maehara T, Tanaka A, Yamauchi M, Sakamoto M, Mochizuki K, Ono Y, Hayashida JN, Sato Y, Kiyoshima T, Yamamoto H, Miyake K, Nakamura S. Activated M2

Macrophages Contribute to the Pathogenesis of IgG4-Related Disease *via* Toll-like Receptor 7/Interleukin-33 Signaling. *Arthritis Rheumatol* 2020; **72**: 166-178 [PMID: 31339007 DOI: 10.1002/art.41052]

31 **Arai Y**, Yamashita K, Kuriyama K, Shiokawa M, Kodama Y, Sakurai T, Mizugishi K, Uchida K, Kadowaki N, Takaori-Kondo A, Kudo M, Okazaki K, Strober W, Chiba T, Watanabe T. Plasmacytoid Dendritic Cell Activation and IFN-α Production Are Prominent Features of Murine Autoimmune Pancreatitis and Human IgG4-Related Autoimmune Pancreatitis. *J Immunol* 2015; **195**: 3033-3044 [PMID: 26297761 DOI: 10.4049/jimmunol.1500971]

32 **Watanabe T**, Yamashita K, Arai Y, Minaga K, Kamata K, Nagai T, Komeda Y, Takenaka M, Hagiwara S, Ida H, Sakurai T, Nishida N, Strober W, Kudo M. Chronic Fibro-Inflammatory Responses in Autoimmune Pancreatitis Depend on IFN-α and IL-33 Produced by Plasmacytoid Dendritic Cells. *J Immunol* 2017; **198**: 3886-3896 [PMID: 28373582 DOI: 10.4049/jimmunol.1700060]

33 **Swiecki M**, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol* 2015; **15**: 471-485 [PMID: 26160613 DOI: 10.1038/nri3865]

34 **Watanabe T**, Sadakane Y, Yagama N, Sakurai T, Ezoe H, Kudo M, Chiba T, Strober W. Nucleotide-binding oligomerization domain 1 acts in concert with the cholecystokinin receptor agonist, cerulein, to induce IL-33-dependent chronic pancreatitis. *Mucosal Immunol* 2016; **9**: 1234-1249 [PMID: 26813347 DOI: 10.1038/mi.2015.144]

35 **Furukawa S**, Moriyama M, Miyake K, Nakashima H, Tanaka A, Maehara T, Iizuka-Koga M, Tsuboi H, Hayashida JN, Ishiguro N, Yamauchi M, Sumida T, Nakamura S. Interleukin-33 produced by M2 macrophages and other immune cells contributes to Th2 immune reaction of IgG4-related disease. *Sci Rep* 2017; **7**: 42413 [PMID: 28205524 DOI: 10.1038/srep42413]

36 **Minaga K**, Watanabe T, Hara A, Kamata K, Omoto S, Nakai A, Otsuka Y, Sekai I, Yoshikawa T, Yamao K, Takenaka M, Chiba Y, Kudo M. Identification of serum IFN-α and IL-33 as novel biomarkers for type 1 autoimmune pancreatitis and IgG4-related disease. *Sci Rep* 2020; **10**: 14879 [PMID: 32938972 DOI: 10.1038/s41598-020-71848-4]

37 **Aalberse RC**, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. *Clin Exp Allergy* 2009; **39**: 469-477 [PMID: 19222496 DOI: 10.1111/j.1365-2222.2009.03207.x]

38 **Shiokawa M**, Kodama Y, Kuriyama K, Yoshimura K, Tomono T, Morita T, Kakiuchi N, Matsumori T, Mima A, Nishikawa Y, Ueda T, Tsuda M, Yamauchi Y, Minami R, Sakuma Y, Ota Y, Maruno T, Kurita A, Sawai Y, Tsuji Y, Uza N, Matsumura K, Watanabe T, Notohara K, Tsuruyama T, Seno H, Chiba T. Pathogenicity of IgG in patients with IgG4-related disease. *Gut* 2016; **65**: 1322-1332 [PMID: 26964842 DOI: 10.1136/gutjnl-2015-310336]

39 **Chan ASY**, Mudhar H, Shen SY, Lang SS, Fernando M, Hilmy MH, Guppy NJ, Rennie I, Dunkley L, Al Jajeh I. Serum IgG2 and tissue IgG2 plasma cell elevation in orbital IgG4-related disease (IgG4-RD): Potential use in IgG4-RD assessment. *Br J Ophthalmol* 2017; **101**: 1576-1582 [PMID: 28351925 DOI: 10.1136/bjophthalmol-2017-310148]

40 **Taguchi M**, Kihara Y, Nagashio Y, Yamamoto M, Otsuki M, Harada M. Decreased production of immunoglobulin M and A in autoimmune pancreatitis. *J Gastroenterol* 2009; **44**: 1133-1139 [PMID: 19626266 DOI: 10.1007/s00535-009-0106-y]

41 **Culver EL**, Sadler R, Bateman AC, Makuch M, Cargill T, Ferry B, Aalberse R, Barnes E, Rispens T. Increases in IgE, Eosinophils, and Mast Cells Can be Used in Diagnosis and to Predict Relapse of IgG4-Related Disease. *Clin Gastroenterol Hepatol* 2017; **15**: 1444-1452. e6 [PMID: 28223204 DOI: 10.1016/j.cgh.2017.02.007]

42 **Shiokawa M**, Kodama Y, Sekiguchi K, Kuwada T, Tomono T, Kuriyama K, Yamazaki H, Morita T, Marui S, Sogabe Y, Kakiuchi N, Matsumori T, Mima A, Nishikawa Y, Ueda T, Tsuda M, Yamauchi Y, Sakuma Y, Maruno T, Uza N, Tsuruyama T, Mimori T, Seno H, Chiba T. Laminin 511 is a target antigen in autoimmune pancreatitis. *Sci Transl Med* 2018; **10**: eaaq0997 [PMID: 30089633 DOI: 10.1126/scitranslmed.aaq0997]

43 **Hubers LM**, Vos H, Schuurman AR, Erken R, Oude Elferink RP, Burgering B, van de Graaf SFJ, Beuers U. Annexin A11 is targeted by IgG4 and IgG1 autoantibodies in IgG4-related disease. *Gut* 2018; **67**: 728-735 [PMID: 28765476 DOI: 10.1136/gutjnl-2017-314548]

44 **Perugino CA**, AlSalem SB, Mattoo H, Della-Torre E, Mahajan V, Ganesh G, Allard-Chamard H, Wallace Z, Montesi SB, Kreuzer J, Haas W, Stone JH, Pillai S. Identification of galectin-3 as an autoantigen in patients with IgG_4-related disease. *J Allergy Clin Immunol* 2019; **143**: 736-745. e6 [PMID: 29852256 DOI: 10.1016/j.jaci.2018.05.011]

45 **Yamamoto M**, Takano KI, Kamekura R, Aochi S, Suzuki C, Ichimiya S, Nakase H, Himi T, Takahashi H. Interleukin 5-producing $ST2^+$ memory Th2 cells in IgG4-related dacryoadenitis and sialadenitis. *Mod Rheumatol* 2019; **29**: 856-860 [PMID: 30354922 DOI: 10.1080/14397595.2018.1526357]

46 **Kubo S**, Nakayamada S, Zhao J, Yoshikawa M, Miyazaki Y, Nawata A, Hirata S, Nakano K, Saito K, Tanaka Y. Correlation of T follicular helper cells and plasmablasts with the development of organ involvement in patients with IgG4-related disease. *Rheumatology (Oxford)* 2018; **57**: 514-524 [PMID: 29253269 DOI: 10.1093/rheumatology/kex455]

47 **Miyoshi H**, Uchida K, Taniguchi T, Yazumi S, Matsushita M, Takaoka M, Okazaki K. Circulating naïve and CD4+CD25high regulatory T cells in patients with autoimmune pancreatitis. *Pancreas* 2008; **36**: 133-140 [PMID: 18376303 DOI: 10.1097/MPA.0b013e3181577553]

48 **Mattoo H**, Della-Torre E, Mahajan VS, Stone JH, Pillai S. Circulating Th2 memory cells in IgG4-

related disease are restricted to a defined subset of subjects with atopy. *Allergy* 2014; **69**: 399-402 [PMID: 24382311 DOI: 10.1111/all.12342]

49 **Yamamoto M**, Takano K, Kamekura R, Suzuki C, Ichimiya S, Himi T, Nakase H, Takahashi H. Stage classification of IgG4-related dacryoadenitis and sialadenitis by the serum cytokine environment. *Mod Rheumatol* 2018; **28**: 1004-1008 [PMID: 29385874 DOI: 10.1080/14397595.2018.1436029]

50 **Müller T**, Beutler C, Picó AH, Otten M, Dürr A, Al-Abadi H, Guckelberger O, Meyer Zum Büschenfelde D, Jöhrens K, Volkmann M, Lankisch T, Voigtländer T, Anders M, Shibolet O, Jefferson DM, Podolsky DK, Fischer A, Veltzke-Schlieker W, Adler A, Baumgart DC, Sturm A, Wiedenmann B, Schott E, Berg T. Increased T-helper 2 cytokines in bile from patients with IgG4-related cholangitis disrupt the tight junction-associated biliary epithelial cell barrier. *Gastroenterology* 2013; **144**: 1116-1128 [PMID: 23391819 DOI: 10.1053/j.gastro.2013.01.055]

51 **Ebbo M**, De Sainte-Marie B, Muller R, Piperoglou C, Grados A, Vély F, Schleinitz N. Comment on article: 'Dupilumab as a novel steroid-sparing treatment for IgG_4-related disease' by Simpson *et al*. *Ann Rheum Dis* 2020; epub ahead of print [PMID: 31996366 DOI: 10.1136/annrheumdis-2020-217010]

52 **Simpson RS**, Lau SKC, Lee JK. Dupilumab as a novel steroid-sparing treatment for IgG4-related disease. *Ann Rheum Dis* 2020; **79**: 549-550 [PMID: 31857343 DOI: 10.1136/annrheumdis-2019-216368]

53 **Minaga K**, Watanabe T, Kamata K, Takenaka M, Yasukawa S, Kudo M. The IFN-α-IL-33 Axis as Possible Biomarkers in IgG4-Related Disease. *Am J Gastroenterol* 2019; **114**: 1002-1003 [PMID: 31058651 DOI: 10.14309/ajg.0000000000000245]

54 **Furie R**, Khamashta M, Merrill JT, Werth VP, Kalunian K, Brohawn P, Illei GG, Drappa J, Wang L, Yoo S; CD1013 Study Investigators. Anifrolumab, an Anti-Interferon-α Receptor Monoclonal Antibody, in Moderate-to-Severe Systemic Lupus Erythematosus. *Arthritis Rheumatol* 2017; **69**: 376-386 [PMID: 28130918 DOI: 10.1002/art.39962]

55 **Khamashta M**, Merrill JT, Werth VP, Furie R, Kalunian K, Illei GG, Drappa J, Wang L, Greth W; CD1067 study investigators. Sifalimumab, an anti-interferon-α monoclonal antibody, in moderate to severe systemic lupus erythematosus: a randomised, double-blind, placebo-controlled study. *Ann Rheum Dis* 2016; **75**: 1909-1916 [PMID: 27009916 DOI: 10.1136/annrheumdis-2015-208562]

56 **Mackay F**, Schneider P. Cracking the BAFF code. *Nat Rev Immunol* 2009; **9**: 491-502 [PMID: 19521398 DOI: 10.1038/nri2572]

57 **Carruthers MN**, Topazian MD, Khosroshahi A, Witzig TE, Wallace ZS, Hart PA, Deshpande V, Smyrk TC, Chari S, Stone JH. Rituximab for IgG4-related disease: a prospective, open-label trial. *Ann Rheum Dis* 2015; **74**: 1171-1177 [PMID: 25667206 DOI: 10.1136/annrheumdis-2014-206605]

58 **Kiyama K**, Kawabata D, Hosono Y, Kitagori K, Yukawa N, Yoshifuji H, Omura K, Fujii T, Mimori T. Serum BAFF and APRIL levels in patients with IgG4-related disease and their clinical significance. *Arthritis Res Ther* 2012; **14**: R86 [PMID: 22531553 DOI: 10.1186/ar3810]

59 **Garbers C**, Heink S, Korn T, Rose-John S. Interleukin-6: designing specific therapeutics for a complex cytokine. *Nat Rev Drug Discov* 2018; **17**: 395-412 [PMID: 29725131 DOI: 10.1038/nrd.2018.45]

60 **Tsukuda S**, Ikeura T, Ito T, Nakamaru K, Masuda M, Hori Y, Ikemune M, Yanagawa M, Tanaka T, Tomiyama T, Yamaguchi T, Ando Y, Uchida K, Fukui T, Nishio A, Terasawa R, Tanigawa N, Okazaki K. Clinical implications of elevated serum interleukin-6 in IgG4-related disease. *PLoS One* 2020; **15**: e0227479 [PMID: 31951598 DOI: 10.1371/journal.pone.0227479]

61 **Sato Y**, Kojima M, Takata K, Morito T, Asaoku H, Takeuchi T, Mizobuchi K, Fujihara M, Kuraoka K, Nakai T, Ichimura K, Tanaka T, Tamura M, Nishikawa Y, Yoshino T. Systemic IgG4-related lymphadenopathy: a clinical and pathologic comparison to multicentric Castleman's disease. *Mod Pathol* 2009; **22**: 589-599 [PMID: 19270642 DOI: 10.1038/modpathol.2009.17]

62 **Kakinuma T**, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, Torii H, Asahina A, Onai N, Matsushima K, Tamaki K. Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. *J Allergy Clin Immunol* 2001; **107**: 535-541 [PMID: 11240957 DOI: 10.1067/mai.2001.113237]

63 **Umeda M**, Origuchi T, Kawashiri SY, Koga T, Ichinose K, Furukawa K, Sato T, Tsuji S, Endo Y, Takatani A, Shimizu T, Fukui S, Iwamoto N, Igawa T, Tamai M, Nakamura H, Kawakami A. Thymus and Activation-regulated Chemokine as a Biomarker for IgG4-related Disease. *Sci Rep* 2020; **10**: 6010 [PMID: 32265499 DOI: 10.1038/s41598-020-62941-9]

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REVIEW

Receptor for advanced glycation end-products axis and coronavirus disease 2019 in inflammatory bowel diseases: A dangerous liaison?

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Abstract

Compelling evidence supports the crucial role of the receptor for advanced glycation end-products (RAGE) axis activation in many clinical entities. Since the beginning of the coronavirus disease 2019 pandemic, there is an increasing concern about the risk and handling of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in inflammatory gastrointestinal disorders, such as inflammatory bowel diseases (IBD). However, clinical data raised during pandemic suggests that IBD patients do not have an increased risk of contracting SARS-CoV-2 infection or develop a more severe course of infection. In the present review, we intend to highlight how two potentially important contributors to the inflammatory response to SARS-CoV-2 infection in IBD patients, the RAGE axis activation as well as the cross-talk with the renin-angiotensin system, are dampened by the high expression of soluble forms of both RAGE and the angiotensin-converting enzyme (ACE) 2. The soluble form of RAGE functions as a decoy for its ligands, and soluble ACE2 seems to be an additionally attenuating contributor to RAGE axis activation, particularly by avoiding the transactivation of the RAGE axis that can be produced by the virus-mediated imbalance of the ACE/angiotensin II/angiotensin II receptor type 1 pathway.

Key Words: COVID-19; Inflammatory bowel diseases; Advanced glycation; Angiotensin-converting enzyme 2; Alarmins; Receptor for advanced glycation end-products; Receptor for advanced glycation end-products axis; Inflammation

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Core Tip: Data raised during the pandemic suggest that inflammatory bowel diseases do not have an increased risk of contracting severe acute respiratory syndrome coronavirus 2 infection or develop a more severe course of infection. These findings are in some way unexpected considering that inflammatory bowel disease is a chronic inflammatory state of the gastrointestinal tract. We herein discuss how the receptor for advanced glycation end-products axis activation as well as the cross-talk with the renin-angiotensin system are dampened by the high expression of soluble forms of both receptor for advanced glycation end-products and angiotensin-converting enzyme 2.

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INTRODUCTION

At the end of 2019, China reported several cases of severe pneumonia of unknown cause; the coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was subsequently identified as the etiological agent[1]. Due to its rapid spread all over the world, the World Health Organization defined coronavirus disease 2019 (COVID-19) as a pandemic on January 30, 2020.

The main symptoms of COVID-19 affect the lower respiratory tract, causing high mortality-rate complications such as acute distress respiratory syndrome[2-6]. However, recent reports reveal that gastrointestinal (GI) manifestations of SARS-CoV-2 infection are common clinical symptoms among patients who develop COVID-19[7-11].

The SARS-CoV-2 uses the cellular transmembrane angiotensin-converting enzyme 2 (ACE2) molecule as the receptor for viral cell entry. Under physiological conditions, epithelial ACE2 is widely expressed in several tissues. However, the expression of epithelial ACE2 in the terminal ileum and colon are amongst the highest in the body, which could explain why COVID-19 patients experience several GI symptoms[12-16].

Consequently, there is an increasing concern about the risk and handling of SARS-CoV-2 infection in inflammatory GI disorders, such as inflammatory bowel disease (IBD). The IBDs are chronic intestinal diseases that comprise Crohn´s disease (CD) and ulcerative colitis, which are characterized by chronic and relapsing intestinal inflammation[17,18]. Thus, since the beginning of the SARS-CoV-2 pandemic, IBD patients were considered a high-risk group for increased severity and adverse outcomes in SARS-CoV-2 infection[19,20].

However, clinical data raised during pandemic suggest that IBD patients do not have an increased risk of contracting SARS-CoV-2 infection or develop a more severe course of infection[21-25]. A compelling body of both clinical and experimental evidence has shed light on the crucial role of the receptor of advanced glycation end-products (RAGE) activation in many chronic inflammatory diseases[26-31]. More recently, the role of RAGE axis activation as a key contributor in the clinical course of SARS-CoV-2 infection has been documented[32].

In the present review, we intend to highlight the role of the RAGE axis activation in the context of SARS-CoV-2 infection and the clinical evolution of the IBD patient.

RAGE AXIS

Firstly described in 1992, the RAGE is a type I single-pass transmembrane protein that can bind advanced glycation-end products (AGEs). This molecule belongs to the immunoglobulin superfamily of cell surface receptors, which is now considered as a pattern recognition receptor and is regarded as a central mediator in chronic inflammatory and immune responses[33-35].

RAGE is usually expressed at low levels in many cell types and tissues, except for the lungs. However, this expression is noticeably increased under inflammatory conditions[36-38].

Besides the transmembrane form of RAGE, several soluble isoforms of this receptor (sRAGE) are generated either by alternative splicing or by the action of membrane associated-proteases, such matrix metalloproteinase-9 (MMP-9), a disintegrin metalloproteases (ADAM)-10, and ADAM-17[39-42]. These soluble variants may function as a decoy receptor for ligands and thus prevent the interaction with the membrane-anchored full-length RAGE. In consequence, a high bioavailability of sRAGE will decreases the inflammatory responses driven by full-length RAGE activation [35,43,44]. Besides AGEs, RAGE can recognize many other ligands including the alarmin high-mobility group box 1 (HMGB1), members of the S100 protein family, glycosaminoglycans, and amyloid β peptides, among many others[35,45].

As a consequence of RAGE engagement by its ligands, multiple signaling pathways are triggered, including reactive oxygen species, p21ras, extracellular signal-regulated protein kinase 1/2 (p44/p42) mitogen-activated protein (MAP) kinases, p38 and stress-activated protein kinases/c-Jun N-terminal kinase mitogen-activated protein kinases, rhoGTPases, phosphoinositol-3 kinase, and the janus kinase/signal transducer and activator of transcription pathway, having crucial downstream inflammatory consequences such as activation of nuclear factor-kappaB (NF-κB), AP-1, and signal transducer and activator of transcription-3[35].

Indeed, the RAGE axis signaling not only triggers pro-inflammatory gene expression but also a positive feed-forward loop, in which the inflammatory stimuli activate NF-κB, which induces RAGE expression, following an enhanced and sustained inflammatory response[35,46-48].

RAGE AXIS ACTIVATION IN IBD

Initially, RAGE axis activation was linked to the complications of diabetes such as macro-and microvascular complications[49,50]. However, a growing body of evidence indicates RAGE as a key molecule involved in many chronic inflammatory diseases[28-30,51].

Many underlying molecular mechanisms are involved in the onset and perpetuation of the disease, particularly those fueling the robust pro-inflammatory signals found in IBD patients[26,52]. Noteworthy, some pieces of evidence reveal an increased expression of RAGE and its ligands on intestinal cells in IBD patients, especially in inflamed areas[53-55]. In this context, it is important to highlight that the release of the RAGE ligand HMGB1 and members of the S100 protein family is increased under inflammation conditions[54-57]. Thus, the engagement of RAGE may play an important role in the maintenance of intestinal injury and inflammatory environment [53-57].

Strikingly, increased levels of both MMP-9 and ADAM17 have been reported in IBD patients[58,59], and both metalloproteases are involved in RAGE shedding, thus increasing the levels of sRAGE, which in turn can modulate the inflammatory responses driven by RAGE axis activation in IBD patients[58]. At present, a compelling body of evidence supports the fact that increased sRAGE levels correlate with a decrease in the RAGE activation-mediated inflammatory responses in many clinical entities[60-63]. In this context, it is important to highlight that CD147 significantly contributes to epithelial inflammation in many clinical entities including IBD[64,65], and it has been recently shown to act as a receptor for SARS-CoV-2[66]. Noteworthy, the inhibition of RAGE activation-mediated inflammatory response leads to a reduced expression of CD147[67].

THE RENIN-ANGIOTENSIN SYSTEM

The renin-angiotensin system (RAS) is a hormonal system regulated by two complementary pathways that mediate opposing effects on inflammation, fibrosis, and cell proliferation[68-70]. Thus, the balance of both pathways determines pro-inflammatory or anti-inflammatory conditions among several systems such as cardiovascular, renal, and respiratory systems[71-74].

The classical pathway mediated *via* ACE, angiotensin II (Ang II) and its receptor Ang II receptor type 1 (AT1R), triggers activation of pro-inflammatory signals such as oxidative and nitrosative stresses, the induction of cytokines and cell adhesion molecules, as well as the activation of transcription factors such NF-κB[75-78]. On the contrary, the alternative pathway predominantly mediated by ACE2, Ang (1-7) and its receptor Mas (MasR), induces the opposite effects of AT1R activation, being an anti-

inflammatory and anti-fibrotic counter regulator of the effects of ACE/Ang II/AT1R[71,75,79,80]. ACE and ACE2 are highly expressed in several tissues such as the lungs, kidneys, and blood vessels. However, the brush border of the ileum and the colon are among the tissues with the highest expression of both enzymes[13-16,81]. Both enzymes can cleave angiotensin, generating different sub-products and regulating the balance between both pathways of the RAS system[79,82,83].

RAS IMBALANCE IN IBD

Recent studies suggest high expression of the major components of both RAS pathways across the ileum and colon[81]. In this sense, the gut could be an especially susceptible organ for the imbalance of RAS pathways. Thus, the dysregulation of these components could have potential implications for inflammation and fibrosis for IBD patients[84,85]. Strikingly, several studies have revealed that the intestinal expression of ACE2 is inversely correlated with fibrosis in IBD patients[81,86].

Additionally, Ang (1-7) ameliorates colonic myofibroblast collagen secretion *via* MasR[81]. Furthermore, angiotensin receptor blockers and ACE inhibitors are reported to decrease mucosal pro-inflammatory cytokines, ameliorate colitis, and were associated with lower rates of complications, surgery, and hospitalization in patients with IBD[87-89].

Normally, ACE2 breaks down Ang II to Ang 1–7 peptide and thus avoiding the activation of the pro-inflammatory pathways of RAS. However, SARS-CoV-2 can hijack ACE2 and use it to gain entry into host cells[12,90]. Noteworthy, high bioavailability of soluble ACE2 has been reported in IBD patients[81,84], mainly ascribed to the increased level of ADAM17 observed in these patients[58,91-93], which in turn may function as a decoy receptor for SARS-CoV-2 and thus avoiding the hijacking of the counterbalancing enzyme.

This is particularly important considering that a novel ligand-independent mechanism for RAGE transactivation has been recently reported to occur following activation of the AT1R by Ang-II, thus leading to NF-κB dependent expression of pro-inflammatory mediators[48].

RAGE AXIS ACTIVATION AND RAS IMBALANCE IN IBD PATIENTS INFECTED WITH SARS-COV-2

Contrary to what is expected, considering the pathophysiology of IBD, there is currently no evidence for an increased risk of worse clinical outcomes in patients with IBD in the context of COVID-19[21-25]. The role of the RAGE axis in the pathophysiology of IBD has been suggested by different reports[53-57]. The colonic expression of RAGE and some RAGE ligands, such as HMGB1 and some members of the S100 protein family, are significantly higher in IBD patients[54-56]. Besides, this receptor has been also considered a key contributor to the dysregulated and misdirected COVID-19 inflammatory response[32,94].

However, a counterbalancing element must be added to this scenario: The soluble RAGE. This molecule is generated by alternative splicing or by cleavage of the ectodomain of the membrane-anchored RAGE by the action of both MMP-9 and ADAM17, which are highly expressed in IBD patients[58,59]. Therefore, the high bioavailability of soluble RAGE may dampen RAGE activation, despite the abundance of both receptor and ligands in the inflamed intestinal mucosa of IBD patients.

On the other hand, the high expression of ACE2 in GI tract, especially among IBD patients, makes this tissue a particularly trophic niche for infection with SARS-CoV-2. Furthermore, the ACE2 exhaustion mediated by the entry of SARS-CoV-2 may then induce a robust RAS imbalance in favor of the pro-inflammatory ACE/Ang II/AT1R pathway[95]. These observations suggest that the inflamed gut of IBD patients represents an optimal doorway for SARS-CoV-2 entry, driving poor clinical outcomes in IBD patients who develop COVID-19.

However, this hypothetical scenario also has an important counterbalancing actor, the soluble form of ACE2, which is also increased in patients with IBD due to the shedding of the membrane-anchored ACE2 by ADAM17[58-59]. This is particularly important considering the non-cognate transactivation mechanism described for RAGE because of AT1R activation by Ang II[48], which is dampened by the preservation of membrane-associated ACE2 exhaustion by its soluble form.

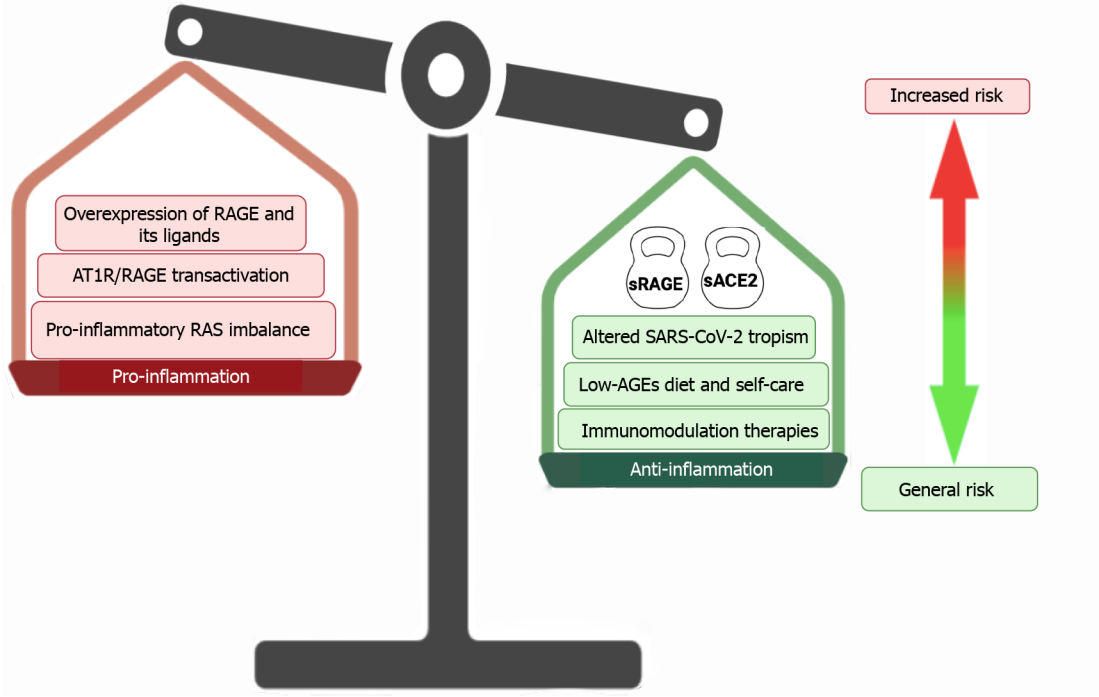


Figure 1 In inflammatory bowel diseases patients, different inflammation–prone mechanisms are known to be activated. Among them, the overexpression of receptor for advanced glycation end-products (RAGE) and the abundance of its ligands may produce a sustained activation of the axis, which can be also fueled by a non-cognate mechanism due to the pro-inflammatory rat sarcoma imbalance. These elements seem to be crucial contributors to the worsening course of inflammatory bowel diseases (IBD) patients with coronavirus disease 2019. However, other elements may dampen these inflammatory contributions, such as the high bioavailability of the soluble forms of both RAGE and angiotensin-converting enzyme 2. Soluble angiotensin-converting enzyme 2 may even interfere with severe acute respiratory syndrome coronavirus 2 entry to epithelial cells. Additionally, most if not all IBD patients are under pharmacological treatments directed to control inflammation. IBD patients deserve special attention to their diets, and as consequence, it is likely the ingestion of dietary advanced glycation-end products is also limited. RAGE: Receptor for advanced glycation end-products; RAS: Renin-angiotensin; ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; AT1R: Angiotensin II receptor type 1; AGEs: Advanced glycation-end products; sRAGE: Several soluble isoforms of this receptor.

A growing body of evidence demonstrates that in IBD patients the use of systemic immunosuppression is not associated with an increased risk of COVID-19 patients with IBD[96-100]. Furthermore, we must also keep in mind that the main objective of pharmacological treatments in IBD is to reduce inflammation levels. In this sense, in addition to interfering with signaling pathways, many drugs used in the current treatments also decrease the expression of RAGE and the bioavailability of some RAGE ligands, particularly the alarmins HMGB1 and S100 protein family members[96,97]. Indeed, several authors remark the possible protective role of IBD therapy against SARS-CoV-2 infection, especially through interfering with cytokine activity observed in the clinical course of COVID-19[98-100].

Additionally, the IBD patients have a high self-care standard and follow diets that help them to maintain good nutritional levels and the disease under control[101]. Some of these nutritional regimens are associated with a low-AGE diet, which may contribute to reducing the proinflammatory intestinal milieu mediated by RAGE activation[102] (Figure 1).

CONCLUSION

The COVID-19 pandemics represent the worst challenge for a century for health systems all over the world. Severity and mortality have been highest in people with underlying morbidities. Therefore, special efforts have been done to understand how SARS-CoV-2 may particularly fuel inflammation in many clinical entities where the chronicity of an inflammatory environment is a relevant part of the pathogenesis of diseases. Based on a particularly inflamed landscape depicted in IBD patients, the activation of the RAGE axis as well the RAS imbalance seem to be crucial contributors to worsen inflammation in the gut. However, data raised during the pandemic suggests that IBD patients have neither an increased risk of contracting SARS-CoV-2 infection nor developing a more severe course of infection.

RAGE axis activation seems to be dampened by the high bioavailability of soluble receptors functioning as a decoy for its ligands. Additionally, soluble ACE2 seems to be another attenuating contributor to RAGE axis activation, particularly by avoiding the transactivation of RAGE axis that can be produced by the virus-mediated imbalance of the ACE/Ang II/ AT1R pathway. Thus, RAGE axis activation in COVID-19 IBD patients does not seem to be a dangerous liaison.

REFERENCES

1 **Zhu N**, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, Wang D, Xu W, Wu G, Gao GF, Tan W; China Novel Coronavirus Investigating and Research Team. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med* 2020; **382**: 727-733 [PMID: 31978945 DOI: 10.1056/NEJMoa2001017]

2 **Goyal P**, Choi JJ, Pinheiro LC, Schenck EJ, Chen R, Jabri A, Satlin MJ, Campion TR Jr, Nahid M, Ringel JB, Hoffman KL, Alshak MN, Li HA, Wehmeyer GT, Rajan M, Reshetnyak E, Hupert N, Horn EM, Martinez FJ, Gulick RM, Safford MM. Clinical Characteristics of Covid-19 in New York City. *N Engl J Med* 2020; **382**: 2372-2374 [PMID: 32302078 DOI: 10.1056/NEJMc2010419]

3 **Hui KPY**, Cheung MC, Perera RAPM, Ng KC, Bui CHT, Ho JCW, Ng MMT, Kuok DIT, Shih KC, Tsao SW, Poon LLM, Peiris M, Nicholls JM, Chan MCW. Tropism, replication competence, and innate immune responses of the coronavirus SARS-CoV-2 in human respiratory tract and conjunctiva: an analysis in ex-vivo and in-vitro cultures. *Lancet Respir Med* 2020; **8**: 687-695 [PMID: 32386571 DOI: 10.1016/S2213-2600(20)30193-4]

4 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

5 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

6 **Lai CC**, Shih TP, Ko WC, Tang HJ, Hsueh PR. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): The epidemic and the challenges. *Int J Antimicrob Agents* 2020; **55**: 105924 [PMID: 32081636 DOI: 10.1016/j.ijantimicag.2020.105924]

7 **Zhou Z**, Zhao N, Shu Y, Han S, Chen B, Shu X. Effect of Gastrointestinal Symptoms in Patients With COVID-19. *Gastroenterology* 2020; **158**: 2294-2297 [PMID: 32199880 DOI: 10.1053/j.gastro.2020.03.020]

8 **Jin X**, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, Hao SR, Jia HY, Cai H, Zhang XL, Yu GD, Xu KJ, Wang XY, Gu JQ, Zhang SY, Ye CY, Jin CL, Lu YF, Yu X, Yu XP, Huang JR, Xu KL, Ni Q, Yu CB, Zhu B, Li YT, Liu J, Zhao H, Zhang X, Yu L, Guo YZ, Su JW, Tao JJ, Lang GJ, Wu XX, Wu WR, Qv TT, Xiang DR, Yi P, Shi D, Chen Y, Ren Y, Qiu YQ, Li LJ, Sheng J, Yang Y. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. *Gut* 2020; **69**: 1002-1009 [PMID: 32213556 DOI: 10.1136/gutjnl-2020-320926]

9 **Xiao F**, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for Gastrointestinal Infection of SARS-CoV-2. *Gastroenterology* 2020; **158**: 1831-1833. e3 [PMID: 32142773 DOI: 10.1053/j.gastro.2020.02.055]

10 **Garg M**, Christensen B, Lubel JS. Gastrointestinal ACE2, COVID-19 and IBD: Opportunity in the Face of Tragedy? *Gastroenterology* 2020; **159**: 1623-1624.e3 [PMID: 32353370 DOI: 10.1053/j.gastro.2020.04.051]

11 **Grassia R**, Testa S, Pan A, Conti CB. SARS-CoV-2 and gastrointestinal tract: The dark side of the pandemic. *Dig Liver Dis* 2020; **52**: 700-701 [PMID: 32423849 DOI: 10.1016/j.dld.2020.04.028]

12 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280. e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]

13 **GTEx Consortium**. . The Genotype-Tissue Expression (GTEx) project. *Nat Genet* 2013; **45**: 580-585 [PMID: 23715323 DOI: 10.1038/ng.2653]

14 **Hamming I**, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol* 2004; **203**: 631-637 [PMID: 15141377 DOI: 10.1002/path.1570]

15 **Harmer D**, Gilbert M, Borman R, Clark KL. Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. *FEBS Lett* 2002; **532**: 107-110 [PMID: 12459472 DOI: 10.1016/s0014-5793(02)03640-2]

16 **Sibony M**, Gasc JM, Soubrier F, Alhenc-Gelas F, Corvol P. Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. *Hypertension* 1993; **21**: 827-835 [PMID:

8388857 DOI: 10.1161/01.hyp.21.6.827]
17 **Graham DB**, Xavier RJ. Pathway paradigms revealed from the genetics of inflammatory bowel disease. *Nature* 2020; **578**: 527-539 [PMID: 32103191 DOI: 10.1038/s41586-020-2025-2]
18 **Zhang YZ**, Li YY. Inflammatory bowel disease: pathogenesis. *World J Gastroenterol* 2014; **20**: 91-99 [PMID: 24415861 DOI: 10.3748/wjg.v20.i1.91]
19 **An P**, Ji M, Ren H, Su J, Ding NS, Kang J, Yin A, Zhou Q, Shen L, Zhao L, Jiang X, Xiao Y, Tan W, Lv X, Li J, Liu S, Zhou J, Chen H, Xu Y, Liu J, Chen M, Cao J, Zhou Z, Tan S, Yu H, Dong W, Ding Y. Prevention of COVID-19 in patients with inflammatory bowel disease in Wuhan, China. *Lancet Gastroenterol Hepatol* 2020; **5**: 525-527 [PMID: 32311321 DOI: 10.1016/S2468-1253(20)30121-7]
20 **Kennedy NA**, Jones GR, Lamb CA, Appleby R, Arnott I, Beattie RM, Bloom S, Brooks AJ, Cooney R, Dart RJ, Edwards C, Fraser A, Gaya DR, Ghosh S, Greveson K, Hansen R, Hart A, Hawthorne AB, Hayee B, Limdi JK, Murray CD, Parkes GC, Parkes M, Patel K, Pollok RC, Powell N, Probert CS, Raine T, Sebastian S, Selinger C, Smith PJ, Stansfield C, Younge L, Lindsay JO, Irving PM, Lees CW. British Society of Gastroenterology guidance for management of inflammatory bowel disease during the COVID-19 pandemic. *Gut* 2020; **69**: 984-990 [PMID: 32303607 DOI: 10.1136/gutjnl-2020-321244]
21 **Macaluso FS**, Orlando A. COVID-19 in patients with inflammatory bowel disease: A systematic review of clinical data. *Dig Liver Dis* 2020; **52**: 1222-1227 [PMID: 32928672 DOI: 10.1016/j.dld.2020.09.002]
22 **Monteleone G**, Ardizzone S. Are Patients with Inflammatory Bowel Disease at Increased Risk for Covid-19 Infection? *J Crohns Colitis* 2020; **14**: 1334-1336 [PMID: 32215548 DOI: 10.1093/ecco-jcc/jjaa061]
23 **Aziz M**, Fatima R, Haghbin H, Lee-Smith W, Nawras A. The Incidence and Outcomes of COVID-19 in IBD Patients: A Rapid Review and Meta-analysis. *Inflamm Bowel Dis* 2020; **26**: e132-e133 [PMID: 32619003 DOI: 10.1093/ibd/izaa170]
24 **Allocca M**, Fiorino G, Zallot C, Furfaro F, Gilardi D, Radice S, Danese S, Peyrin-Biroulet L. Incidence and Patterns of COVID-19 Among Inflammatory Bowel Disease Patients From the Nancy and Milan Cohorts. *Clin Gastroenterol Hepatol* 2020; **18**: 2134-2135 [PMID: 32360811 DOI: 10.1016/j.cgh.2020.04.071]
25 **Neurath MF**. COVID-19 and immunomodulation in IBD. *Gut* 2020; **69**: 1335-1342 [PMID: 32303609 DOI: 10.1136/gutjnl-2020-321269]
26 **Moura FA**, Goulart MOF, Campos SBG, da Paz Martins AS. The Close Interplay of Nitro-Oxidative Stress, Advanced Glycation end Products and Inflammation in Inflammatory Bowel Diseases. *Curr Med Chem* 2020; **27**: 2059-2076 [PMID: 30182837 DOI: 10.2174/0929867325666180904115633]
27 **Schmidt AM**, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest* 2001; **108**: 949-955 [PMID: 11581294 DOI: 10.1172/JCI14002]
28 **Bierhaus A**, Stern DM, Nawroth PP. RAGE in inflammation: a new therapeutic target? *Curr Opin Investig Drugs* 2006; **7**: 985-991 [PMID: 17117586]
29 **Sparvero LJ**, Asafu-Adjei D, Kang R, Tang D, Amin N, Im J, Rutledge R, Lin B, Amoscato AA, Zeh HJ, Lotze MT. RAGE (Receptor for Advanced Glycation Endproducts), RAGE ligands, and their role in cancer and inflammation. *J Transl Med* 2009; **7**: 17 [PMID: 19292913 DOI: 10.1186/1479-5876-7-17]
30 **Chuah YK**, Basir R, Talib H, Tie TH, Nordin N. Receptor for advanced glycation end products and its involvement in inflammatory diseases. *Int J Inflam* 2013; **2013**: 403460 [PMID: 24102034 DOI: 10.1155/2013/403460]
31 **Chavakis T**, Bierhaus A, Nawroth PP. RAGE (receptor for advanced glycation end products): a central player in the inflammatory response. *Microbes Infect* 2004; **6**: 1219-1225 [PMID: 15488742 DOI: 10.1016/j.micinf.2004.08.004]
32 **Yalcin Kehribar D**, Cihangiroglu M, Sehmen E, Avci B, Capraz A, Yildirim Bilgin A, Gunaydin C, Ozgen M. The receptor for advanced glycation end product (RAGE) pathway in COVID-19. *Biomarkers* 2021; **26**: 114-118 [PMID: 33284049 DOI: 10.1080/1354750X.2020.1861099]
33 **Schmidt AM**, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, Esposito C, Hegarty H, Hurley W, Clauss M. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *J Biol Chem* 1992; **267**: 14987-14997 [PMID: 1321822]
34 **Yan SF**, Ramasamy R, Schmidt AM. Mechanisms of disease: advanced glycation end-products and their receptor in inflammation and diabetes complications. *Nat Clin Pract Endocrinol Metab* 2008; **4**: 285-293 [PMID: 18332897 DOI: 10.1038/ncpendmet0786]
35 **Rojas A**, Delgado-López F, González I, Pérez-Castro R, Romero J, Rojas I. The receptor for advanced glycation end-products: a complex signaling scenario for a promiscuous receptor. *Cell Signal* 2013; **25**: 609-614 [PMID: 23200851 DOI: 10.1016/j.cellsig.2012.11.022]
36 **Zen K**, Chen CX, Chen YT, Wilton R, Liu Y. Receptor for advanced glycation endproducts mediates neutrophil migration across intestinal epithelium. *J Immunol* 2007; **178**: 2483-2490 [PMID: 17277156 DOI: 10.4049/jimmunol.178.4.2483]
37 **González I**, Romero J, Rodríguez BL, Pérez-Castro R, Rojas A. The immunobiology of the receptor of advanced glycation end-products: trends and challenges. *Immunobiology* 2013; **218**: 790-797

[PMID: 23182709 DOI: 10.1016/j.imbio.2012.09.005]
38 **Oczypok EA**, Perkins TN, Oury TD. All the "RAGE" in lung disease: The receptor for advanced glycation endproducts (RAGE) is a major mediator of pulmonary inflammatory responses. *Paediatr Respir Rev* 2017; **23**: 40-49 [PMID: 28416135 DOI: 10.1016/j.prrv.2017.03.012]
39 **Raucci A**, Cugusi S, Antonelli A, Barabino SM, Monti L, Bierhaus A, Reiss K, Saftig P, Bianchi ME. A soluble form of the receptor for advanced glycation endproducts (RAGE) is produced by proteolytic cleavage of the membrane-bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10). *FASEB J* 2008; **22**: 3716-3727 [PMID: 18603587 DOI: 10.1096/fj.08-109033]
40 **Zhang L**, Bukulin M, Kojro E, Roth A, Metz VV, Fahrenholz F, Nawroth PP, Bierhaus A, Postina R. Receptor for advanced glycation end products is subjected to protein ectodomain shedding by metalloproteinases. *J Biol Chem* 2008; **283**: 35507-35516 [PMID: 18952609 DOI: 10.1074/jbc.M806948200]
41 **Deuss M**, Reiss K, Hartmann D. Part-time alpha-secretases: the functional biology of ADAM 9, 10 and 17. *Curr Alzheimer Res* 2008; **5**: 187-201 [PMID: 18393804 DOI: 10.2174/156720508783954686]
42 **Metz VV**, Kojro E, Rat D, Postina R. Induction of RAGE shedding by activation of G protein-coupled receptors. *PLoS One* 2012; **7**: e41823 [PMID: 22860017 DOI: 10.1371/journal.pone.0041823]
43 **Grauen Larsen H**, Marinkovic G, Nilsson PM, Nilsson J, Engström G, Melander O, Orho-Melander M, Schiopu A. High Plasma sRAGE (Soluble Receptor for Advanced Glycation End Products) Is Associated With Slower Carotid Intima-Media Thickness Progression and Lower Risk for First-Time Coronary Events and Mortality. *Arterioscler Thromb Vasc Biol* 2019; **39**: 925-933 [PMID: 30917679 DOI: 10.1161/ATVBAHA.118.312319]
44 **Geroldi D**, Falcone C, Emanuele E. Soluble receptor for advanced glycation end products: from disease marker to potential therapeutic target. *Curr Med Chem* 2006; **13**: 1971-1978 [PMID: 16842191 DOI: 10.2174/092986706777585013]
45 **Bucciarelli LG**, Wendt T, Rong L, Lalla E, Hofmann MA, Goova MT, Taguchi A, Yan SF, Yan SD, Stern DM, Schmidt AM. RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease. *Cell Mol Life Sci* 2002; **59**: 1117-1128 [PMID: 12222959 DOI: 10.1007/s00018-002-8491-x]
46 **Bierhaus A**, Schiekofer S, Schwaninger M, Andrassy M, Humpert PM, Chen J, Hong M, Luther T, Henle T, Klöting I, Morcos M, Hofmann M, Tritschler H, Weigle B, Kasper M, Smith M, Perry G, Schmidt AM, Stern DM, Häring HU, Schleicher E, Nawroth PP. Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. *Diabetes* 2001; **50**: 2792-2808 [PMID: 11723063 DOI: 10.2337/diabetes.50.12.2792]
47 **Hudson BI**, Lippman ME. Targeting RAGE Signaling in Inflammatory Disease. *Annu Rev Med* 2018; **69**: 349-364 [PMID: 29106804 DOI: 10.1146/annurev-med-041316-085215]
48 **Pickering RJ**, Tikellis C, Rosado CJ, Tsorotes D, Dimitropoulos A, Smith M, Huet O, Seeber RM, Abhayawardana R, Johnstone EK, Golledge J, Wang Y, Jandeleit-Dahm KA, Cooper ME, Pfleger KD, Thomas MC. Transactivation of RAGE mediates angiotensin-induced inflammation and atherogenesis. *J Clin Invest* 2019; **129**: 406-421 [PMID: 30530993 DOI: 10.1172/JCI99987]
49 **Ramasamy R**, Yan SF, Schmidt AM. The RAGE axis and endothelial dysfunction: maladaptive roles in the diabetic vasculature and beyond. *Trends Cardiovasc Med* 2005; **15**: 237-243 [PMID: 16226677 DOI: 10.1016/j.tcm.2005.08.003]
50 **Manigrasso MB**, Juranek J, Ramasamy R, Schmidt AM. Unlocking the biology of RAGE in diabetic microvascular complications. *Trends Endocrinol Metab* 2014; **25**: 15-22 [PMID: 24011512 DOI: 10.1016/j.tem.2013.08.002]
51 **Sims GP**, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol* 2010; **28**: 367-388 [PMID: 20192808 DOI: 10.1146/annurev.immunol.021908.132603]
52 **Yadav V**, Varum F, Bravo R, Furrer E, Bojic D, Basit AW. Inflammatory bowel disease: exploring gut pathophysiology for novel therapeutic targets. *Transl Res* 2016; **176**: 38-68 [PMID: 27220087 DOI: 10.1016/j.trsl.2016.04.009]
53 **Ciccocioppo R**, Vanoli A, Klersy C, Imbesi V, Boccaccio V, Manca R, Betti E, Cangemi GC, Strada E, Besio R, Rossi A, Falcone C, Ardizzone S, Fociani P, Danelli P, Corazza GR. Role of the advanced glycation end products receptor in Crohn's disease inflammation. *World J Gastroenterol* 2013; **19**: 8269-8281 [PMID: 24363518 DOI: 10.3748/wjg.v19.i45.8269]
54 **Hu Z**, Wang X, Gong L, Wu G, Peng X, Tang X. Role of high-mobility group box 1 protein in inflammatory bowel disease. *Inflamm Res* 2015; **64**: 557-563 [PMID: 26077468 DOI: 10.1007/s00011-015-0841-x]
55 **Foell D**, Kucharzik T, Kraft M, Vogl T, Sorg C, Domschke W, Roth J. Neutrophil derived human S100A12 (EN-RAGE) is strongly expressed during chronic active inflammatory bowel disease. *Gut* 2003; **52**: 847-853 [PMID: 12740341 DOI: 10.1136/gut.52.6.847]
56 **Yamasaki H**, Mitsuyama K, Masuda J, Kuwaki K, Takedatsu H, Sugiyama G, Yamada S, Sata M. Roles of high-mobility group box 1 in murine experimental colitis. *Mol Med Rep* 2009; **2**: 23-27 [PMID: 21475785 DOI: 10.3892/mmr_00000056]
57 **Manolakis AC**, Kapsoritakis AN, Tiaka EK, Potamianos SP. Calprotectin, calgranulin C, and other members of the s100 protein family in inflammatory bowel disease. *Dig Dis Sci* 2011; **56**: 1601-1611 [PMID: 21203903 DOI: 10.1007/s10620-010-1494-9]

58 **Cesaro A**, Abakar-Mahamat A, Brest P, Lassalle S, Selva E, Filippi J, Hébuterne X, Hugot JP, Doglio A, Galland F, Naquet P, Vouret-Craviari V, Mograbi B, Hofman PM. Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G1332-G1343 [PMID: 19299578 DOI: 10.1152/ajpgi.90641.2008]

59 **Meijer MJ**, Mieremet-Ooms MA, van der Zon AM, van Duijn W, van Hogezand RA, Sier CF, Hommes DW, Lamers CB, Verspaget HW. Increased mucosal matrix metalloproteinase-1, -2, -3 and -9 activity in patients with inflammatory bowel disease and the relation with Crohn's disease phenotype. *Dig Liver Dis* 2007; **39**: 733-739 [PMID: 17602907 DOI: 10.1016/j.dld.2007.05.010]

60 **Park L**, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS, Stern D, Schmidt AM. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med* 1998; **4**: 1025-1031 [PMID: 9734395 DOI: 10.1038/2012]

61 **Lalla E**, Lamster IB, Feit M, Huang L, Spessot A, Qu W, Kislinger T, Lu Y, Stern DM, Schmidt AM. Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. *J Clin Invest* 2000; **105**: 1117-1124 [PMID: 10772656 DOI: 10.1172/JCI8942]

62 **Wendt T**, Harja E, Bucciarelli L, Qu W, Lu Y, Rong LL, Jenkins DG, Stein G, Schmidt AM, Yan SF. RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes. *Atherosclerosis* 2006; **185**: 70-77 [PMID: 16076470 DOI: 10.1016/j.atherosclerosis.2005.06.013]

63 **Bucciarelli LG**, Wendt T, Qu W, Lu Y, Lalla E, Rong LL, Goova MT, Moser B, Kislinger T, Lee DC, Kashyap Y, Stern DM, Schmidt AM. RAGE blockade stabilizes established atherosclerosis in diabetic apolipoprotein E-null mice. *Circulation* 2002; **106**: 2827-2835 [PMID: 12451010 DOI: 10.1161/01.cir.0000039325.03698.36]

64 **Wang H**, Ye J, Liu R, Chen G, Zhao J, Huang L, Yang F, Li M, Zhang S, Jingxie, Xiong L, Chen H, Xu Y, Su M, Xie Y, Zheng F, Geng L, Xu W, Gong S. Clinical Significance of CD147 in Children with Inflammatory Bowel Disease. *Biomed Res Int* 2020; **2020**: 7647181 [PMID: 33015178 DOI: 10.1155/2020/7647181]

65 **Xu Z**, Liu R, Huang L, Xu Y, Su M, Chen J, Geng L, Xu W, Gong S. CD147 Aggravated Inflammatory Bowel Disease by Triggering NF-kB-Mediated Pyroptosis. *Biomed Res Int* 2020; **2020**: 5341247 [PMID: 32714980 DOI: 10.1155/2020/5341247]

66 **Wang K**, Chen W, Zhang Z, Deng Y, Lian JQ, Du P, Wei D, Zhang Y, Sun XX, Gong L, Yang X, He L, Zhang L, Yang Z, Geng JJ, Chen R, Zhang H, Wang B, Zhu YM, Nan G, Jiang JL, Li L, Wu J, Lin P, Huang W, Xie L, Zheng ZH, Zhang K, Miao JL, Cui HY, Huang M, Zhang J, Fu L, Yang XM, Zhao Z, Sun S, Gu H, Wang Z, Wang CF, Lu Y, Liu YY, Wang QY, Bian H, Zhu P, Chen ZN. CD147-spike protein is a novel route for SARS-CoV-2 infection to host cells. *Signal Transduct Target Ther* 2020; **5**: 283 [PMID: 33277466 DOI: 10.1038/s41392-020-00426-x]

67 **Bao W**, Min D, Twigg SM, Shackel NA, Warner FJ, Yue DK, McLennan SV. Monocyte CD147 is induced by advanced glycation end products and high glucose concentration: possible role in diabetic complications. *Am J Physiol Cell Physiol* 2010; **299**: C1212-C1219 [PMID: 20810913 DOI: 10.1152/ajpcell.00228.2010]

68 **Garg M**, Angus PW, Burrell LM, Herath C, Gibson PR, Lubel JS. Review article: the pathophysiological roles of the renin-angiotensin system in the gastrointestinal tract. *Aliment Pharmacol Ther* 2012; **35**: 414-428 [PMID: 22221317 DOI: 10.1111/j.1365-2036.2011.04971.x]

69 **Khajah MA**, Fateel MM, Ananthalakshmi KV, Luqmani YA. Anti-Inflammatory Action of Angiotensin 1-7 in Experimental Colitis. *PLoS One* 2016; **11**: e0150861 [PMID: 26963721 DOI: 10.1371/journal.pone.0150861]

70 **Santos RAS**, Oudit GY, Verano-Braga T, Canta G, Steckelings UM, Bader M. The renin-angiotensin system: going beyond the classical paradigms. *Am J Physiol Heart Circ Physiol* 2019; **316**: H958-H970 [PMID: 30707614 DOI: 10.1152/ajpheart.00723.2018]

71 **Gaddam RR**, Chambers S, Bhatia M. ACE and ACE2 in inflammation: a tale of two enzymes. *Inflamm Allergy Drug Targets* 2014; **13**: 224-234 [PMID: 25019157 DOI: 10.2174/1871528113666140713164506]

72 **Mehta PK**, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* 2007; **292**: C82-C97 [PMID: 16870827 DOI: 10.1152/ajpcell.00287.2006]

73 **Simões E Silva AC**, Teixeira MM. ACE inhibition, ACE2 and angiotensin-(1-7) axis in kidney and cardiac inflammation and fibrosis. *Pharmacol Res* 2016; **107**: 154-162 [PMID: 26995300 DOI: 10.1016/j.phrs.2016.03.018]

74 **Tan WSD**, Liao W, Zhou S, Mei D, Wong WF. Targeting the renin-angiotensin system as novel therapeutic strategy for pulmonary diseases. *Curr Opin Pharmacol* 2018; **40**: 9-17 [PMID: 29288933 DOI: 10.1016/j.coph.2017.12.002]

75 **Fyhrquist F**, Saijonmaa O. Renin-angiotensin system revisited. *J Intern Med* 2008; **264**: 224-236 [PMID: 18793332 DOI: 10.1111/j.1365-2796.2008.01981.x]

76 **Suzuki Y**, Ruiz-Ortega M, Lorenzo O, Ruperez M, Esteban V, Egido J. Inflammation and angiotensin II. *Int J Biochem Cell Biol* 2003; **35**: 881-900 [PMID: 12676174 DOI: 10.1016/s1357-2725(02)00271-6]

77 **Husain K**, Hernandez W, Ansari RA, Ferder L. Inflammation, oxidative stress and renin angiotensin system in atherosclerosis. *World J Biol Chem* 2015; **6**: 209-217 [PMID: 26322175 DOI: 10.4331/wjbc.v6.i3.209]

78 **Capettini LS**, Montecucco F, Mach F, Stergiopulos N, Santos RA, da Silva RF. Role of renin-angiotensin system in inflammation, immunity and aging. *Curr Pharm Des* 2012; **18**: 963-970 [PMID: 22283774 DOI: 10.2174/138161212799436593]

79 **Donoghue M**, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* 2000; **87**: E1-E9 [PMID: 10969042 DOI: 10.1161/01.res.87.5.e1]

80 **Ruiz-Ortega M**, Ruperez M, Esteban V, Rodriguez-Vita J, Sanchez-Lopez E, Egido J. Modulation of angiotensin II effects, A potential novel approach to inflammatory and immune diseases. *Curr Med Chem* 2003; **2**: 379-394 [DOI: 10.2174/1568014033483626]

81 **Garg M**, Royce SG, Tikellis C, Shallue C, Batu D, Velkoska E, Burrell LM, Patel SK, Beswick L, Jackson A, Britto K, Lukies M, Sluka P, Wardan H, Hirokawa Y, Tan CW, Faux M, Burgess AW, Hosking P, Monagle S, Thomas M, Gibson PR, Lubel J. Imbalance of the renin-angiotensin system may contribute to inflammation and fibrosis in IBD: a novel therapeutic target? *Gut* 2020; **69**: 841-851 [PMID: 31409604 DOI: 10.1136/gutjnl-2019-318512]

82 **Santos RAS**, Sampaio WO, Alzamora AC, Motta-Santos D, Alenina N, Bader M, Campagnole-Santos MJ. The ACE2/Angiotensin-(1-7)/MAS Axis of the Renin-Angiotensin System: Focus on Angiotensin-(1-7). *Physiol Rev* 2018; **98**: 505-553 [PMID: 29351514 DOI: 10.1152/physrev.00023.2016]

83 **Patel S**, Rauf A, Khan H, Abu-Izneid T. Renin-angiotensin-aldosterone (RAAS): The ubiquitous system for homeostasis and pathologies. *Biomed Pharmacother* 2017; **94**: 317-325 [PMID: 28772209 DOI: 10.1016/j.biopha.2017.07.091]

84 **Garg M**, Burrell LM, Velkoska E, Griggs K, Angus PW, Gibson PR, Lubel JS. Upregulation of circulating components of the alternative renin-angiotensin system in inflammatory bowel disease: A pilot study. *J Renin Angiotensin Aldosterone Syst* 2015; **16**: 559-569 [PMID: 24505094 DOI: 10.1177/1470320314521086]

85 **Hirasawa K**, Sato Y, Hosoda Y, Yamamoto T, Hanai H. Immunohistochemical localization of angiotensin II receptor and local renin-angiotensin system in human colonic mucosa. *J Histochem Cytochem* 2002; **50**: 275-282 [PMID: 11799146 DOI: 10.1177/002215540205000215]

86 **Ferreira-Duarte M**, Estevinho MM, Duarte-Araújo M, Magro F, Morato M. Unraveling the Role of ACE2, the Binding Receptor for SARS-CoV-2, in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2020; **26**: 1787-1795 [PMID: 33064147 DOI: 10.1093/ibd/izaa249]

87 **Jacobs JD**, Wagner T, Gulotta G, Liao C, Li YC, Bissonnette M, Pekow J. Impact of Angiotensin II Signaling Blockade on Clinical Outcomes in Patients with Inflammatory Bowel Disease. *Dig Dis Sci* 2019; **64**: 1938-1944 [PMID: 30725290 DOI: 10.1007/s10620-019-5474-4]

88 **Wengrower D**, Zanninelli G, Pappo O, Latella G, Sestieri M, Villanova A, Faitelson Y, Pines M, Goldin E. Prevention of fibrosis in experimental colitis by captopril: the role of tgf-beta1. *Inflamm Bowel Dis* 2004; **10**: 536-545 [PMID: 15472513 DOI: 10.1097/00054725-200409000-00007]

89 **Mantaka A**, Tsoukali E, Fragkaki M, Karmiris K, Viazis N, Mantzaris GJ, Koutroubakis IE. Is there any role of renin-angiotensin system inhibitors in modulating inflammatory bowel disease outcome? *Eur J Gastroenterol Hepatol* 2021; **33**: 364-371 [PMID: 32925506 DOI: 10.1097/MEG.0000000000001912]

90 **Wang Q**, Zhang Y, Wu L, Niu S, Song C, Zhang Z, Lu G, Qiao C, Hu Y, Yuen KY, Wang Q, Zhou H, Yan J, Qi J. Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. *Cell* 2020; **181**: 894-904. e9 [PMID: 32275855 DOI: 10.1016/j.cell.2020.03.045]

91 **Colón AL**, Menchén LA, Hurtado O, De Cristóbal J, Lizasoain I, Leza JC, Lorenzo P, Moro MA. Implication of TNF-alpha convertase (TACE/ADAM17) in inducible nitric oxide synthase expression and inflammation in an experimental model of colitis. *Cytokine* 2001; **16**: 220-226 [PMID: 11884025 DOI: 10.1006/cyto.2001.0969]

92 **Brynskov J**, Foegh P, Pedersen G, Ellervik C, Kirkegaard T, Bingham A, Saermark T. Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease. *Gut* 2002; **51**: 37-43 [PMID: 12077089 DOI: 10.1136/gut.51.1.37]

93 **He L**, Du J, Chen Y, Liu C, Zhou M, Adhikari S, Rubin DT, Pekow J, Li YC. Renin-angiotensin system promotes colonic inflammation by inducing T_H17 activation *via* JAK2/STAT pathway. *Am J Physiol Gastrointest Liver Physiol* 2019; **316**: G774-G784 [PMID: 30995068 DOI: 10.1152/ajpgi.00053.2019]

94 **De Francesco EM**, Vella V, Belfiore A. COVID-19 and Diabetes: The Importance of Controlling RAGE. *Front Endocrinol (Lausanne)* 2020; **11**: 526 [PMID: 32760352 DOI: 10.3389/fendo.2020.00526]

95 **Rojas A**, Gonzalez I, Morales MA. SARS-CoV-2-mediated inflammatory response in lungs: should we look at RAGE? *Inflamm Res* 2020; **69**: 641-643 [PMID: 32372149 DOI: 10.1007/s00011-020-01353-x]

96 **Bezzio C**, Saibeni S, Variola A, Allocca M, Massari A, Gerardi V, Casini V, Ricci C, Zingone F, Amato A, Caprioli F, Lenti MV, Viganò C, Ascolani M, Bossa F, Castiglione F, Cortelezzi C, Grossi L, Milla M, Morganti D, Pastorelli L, Ribaldone DG, Sartini A, Soriano A, Manes G, Danese S, Fantini MC, Armuzzi A, Daperno M, Fiorino G; Italian Group for the Study of Inflammatory Bowel Disease (IG-IBD). Outcomes of COVID-19 in 79 patients with IBD in Italy: an IG-IBD study. *Gut* 2020; **69**: 1213-1217 [PMID: 32354990 DOI: 10.1136/gutjnl-2020-321411]

97 **Burke KE**, Kochar B, Allegretti JR, Winter RW, Lochhead P, Khalili H, Colizzo FP, Hamilton MJ,

Chan WW, Ananthakrishnan AN. Immunosuppressive Therapy and Risk of COVID-19 Infection in Patients With Inflammatory Bowel Diseases. *Inflamm Bowel Dis* 2021; **27**: 155-161 [PMID: 33089863 DOI: 10.1093/ibd/izaa278]

98 **Occhipinti V**, Pastorelli L. Challenges in the Care of IBD Patients During the CoViD-19 Pandemic: Report From a "Red Zone" Area in Northern Italy. *Inflamm Bowel Dis* 2020; **26**: 793-796 [PMID: 32314792 DOI: 10.1093/ibd/izaa084]

99 **Tursi A**, Angarano G, Monno L, Saracino A, Signorile F, Ricciardi A, Papa A. COVID-19 infection in Crohn's disease under treatment with adalimumab. *Gut* 2020; **69**: 1364-1365 [PMID: 32312788 DOI: 10.1136/gutjnl-2020-321240]

100 **Bezzio C**, Pellegrini L, Manes G, Arena I, Picascia D, Della Corte C, Devani M, Schettino M, Saibeni S. Biologic Therapies May Reduce the Risk of COVID-19 in Patients With Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2020; **26**: e107-e109 [PMID: 32869831 DOI: 10.1093/ibd/izaa242]

101 **Sigall-Boneh R**, Levine A, Lomer M, Wierdsma N, Allan P, Fiorino G, Gatti S, Jonkers D, Kierkus J, Katsanos KH, Melgar S, Yuksel ES, Whelan K, Wine E, Gerasimidis K. Research Gaps in Diet and Nutrition in Inflammatory Bowel Disease. A Topical Review by D-ECCO Working Group [Dietitians of ECCO]. *J Crohns Colitis* 2017; **11**: 1407-1419 [PMID: 28961811 DOI: 10.1093/ecco-jcc/jjx109]

102 **Uribarri J**, del Castillo MD, de la Maza MP, Filip R, Gugliucci A, Luevano-Contreras C, Macías-Cervantes MH, Markowicz Bastos DH, Medrano A, Menini T, Portero-Otin M, Rojas A, Sampaio GR, Wrobel K, Garay-Sevilla ME. Dietary advanced glycation end products and their role in health and disease. *Adv Nutr* 2015; **6**: 461-473 [PMID: 26178030 DOI: 10.3945/an.115.008433]

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REVIEW

Individualized treatment options for patients with non-cirrhotic and cirrhotic liver disease

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Abstract

The obesity pandemic has led to a significant increase in patients with metabolic dysfunction-associated fatty liver disease (MAFLD). While dyslipidemia, type 2 diabetes mellitus and cardiovascular diseases guide treatment in patients without signs of liver fibrosis, liver related morbidity and mortality becomes relevant for MAFLD's progressive form, non-alcoholic steatohepatitis (NASH), and upon development of liver fibrosis. Statins should be prescribed in patients without significant fibrosis despite concomitant liver diseases but are underutilized in the real-world setting. Bariatric surgery, especially Y-Roux bypass, has been proven to be superior to conservative and/or medical treatment for weight loss and resolution of obesity-associated diseases, but comes at a low but existent risk of surgical complications, reoperations and very rarely, paradoxical progression of NASH. Once end-stage liver disease develops, obese patients benefit from liver transplantation (LT), but may be at increased risk of perioperative infectious complications. After LT, metabolic comorbidities are commonly observed, irrespective of the underlying liver disease, but MAFLD/NASH patients are at even higher risk of disease recurrence. Few studies with low patient numbers evaluated if, and when, bariatric surgery may be an option to avoid disease recurrence but more high-quality studies are needed to establish clear recom-

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mendations. In this review, we summarize the most recent literature on treatment options for MAFLD and NASH and highlight important considerations to tailor therapy to individual patient's needs in light of their risk profile.

Key Words: Metabolic dysfunction-associated fatty liver disease; Non-alcoholic fatty liver disease; Portal hypertension; Cirrhosis; Bariatric surgery; Metabolism

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Core Tip: No single therapy fits all needs, sometimes resulting in complex clinical decision making. While some etiologies can distinctly be characterized, a multifactorial disease such as metabolic dysfunction-associated fatty liver disease requires thorough assessment of comorbidities and severity of concomitant fibrosis to assess a patient's overall risk. While (guided) physical exercise is usually safe and well tolerated and strict treatment of diabetes and dyslipidemia is warranted, patients often fail to change their lifestyle, resulting in life-long drug dependency for comorbidities. Bariatric surgery has therefore become a valid option for obese patients and should be offered in eligible patients before liver fibrosis develops.

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INTRODUCTION

In recent decades, the fractional contribution of different etiologies to the total burden of chronic liver disease (CLD) has shifted. On the one hand these changes are driven by a decrease in hepatitis C virus (HCV) related morbidity which has decreased by 40% in the United States[1] and led to HCV becoming a less common indication for liver transplantation (LT) in Europe[2], a trend that will likely be seen globally in the near future. On the other hand there is a steady and significant increase in non-alcoholic fatty liver disease (NAFLD), overall resulting in a relative shift of CLD etiologies, and an even further absolute increase in NAFLD related morbidity. While HCV related liver disease is a domain of hepatologists and transplant units, NAFLD, recently proposed to be re-named metabolic dysfunction-associated fatty liver disease (MAFLD)[3,4], is associated with extrahepatic diseases, such as central obesity[5], sleep apnea, type 2 diabetes mellitus (T2DM), cardiovascular diseases, and bone and joint disorders, all contributing to relevant morbidity and affecting different specialties[6].

Mirroring the obesity pandemic and in line with CLD etiology shifts, the number of LTs due to non-alcoholic steatohepatitis (NASH)-related cirrhosis, which results from progression of MAFLD, has markedly increased[1,2,7,8], with NASH already representing the second most frequent cause for LT in the United States[1,9,10]. In addition, the prevalence of hepatocellular carcinoma due to NASH is also rapidly increasing [2,8,11,12], probably resulting in an even higher need for LT due to MAFLD/NASH in the future. Thus, this review summarizes current treatment options in MAFLD, tailored to individual patient's disease stage in light of the most recent evidence. We provide a short overview of the core messages in Figure 1 and highlight several studies on the most important topics, which are discussed in further detail below, in Table 1.

NAFLD/MAFLD AND THE METABOLIC SYNDROME

MAFLD is commonly considered a hepatic manifestation of the metabolic syndrome (MS)[13,14]. It is defined as excessive hepatic fat accumulation with insulin resistance, steatosis in > 5% of hepatocytes in histological analysis (or > 5.6% by quantitative fat/water-selective magnetic resonance imaging or proton magnetic resonance

Table 1 Overview of important studies concerning the management of metabolic dysfunction-associated fatty liver disease/non-alcoholic steatohepatitis patients

	Ref.	Study design	No. of patients	Liver disease	Main findings
Diet/physical exercise	Berzigotti *et al*[57], 2017	Prospective, uncontrolled	60 (50 completed the study)	Cirrhosis, BMI ≥ 26 kg/m^2, portal hypertension	Moderate exercise was safe in patients with compensated cirrhosis Diet and moderate exercise reduced body weight and portal pressure Weight loss ≥ 10% is associated with more pronounced portal pressure reduction
	Wong *et al*[55], 2018	Randomized controlled trial	154	NAFLD	Regular exercise associated with significantly more frequent remission of NAFLD (assessed by proton-magnetic MR-spectroscopy) NAFLD remission in 67% of non-overweight patients (baseline BMI < 25 kg/m^2) with lifestyle intervention
Dyslipidemia	Unger *et al*[150], 2019	Retrospective	1265	CLD	34.2% of non-advanced and 48.2% of advanced CLD patients did not receive guideline-conform statin therapy Guideline-conform statin use was associated with improved overall survival in compensated, but not in decompensated CLD patients
	Abraldes *et al*[69], 2009	Randomized controlled trial	59	Cirrhosis and portal hypertension	Simvastatin reduced portal pressure (-8.3) in both patients, who did and did not also receive beta-blockers Simvastatin improved liver perfusion The effects of simvastatin were additive to beta-adrenergic blockade
	Nelson *et al*[72], 2009	Randomized controlled trial	16	NASH	Simvastatin reduced low-density lipoprotein by 26% Simvastatin was well-tolerated Simvastatin did not histologically improve NASH (but small sample size, only n = 10 follow-up biopsies)
T2DM	Lavine *et al*[108], 2011	Randomized controlled trial	173	NAFLD	Sustained ALT level reduction was similar in the metformin and placebo group Metformin did not change the NAFLD activity score
	Cusi *et al*[102], 2016	Randomized controlled trial	101	NASH and prediabetes/T2DM	Significantly more patients receiving pioglitazone (59%) resolved NASH compared to placebo (23%) Pioglitazone improved fibrosis score (-0.9 *vs* placebo 0.0) Pioglitazone improved insulin sensitivity in liver, muscle and adipose tissue
	Armstrong *et al*[104], 2016	Randomized controlled trial	52	NASH	Significantly more patients receiving liraglutide (39%) resolved NASH compared to placebo (9%) Significantly less patients receiving liraglutide (9%) exhibited fibrosis progression compared to placebo (36%) Liraglutide was safe and well-tolerated
Bariatric surgery	Lassailly *et al*[114], 2015	Prospective	109	NASH	NASH was resolved in 85% of patients one year after surgery and even in 94% with mild NASH before surgery (assessed *via* biopsy) NASH persistence was higher in patients after gastric banding (30.4%) compared to gastric bypass (7.6%)

	Goossens *et al*[117], 2016	Retrospective	59	NASH	NASH is an independent predictor of overall mortality after bariatric surgery NASH may reduce the overall survival benefit of bariatric surgery
	Eilenberg *et al*[118], 2018	Retrospective	10	NAFLD/NASH	Liver dysfunction, liver steatosis/fibrosis and cirrhosis may occur after bariatric surgery Lengthening of the alimentary or common limb may lead to a clinical improvement in these patients
Post-LT	Krasnoff *et al*[151], 2006	Randomized controlled trial	151	Post-LT	Exercise and dietary counseling intervention improved exercise capacity and self-reported general health Adherence to the intervention was associated with positive trends in exercise capacity and body composition (% body fat)
	Zamora-Valdes *et al*[152], 2018	Prospective	29	NAFLD/NASH/obese ACLD	Patients, who received sleeve gastrectomy at the time of LT had more pronounced and sustained weight loss They also had a lower prevalences of hepatic steatosis, hypertension and insulin resistance 3 yr after LT
	Patel *et al*[139], 2019	Retrospective	495	Post-LT	Statins were underused after LT (54.3% of patients with known coronary artery disease did not receive statin therapy) Statin use was well-tolerated Statin therapy was associated with improved overall survival

BMI: Body mass index; NAFLD: Non-alcoholic fatty liver disease; CLD: Chronic liver disease; NASH: Non-alcoholic steatohepatitis; ALT: Alanine aminotransferase; T2DM: Type 2 diabetes mellitus; LT: Liver transplantation; ACLD: Advanced chronic liver disease.

spectroscopy), and exclusion of secondary causes as well as alcoholic fatty liver disease, *e.g.* daily alcohol consumption of < 30 g for men and < 20 g for women, commonly resulting in difficulties to differentiate between alcoholic fatty liver disease and MAFLD in retrospective studies[15]. The severity of MAFLD can vary, ranging from simple steatosis[16] to NASH with chronic inflammation and fibrosis to liver cirrhosis[17,18]. Unfortunately, NASH diagnosis can, to date, only be made histologically by presence of macrovesicular steatosis, ballooning degeneration of hepatocytes, scattered inflammation, and Mallory-Denk bodies[19]. This limitation has led to the search for alternative non-invasive diagnostic procedures that avoid the need for liver biopsy, reviewed by *e.g.* Paternostro *et al*[20], to identify patients that are most likely to suffer from liver-related complications[21].

Before significant fibrosis develops, however, several factors contribute to the development of MAFLD, such as nutrition[22-24], insulin resistance[25,26], adipokines [27], gut microbiota[28,29], and genetic as well as epigenetic factors[30,31]. The close association of energy metabolism and fatty liver disease is illustrated by the fact that MAFLD patients suffer from increased risk for cardiovascular disease[32,33], T2DM[34-36], as well as chronic kidney disease[37]. According to a meta-analysis by Younossi *et al*[38], 51.3% of NAFLD and 81.8% of NASH patients are obese, 22.5% and 43.6% suffer from T2DM, and 69.2% and 72.1% from dyslipidemia, respectively[38]. This indicates that neither of the diseases should be addressed in an isolated fashion as they impact each other and contribute to disease progression. Thus, MAFLD patients must be seen as metabolically multimorbid, which is reflected by increased cardiovascular mortality compared to liver-related mortality in individuals without significant liver fibrosis[38]. Once liver fibrosis develops, however, liver-related mortality becomes more relevant. Recent evidence from high quality studies suggests that concomitant fibrosis, and especially cirrhosis, rather than NASH *per se* significantly increase liver-related morbidity and mortality[39-41]. Thus, well-established tools such as transient elastography with adapted cutoff values may allow risk stratification, and identification of significant fibrosis should result in state-of-the-art therapy with a liver-centered approach[20].

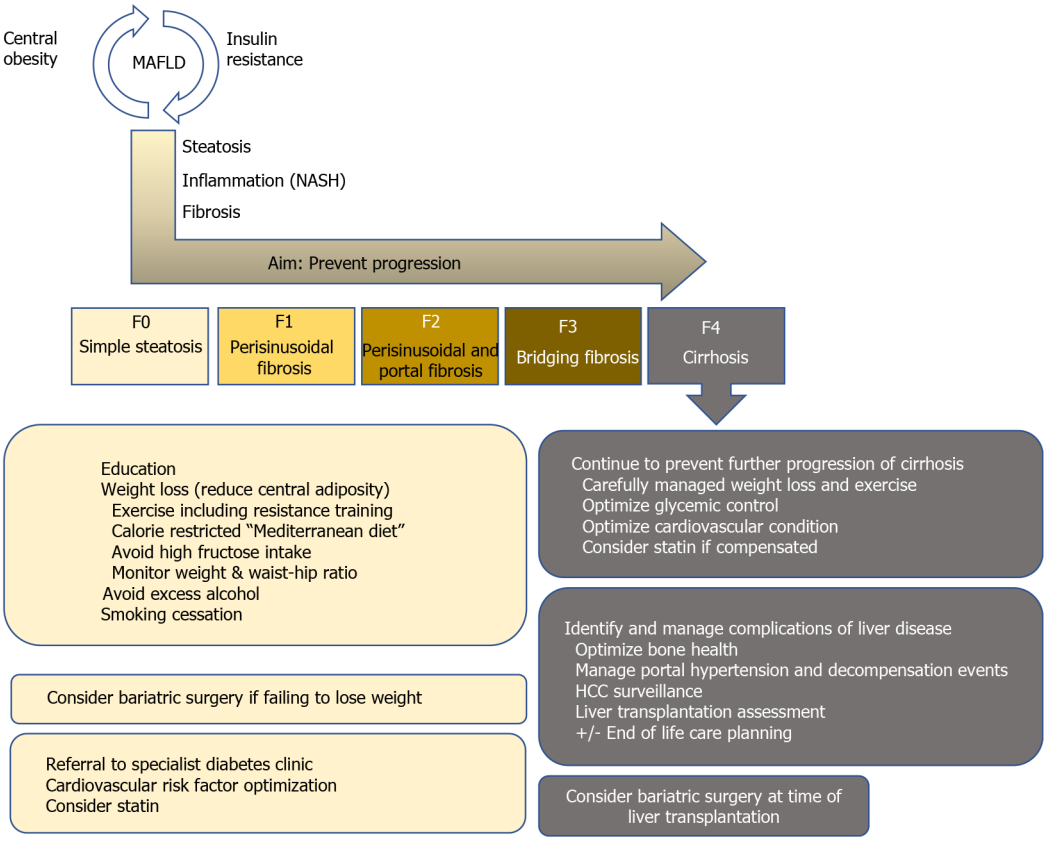


Figure 1 Treatment recommendations based on liver fibrosis severity in metabolic dysfunction-associated fatty liver disease patients.
HCC: Hepatocellular carcinoma; MAFLD: Metabolic dysfunction-associated fatty liver disease; NASH: Non-alcoholic steatohepatitis.

THERAPEUTIC OPTIONS IN MAFLD/NASH

As mentioned above, the first step in risk stratification for individual patients should be assessment of presence/absence of liver fibrosis. In case of absence of liver fibrosis, regardless of the underlying etiology, removal of the damaging agent is vital to prevent development of fibrosis and subsequent portal-hypertensive decompensation events. In MAFLD, lifestyle modifications should be seen as the cornerstone of causative treatment, as obesity, high-fat diet and physical inactivity are strongly associated with development as well as progression of the disease[42]. Unfortunately, to date, no pharmacological treatment has specifically been approved for MAFLD, and current trials on drugs for MAFLD or NASH target mostly metabolic pathways to improve insulin resistance or dyslipidemia. As of 2018, more than 300 substances were in clinical trials for MAFLD/NASH[43,44]. However, the majority of trials have fallen short of proving efficacy and the most effective, to date, are repurposed drugs such as statins[45]. In terms of newly developed compounds, a recent prospective, placebo-controlled study of obeticholic acid (OCA), which is a farnesoid X receptor agonist that was shown to decrease hepatic fibrosis and reduce inflammation in preclinical studies, found that OCA improved fibrosis severity in patients with NASH[46]. Of note, however, complete NASH resolution was not more common in patients treated with either OCA dosing intensity (placebo: 8%; OCA 10 mg daily: 11%; OCA 25 mg daily, 12%), and overall fibrosis improvement was still only achieved in approximately 1/4 of patients (fibrosis improvement of ≥ 1 stage: Placebo: 12%, OCA 10 mg daily: 18%, and OCA 25 mg daily: 23%), highlighting the complexity of NASH treatment. Nevertheless, with this first successful trial, a broader repertoire of pharmacological agents will hopefully be available in the near future.

OBESITY MANAGEMENT, DIET AND EXERCISE

Adequate therapy for obesity is of utmost relevance, as obesity *per se* independently increases the risk for cardiovascular disease[47] and independently predicted clinical decompensation in a subgroup-analysis of a placebo-controlled trial assessing beta

blockers for the prevention of esophageal varices, irrespective of the underlying etiology[48]. Furthermore, morbidly obese patients, defined as patients with a body mass index (BMI) ≥ 40 kg/m², have a significantly higher LT waiting list mortality, and benefit more from LT according to Schlansky *et al*[49], although the cause of death was not available from this United Network for Organ Sharing registry based study[49].

Lifestyle interventions are crucial, as a weight loss of 7%-10% of initial body weight is already associated with histological improvement in MAFLD with a reduction of steatosis, ballooning and lobular inflammation[50,51]. Even lower rates of sustained weight loss (about 5%) can decrease steatosis[52], liver enzymes[53] and the risk of developing T2DM[54]. Remission of MAFLD due to lifestyle interventions has also been demonstrated in non-obese patients with MAFLD[55] despite the fact that the underlying causes of lean MAFLD are unclear[56]. Guidelines suggest that the lifestyle modifications recommended to patients with MAFLD should be structured and include prescribed physical activity including resistance training, a calory restricted "Mediterranean" diet, avoidance of high fructose foods and avoidance of excess alcohol consumption. In addition, smoking cessation is important to improve the cardiovascular risk profile.

Both diet and exercise are safe in patients with compensated cirrhosis[57], have been shown to be highly effective for treatment of risk factors (cardiovascular disease and T2DM, respectively)[50,51,58], and lower portal pressure in overweight CLD patients regardless of etiology[57]. Importantly, however, recommendations for weight loss in obese NAFLD/NASH patients with cirrhosis are more cautious, as uncontrolled weight loss in decompensated patients may worsen sarcopenia and frailty[47]. Thus, diligent planning of diet and exercise is required to ensure weight loss with an adequate intake of nutrients, especially proteins. It should also be considered to be mandatory to investigate, whether patients have an indication for non-selective beta-blocker (NSBB) prophylaxis against variceal hemorrhage before enrollment into an exercise program, as NSBB counteract exercise-mediated increases of hepatic venous pressure gradient (HVPG)[59,60].

Importantly, evidence from a recently published randomized controlled trial suggests that once-weekly subcutaneous semaglutide leads to sustained and clinically relevant weight reduction (mean weight loss -14.9% in semaglutide-treated patients compared to -2.4% in the placebo group, respectively), with a more pronounced amelioration of cardiometabolic risk factors and patient-reported physical functioning in non-diabetic obese individuals[61]. Thus, these first encouraging results suggest that more effective pharmacological therapies may become available in the future.

DYSLIPIDEMIA

Dyslipidemia is a major risk factor for the development and progression of atherosclerotic cardiovascular disease[62] and often presents as a comorbidity in patients with CLD[63]. Lipid profiles can be altered by liver diseases due to impaired cholesterol synthesis, leading to a seemingly improved lipid profile with CLD disease progression[63]. Nevertheless, pharmacologically, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibition *via* statins is by far the most important treatment option for dyslipidemia, leading to a decrease of systemic levels of low-density lipoprotein (LDL) cholesterol, as well as other pleiotropic effects[64]. Generally, statins are well-tolerated, however, 10%-15% of patients experience adverse events such as myalgia with or without increase of creatin kinase[64,65]. From a liver perspective, the long-standing dogma that statin therapy is contraindicated in patients with CLD has been proven to be outdated[63]. We and others could show that in real-life settings, statins are underutilized in CLD patients[63,66]. Despite clear indications for statin utilization to reduce cardiovascular morbidity and mortality, outlined in the American College of Cardiology/American Heart Association guidelines, we found that 34.2% of patients with non-advanced CLD and 48.2% patients with advanced CLD did not receive statins despite having a clear indication, and we found that guideline-conformed statin use translated to improved overall survival of compensated CLD, but not decompensated CLD patients[63]. Others have found that statins directly influence liver-specific outcome by lowering the risk of hepatic decompensation [67,68], potentially by reducing HVPG, improving hepatocyte function[69] and ameliorating sinusoidal endothelial dysfunction[70,71], overall indicating that statins should at least be prescribed in patients with non-cirrhotic CLD with cardiovascular risk profiles. In a small pilot trial, simvastatin did improve lipid profiles, but did not affect steatosis levels and necroinflammation in 16 NASH patients. However, it also

did not do any harm although results have to be interpreted with caution due to the small sample size[72].

Overall, most studies have found that, if adhering to available guidelines for statin initiation in patients without decompensated liver disease, adverse events rates are low, and the majority of studies reported beneficial effects of statins in compensated CLD, irrespective of CLD etiology[73-79].

T2DM

An association between MAFLD and T2DM is well-established[80]. MAFLD and T2DM commonly coexist[81,82] and even in T2DM patients with normal serum alanine aminotransferase levels, the prevalence of liver steatosis is high[83]. Conversely, many studies demonstrated high rates of NASH in T2DM patients[84-86], and it has also been shown that T2DM is strongly associated with liver fibrosis[87-90]. Two studies based on liver histology found that MAFLD patients with T2DM commonly develop severe fibrosis, namely 40.3% and 41.0%, respectively[85,86]. Other studies, assessing liver stiffness by transient elastography, showed that 17.7% and 5.6% of diabetic patients suffer from advanced fibrosis[91,92]. This is of high importance, as liver fibrosis is the crucial factor associated with long-term outcome in MAFLD patients[93,94] and indeed, MAFLD and T2DM synergistically lead to an increased rate of adverse outcomes[95] including increased liver-related and overall mortality[96,97].

Thus, regulation of insulin sensitivity is essential in patients with MAFLD and there is growing evidence for pharmacological treatments that are effective for treating both T2DM and MAFLD[93]. Pioglitazone, an insulin sensitizer that stimulates adipocyte differentiation by peroxisome proliferator-activated receptor *g* agonism[98], has, for example, shown beneficial effects on NAFLD. Pioglitazone reduced biopsy-assessed NAFLD severity and liver fat content in patients with[99], but also without T2DM[100] upon short-term treatment. Moreover, a randomized controlled trial showed significantly more frequent resolution of NASH in patients treated with pioglitazone (34%) than with placebo (19%)[101]. However, fibrosis was not ameliorated and also insulin resistance only partially decreased, which may be attributable to the low administered pioglitazone dose of 30 mg *per* day[93]. In another randomized controlled trial in NASH patients with T2DM or prediabetes, 45 mg pioglitazone *per* day improved histological NAFLD activity score, fibrosis and insulin sensitivity[102]. Importantly, side effects of pioglitazone include weight gain, fluid retention with increased risk of congestive heart failure, as well as decrease of bone mineral density, resulting in atypical fractures[98], which has to be actively screened for when prescribing pioglitazone in MAFLD patients.

Glucagon-like peptide-1 (GLP-1) receptor agonists also represent a valuable treatment option for patients with MAFLD, as they improve glucose-dependent insulin secretion, but also promote weight loss and lower liver transaminase levels[103]. In a pilot trial, subcutaneous liraglutide decreased liver fat content and was associated with more frequent NASH resolution, as compared to placebo (39% *vs* 9%)[104]. In contrast, metformin, the first-line T2DM medication, does not consistently improve hepatic steatosis or inflammation in patients with NASH[105-109]. Overall, however, antidiabetic drugs show great promise for treatment of MAFLD/NASH (and weight loss) but more adequately designed randomized controlled trials, are needed.

BARIATRIC SURGERY AND MAFLD/NASH REGRESSION

As mentioned above, the co-existence of several metabolic diseases, summarized as metabolic syndrome, has led to the development of invasive/surgical treatment options. While bariatric surgery was a niche phenomenon for several years, its benefit with regards to weight loss and subsequent improvement of insulin resistance/T2DM is established by now[110]. Moreover, due to improved success rates with regards to weight loss compared to conservative approaches, bariatric surgery patients have a significantly better 10-year[111] and 20-year overall survival than comparable patients that were treated conservatively, although, despite this improvement, their life expectancy is still lower than the general population's[112]. Recent evidence suggests that the major benefit results from weight loss itself and is not attributed to any other metabolic effects of bypass surgery. These assumptions come from a study that compared patients with Y-Roux bypass to patients who lost the same amount of

weight by dietary/lifestyle changes and observed similar effects, indicating that bypass surgery *per se* does not alter metabolism more than weight loss itself[113]. In terms of liver-specific outcomes, bariatric surgery has not been taken into account as treatment option in several meta-analyses on NASH resolution, despite available properly designed studies. In general, bariatric surgery results in resolution of NASH in the majority of patients (85% in a study by Lassailly *et al*[114], with 64.2% of patients undergoing bypass surgery and 5.5% of sleeve gastrectomiy) and regression of fibrosis[114]. However, not all procedures are equal, and Y-Roux bypass is considered to be the most effective strategy for sustainable weight loss to date[115]. A recent hierarchical network meta-analysis included 48 high-quality trials and found that pioglitazone and Y-Roux gastric bypass had the best effect on improvement of NAFLD Activity Score[116], suggesting a causative connection between glucose metabolism and fatty liver development. While bariatric surgery impacts on NASH, NASH and liver fibrosis, expectedly, also impact on postoperative outcome after bariatric surgery[117]. This, again, highlights that metabolic diseases do not exist as isolated diseases but must be treated together. Importantly, bariatric surgery is only offered to severely obese patients, while the general population is often overweight, but not obese, and thus not eligible for surgery, warranting further basic research studies disentangling the mechanisms of MAFLD/NASH development. Noteworthy, a very small fraction of patients develops NASH or suffers from NASH/fibrosis aggravation after bariatric surgery, requiring adequate post-operative care for early detection of complications and further emphasizing the need for ongoing research[118]. Considering that bariatric surgery is increasingly utilized, prospective studies answering the remaining questions on the connection of insulin resistance, fatty liver, and fibrosis progression should become available in the near future.

OBESITY AND MAFLD/NASH BEFORE AND AFTER LT

In general, patients with cirrhosis/end-stage liver disease should be managed according to available guidelines for the treatment of portal hypertension, as liver-related mortality is the main cause of death in end-stage liver disease, with special regard to the above-mentioned pitfalls in obese patients[119]. According to the 2018 Organ Procurement and Transplantation Network/Scientific Registry of Transplant Recipients LT report, 36.9% of adult patients undergoing LT were obese [BMI (30 kg/m^2)] including 14.8% with a BMI of more than 35 (kg/m^2)[120]. Despite the caveat that BMI is not an ideal parameter in patients with end-stage liver disease due to ascites, these data still highlight obesity as an important comorbidity in LT. Due to increasing experience in treatment of these patients, morbid obesity [BMI (40 kg/m^2)] is no longer seen as a contraindication for LT[47], as morbidly obese patients clearly profit from LT[49,121]. However, specific challenges include technical difficulties during surgery, as well as higher morbidity in the postoperative course, especially due to an increased risk of infections[122-125]. Ultimately, these challenges translate to an increased 30 d mortality[126]. However, outcomes seem to be gradually improving, as Schlansky *et al*[49] could detect impaired post-OP survival before but not after 2007[49]. In terms of long-term outcomes of NASH LT recipients, survival rates are comparable to other etiologies despite the fact that Malik *et al*[127] found an alarming 50% 1-year mortality rate among obese NASH patients ≥ 60 years old with T2DM and arterial hypertension[127]. Thus, pre-transplant work-up warrants extensive risk-benefit evaluation on a case-to-case basis before listing for LT to avoid unexpected complications[128].

Following LT, weight gain is common irrespective of the underlying CLD and type of transplanted organ. In general, approximately one in three LT recipients becomes overweight or obese within 3 years[129] and decreased physical activity, excess energy intake and older age favor development of sarcopenic obesity with increased risk of cardiovascular and metabolic comorbidities[130,131]. Although a clear research agenda has been set out in 2014 by the American Society for Transplantation[132], outcome measures are heterogeneous, and liver transplant recipients are underrepresented in these studies. A recent review of 2 observational and 3 randomized controlled trials by Dunn *et al*[133] reported that exercise intervention groups generally performed better at strength testing, energy expenditure in metabolic equivalents, and peak or maximal oxygen uptake[133]. An even more recently published prospective study reported that financial incentives resulted in more patients achieving their target of > 7000 steps *per* day, which, however, did not translate into less weight gain[134]. Another study using a smartphone app found that

35% of participants significantly increased their physical performance, but did not report whether this translated into an outcome benefit[135]. Thus, despite positive impacts on surrogate parameters, little to no high-quality evidence is available on whether exercise directly affects overall survival or liver related outcome after transplantation.

Similar to a lack of high-quality data on exercise programs, more prospective studies are needed to evaluate the effect of bariatric surgery at the time of LT. Recently, a meta-analysis of available studies on bariatric surgery during or after LT found that sleeve gastrectomy is the most commonly performed procedure and that bariatric surgery-related morbidity and mortality rates were 37% and 0.6%, respectively. Regarding outcome parameters, BMI was significantly lower in bariatric surgery patients 2 years after LT, with significantly lower rates of arterial hypertension and diabetes mellitus[136]. Of note, however, prospective randomized studies are needed to compare whether the benefits outweigh the risks in terms of overall outcome, which poses several difficulties in this setting.

In addition to weight gain, prevalence of dyslipidemia is high in the post-LT setting and affects approximately 40%-70%[137]. Partly, dyslipidemia and impaired glucose tolerance are metabolic adverse effects of immunosuppressants such as calcineurin inhibitors, mammalian target of rapamycin inhibitors and corticosteroids[8,138]. Thus, statins are commonly used after LT, however, data regarding statin therapy and potential effects on portal pressure and hepatocyte function in the post-transplant setting are scarce and a clear guideline for post-transplant statin use is not available[138]. Nevertheless, it has been shown that dyslipidemia is linked to increased morbidity and mortality in LT recipients and recently, a study by Patel *et al*[139] demonstrated good tolerance of statins and a survival benefit of statin-treated patients after LT, favoring statin use also in this setting[139]. Moreover, experimental studies in rats have demonstrated a graft-protecting effect of statins, when added to the cold storage solution[140,141]. Overall, prospective high-quality studies defining cut-offs are lacking, but available evidence suggests beneficial effects of statins in the post-LT setting.

Despite ameliorated glycogen synthesis, only few patients exhibit improved insulin sensitivity after LT[8]. Contrarily, 10% to 30% of patients suffer from new onset T2DM after LT, which is linked to the use of corticosteroids and tacrolimus[142,143]. In the immediate post-transplant period, insulin is considered the safest and most effective choice for anti-hyperglycemic therapy[144-147]. For the management of persistent T2DM after LT, however, evidence is scarce. A recent meta-analysis concluded that safety and efficacy cannot be concluded for various anti-hyperglycemic agents in the post-transplant setting, as the available studies are not of high enough quality[148]. Thus, anti-hyperglycemic therapy after the first-line metformin should be selected according to patient preference, as well as clinical characteristics such as presence of chronic kidney disease, heart failure or obesity[147,149].

AUTHOR'S PERSPECTIVE

MAFLD/NASH is a complex disease entity that poses challenges for clinical practice and requires interdisciplinary management for optimal patient care. In recent years, several novel concepts have been established, and bariatric surgery has been proven to be an effective treatment option. Additionally, recent trial results suggest that novel therapeutics, or repurposed drugs, may be effective to improve MAFLD or achieve sustainable weight loss and potentially secondary improvement of MAFLD/NASH. Thus, the multifactorial nature of the disease and the interconnectedness of different aspects require up-to-date knowledge, especially as more therapeutics will likely become available. These developments require an individualized treatment plan and should be based on patients' preferences, as compliance is of utmost importance.

In patients with advanced CLD or end-stage NASH, eligibility assessment for LT should be conducted in due time. Once patients undergo orthotopic LT, metabolic comorbidities should be closely monitored and adequately treated. In the future, the special metabolic vulnerability of LT patients will become even more relevant, as NASH as indication for LT is rapidly increasing, emphasizing the importance of future trials in this special patient population.

CONCLUSION

With the growing obesity epidemic and the rising prevalence of MAFLD/NASH, management of patients with CLD has become quite complex. MAFLD/NASH patients are often multimorbid, exhibiting various features of the metabolic syndrome, which altogether increase the risk of cardiovascular morbidity and mortality. In the early stages of liver disease without signs of liver fibrosis (MAFLD), management of comorbidities guides the therapy, while in patients who develop NASH and liver fibrosis, liver-related complications and mortality become relevant.

Unfortunately, there is a general lack of high-quality studies reporting important end points, such as fibrosis severity, which impedes comparability of the available results. Lifestyle interventions such as specific diets and exercise represent an etiological treatment for MAFLD/NASH patients and have been proven to be safe even for patients with cirrhosis and portal hypertension. Moreover, it has been shown that even moderate weight loss can lead to histological improvement, making lifestyle intervention an essential part of MAFLD/NASH management. Bariatric surgery is superior for weight loss of morbidly obese patients compared to conservative weight loss regimen, however, the risk of bariatric surgery is higher in patients with CLD and in some patients, severe liver dysfunction after bariatric surgery does occur.

Statins should be prescribed for all compensated patients with dyslipidemia or other risk factors like cardiovascular disease, but are heavily underutilized. While there is evidence that statin therapy is safe and also effective in MAFLD/NASH patients, large randomized controlled trials are still lacking. Concerning T2DM therapy, new anti-hyperglycemic agents such as pioglitazone or GLP-1 agonists are promising, but specific side effects may be detrimental and have to be considered. Metformin remains the first-line antihyperglycemic therapy.

Once end-stage liver disease has developed, obese patients benefit from LT, but also have increased perioperative risk, especially due to infections. After LT, metabolic complications are common. However, to date, there is little high-quality data concerning management of post-LT dyslipidemia and T2DM. Randomized controlled trials are needed to ensure the best possible care for these patient groups.

REFERENCES

1 **Cotter TG**, Charlton M. Nonalcoholic Steatohepatitis After Liver Transplantation. *Liver Transpl* 2020; **26**: 141-159 [PMID: 31610081 DOI: 10.1002/lt.25657]

2 **Adam R**, Karam V, Cailliez V, O Grady JG, Mirza D, Cherqui D, Klempnauer J, Salizzoni M, Pratschke J, Jamieson N, Hidalgo E, Paul A, Andujar RL, Lerut J, Fisher L, Boudjema K, Fondevila C, Soubrane O, Bachellier P, Pinna AD, Berlakovich G, Bennet W, Pinzani M, Schemmer P, Zieniewicz K, Romero CJ, De Simone P, Ericzon BG, Schneeberger S, Wigmore SJ, Prous JF, Colledan M, Porte RJ, Yilmaz S, Azoulay D, Pirenne J, Line PD, Trunecka P, Navarro F, Lopez AV, De Carlis L, Pena SR, Kochs E, Duvoux C; all the other 126 contributing centers (www. eltr.org) and the European Liver and Intestine Transplant Association (ELITA). 2018 Annual Report of the European Liver Transplant Registry (ELTR) - 50-year evolution of liver transplantation. *Transpl Int* 2018; **31**: 1293-1317 [PMID: 30259574 DOI: 10.1111/tri.13358]

3 **Eslam M**, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-Gomez M, Zelber-Sagi S, Wai-Sun Wong V, Dufour JF, Schattenberg JM, Kawaguchi T, Arrese M, Valenti L, Shiha G, Tiribelli C, Yki-Järvinen H, Fan JG, Grønbæk H, Yilmaz Y, Cortez-Pinto H, Oliveira CP, Bedossa P, Adams LA, Zheng MH, Fouad Y, Chan WK, Mendez-Sanchez N, Ahn SH, Castera L, Bugianesi E, Ratziu V, George J. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *J Hepatol* 2020; **73**: 202-209 [PMID: 32278004 DOI: 10.1016/j.jhep.2020.03.039]

4 **Eslam M**, Sanyal AJ, George J; International Consensus Panel. MAFLD: A Consensus-Driven Proposed Nomenclature for Metabolic Associated Fatty Liver Disease. *Gastroenterology* 2020; **158**: 1999-2014. e1 [PMID: 32044314 DOI: 10.1053/j.gastro.2019.11.312]

5 **Pang Q**, Zhang JY, Song SD, Qu K, Xu XS, Liu SS, Liu C. Central obesity and nonalcoholic fatty liver disease risk after adjusting for body mass index. *World J Gastroenterol* 2015; **21**: 1650-1662 [PMID: 25663786 DOI: 10.3748/wjg.v21.i5.1650]

6 **Younossi Z**, Tacke F, Arrese M, Chander Sharma B, Mostafa I, Bugianesi E, Wai-Sun Wong V, Yilmaz Y, George J, Fan J, Vos MB. Global Perspectives on Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. *Hepatology* 2019; **69**: 2672-2682 [PMID: 30179269 DOI: 10.1002/hep.30251]

7 **Mathurin P**, Lucey MR. Liver transplantation in patients with alcohol-related liver disease: current status and future directions. *Lancet Gastroenterol Hepatol* 2020; **5**: 507-514 [PMID: 32277903 DOI: 10.1016/S2468-1253(19)30451-0]

8 **Pais R**, Barritt AS 4th, Calmus Y, Scatton O, Runge T, Lebray P, Poynard T, Ratziu V, Conti F.

NAFLD and liver transplantation: Current burden and expected challenges. *J Hepatol* 2016; **65**: 1245-1257 [PMID: 27486010 DOI: 10.1016/j.jhep.2016.07.033]

9 **Burra P**, Becchetti C, Germani G. NAFLD and liver transplantation: Disease burden, current management and future challenges. *JHEP Rep* 2020; **2**: 100192 [PMID: 33163950 DOI: 10.1016/j.jhepr.2020.100192]

10 **Wong RJ**, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, Ahmed A. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. *Gastroenterology* 2015; **148**: 547-555 [PMID: 25461851 DOI: 10.1053/j.gastro.2014.11.039]

11 **Younossi Z**, Stepanova M, Ong JP, Jacobson IM, Bugianesi E, Duseja A, Eguchi Y, Wong VW, Negro F, Yilmaz Y, Romero-Gomez M, George J, Ahmed A, Wong R, Younossi I, Ziayee M, Afendy A; Global Nonalcoholic Steatohepatitis Council. Nonalcoholic Steatohepatitis Is the Fastest Growing Cause of Hepatocellular Carcinoma in Liver Transplant Candidates. *Clin Gastroenterol Hepatol* 2019; **17**: 748-755. e3 [PMID: 29908364 DOI: 10.1016/j.cgh.2018.05.057]

12 **Rinella ME**. Nonalcoholic fatty liver disease: a systematic review. *JAMA* 2015; **313**: 2263-2273 [PMID: 26057287 DOI: 10.1001/jama.2015.5370]

13 **Marchesini G**, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, Natale S, Forlani G, Melchionda N. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 2001; **50**: 1844-1850 [PMID: 11473047 DOI: 10.2337/diabetes.50.8.1844]

14 **Tilg H**, Effenberger M. From NAFLD to MAFLD: when pathophysiology succeeds. *Nat Rev Gastroenterol Hepatol* 2020; **17**: 387-388 [PMID: 32461575 DOI: 10.1038/s41575-020-0316-6]

15 **European Association for the Study of the Liver (EASL)**. European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* 2016; **64**: 1388-1402 [PMID: 27062661 DOI: 10.1016/j.jhep.2015.11.004]

16 **Papatheodoridi M**, Cholongitas E. Diagnosis of Non-alcoholic Fatty Liver Disease (NAFLD): Current Concepts. *Curr Pharm Des* 2018; **24**: 4574-4586 [PMID: 30652642 DOI: 10.2174/1381612825666190117102111]

17 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438 [PMID: 7382552]

18 **Fleming KA**, Morton JA, Barbatis C, Burns J, Canning S, McGee JO. Mallory bodies in alcoholic and non-alcoholic liver disease contain a common antigenic determinant. *Gut* 1981; **22**: 341-344 [PMID: 6166516 DOI: 10.1136/gut.22.5.341]

19 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461 DOI: 10.1002/hep.20701]

20 **Paternostro R**, Reiberger T, Bucsics T. Elastography-based screening for esophageal varices in patients with advanced chronic liver disease. *World J Gastroenterol* 2019; **25**: 308-329 [PMID: 30686900 DOI: 10.3748/wjg.v25.i3.308]

21 **Vilar-Gomez E**, Chalasani N. Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. *J Hepatol* 2018; **68**: 305-315 [PMID: 29154965 DOI: 10.1016/j.jhep.2017.11.013]

22 **Kechagias S**, Ernersson A, Dahlqvist O, Lundberg P, Lindström T, Nystrom FH; Fast Food Study Group. Fast-food-based hyper-alimentation can induce rapid and profound elevation of serum alanine aminotransferase in healthy subjects. *Gut* 2008; **57**: 649-654 [PMID: 18276725 DOI: 10.1136/gut.2007.131797]

23 **Bergheim I**, Weber S, Vos M, Krämer S, Volynets V, Kaserouni S, McClain CJ, Bischoff SC. Antibiotics protect against fructose-induced hepatic lipid accumulation in mice: role of endotoxin. *J Hepatol* 2008; **48**: 983-992 [PMID: 18395289 DOI: 10.1016/j.jhep.2008.01.035]

24 **Moore JB**, Gunn PJ, Fielding BA. The role of dietary sugars and de novo lipogenesis in non-alcoholic fatty liver disease. *Nutrients* 2014; **6**: 5679-5703 [PMID: 25514388 DOI: 10.3390/nu6125679]

25 **Bugianesi E**, Moscatiello S, Ciaravella MF, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. *Curr Pharm Des* 2010; **16**: 1941-1951 [PMID: 20370677 DOI: 10.2174/138161210791208875]

26 **Guilherme A**, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* 2008; **9**: 367-377 [PMID: 18401346 DOI: 10.1038/nrm2391]

27 **Kucukoglu O**, Sowa JP, Mazzolini GD, Syn WK, Canbay A. Hepatokines and adipokines in NASH-related hepatocellular carcinoma. *J Hepatol* 2021; **74**: 442-457 [PMID: 33161047 DOI: 10.1016/j.jhep.2020.10.030]

28 **Kolodziejczyk AA**, Zheng D, Shibolet O, Elinav E. The role of the microbiome in NAFLD and NASH. *EMBO Mol Med* 2019; **11** [PMID: 30591521 DOI: 10.15252/emmm.201809302]

29 **Safari Z**, Gérard P. The links between the gut microbiome and non-alcoholic fatty liver disease (NAFLD). *Cell Mol Life Sci* 2019; **76**: 1541-1558 [PMID: 30683985 DOI: 10.1007/s00018-019-03011-w]

30 **Eslam M**, Valenti L, Romeo S. Genetics and epigenetics of NAFLD and NASH: Clinical impact. *J Hepatol* 2018; **68**: 268-279 [PMID: 29122391 DOI: 10.1016/j.jhep.2017.09.003]

31 **Carlsson B**, Lindén D, Brolén G, Liljeblad M, Bjursell M, Romeo S, Loomba R. Review article: the emerging role of genetics in precision medicine for patients with non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2020; **51**: 1305-1320 [PMID: 32383295 DOI: 10.1111/apt.15738]

32 **Targher G**, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; **363**: 1341-1350 [PMID: 20879883 DOI: 10.1056/NEJMra0912063]

33 **Targher G**, Byrne CD, Lonardo A, Zoppini G, Barbui C. Non-alcoholic fatty liver disease and risk of incident cardiovascular disease: A meta-analysis. *J Hepatol* 2016; **65**: 589-600 [PMID: 27212244 DOI: 10.1016/j.jhep.2016.05.013]

34 **Yamada T**, Fukatsu M, Suzuki S, Wada T, Yoshida T, Joh T. Fatty liver predicts impaired fasting glucose and type 2 diabetes mellitus in Japanese undergoing a health checkup. *J Gastroenterol Hepatol* 2010; **25**: 352-356 [PMID: 19817963 DOI: 10.1111/j.1440-1746.2009.05998.x]

35 **Fan JG**, Li F, Cai XB, Peng YD, Ao QH, Gao Y. Effects of nonalcoholic fatty liver disease on the development of metabolic disorders. *J Gastroenterol Hepatol* 2007; **22**: 1086-1091 [PMID: 17608855 DOI: 10.1111/j.1440-1746.2006.04781.x]

36 **Adams LA**, Waters OR, Knuiman MW, Elliott RR, Olynyk JK. NAFLD as a risk factor for the development of diabetes and the metabolic syndrome: an eleven-year follow-up study. *Am J Gastroenterol* 2009; **104**: 861-867 [PMID: 19293782 DOI: 10.1038/ajg.2009.67]

37 **Mantovani A**, Petracca G, Beatrice G, Csermely A, Lonardo A, Schattenberg JM, Tilg H, Byrne CD, Targher G. Non-alcoholic fatty liver disease and risk of incident chronic kidney disease: an updated meta-analysis. *Gut* 2020 [PMID: 33303564 DOI: 10.1136/gutjnl-2020-323082]

38 **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]

39 **Dulai PS**, Singh S, Patel J, Soni M, Prokop LJ, Younossi Z, Sebastiani G, Ekstedt M, Hagstrom H, Nasr P, Stal P, Wong VW, Kechagias S, Hultcrantz R, Loomba R. Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: Systematic review and meta-analysis. *Hepatology* 2017; **65**: 1557-1565 [PMID: 28130788 DOI: 10.1002/hep.29085]

40 **Hagström H**, Nasr P, Ekstedt M, Hammar U, Stål P, Hultcrantz R, Kechagias S. Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in biopsy-proven NAFLD. *J Hepatol* 2017; **67**: 1265-1273 [PMID: 28803953 DOI: 10.1016/j.jhep.2017.07.027]

41 **Mann JP**, Carter P, Armstrong MJ, Abdelaziz HK, Uppal H, Patel B, Chandran S, More R, Newsome PN, Potluri R. Hospital admission with non-alcoholic fatty liver disease is associated with increased all-cause mortality independent of cardiovascular risk factors. *PLoS One* 2020; **15**: e0241357 [PMID: 33108366 DOI: 10.1371/journal.pone.0241357]

42 **Stavropoulos K**, Imprialos K, Pittaras A, Faselis C, Narayan P, Kokkinos P. Lifestyle Modifications in Non-Alcoholic Fatty Liver Disease and Non- Alcoholic Steatohepatitis. *Curr Vasc Pharmacol* 2018; **16**: 239-245 [PMID: 28637408 DOI: 10.2174/1570161115666170621080835]

43 **Drew L**. Drug development: Sprint finish. *Nature* 2017; **551**: S86-S89 [PMID: 32080575 DOI: 10.1038/d41586-017-06926-1]

44 **Eslam M**, Alvani R, Shiha G. Obeticholic acid: towards first approval for NASH. *Lancet* 2019; **394**: 2131-2133 [PMID: 31813639 DOI: 10.1016/S0140-6736(19)32963-0]

45 **Jalili R**, Somi MH, Hosseinifard H, Salehnia F, Ghojazadeh M, Makhdami N, Shirmohammadi M. The Evaluation of Effective Drugs for the Treatment of Non-Alcoholic Fatty Liver Disease: A Systematic Review and Network Meta-Analysis. *Adv Pharm Bull* 2020; **10**: 542-555 [PMID: 33072533 DOI: 10.34172/apb.2020.065]

46 **Younossi ZM**, Ratziu V, Loomba R, Rinella M, Anstee QM, Goodman Z, Bedossa P, Geier A, Beckebaum S, Newsome PN, Sheridan D, Sheikh MY, Trotter J, Knapple W, Lawitz E, Abdelmalek MF, Kowdley KV, Montano-Loza AJ, Boursier J, Mathurin P, Bugianesi E, Mazzella G, Olveira A, Cortez-Pinto H, Graupera I, Orr D, Gluud LL, Dufour JF, Shapiro D, Campagna J, Zaru L, MacConell L, Shringarpure R, Harrison S, Sanyal AJ; REGENERATE Study Investigators. Obeticholic acid for the treatment of non-alcoholic steatohepatitis: interim analysis from a multicentre, randomised, placebo-controlled phase 3 trial. *Lancet* 2019; **394**: 2184-2196 [PMID: 31813633 DOI: 10.1016/S0140-6736(19)33041-7]

47 **Moctezuma-Velazquez C**, Márquez-Guillén E, Torre A. Obesity in the Liver Transplant Setting. *Nutrients* 2019; **11** [PMID: 31652761 DOI: 10.3390/nu11112552]

48 **Berzigotti A**, Garcia-Tsao G, Bosch J, Grace ND, Burroughs AK, Morillas R, Escorsell A, Garcia-Pagan JC, Patch D, Matloff DS, Groszmann RJ; Portal Hypertension Collaborative Group. Obesity is an independent risk factor for clinical decompensation in patients with cirrhosis. *Hepatology* 2011; **54**: 555-561 [PMID: 21567436 DOI: 10.1002/hep.24418]

49 **Schlansky B**, Naugler WE, Orloff SL, Enestvedt CK. Higher Mortality and Survival Benefit in Obese Patients Awaiting Liver Transplantation. *Transplantation* 2016; **100**: 2648-2655 [PMID: 27575690 DOI: 10.1097/TP.0000000000001461]

50 **Uusitupa M**. Lifestyle changes and cardiovascular risk reduction in diabetes. *Lancet Diabetes Endocrinol* 2016; **4**: 877-878 [PMID: 27595919 DOI: 10.1016/S2213-8587(16)30185-1]

51 **Peng L**, Wang J, Li F. Weight reduction for non-alcoholic fatty liver disease. *Cochrane Database Syst Rev* 2011; **6**: CD003619 [PMID: 21678341 DOI: 10.1002/14651858.CD003619.pub3]

52 **Lazo M**, Solga SF, Horska A, Bonekamp S, Diehl AM, Brancati FL, Wagenknecht LE, Pi-Sunyer FX, Kahn SE, Clark JM; Fatty Liver Subgroup of the Look AHEAD Research Group. Effect of a

12-month intensive lifestyle intervention on hepatic steatosis in adults with type 2 diabetes. *Diabetes Care* 2010; **33**: 2156-2163 [PMID: 20664019 DOI: 10.2337/dc10-0856]

53 **Suzuki A**, Lindor K, St Saver J, Lymp J, Mendes F, Muto A, Okada T, Angulo P. Effect of changes on body weight and lifestyle in nonalcoholic fatty liver disease. *J Hepatol* 2005; **43**: 1060-1066 [PMID: 16140415 DOI: 10.1016/j.jhep.2005.06.008]

54 **Jensen MD**, Ryan DH, Apovian CM, Ard JD, Comuzzie AG, Donato KA, Hu FB, Hubbard VS, Jakicic JM, Kushner RF, Loria CM, Millen BE, Nonas CA, Pi-Sunyer FX, Stevens J, Stevens VJ, Wadden TA, Wolfe BM, Yanovski SZ; American College of Cardiology/American Heart Association Task Force on Practice Guidelines; Obesity Society. 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society. *J Am Coll Cardiol* 2014; **63**: 2985-3023 [PMID: 24239920 DOI: 10.1016/j.jacc.2013.11.004]

55 **Wong VW**, Wong GL, Chan RS, Shu SS, Cheung BH, Li LS, Chim AM, Chan CK, Leung JK, Chu WC, Woo J, Chan HL. Beneficial effects of lifestyle intervention in non-obese patients with non-alcoholic fatty liver disease. *J Hepatol* 2018; **69**: 1349-1356 [PMID: 30142427 DOI: 10.1016/j.jhep.2018.08.011]

56 **Unger LW**, Forstner B, Muckenhuber M, Scheuba K, Eigenbauer E, Scheiner B, Pfisterer N, Paternostro R, Trauner M, Mandorfer M, Reiberger T. Hepatic Steatosis in Lean Patients: Risk Factors and Impact on Mortality. *Dig Dis Sci* 2020; **65**: 2712-2718 [PMID: 31875288 DOI: 10.1007/s10620-019-06000-y]

57 **Berzigotti A**, Albillos A, Villanueva C, Genescá J, Ardevol A, Augustín S, Calleja JL, Bañares R, García-Pagán JC, Mesonero F, Bosch J; Ciberehd SportDiet Collaborative Group. Effects of an intensive lifestyle intervention program on portal hypertension in patients with cirrhosis and obesity: The SportDiet study. *Hepatology* 2017; **65**: 1293-1305 [PMID: 27997989 DOI: 10.1002/hep.28992]

58 **Romero-Gómez M**, Zelber-Sagi S, Trenell M. Treatment of NAFLD with diet, physical activity and exercise. *J Hepatol* 2017; **67**: 829-846 [PMID: 28545937 DOI: 10.1016/j.jhep.2017.05.016]

59 **Bandi JC**, García-Pagán JC, Escorsell A, François E, Moitinho E, Rodés J, Bosch J. Effects of propranolol on the hepatic hemodynamic response to physical exercise in patients with cirrhosis. *Hepatology* 1998; **28**: 677-682 [PMID: 9731558 DOI: 10.1002/hep.510280312]

60 **Albillos A**, Zamora J, Martínez J, Arroyo D, Ahmad I, De-la-Peña J, Garcia-Pagán JC, Lo GH, Sarin S, Sharma B, Abraldes JG, Bosch J, Garcia-Tsao G; Baveno Cooperation. Stratifying risk in the prevention of recurrent variceal hemorrhage: Results of an individual patient meta-analysis. *Hepatology* 2017; **66**: 1219-1231 [PMID: 28543862 DOI: 10.1002/hep.29267]

61 **Wilding JPH**, Batterham RL, Calanna S, Davies M, Van Gaal LF, Lingvay I, McGowan BM, Rosenstock J, Tran MTD, Wadden TA, Wharton S, Yokote K, Zeuthen N, Kushner RF; STEP 1 Study Group. Once-Weekly Semaglutide in Adults with Overweight or Obesity. *N Engl J Med* 2021; **384**: 989 [PMID: 33567185 DOI: 10.1056/NEJMoa2032183]

62 **Benjamin EJ**, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, Isasi CR, Jiménez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME, Nasir K, Neumar RW, Palaniappan L, Pandey DK, Thiagarajan RR, Reeves MJ, Ritchey M, Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation* 2017; **135**: e146-e603 [PMID: 28122885 DOI: 10.1161/CIR.0000000000000485]

63 **Unger LW**, Forstner B, Schneglberger S, Muckenhuber M, Eigenbauer E, Scheiner B, Mandorfer M, Trauner M, Reiberger T. Patterns and prevalence of dyslipidemia in patients with different etiologies of chronic liver disease. *Wien Klin Wochenschr* 2019; **131**: 395-403 [PMID: 31493100 DOI: 10.1007/s00508-019-01544-5]

64 **Cholesterol Treatment Trialists' (CTT) Collaboration**, Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keech A, Simes J, Collins R. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet* 2010; **376**: 1670-1681 [PMID: 21067804 DOI: 10.1016/S0140-6736(10)61350-5]

65 **Abd TT**, Jacobson TA. Statin-induced myopathy: a review and update. *Expert Opin Drug Saf* 2011; **10**: 373-387 [PMID: 21342078 DOI: 10.1517/14740338.2011.540568]

66 **Blais P**, Lin M, Kramer JR, El-Serag HB, Kanwal F. Statins Are Underutilized in Patients with Nonalcoholic Fatty Liver Disease and Dyslipidemia. *Dig Dis Sci* 2016; **61**: 1714-1720 [PMID: 26707137 DOI: 10.1007/s10620-015-4000-6]

67 **Wong JC**, Chan HL, Tse YK, Yip TC, Wong VW, Wong GL. Statins reduce the risk of liver decompensation and death in chronic viral hepatitis: a propensity score weighted landmark analysis. *Aliment Pharmacol Ther* 2017; **46**: 1001-1010 [PMID: 28940673 DOI: 10.1111/apt.14341]

68 **Kim RG**, Loomba R, Prokop LJ, Singh S. Statin Use and Risk of Cirrhosis and Related Complications in Patients With Chronic Liver Diseases: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2017; **15**: 1521-1530. e8 [PMID: 28479502 DOI: 10.1016/j.cgh.2017.04.039]

69 **Abraldes JG**, Albillos A, Bañares R, Turnes J, González R, García-Pagán JC, Bosch J. Simvastatin lowers portal pressure in patients with cirrhosis and portal hypertension: a randomized controlled

trial. *Gastroenterology* 2009; **136**: 1651-1658 [PMID: 19208350 DOI: 10.1053/j.gastro.2009.01.043]

70 **La Mura V**, Pasarín M, Meireles CZ, Miquel R, Rodríguez-Vilarrupla A, Hide D, Gracia-Sancho J, García-Pagán JC, Bosch J, Abraldes JG. Effects of simvastatin administration on rodents with lipopolysaccharide-induced liver microvascular dysfunction. *Hepatology* 2013; **57**: 1172-1181 [PMID: 23184571 DOI: 10.1002/hep.26127]

71 **Marrone G**, Russo L, Rosado E, Hide D, García-Cardeña G, García-Pagán JC, Bosch J, Gracia-Sancho J. The transcription factor KLF2 mediates hepatic endothelial protection and paracrine endothelial-stellate cell deactivation induced by statins. *J Hepatol* 2013; **58**: 98-103 [PMID: 22989565 DOI: 10.1016/j.jhep.2012.08.026]

72 **Nelson A**, Torres DM, Morgan AE, Fincke C, Harrison SA. A pilot study using simvastatin in the treatment of nonalcoholic steatohepatitis: A randomized placebo-controlled trial. *J Clin Gastroenterol* 2009; **43**: 990-994 [PMID: 19448566 DOI: 10.1097/MCG.0b013e31819c392e]

73 **Yang YH**, Chen WC, Tsan YT, Chen MJ, Shih WT, Tsai YH, Chen PC. Statin use and the risk of cirrhosis development in patients with hepatitis C virus infection. *J Hepatol* 2015; **63**: 1111-1117 [PMID: 26196278 DOI: 10.1016/j.jhep.2015.07.006]

74 **Mohanty A**, Tate JP, Garcia-Tsao G. Statins Are Associated With a Decreased Risk of Decompensation and Death in Veterans With Hepatitis C-Related Compensated Cirrhosis. *Gastroenterology* 2016; **150**: 430-40. e1 [PMID: 26484707 DOI: 10.1053/j.gastro.2015.10.007]

75 **Tsan YT**, Lee CH, Ho WC, Lin MH, Wang JD, Chen PC. Statins and the risk of hepatocellular carcinoma in patients with hepatitis C virus infection. *J Clin Oncol* 2013; **31**: 1514-1521 [PMID: 23509319 DOI: 10.1200/JCO.2012.44.6831]

76 **Simon TG**, Bonilla H, Yan P, Chung RT, Butt AA. Atorvastatin and fluvastatin are associated with dose-dependent reductions in cirrhosis and hepatocellular carcinoma, among patients with hepatitis C virus: Results from ERCHIVES. *Hepatology* 2016; **64**: 47-57 [PMID: 26891205 DOI: 10.1002/hep.28506]

77 **Huang YW**, Lee CL, Yang SS, Fu SC, Chen YY, Wang TC, Hu JT, Chen DS. Statins Reduce the Risk of Cirrhosis and Its Decompensation in Chronic Hepatitis B Patients: A Nationwide Cohort Study. *Am J Gastroenterol* 2016; **111**: 976-985 [PMID: 27166128 DOI: 10.1038/ajg.2016.179]

78 **Stojakovic T**, Claudel T, Putz-Bankuti C, Fauler G, Scharnagl H, Wagner M, Sourij H, Stauber RE, Winkler K, März W, Wascher TC, Trauner M. Low-dose atorvastatin improves dyslipidemia and vascular function in patients with primary biliary cirrhosis after one year of treatment. *Atherosclerosis* 2010; **209**: 178-183 [PMID: 19782361 DOI: 10.1016/j.atherosclerosis.2009.08.052]

79 **Cash WJ**, O'Neill S, O'Donnell ME, McCance DR, Young IS, McEneny J, McDougall NI, Callender ME. Randomized controlled trial assessing the effect of simvastatin in primary biliary cirrhosis. *Liver Int* 2013; **33**: 1166-1174 [PMID: 23672463 DOI: 10.1111/liv.12191]

80 **Marchesini G**, Brizi M, Morselli-Labate AM, Bianchi G, Bugianesi E, McCullough AJ, Forlani G, Melchionda N. Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 1999; **107**: 450-455 [PMID: 10569299 DOI: 10.1016/s0002-9343(99)00271-5]

81 **Williamson RM**, Price JF, Glancy S, Perry E, Nee LD, Hayes PC, Frier BM, Van Look LA, Johnston GI, Reynolds RM, Strachan MW; Edinburgh Type 2 Diabetes Study Investigators. Prevalence of and risk factors for hepatic steatosis and nonalcoholic Fatty liver disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabetes Care* 2011; **34**: 1139-1144 [PMID: 21478462 DOI: 10.2337/dc10-2229]

82 **Portillo-Sanchez P**, Bril F, Maximos M, Lomonaco R, Biernacki D, Orsak B, Subbarayan S, Webb A, Hecht J, Cusi K. High Prevalence of Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes Mellitus and Normal Plasma Aminotransferase Levels. *J Clin Endocrinol Metab* 2015; **100**: 2231-2238 [PMID: 25885947 DOI: 10.1210/jc.2015-1966]

83 **Fracanzani AL**, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, Bertelli C, Fatta E, Bignamini D, Marchesini G, Fargion S. Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. *Hepatology* 2008; **48**: 792-798 [PMID: 18752331 DOI: 10.1002/hep.22429]

84 **Abrams GA**, Kunde SS, Lazenby AJ, Clements RH. Portal fibrosis and hepatic steatosis in morbidly obese subjects: A spectrum of nonalcoholic fatty liver disease. *Hepatology* 2004; **40**: 475-483 [PMID: 15368453 DOI: 10.1002/hep.20323]

85 **Goh GB**, Pagadala MR, Dasarathy J, Unalp-Arida A, Sargent R, Hawkins C, Sourianarayanane A, Khiyami A, Yerian L, Pai RK, Dasarathy S, McCullough AJ. Clinical spectrum of non-alcoholic fatty liver disease in diabetic and non-diabetic patients. *BBA Clin* 2015; **3**: 141-145 [PMID: 26675585 DOI: 10.1016/j.bbacli.2014.09.001]

86 **Bazick J**, Donithan M, Neuschwander-Tetri BA, Kleiner D, Brunt EM, Wilson L, Doo E, Lavine J, Tonascia J, Loomba R. Clinical Model for NASH and Advanced Fibrosis in Adult Patients With Diabetes and NAFLD: Guidelines for Referral in NAFLD. *Diabetes Care* 2015; **38**: 1347-1355 [PMID: 25887357 DOI: 10.2337/dc14-1239]

87 **Hossain N**, Afendy A, Stepanova M, Nader F, Srishord M, Rafiq N, Goodman Z, Younossi Z. Independent predictors of fibrosis in patients with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2009; **7**: 1224-1229, 1229.e1-1229. e2 [PMID: 19559819 DOI: 10.1016/j.cgh.2009.06.007]

88 **Doycheva I**, Cui J, Nguyen P, Costa EA, Hooker J, Hofflich H, Bettencourt R, Brouha S, Sirlin CB, Loomba R. Non-invasive screening of diabetics in primary care for NAFLD and advanced fibrosis

by MRI and MRE. *Aliment Pharmacol Ther* 2016; **43**: 83-95 [PMID: 26369383 DOI: 10.1111/apt.13405]

89 **Williams CD**, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; **140**: 124-131 [PMID: 20858492 DOI: 10.1053/j.gastro.2010.09.038]

90 **Loomba R**, Abraham M, Unalp A, Wilson L, Lavine J, Doo E, Bass NM; Nonalcoholic Steatohepatitis Clinical Research Network. Association between diabetes, family history of diabetes, and risk of nonalcoholic steatohepatitis and fibrosis. *Hepatology* 2012; **56**: 943-951 [PMID: 22505194 DOI: 10.1002/hep.25772]

91 **Kwok R**, Choi KC, Wong GL, Zhang Y, Chan HL, Luk AO, Shu SS, Chan AW, Yeung MW, Chan JC, Kong AP, Wong VW. Screening diabetic patients for non-alcoholic fatty liver disease with controlled attenuation parameter and liver stiffness measurements: a prospective cohort study. *Gut* 2016; **65**: 1359-1368 [PMID: 25873639 DOI: 10.1136/gutjnl-2015-309265]

92 **Koehler EM**, Plompen EP, Schouten JN, Hansen BE, Darwish Murad S, Taimr P, Leebeek FW, Hofman A, Stricker BH, Castera L, Janssen HL. Presence of diabetes mellitus and steatosis is associated with liver stiffness in a general population: The Rotterdam study. *Hepatology* 2016; **63**: 138-147 [PMID: 26171685 DOI: 10.1002/hep.27981]

93 **Tilg H**, Moschen AR, Roden M. NAFLD and diabetes mellitus. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 32-42 [PMID: 27729660 DOI: 10.1038/nrgastro.2016.147]

94 **Angulo P**, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P, Mills PR, Keach JC, Lafferty HD, Stahler A, Haflidadottir S, Bendtsen F. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2015; **149**: 389-97. e10 [PMID: 25935633 DOI: 10.1053/j.gastro.2015.04.043]

95 **Hazlehurst JM**, Woods C, Marjot T, Cobbold JF, Tomlinson JW. Non-alcoholic fatty liver disease and diabetes. *Metabolism* 2016; **65**: 1096-1108 [PMID: 26856933 DOI: 10.1016/j.metabol.2016.01.001]

96 **de Marco R**, Locatelli F, Zoppini G, Verlato G, Bonora E, Muggeo M. Cause-specific mortality in type 2 diabetes. The Verona Diabetes Study. *Diabetes Care* 1999; **22**: 756-761 [PMID: 10332677 DOI: 10.2337/diacare.22.5.756]

97 **Younossi ZM**, Gramlich T, Matteoni CA, Boparai N, McCullough AJ. Nonalcoholic fatty liver disease in patients with type 2 diabetes. *Clin Gastroenterol Hepatol* 2004; **2**: 262-265 [PMID: 15017611 DOI: 10.1016/s1542-3565(04)00014-x]

98 **Phielix E**, Szendroedi J, Roden M. The role of metformin and thiazolidinediones in the regulation of hepatic glucose metabolism and its clinical impact. *Trends Pharmacol Sci* 2011; **32**: 607-616 [PMID: 21824668 DOI: 10.1016/j.tips.2011.06.006]

99 **Belfort R**, Harrison SA, Brown K, Darland C, Finch J, Hardies J, Balas B, Gastaldelli A, Tio F, Pulcini J, Berria R, Ma JZ, Dwivedi S, Havranek R, Fincke C, DeFronzo R, Bannayan GA, Schenker S, Cusi K. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med* 2006; **355**: 2297-2307 [PMID: 17135584 DOI: 10.1056/NEJMoa060326]

100 **Aithal GP**, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, Austin AS, Freeman JG, Morgan L, Webber J. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. *Gastroenterology* 2008; **135**: 1176-1184 [PMID: 18718471 DOI: 10.1053/j.gastro.2008.06.047]

101 **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR; NASH CRN. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]

102 **Cusi K**, Orsak B, Bril F, Lomonaco R, Hecht J, Ortiz-Lopez C, Tio F, Hardies J, Darland C, Musi N, Webb A, Portillo-Sanchez P. Long-Term Pioglitazone Treatment for Patients With Nonalcoholic Steatohepatitis and Prediabetes or Type 2 Diabetes Mellitus: A Randomized Trial. *Ann Intern Med* 2016; **165**: 305-315 [PMID: 27322798 DOI: 10.7326/M15-1774]

103 **Fruci B**, Giuliano S, Mazza A, Malaguarnera R, Belfiore A. Nonalcoholic Fatty liver: a possible new target for type 2 diabetes prevention and treatment. *Int J Mol Sci* 2013; **14**: 22933-22966 [PMID: 24264040 DOI: 10.3390/ijms141122933]

104 **Armstrong MJ**, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, Guo K; LEAN trial team, Abouda G, Aldersley MA, Stocken D, Gough SC, Tomlinson JW, Brown RM, Hübscher SG, Newsome PN. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet* 2016; **387**: 679-690 [PMID: 26608256 DOI: 10.1016/S0140-6736(15)00803-X]

105 **Bugianesi E**, Gentilcore E, Manini R, Natale S, Vanni E, Villanova N, David E, Rizzetto M, Marchesini G. A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. *Am J Gastroenterol* 2005; **100**: 1082-1090 [PMID: 15842582 DOI: 10.1111/j.1572-0241.2005.41583.x]

106 **Haukeland JW**, Konopski Z, Eggesbø HB, von Volkmann HL, Raschpichler G, Bjøro K, Haaland T, Løberg EM, Birkeland K. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. *Scand J Gastroenterol* 2009; **44**: 853-860 [PMID: 19811343 DOI: 10.1080/00365520902845268]

107 **Shields WW**, Thompson KE, Grice GA, Harrison SA, Coyle WJ. The Effect of Metformin and Standard Therapy versus Standard Therapy alone in Nondiabetic Patients with Insulin Resistance and Nonalcoholic Steatohepatitis (NASH): A Pilot Trial. *Therap Adv Gastroenterol* 2009; **2**: 157-163 [PMID: 21180541 DOI: 10.1177/1756283X09105462]

108 **Lavine JE**, Schwimmer JB, Van Natta ML, Molleston JP, Murray KF, Rosenthal P, Abrams SH, Scheimann AO, Sanyal AJ, Chalasani N, Tonascia J, Ünalp A, Clark JM, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR; Nonalcoholic Steatohepatitis Clinical Research Network. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. *JAMA* 2011; **305**: 1659-1668 [PMID: 21521847 DOI: 10.1001/jama.2011.520]

109 **Torres DM**, Jones FJ, Shaw JC, Williams CD, Ward JA, Harrison SA. Rosiglitazone versus rosiglitazone and metformin versus rosiglitazone and losartan in the treatment of nonalcoholic steatohepatitis in humans: a 12-month randomized, prospective, open- label trial. *Hepatology* 2011; **54**: 1631-1639 [PMID: 21748770 DOI: 10.1002/hep.24558]

110 **Sjöström L**, Lindroos AK, Peltonen M, Torgerson J, Bouchard C, Carlsson B, Dahlgren S, Larsson B, Narbro K, Sjöström CD, Sullivan M, Wedel H; Swedish Obese Subjects Study Scientific Group. Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery. *N Engl J Med* 2004; **351**: 2683-2693 [PMID: 15616203 DOI: 10.1056/NEJMoa035622]

111 **Sjöström L**, Narbro K, Sjöström CD, Karason K, Larsson B, Wedel H, Lystig T, Sullivan M, Bouchard C, Carlsson B, Bengtsson C, Dahlgren S, Gummesson A, Jacobson P, Karlsson J, Lindroos AK, Lönroth H, Näslund I, Olbers T, Stenlöf K, Torgerson J, Agren G, Carlsson LM; Swedish Obese Subjects Study. Effects of bariatric surgery on mortality in Swedish obese subjects. *N Engl J Med* 2007; **357**: 741-752 [PMID: 17715408 DOI: 10.1056/NEJMoa066254]

112 **Carlsson LMS**, Sjöholm K, Jacobson P, Andersson-Assarsson JC, Svensson PA, Taube M, Carlsson B, Peltonen M. Life Expectancy after Bariatric Surgery in the Swedish Obese Subjects Study. *N Engl J Med* 2020; **383**: 1535-1543 [PMID: 33053284 DOI: 10.1056/NEJMoa2002449]

113 **Yoshino M**, Kayser BD, Yoshino J, Stein RI, Reeds D, Eagon JC, Eckhouse SR, Watrous JD, Jain M, Knight R, Schechtman K, Patterson BW, Klein S. Effects of Diet versus Gastric Bypass on Metabolic Function in Diabetes. *N Engl J Med* 2020; **383**: 721-732 [PMID: 32813948 DOI: 10.1056/NEJMoa2003697]

114 **Lassailly G**, Caiazzo R, Buob D, Pigeyre M, Verkindt H, Labreuche J, Raverdy V, Leteurtre E, Dharancy S, Louvet A, Romon M, Duhamel A, Pattou F, Mathurin P. Bariatric Surgery Reduces Features of Nonalcoholic Steatohepatitis in Morbidly Obese Patients. *Gastroenterology* 2015; **149**: 379-88; quiz e15 [PMID: 25917783 DOI: 10.1053/j.gastro.2015.04.014]

115 **Adams TD**, Davidson LE, Litwin SE, Kim J, Kolotkin RL, Nanjee MN, Gutierrez JM, Frogley SJ, Ibele AR, Brinton EA, Hopkins PN, McKinlay R, Simper SC, Hunt SC. Weight and Metabolic Outcomes 12 Years after Gastric Bypass. *N Engl J Med* 2017; **377**: 1143-1155 [PMID: 28930514 DOI: 10.1056/NEJMoa1700459]

116 **Panunzi S**, Maltese S, Verrastro O, Labbate L, De Gaetano A, Pompili M, Capristo E, Bornstein SR, Mingrone G. Pioglitazone and bariatric surgery are the most effective treatments for non-alcoholic steatohepatitis: A hierarchical network meta-analysis. *Diabetes Obes Metab* 2021; **23**: 980-990 [PMID: 33368954 DOI: 10.1111/dom.14304]

117 **Goossens N**, Hoshida Y, Song WM, Jung M, Morel P, Nakagawa S, Zhang B, Frossard JL, Spahr L, Friedman SL, Negro F, Rubbia-Brandt L, Giostra E. Nonalcoholic Steatohepatitis Is Associated With Increased Mortality in Obese Patients Undergoing Bariatric Surgery. *Clin Gastroenterol Hepatol* 2016; **14**: 1619-1628 [PMID: 26492845 DOI: 10.1016/j.cgh.2015.10.010]

118 **Eilenberg M**, Langer FB, Beer A, Trauner M, Prager G, Staufer K. Significant Liver-Related Morbidity After Bariatric Surgery and Its Reversal-a Case Series. *Obes Surg* 2018; **28**: 812-819 [PMID: 28965313 DOI: 10.1007/s11695-017-2925-x]

119 **Reiberger T**, Püspök A, Schoder M, Baumann-Durchschein F, Bucsics T, Datz C, Dolak W, Ferlitsch A, Finkenstedt A, Graziadei I, Hametner S, Karnel F, Krones E, Maieron A, Mandorfer M, Peck-Radosavljevic M, Rainer F, Schwabl P, Stadlbauer V, Stauber R, Tilg H, Trauner M, Zoller H, Schöfl R, Fickert P. Austrian consensus guidelines on the management and treatment of portal hypertension (Billroth III). *Wien Klin Wochenschr* 2017; **129**: 135-158 [PMID: 29063233 DOI: 10.1007/s00508-017-1262-3]

120 **Kwong A**, Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, Noreen SM, Foutz J, Miller E, Snyder JJ, Israni AK, Kasiske BL. OPTN/SRTR 2018 Annual Data Report: Liver. *Am J Transplant* 2020; **20** Suppl s1: 193-299 [PMID: 31898413 DOI: 10.1111/ajt.15674]

121 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Liver transplantation. *J Hepatol* 2016; **64**: 433-485 [PMID: 26597456 DOI: 10.1016/j.jhep.2015.10.006]

122 **Hakeem AR**, Cockbain AJ, Raza SS, Pollard SG, Toogood GJ, Attia MA, Ahmad N, Hidalgo EL, Prasad KR, Menon KV. Increased morbidity in overweight and obese liver transplant recipients: a single-center experience of 1325 patients from the United Kingdom. *Liver Transpl* 2013; **19**: 551-562 [PMID: 23408499 DOI: 10.1002/lt.23618]

123 **LaMattina JC**, Foley DP, Fernandez LA, Pirsch JD, Musat AI, D'Alessandro AM, Mezrich JD. Complications associated with liver transplantation in the obese recipient. *Clin Transplant* 2012; **26**: 910-918 [PMID: 22694047 DOI: 10.1111/j.1399-0012.2012.01669.x]

124 **Sawyer RG**, Pelletier SJ, Pruett TL. Increased early morbidity and mortality with acceptable long-term function in severely obese patients undergoing liver transplantation. *Clin Transplant* 1999; **13**:

126-130 [PMID: 10081649 DOI: 10.1034/j.1399-0012.1999.130111.x]
125 **Sundaram V**, Kaung A, Rajaram A, Lu SC, Tran TT, Nissen NN, Klein AS, Jalan R, Charlton MR, Jeon CY. Obesity is independently associated with infection in hospitalised patients with end-stage liver disease. *Aliment Pharmacol Ther* 2015; **42**: 1271-1280 [PMID: 26510540 DOI: 10.1111/apt.13426]
126 **Barone M**, Viggiani MT, Losurdo G, Principi M, Leandro G, Di Leo A. Systematic review with meta-analysis: post-operative complications and mortality risk in liver transplant candidates with obesity. *Aliment Pharmacol Ther* 2017; **46**: 236-245 [PMID: 28488418 DOI: 10.1111/apt.14139]
127 **Malik SM**, deVera ME, Fontes P, Shaikh O, Ahmad J. Outcome after liver transplantation for NASH cirrhosis. *Am J Transplant* 2009; **9**: 782-793 [PMID: 19344467 DOI: 10.1111/j.1600-6143.2009.02590.x]
128 **Unger LW**, Mandorfer M, Reiberger T. Portal Hypertension after Liver Transplantation—Causes and Management. *Curr Hepatol Rep* 2019; **18**: 59-66 [DOI: 10.1007/s11901-019-00450-8]
129 **Richards J**, Gunson B, Johnson J, Neuberger J. Weight gain and obesity after liver transplantation. *Transpl Int* 2005; **18**: 461-466 [PMID: 15773968 DOI: 10.1111/j.1432-2277.2004.00067.x]
130 **Choudhary NS**, Saigal S, Saraf N, Mohanka R, Rastogi A, Goja S, Menon PB, Mishra S, Mittal A, Soin AS. Sarcopenic obesity with metabolic syndrome: a newly recognized entity following living donor liver transplantation. *Clin Transplant* 2015; **29**: 211-215 [PMID: 25594826 DOI: 10.1111/ctr.12505]
131 **Hong HC**, Hwang SY, Choi HY, Yoo HJ, Seo JA, Kim SG, Kim NH, Baik SH, Choi DS, Choi KM. Relationship between sarcopenia and nonalcoholic fatty liver disease: the Korean Sarcopenic Obesity Study. *Hepatology* 2014; **59**: 1772-1778 [PMID: 23996808 DOI: 10.1002/hep.26716]
132 **Mathur S**, Janaudis-Ferreira T, Wickerson L, Singer LG, Patcai J, Rozenberg D, Blydt-Hansen T, Hartmann EL, Haykowsky M, Helm D, High K, Howes N, Kamath BM, Lands L, Marzolini S, Sonnenday C. Meeting report: consensus recommendations for a research agenda in exercise in solid organ transplantation. *Am J Transplant* 2014; **14**: 2235-2245 [PMID: 25135579 DOI: 10.1111/ajt.12874]
133 **Dunn MA**, Rogal SS, Duarte-Rojo A, Lai JC. Physical Function, Physical Activity, and Quality of Life After Liver Transplantation. *Liver Transpl* 2020; **26**: 702-708 [PMID: 32128971 DOI: 10.1002/lt.25742]
134 **Serper M**, Barankay I, Chadha S, Shults J, Jones LS, Olthoff KM, Reese PP. A randomized, controlled, behavioral intervention to promote walking after abdominal organ transplantation: results from the LIFT study. *Transpl Int* 2020; **33**: 632-643 [PMID: 31925833 DOI: 10.1111/tri.13570]
135 **Duarte-Rojo A**, Bloomer PM, Rogers RJ, Hassan MA, Dunn MA, Tevar AD, Vivis SL, Bataller R, Hughes CB, Ferrando AA, Jakicic JM, Kim WR. Introducing EL-FIT (Exercise and Liver FITness): A Smartphone App to Prehabilitate and Monitor Liver Transplant Candidates. *Liver Transpl* 2021; **27**: 502-512 [PMID: 33232547 DOI: 10.1002/lt.25950]
136 **Lopez-Lopez V**, Ruiz-Manzanera JJ, Eshmuminov D, Lehmann K, Schneider M, von der Groeben M, de Angulo DR, Gajownik U, Pons JA, Sanchez-Bueno F, Robles-Campos R, Ramirez-Romero P. Are We Ready for Bariatric Surgery in a Liver Transplant Program? *Obes Surg* 2020; **31**: 1214-1222 [PMID: 33225408 DOI: 10.1007/s11695-020-05118-7]
137 **Johnston SD**, Morris JK, Cramb R, Gunson BK, Neuberger J. Cardiovascular morbidity and mortality after orthotopic liver transplantation. *Transplantation* 2002; **73**: 901-906 [PMID: 11923689 DOI: 10.1097/00007890-200203270-00012]
138 **Unger LW**, Berlakovich GA, Trauner M, Reiberger T. Management of portal hypertension before and after liver transplantation. *Liver Transpl* 2018; **24**: 112-121 [PMID: 28752925 DOI: 10.1002/lt.24830]
139 **Patel SS**, Rodriguez VA, Siddiqui MB, Faridnia M, Lin FP, Chandrakumaran A, Laurenzano J, Clinton J, Kowlgi GN, Kirkman D, Sima AP, Liptrap E, Bhati C, Siddiqui MS. The Impact of Coronary Artery Disease and Statins on Survival After Liver Transplantation. *Liver Transpl* 2019; **25**: 1514-1523 [PMID: 31344758 DOI: 10.1002/lt.25613]
140 **Gracia-Sancho J**, García-Calderó H, Hide D, Marrone G, Guixé-Muntet S, Peralta C, García-Pagán JC, Abraldes JG, Bosch J. Simvastatin maintains function and viability of steatotic rat livers procured for transplantation. *J Hepatol* 2013; **58**: 1140-1146 [PMID: 23428876 DOI: 10.1016/j.jhep.2013.02.005]
141 **Russo L**, Gracia-Sancho J, García-Calderó H, Marrone G, García-Pagán JC, García-Cardeña G, Bosch J. Addition of simvastatin to cold storage solution prevents endothelial dysfunction in explanted rat livers. *Hepatology* 2012; **55**: 921-930 [PMID: 22031447 DOI: 10.1002/hep.24755]
142 **Bianchi G**, Marchesini G, Marzocchi R, Pinna AD, Zoli M. Metabolic syndrome in liver transplantation: relation to etiology and immunosuppression. *Liver Transpl* 2008; **14**: 1648-1654 [PMID: 18975273 DOI: 10.1002/lt.21588]
143 **Heisel O**, Heisel R, Balshaw R, Keown P. New onset diabetes mellitus in patients receiving calcineurin inhibitors: a systematic review and meta-analysis. *Am J Transplant* 2004; **4**: 583-595 [PMID: 15023151 DOI: 10.1046/j.1600-6143.2003.00372.x]
144 **Wallia A**, Parikh ND, O'Shea-Mahler E, Schmidt K, DeSantis AJ, Tian L, Levitsky J, Molitch ME. Glycemic control by a glucose management service and infection rates after liver transplantation. *Endocr Pract* 2011; **17**: 546-551 [PMID: 21324822 DOI: 10.4158/EP10343.OR]
145 **Park C**, Hsu C, Neelakanta G, Nourmand H, Braunfeld M, Wray C, Steadman RH, Hu KQ, Cheng RT, Xia VW. Severe intraoperative hyperglycemia is independently associated with surgical site

infection after liver transplantation. *Transplantation* 2009; **87**: 1031-1036 [PMID: 19352123 DOI: 10.1097/TP.0b013e31819cc3e6]

146 **Wallia A**, Parikh ND, Molitch ME, Mahler E, Tian L, Huang JJ, Levitsky J. Posttransplant hyperglycemia is associated with increased risk of liver allograft rejection. *Transplantation* 2010; **89**: 222-226 [PMID: 20098286 DOI: 10.1097/TP.0b013e3181c3c2ff]

147 **Grancini V**, Resi V, Palmieri E, Pugliese G, Orsi E. Management of diabetes mellitus in patients undergoing liver transplantation. *Pharmacol Res* 2019; **141**: 556-573 [PMID: 30690071 DOI: 10.1016/j.phrs.2019.01.042]

148 **Lo C**, Jun M, Badve SV, Pilmore H, White SL, Hawley C, Cass A, Perkovic V, Zoungas S. Glucose-lowering agents for treating pre-existing and new-onset diabetes in kidney transplant recipients. *Cochrane Database Syst Rev* 2017; **2**: CD009966 [PMID: 28238223 DOI: 10.1002/14651858.CD009966.pub2]

149 **Davies MJ**, D'Alessio DA, Fradkin J, Kernan WN, Mathieu C, Mingrone G, Rossing P, Tsapas A, Wexler DJ, Buse JB. Management of Hyperglycemia in Type 2 Diabetes, 2018. A Consensus Report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetes Care* 2018; **41**: 2669-2701 [PMID: 30291106 DOI: 10.2337/dci18-0033]

150 **Unger LW**, Forstner B, Schneglberger S, Muckenhuber M, Eigenbauer E, Bauer D, Scheiner B, Mandorfer M, Trauner M, Reiberger T. Guideline-conform statin use reduces overall mortality in patients with compensated liver disease. *Sci Rep* 2019; **9**: 11674 [PMID: 31406146 DOI: 10.1038/s41598-019-47943-6]

151 **Krasnoff JB**, Vintro AQ, Ascher NL, Bass NM, Paul SM, Dodd MJ, Painter PL. A randomized trial of exercise and dietary counseling after liver transplantation. *Am J Transplant* 2006; **6**: 1896-1905 [PMID: 16889545 DOI: 10.1111/j.1600-6143.2006.01391.x]

152 **Zamora-Valdes D**, Watt KD, Kellogg TA, Poterucha JJ, Di Cecco SR, Francisco-Ziller NM, Taner T, Rosen CB, Heimbach JK. Long-term outcomes of patients undergoing simultaneous liver transplantation and sleeve gastrectomy. *Hepatology* 2018; **68**: 485-495 [PMID: 29457842 DOI: 10.1002/hep.29848]

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MINIREVIEWS

Clinical characteristics and outcomes of patients with hepatic angiomyolipoma: A literature review

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Abstract

First reported in 1976, hepatic angiomyolipoma (HAML) is a rare mesenchymal liver tumor occurring mostly in middle-aged women. Diagnosis of the liver mass is often incidental on abdominal imaging due to the frequent absence of specific symptoms. Nearly 10% of HAMLs are associated with tuberous sclerosis complex. HAML contains variable proportions of blood vessels, smooth muscle cells and adipose tissue, which renders radiological diagnosis hazardous. Cells express positivity for HMB-45 and actin, thus these tumors are integrated into the group of perivascular epithelioid cell tumors. Typically, a HAML appears on magnetic resonance imaging (or computed tomography scan) as a hypervascular solid tumor with fatty areas and with washout, and can easily be misdiagnosed as other liver tumors, particularly hepatocellular carcinoma. The therapeutic strategy is not clearly defined, but surgical resection is indicated for symptomatic patients, for tumors showing an aggressive pattern (*i.e.*, changes in size on imaging or high proliferation activity and atypical epithelioid pattern on liver biopsy), for large (> 5 cm) biopsy-proven HAML, and if doubts remain on imaging or histology. Conservative management may be justified in other conditions, since most cases follow a benign clinical course. In summary, the correct diagnosis of HAML is challenging on imaging and relies mainly on pathological findings.

Key Words: Angiomyolipoma; Liver; Tuberous sclerosis complex; Imaging; Pathology;

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Core Tip: Hepatic angiomyolipoma (HAML) is a rare, but not exceptional, mesenchymal liver tumor. HAML contains variable proportions of blood vessels, smooth muscle cells and adipose tissue, which renders its radiological diagnosis challenging. In most cases, this tumor follows a benign clinical course but more aggressive behavior may complicate management, which remains poorly codified. This review presents the main demographic and histological characteristics of HAML, summarizes reported cases of HAML with spontaneous rupture and aggressive behavior, and finally proposes a pragmatic algorithm for the management of HAML based on the most recent knowledge.

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INTRODUCTION

Angiomyolipoma (AML) is a solid mesenchymal tumor, mainly described in the kidney, and belongs to the group of perivascular epithelioid cell tumors (PEComas)[1]. Hepatic localization of AML, described for the first time in 1976[2], is rare, since only around 600 cases were reported after an exhaustive search of the literature up to the year 2017[3]. Hepatic AML (HAML) poses a veritable diagnostic challenge in radiological terms, especially when fat content is low, because this type of tumor may appear as a hypervascular tumor associated with a washout phase that mimics other, more common hypervascular hepatic tumors, such as hepatocellular carcinoma[4-7]. The natural course of HAML is mostly benign, although several cases have been reported to exhibit aggressive behavior with metastasis or recurrence after surgery[8-24], or spontaneous rupture[8,25-33]. These rare but dramatic observations unavoidably compound the complexity of managing patients with HAML. Due to its difficult radiological diagnosis, its potentially aggressive behavior, and its poorly codified management, we aimed to analyze the recent literature regarding this rare, but not exceptional liver tumor, and to provide a pragmatic algorithm for its management according to the most recent knowledge.

PATIENT CHARACTERISTICS

HAML is a tumor usually occurring in a non-cirrhotic liver, and mainly affects middle-aged women. A retrospective analysis of the literature carried out up to 2016 identified 292 patients with one or more HAML, and most of them (nearly 74%) were women, with a median age ranging between 24 years and 53 years across studies[3]. HAML mainly locates in the right liver (60% of cases[7]), is unique in 84% of cases, and median size ranges from 2 cm to 12.7 cm[3,34]. This type of tumor is often detected incidentally during medical check-ups (42% to 72% of cases) since most subjects are asymptomatic[3,34,35]. Symptoms revealing HAML may include abdominal pain or discomfort, bloating, weight loss or, more rarely, discovery of an abdominal mass on palpation[35]. A few cases of spontaneous rupture of HAML have been reported (Table 1)[8,25-33]. Tumor size (≥ 4 cm) and pregnancy are two recognized conditions favoring rupture of renal AML[36,37], but the small number of reported cases of HAML rupture precludes identification of predictors of rupture in the liver. In the ten cases reported in Table 1, tumor size was usually large, between 5 cm and 12 cm (except for one case due to an inflammatory variant of HAML with a subcapsular location[32]), and there was no age preference (mean age: 48 years; range: 22 years to 77 years). All patients underwent emergent or delayed resection of the tumor,

Table 1 Cases of spontaneous rupture of hepatic angiomyolipoma

Ref.	Sex	Age, yr	Symptoms	Abdominal radiological findings	Treatment	Outcomes
Huber *et al*[25] 1996	F	22	Hemorrhagic shock with clinical symptoms of acute abdomen	CT scan: multiple tumors of the liver (the largest in segment III measured 8 cm) and both kidneys and a splenic lesion with a diameter of 4 cm	Surgical resection of segments II and III	Postoperative course was uneventful. Discharge from hospital 12 d later
Guidi *et al*[26] 1997	M	74	Sudden onset of upper-quadrant pain	CT scan: liver tumor of 10 cm × 8 cm in the segments I and V and another small mass of 4 cm × 3 cm in segment IV. Fluid was present in the upper abdominal compartments	Surgical resection of the hemorrhagic hepatic mass	Postoperative course was uneventful. Discharge from hospital 8 d later
Tsui *et al*[27] 1999	F	41	Acute rupture of a subcapsular tumor	9 cm	Surgical resection	Patient in healthy condition 4 yr after surgery
Zhou *et al*[28] 2008	ND	ND	Hemorrhagic shock	Ultrasonography showed a 5-cm "cavernous hemangioma" in the right hepatic lobe	Emergency laparotomy for hemostasis	No tumor recurrence or metastasis was found during follow-up of 2-3 yr
Ding *et al*[8] 2011	F	56	ND	A rupture of the tumor measuring 6 cm × 6 cm in segment VI was confirmed by emergent laparotomy	Liver suture followed by segmentectomy	No serious morbidity in the postoperative course
Occhionorelli *et al*[29] 2013	F	25	Sudden onset of abdominal upper-quadrant pain and hypotension, after two recent syncopal episodes	CT scan showed a hepatic tumor in the left lobe (8.6 cm × 7.2 cm) with suspected peritoneal blood leakage	Hemorrhage initially managed by manual compression, followed by deep and pro-coagulant tissue adhesives. After 48 hours, the patient underwent left-liver lobectomy	Postoperative course was uneventful. Discharge from hospital 9 d later
Aoki *et al*[30] 2014	F	70	Sudden onset of back pain on the right side	CT scan: hepatic tumor in segment VII measuring 7 cm in diameter accompanied by subcapsular hematoma with extravasation	Transcatheter arterial embolization. Right hepatic lobectomy was carried out 39 d later	Five days after surgery, she had thrombi in the left popliteal vein and the left pulmonary artery. Insertion of an IVC filter which was removed due to sepsis. She was discharged 24 d after surgery. There was no recurrence 42 mo following surgery
Tajima *et al*[31] 2014	M	38	Upper abdominal pain	CT scan showed a tumor measuring 10.5 cm × 9.5 cm × 7 cm in the posterior segment of the right hepatic lobe that had ruptured into the space between the liver and the diaphragm	Transcatheter arterial embolization was performed. The patient developed fever and the hematoma surrounding the liver was drained. No infection was confirmed but right lobectomy was performed	ND
Kai *et al*[32] 2015	F	77	Sudden abdominal pain and transient loss of consciousness	CT scan: hemoperitoneum with subcapsular hematoma at the left lobe and a hepatic nodule measuring 2.3 cm in diameter in segment II	Conservative initial treatment with periodic imaging studies. Transcatheter arterial chemoembolization was performed because a diagnosis of HCC was suggested. Surgical resection (laparoscopic left lateral segmentectomy) was performed 4 mo later	Postoperative course was uneventful. Discharge from hospital 7 d later No signs of recurrence at 3.5 yr after surgery
Kim *et al*[33] 2017	M	31	Sudden onset severe abdominal pain in the right upper quadrant area	CT scan: Mass of approximately 12 cm in the right hepatic lobe with hemorrhage along the perihepatic space	Emergent angiography with embolization.Hepatic resection was performed 15 d later	Postoperative course was uneventful

F: Female; M: Male. ND: Not determined; CT: Computed tomography; HCC: Hepatocellular carcinoma; IVC: Inferior vena cava.

sometimes preceded by preoperative embolization. Routine laboratory tests (including liver tests) are usually normal, as are serum tumor markers (alpha-fetoprotein, carcinoembryonic antigen and carbohydrate antigen 19-9[38].

The association between tuberous sclerosis complex (TSC) and renal AML, first described in 1911[39], is observed in 50% of cases, while the association between TSC and HAML is only observed in 5% to 15% of cases[3,40]. TSC is an autosomal dominant genetic disorder with a birth incidence of 1:6000[41], although sporadic cases due to *de novo* mutation are the most frequent presentation in the absence of a family history. TSC results from a mutation of *TSC1* or *TSC2*, which code for hamartin and tuberin, respectively[42]. These proteins are critical regulators of cell growth and proliferation, potentially through their upstream modulator, mammalian target of rapamycin (mTOR). Loss of function or dysfunction of either protein results in the development of hamartomas in numerous organ systems, including the brain, kidneys, heart and liver[43]. In patients with TSC, HAML is frequently associated with renal AML. A recent retrospective study showed that among 25 patients with HAML, 88% also had renal AML, and *TSC2* patients had a higher frequency of HAML compared to *TSC1* patients (18% *vs* 5%; $P = 0.037$)[42]. In contrast to previous reports, the predominance of female gender observed in patients with HAML but no TSC was not observed in patients with the TSC-HAML association[42,44].

IMMUNOHISTOLOGICAL CHARACTERISTICS OF HAML

Histological examination is the gold standard for HAML diagnosis, since diagnosis by imaging is difficult. Of note, even histological analysis of liver biopsy was shown to misdiagnose HAML in about 15% of cases in a recent multicenter study[34]. The World Health Organization defines PEComas as "mesenchymal tumors containing distinctive perivascular epithelioid cells". AML, which belongs to the PEComa group, is composed of adipose tissue, smooth muscle and vessels with dystrophic walls (Figure 1). The histological and immunohistochemical characteristics class HAML in the group of PEComas, an entity that brings together tumors of different histology, but with a common immunohistochemical signature, namely co-expression of melanocytic and muscle markers (see below)[1,40,45]. The PEComa family includes AML, clear cell "sugar" tumor of the lung, lymphangioleiomyomatosis (LAM), and a variety of unusual visceral, intra-abdominal, and soft tissue/bone tumors, described under the term "clear cell myomelanocytic tumor of the falciform ligament/Ligamentum teres"[46]. There is a strong association between AML and TSC, and between LAM and TSC[47], although the link is less marked for other members of the PEComa family[46].

Perivascular epithelioid cells are characterized by their perivascular location, often with a radial arrangement of cells around vessels. Typically, these cells are mostly epithelioid when they are just around the vessels, whereas spindle cells resembling smooth muscle are seen further away from the vessels. In the liver, the aspect is most often only epithelioid without spindle cells. Adipose cells are usually found distant from the blood vessels. Wide variation is seen in the relative proportion of epithelioid, spindle, and lipid-distended cells. Depending on the relative proportion of these different tissues, there are, on the one hand, conventional AMLs with a predominance of lipomatous or myomatous cells or vessels, and, on the other hand, epithelioid AMLs containing at least 10% epithelioid cells[48]. Mixed and myomatous AMLs are the most frequent (respectively 36% and 42% of the 151 HAML cases with informative data)[3]. Another subtype of HAML, namely inflammatory HAML, has also been recognized, although only 14 cases have been reported in the English-language literature[49]. Overall, this tumor is characterized by inflammatory infiltration exceeding 50% of tumor area, and the main types of inflammatory cells are lymphocytes (100%), plasma cells (93%) and histiocytes (71%)[49].

Macroscopically, HAML is well circumscribed, unencapsulated, smooth and brownish in color; however, the existence of hemorrhage or intra-tumor necrosis can change its appearance. On microscopic examination, cells typically have clear or slightly eosinophilic cytoplasms, small, central, round or oval nuclei, with a small nucleolus. "Atypical" AML presents cytological atypia, a multinucleated nucleus, focal necrosis and an increase in the number of mitoses[48].

By immunohistochemistry, these tumors are positive for both melanocytic markers (HBM-45 and melan-A are the more sensitive markers) and smooth muscle markers (actin and/or desmin) with variable extent of staining[46,50]. HBM-45 is the most specific marker of AML[38]. Actin non-immunoreactivity does not exclude a tumor from the PEComa group[51]. Classically, AML does not express epithelial markers (like cytokeratin), S100 protein or alpha-fetoprotein[52]. Estrogen and progesterone receptors are frequently positive in classic renal AML but are only rarely positive in

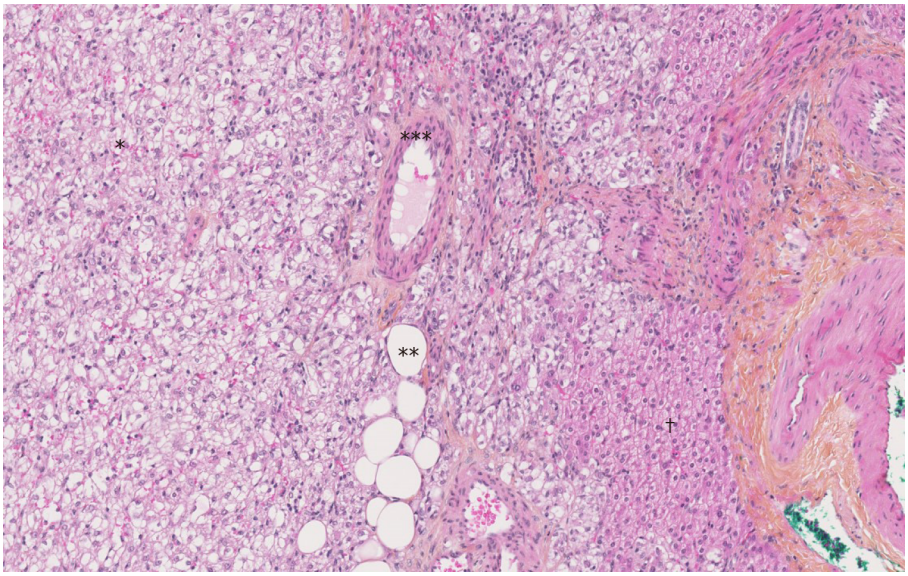


Figure 1 The Hematoxylin-Eosin-Saffron staining image of hepatic angiomyolipoma. There are three components of hepatic angiomyolipoma: vessel (*), adipocytes (**) and numerous epithelioid cells (***). There are fewer hepatocytes (†) (magnification × 10).

extrarenal PEComas, including HAML, suggesting the absence of the role of sex hormones in the pathogenesis and growth of HAML, despite a clear predominance in women[45,53].

There are numerous possible differential diagnoses of PEComas depending on the location and the predominant tissue composing the tumor. Given their uniform expression of melanocytic markers, PEComas may be confused with both conventional melanoma and clear cell sarcoma, but these latter typically have strong expression of S100 protein, and do not stain with smooth muscle actin. Due to its preferential abdominal location, the presence of epithelioid and spindle cells, and the occasional positive KIT (CD117) staining in HAML, the diagnosis of gastrointestinal stromal tumor is sometimes discussed. Depending on the size of the contingent of epithelioid cells, spindle-shaped cells or adipocytes, AML can also be confused with carcinoma, smooth muscle neoplasm or adipocytic tumor[45,54].

PROGNOSIS

The scarcity of PEComas precludes the identification of robust criteria to discriminate benign AML from other tumors with more aggressive behavior. The first description of a likely "malignant" HAML is recent[55], and although the authors do not clearly indicate the malignant nature of this tumor, the reported characteristics (*i.e.*, large size, cytological atypia and presence of necrosis) and the tumor-related death of the patient are robust arguments in favor of a "malignant" case. From a series of 24 PEComas of the soft tissue and gynecologic tract (not including AML) with a median follow-up of 30 mo (range: 10-84), Folpe *et al*[46] observed 3 local recurrences and 5 distant metastases (8/24, 33% of cases), 2 deaths (8%), 4 patients (17%) alive with metastatic or unresectable local disease, and 18 patients (75%) alive with no evidence of disease. A combined analysis of these 24 cases plus 45 other reported cases in the literature with sufficient available follow-up information identified the following variables associated with an increased risk of recurrence or metastasis: tumor size greater than 5 cm, infiltrative growth pattern, high nuclear grade, necrosis, and mitotic activity > 1/50 high power field. Consequently, these authors developed a provisional classification of PEComas with increasing aggressive potential (Table 2). Cases of HAML with aggressive behavior are reported in Table 3[8-24]. Regarding HAML, it is mainly the epithelioid type that confers a risk of aggressive behavior[34]. In the review published in 2017, the mortality rate associated with HAML was 0.8%[3].

Imaging findings

The imaging features of HAML vary greatly depending on the highly variable proportion of fat, smooth muscle and vascular elements. Diagnosis can be challenging, and depends mainly on the amount of fat present, which is the key to HAML

Table 2 Classification of perivascular epithelioid cell tumors according to their malignant potential[1,27]

Classification	Criteria
Benign	No worrisome features: (1) Tumor size < 5 cm; (2) No infiltration; (3) Non-high nuclear grade and cellularity; (4) Mitotic activity ≤ 1/50 HPF; (5) No necrosis; and (6) No vascular invasion
Uncertain malignant potential	Tumor with: (1) Pleomorphism/multinucleated giant cells only; or (2) Size > 5 cm only
Aggressive behavior	Two or more worrisome features: (1) Size > 5 cm; (2) Peripheral infiltration; (3) High nuclear grade and cellularity; (4) Mitotic activity > 1/50 HPF; (5) Ischemic tumor necrosis for large tumor; and (6) Vascular invasion
According to the WHO classification of tumors[1]	As with GISTs, the main predictors of a risk of metastatic behavior are marked nuclear atypia, diffuse pleomorphism and mitotic activity of more than 1 mitosis per 1 mm^2

GIST: Gastrointestinal stromal tumor; HPF: High-power field.

diagnosis. On ultrasound, the lesion is usually well circumscribed, hyperechoic or mixed echoic and after injection of ultrasound contrast, presents rapid enhancement in the arterial phase compared to the adjacent liver. In the portal and delayed phase, HAML can display either hypo, iso or hyperenhancement[4]. Computed tomography (CT) shows a hypodense tumor with fatty areas within the lesion (density around -50 HU). Classically, this solid tumor is hypervascular (Figure 2) with wash-out in the portal and late portal phase [CT or magnetic resonance imaging (MRI)]. HAML with few or no vessels on histologic examination show persistent portal and late-phase enhancement, whereas HAML with richly vascularized tissue is more likely to show wash-out[5]. The tumor signal on MRI is hyperintense in T2 weighted sequences and variable in T1 weighted sequences. MRI is the most sensitive imaging technique to detect liver fat using in-phase and opposed-phase T1 gradient echo sequences. The drop-out signal within a liver lesion on the opposed-phase sequences indicates the presence of fat within the lesion (Figure 3A and B). The imaging features on MRI after injection of contrast medium are similar to those observed on CT scan. When using a hepatocyte specific agent (gadoxetic acid or gadobenate dimeglumine), the lesion shows a hyposignal in the hepatobiliary phase (Figure 4).

Data regarding HAML evaluation using fluorine-18-fluorodeoxyglucose positron emission tomography (^{18}F-FDG-PET) are limited. FDG uptake in HAML is variable and the value of ^{18}F-FDG-PET for diagnosing or managing this type of tumor is unclear[56].

Given the imaging characteristics of HAML (hypervascular lesion with a fat component in a healthy liver and with frequent wash-out), differential diagnoses are benign hepatocytic tumors (steatotic or telangiectasia adenoma, fat focal nodular hyperplasia) and malignant hepatocytic tumors (mainly hepatocellular carcinoma). When the diagnosis is challenging, especially with hepatocellular carcinoma, the absence of a capsule and the visualization of a drainage vein are two useful radiological features that can be helpful for HAML diagnosis when they are present[6].

MANAGEMENT OF HAML

Due to its rarity, the diagnosis of HAML on imaging (and even histological examination[34]) is difficult. Consequently, the clinical management of HAML patients should take place in expert centers for a multidisciplinary work-up involving radiologists, pathologists and hepatologists. Obtaining a liver biopsy is strongly advised to better balance the risk of surgery (resection of centrohepatic tumor will be at higher risk, for instance) against the risk of tumor-related complications. Thus, in the presence of asymptomatic HAML, without cytological atypia on biopsy, but at high risk of complicated resection, regular radiological monitoring will be preferred[34].

Analysis of the literature shows that the majority of patients are treated with surgical resection (84% and 76% of patients in two large case series[3,34]). For other patients, regular radiological monitoring is justified by the uncertainty surrounding the risk of HAML progression. Although the risk of tumor recurrence after resection or metastases has rarely been described, the identification of radiological and especially histological factors predicting an unfavorable course (Table 1) is essential for a collegial therapeutic decision. Furthermore, patient compliance with regular radiological monitoring will also be an important argument in decision-making[3,35].

Table 3 Reported cases of hepatic angiomyolipoma with aggressive behavior

Ref.	Sex	Age, yr	Size	Types	Treatment	Duration of follow-up	Outcome
Croquet *et al*[9] 2000	F	16	19 cm × 12 cm × 8 cm	Epithelioid	SR	6 yr	Recurrence in the liver, associated with renal angiomyolipoma
Dalle *et al* [10] 2000	F	70	15 cm	Epithelioid	SR	5 mo	Recurrence in the liver with a lesion measuring 15 cm and presence of multiple metastases in the liver
Flemming *et al*[11] 2000	F	51	2 nodules: 0.5 cm and 15 cm	Epithelioid	SR	3 yr	Recurrence in the right hepatic lobe and presence of multiple metastases
McKinney *et al*[12] 2005	F	14	11 cm × 7 cm × 8 cm	NS	SR, interferon α	1 yr	Recurrence with a hepatic lesion measuring 9 cm × 6 cm × 14 cm, appearance of lymph nodes and hepatic metastases. Death after disease progression
Parfitt *et al* [13] 2007	F	60	14 cm × 11 cm	Epithelioid	SR	9 yr	Recurrence in the liver and appearance of metastases in the trapezius muscle, the left lung and the tail of the pancreas
Yang *et al* [14] 2007	F	37	13 cm × 9 cm × 9 cm	Classic	SR	14 mo	Recurrence in the right hepatic lobe 6 months after SR, appearance of pulmonary metastases 11 mo after SR and death occurred at 14 mo
Deng *et al* [15] 2008	M	30	18 cm × 14 cm	Classic	SR, Chemotherapy	3 yr and 4 mo	Recurrence with a hepatic lesion measuring 11 cm and metastases in pancreatic tail and portal vein thrombosis 3 yr after SR. Chemotherapy was initiated but 4 mo later pulmonary metastases appeared. Death occurred after disease progression
Nguyen *et al*[16] 2008	F	43	11 cm × 7.5 cm × 7.5 cm	Classic	SR	6 mo	Recurrence in the liver 6 mo after SR, together with metastases in the peritoneum, omentum, stomach and spleen. Death after disease progression
Xu *et al*[17] 2009	F	33	2 nodules: 1 cm and 6 cm	Epithelioid	SR	1 yr	Recurrence in the left hepatic lobe
Zeng *et al* [18] 2010	NS	NS	6 cm	NS	SR	9 yr	Recurrence in the right hepatic lobe with a lesion measuring 6 cm
Butte *et al* [19] 2011	F;M	54; 41	NS; 9 cm	NS; Epithelioid	SR; SR	53 mo; 41 mo	Recurrence in the liver 53 mo after SR; Occurrence of pulmonary and retroperitoneal metastases 41 mo after SR
Hu *et al*[20] 2011	F	NS	NS	NS	SR	14 mo	Appearance of local and distant metastases 6 mo after SR. Death occurred 14 mo after SR
Ding *et al* [8] 2011	F	31	8 cm × 8 cm	NS	SR	7 yr	Recurrence in the right hepatic lobe 6 yr after SR and death occurred one year later
Wang *et al* [21] 2015	F	37	7 cm × 9 cm	Classic	SR	3 yr	Recurrence of two hepatic nodules in the right lobe (13 cm × 12 cm and 2.3 cm × 1.8 cm) 3 yr after SR. Arterial chemoembolization was performed, followed by liver transplantation
Fukuda *et al*[22] 2016	M	58	6.3 cm	Epithelioid	SR	9 yr	Metastases occurred in the right lung 7 yr after SR and were treated by pneumonectomy. No recurrence was observed after 2 yr of follow-up
Marcuzzi *et al*[23] 2018	F	47	3.8 cm × 4.6 cm × 4.7 cm+ 2 hepatic lesions measuring 6 mm and 5 mm	Epithelioid	SR	8 yr and 8 mo	CT scan was performed 6 yr and 4 mo after the initial presentation: the hepatic lesion had grown in size to an estimated 10.9 cm × 9.7 cm × 11.2 cm and the adjacent lesions had grown to 1.9 cm and 2.4 cm with a new lesion on the kidney of 4.6 cm × 5.1 cm. 16 mo later, MRI showed an increase in size of the hepatic lesion (12 cm × 11 cm), and kidney lesion (6.2 cm × 5.6 cm). SR performed 2 mo later. 6 mo after SR, recurrence in the resection line and in the hepatic segment II
Yan *et al* [24] 2018	NS	NS	15 cm	Epithelioid	SR	9 yr	Recurrence in the liver 9 yr after SR with invasion of the inferior vena cava and diaphragm, and appearance of pulmonary metastases

F: Female; M: Male; NS: Not specified; SR: Surgical resection; CT: Computed tomography; MRI: Magnetic resonance imaging.

The optimal radiological follow-up is not well defined, but the first radiological evaluation may take place at one year, since HAMLs were described to increase by only 0.77 cm per year in a series of 29 patients followed radiologically[3], and radiological progression affected only 6 of the 29 patients (20%). Later, radiological monitoring could be performed twice a year[3], but the frequency will depend on the

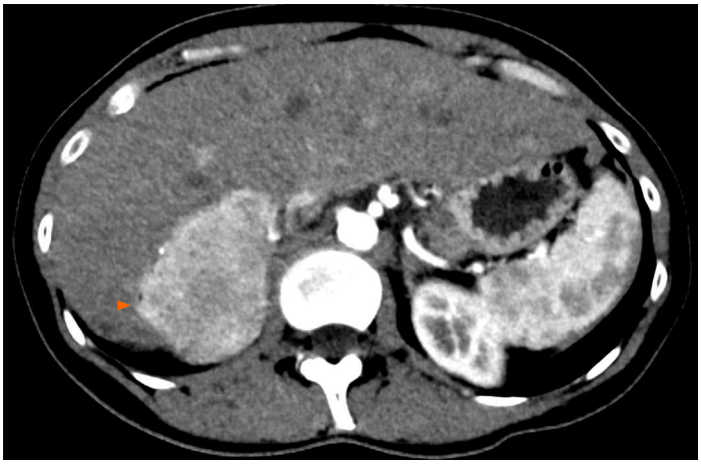


Figure 2 Angiomyolipoma in a healthy 33-year-old woman. Abdominal computed tomography on arterial phase showed a hypervascular solid tumor localized in the right posterior segment (arrowheads).

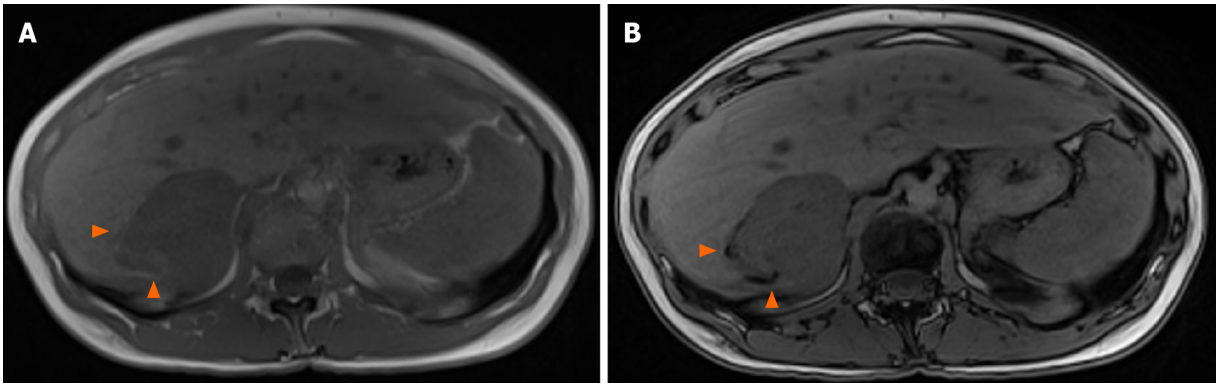


Figure 3 T1 weighted magnetic resonance images. Signal dropout at the periphery of the lesion due to fat contingents (arrowhead). A: In-phase; B: Opposed-phase.

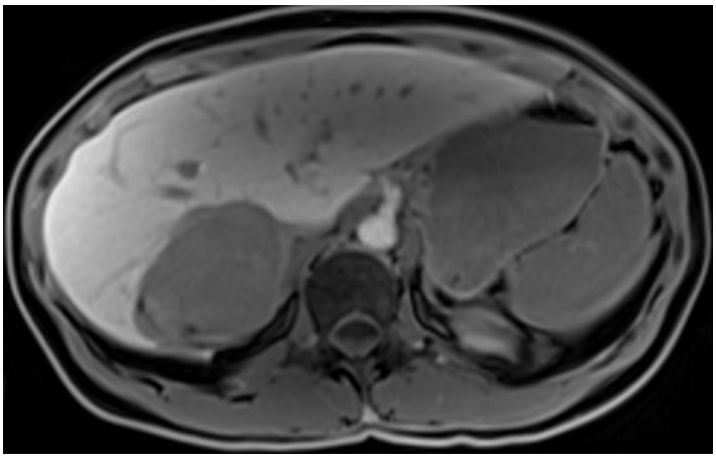


Figure 4 T1 weighted images one hour after hepatocyte-specific agent injection (gadobenate dimeglumine). Hyposignal of the lesion indicates that this is not a hepatocytic tumor.

magnitude of tumor progression during the first years of monitoring. Evidently, persistent tumor progression on successive imaging will require a surgical approach. Surgical resection is therefore recommended when there is uncertainty regarding the histological nature of the lesion after liver biopsy, tumor progression on imaging, tumor-related symptoms, and when the tumor exceeds 5 cm[34,38]. The recurrence rate after surgical resection was 2.4% (6 of 246 patients in the series reported by

Klompenhouwer *et al*[3]). The local or distant post-resection recurrence rate is 10% in the case of epithelioid-type HAML[52].

Liver transplantation (LT) has sometimes been erroneously performed for a suspected diagnosis of cholangiocarcinoma or hemangiosarcoma mimicking a hepatocellular carcinoma[3,34]. These flawed diagnoses further underline the interest of systematic liver biopsy as well as radiological and anatomopathological expertise. Since the first reported case of LT for HAML in 2010[57], other exceptional cases have been added[21,58]. LT was performed as a last resort treatment for unresectable HAML due to excessive size or a significant number of hepatic tumors.

Other therapeutic alternatives have been reported, such as radiofrequency ablation, arterial embolization or the use of sirolimus[35,42]. mTOR inhibitors, which include sirolimus and everolimus, are immunosuppressive molecules used in transplantation, and which also have antiproliferative properties. In a multicenter, double-blind, placebo-controlled, phase 3 trial (EXIST-2), 118 patients with at least one renal AML larger than 3 cm associated with a definite TSC diagnosis or sporadic LAM were randomized to receive oral everolimus 10 mg/d (n = 79, mean dosage: 8.6 mg/d, median duration: 38 wk) or placebo (n = 39). The trial showed a beneficial effect of everolimus in reducing the size of AML (response rate: 42% *vs* 0% in the placebo group; P < 0.0001). Response was evaluated as a composite endpoint including a reduction ≥ 50% of the AML volume[59].

The favorable outcomes reported in the EXIST-2 trial led to an open label extension undertaken by the same authors. This study demonstrated a pronounced benefit of everolimus for the patients who continued on this drug. The response rate improved from 42% in the primary analysis (median exposure 8.7 mo)[59] to 54% (median exposure 28.9 mo), and the long-term use of everolimus appeared safe[60].

The pooled analysis of two randomized trials[59,61] comparing 109 and 53 patients with renal AML treated respectively with everolimus and placebo for 6 mo confirmed the efficacy of everolimus in reducing tumor volume by 50% or more (risk ratio = 24.69; P = 0.001)[62]. Everolimus is currently indicated for the treatment of adult patients with renal AML and TSC not requiring immediate surgery. Some patients with HAML associated with TSC have been treated with sirolimus, which also proved efficacious in reducing tumor volume[54]. The role of mTOR inhibitors for patients with HAML remains undefined, but these molecules could be used, as for the kidney, in a palliative context. The long-term safety profile is consistent with that previously reported and no new safety issues have raised concern[60].

In a retrospective Chinese series (2009-2016) of 92 patients diagnosed with histologically proven HAML measuring between 2 cm and 5 cm, ultrasonography-guided radiofrequency ablation after liver biopsy was used in 22/92 patients. No tumor recurrence was reported, but the duration of follow-up was not indicated[35]. Radiofrequency ablation can therefore advantageously compete with surgery when HAML is relatively small (< 5 cm), and when the location in the liver or the patient's comorbidities are not amenable to safe hepatic surgery.

Arterial embolization[33] is sometimes necessary in the presence of hemorrhagic HAML. There are only eight reported cases of HAML presenting as spontaneous rupture and hemorrhage; the median size of these tumors was 8.5 cm (range: 2.5 cm to 12.5 cm) and three of them were treated with arterial embolization followed by liver resection enabling formal diagnosis of HAML. The main differential diagnosis of hemorrhagic liver tumor in a non-cirrhotic liver is adenoma, which is outside the scope of this review. Therapeutic arterial embolization was used in three other patients with histologically proven HAML (size: 11, 12 and 17 cm) in a retrospective American series[19], and no progression was observed after an average follow-up of 12.7 mo (range, 1-36 mo). The risk of spontaneous hemorrhage seems to be lower for HAML than for kidney AML, which are usually supplied by a single vessel and associated with aneurysms[19]. We propose a decisional algorithm for the management of HAML (Figure 5).

CONCLUSION

HAML is a rare but not exceptional tumor, and usually has a benign course. However, this tumor may display more aggressive behavior with recurrence or metastasis, although there are no robust histological or radiological characteristics to predict the natural course of this type of tumor. Radiological diagnosis is often hazardous due to the variable proportions of the tissues that comprise HAML. Therefore, histological analysis of the tumor and multidisciplinary consultation, whenever possible in an

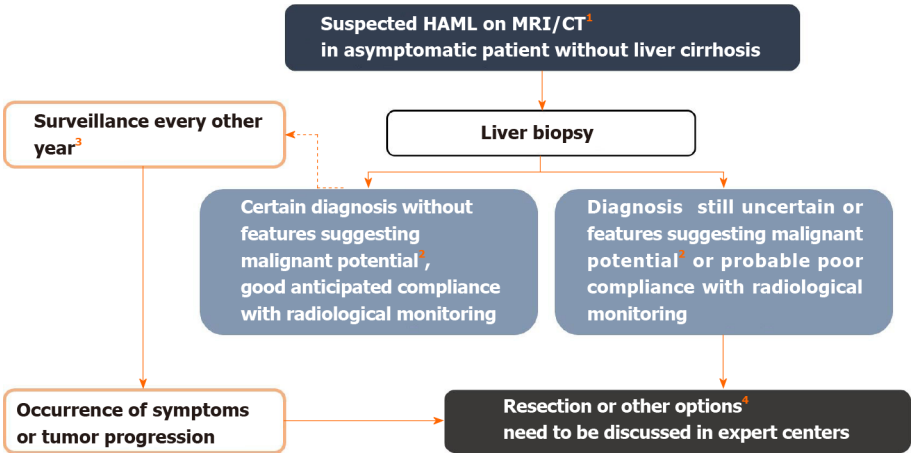


Figure 5 Management algorithm for suspected hepatic angiomyolipoma on imaging. [1]Hepatic angiomyolipoma diagnosis is suggested in the presence of fatty tissue within the solid lesion or presence of wash-out. In the presence of tumor-related symptoms, surgical resection is considered first. [2]Features suggesting malignant potential are reported in Table 2. Some authors also recommend surgery in the case of epithelioid-type hepatic angiomyolipoma, which would be at greater risk of progression. Likewise, an association with tuberous sclerosis complex is a condition that increases the risk of malignant transformation, by analogy with renal angiomyolipoma[3]. [3]Monitoring maintained despite the benign nature of the initial diagnosis because the aggressive behavior of the tumor is difficult to predict. [4]Other possible therapeutic options include mTOR inhibitors, radiofrequency ablation, arterial embolization in cases of hemorrhagic rupture, and liver transplantation. Citation: Klompenhouwer AJ, Verver D, Janki S, Bramer WM, Doukas M, Dwarkasing RS, de Man RA, IJzermans JNM. Management of hepatic angiomyolipoma: A systematic review. Liver Int 2017; 37(9): 1272-1280. Copyright ©The Author(s) 2017. Published by John Wiley and Sons[3]. MRI: Magnetic resonance imaging; CT: Computed tomography.

expert center, are essential for optimal care of these patients.

REFERENCES

1 **World Health Organization Classification of Tumours Editorial Board**. Digestive System Tumours. 5th ed. World Health Organization: International Agency for Research on Cancer Publications, 2019

2 **Ishak K**. Mesenchymal tumors of the liver. In: Okuda K, Peter R. Hepatocellular carcinoma. New York: John Wiley & Sons, 1976: 247-275

3 **Klompenhouwer AJ**, Verver D, Janki S, Bramer WM, Doukas M, Dwarkasing RS, de Man RA, IJzermans JNM. Management of hepatic angiomyolipoma: A systematic review. *Liver Int* 2017; **37**: 1272-1280 [PMID: 28177188 DOI: 10.1111/liv.13381]

4 **Huang Z**, Zhou P, Li S, Li K. Hepatic Angiomyolipoma: Clinical Features and Imaging Findings of Quantitative Contrast-Enhanced Ultrasound Perfusion Analysis and Magnetic Resonance Imaging. *J Ultrasound Med* 2020; **39**: 2111-2122 [PMID: 32383807 DOI: 10.1002/jum.15316]

5 **Jung DH**, Hwang S, Hong SM, Kim KH, Ahn CS, Moon DB, Alshahrani AA, Lee SG. Clinico-pathological correlation of hepatic angiomyolipoma: a series of 23 resection cases. *ANZ J Surg* 2018; **88**: E60-E65 [PMID: 28122404 DOI: 10.1111/ans.13880]

6 **Seow J**, McGill M, Wang W, Smith P, Goodwin M. Imaging hepatic angiomyolipomas: key features and avoiding errors. *Clin Radiol* 2020; **75**: 88-99 [PMID: 31677881 DOI: 10.1016/j.crad.2019.09.135]

7 **Liu W**, Wang J, Huang Q, Lu Q, Liang W. Comparison of MRI Features of Epithelioid Hepatic Angiomyolipoma and Hepatocellular Carcinoma: Imaging Data From Two Centers. *Front Oncol* 2018; **8**: 600 [PMID: 30619742 DOI: 10.3389/fonc.2018.00600]

8 **Ding GH**, Liu Y, Wu MC, Yang GS, Yang JM, Cong WM. Diagnosis and treatment of hepatic angiomyolipoma. *J Surg Oncol* 2011; **103**: 807-812 [PMID: 21283992 DOI: 10.1002/jso.21814]

9 **Croquet V**, Pilette C, Aubé C, Bouju B, Oberti F, Cervi C, Arnaud JP, Rousselet MC, Boyer J, Calès P. Late recurrence of a hepatic angiomyolipoma. *Eur J Gastroenterol Hepatol* 2000; **12**: 579-582 [PMID: 10833105 DOI: 10.1097/00042737-200012050-00018]

10 **Dalle I**, Sciot R, de Vos R, Aerts R, van Damme B, Desmet V, Roskams T. Malignant angiomyolipoma of the liver: a hitherto unreported variant. *Histopathology* 2000; **36**: 443-450 [PMID: 10792486 DOI: 10.1046/j.1365-2559.2000.00891.x]

11 **Flemming P**, Lehmann U, Becker T, Klempnauer J, Kreipe H. Common and epithelioid variants of hepatic angiomyolipoma exhibit clonal growth and share a distinctive immunophenotype. *Hepatology* 2000; **32**: 213-217 [PMID: 10915726 DOI: 10.1053/jhep.2000.9142]

12 **McKinney CA**, Geiger JD, Castle VP, Ruiz RE, Strouse PJ. Aggressive hepatic angiomyolipoma in a child. *Pediatr Hematol Oncol* 2005; **22**: 17-24 [PMID: 15770828 DOI: 10.1080/08880010590896206]

13 **Parfitt JR**, Bella AJ, Izawa JI, Wehrli BM. Malignant neoplasm of perivascular epithelioid cells of the liver. *Arch Pathol Lab Med* 2006; **130**: 1219-1222 [PMID: 16879028]

14 **Yang CY**, Ho MC, Jeng YM, Hu RH, Wu YM, Lee PH. Management of hepatic angiomyolipoma. *J Gastrointest Surg* 2007; **11**: 452-457 [PMID: 17436129 DOI: 10.1007/s11605-006-0037-3]

15 **Deng YF**, Lin Q, Zhang SH, Ling YM, He JK, Chen XF. Malignant angiomyolipoma in the liver: a case report with pathological and molecular analysis. *Pathol Res Pract* 2008; **204**: 911-918 [PMID: 18723294 DOI: 10.1016/j.prp.2008.06.007]

16 **Nguyen TT**, Gorman B, Shields D, Goodman Z. Malignant hepatic angiomyolipoma: report of a case and review of literature. *Am J Surg Pathol* 2008; **32**: 793-798 [PMID: 18391749 DOI: 10.1097/PAS.0b013e3181607349]

17 **Xu PJ**, Shan Y, Yan FH, Ji Y, Ding Y, Zhou ML. Epithelioid angiomyolipoma of the liver: cross-sectional imaging findings of 10 immunohistochemically-verified cases. *World J Gastroenterol* 2009; **15**: 4576-4581 [PMID: 19777618 DOI: 10.3748/wjg.15.4576]

18 **Zeng JP**, Dong JH, Zhang WZ, Wang J, Pang XP. Hepatic angiomyolipoma: a clinical experience in diagnosis and treatment. *Dig Dis Sci* 2010; **55**: 3235-3240 [PMID: 20165978 DOI: 10.1007/s10620-010-1144-2]

19 **Butte JM**, Do RK, Shia J, Gönen M, D'Angelica MI, Getrajdman GI, Allen PJ, Fong Y, Dematteo RP, Klimstra DS, Jarnagin WR. Liver angiomyolipomas: a clinical, radiologic, and pathologic analysis of 22 patients from a single center. *Surgery* 2011; **150**: 557-567 [PMID: 21621235 DOI: 10.1016/j.surg.2011.03.006]

20 **Hu WG**, Lai EC, Liu H, Li AJ, Zhou WP, Fu SY, Pan ZY, Huang G, Lei Y, Lau WY, Wu MC. Diagnostic difficulties and treatment strategy of hepatic angiomyolipoma. *Asian J Surg* 2011; **34**: 158-162 [PMID: 22464831 DOI: 10.1016/j.asjsur.2011.11.005]

21 **Wang WT**, Li ZQ, Zhang GH, Guo Y, Teng MJ. Liver transplantation for recurrent posthepatectomy malignant hepatic angiomyolipoma: a case report. *World J Gastroenterol* 2015; **21**: 3755-3758 [PMID: 25834347 DOI: 10.3748/wjg.v21.i12.3755]

22 **Fukuda Y**, Omiya H, Takami K, Mori K, Kodama Y, Mano M, Nomura Y, Akiba J, Yano H, Nakashima O, Ogawara M, Mita E, Nakamori S, Sekimoto M. Malignant hepatic epithelioid angiomyolipoma with recurrence in the lung 7 years after hepatectomy: a case report and literature review. *Surg Case Rep* 2016; **2**: 31 [PMID: 27037804 DOI: 10.1186/s40792-016-0158-1]

23 **Marcuzzi A**, Haider EA, Salmi ISA. Hepatic epithelioid angiomyolipoma with renal metastasis: radiologic-pathologic correlation. *Radiol Case Rep* 2018; **13**: 829-833 [PMID: 29955240 DOI: 10.1016/j.radcr.2018.05.007]

24 **Yan Z**, Grenert JP, Joseph NM, Ren C, Chen X, Shafizadeh N, Kakar S. Hepatic angiomyolipoma: mutation analysis and immunohistochemical pitfalls in diagnosis. *Histopathology* 2018; **73**: 101-108 [PMID: 29512829 DOI: 10.1111/his.13509]

25 **Huber C**, Treutner KH, Steinau G, Schumpelick V. Ruptured hepatic angiolipoma in tuberous sclerosis complex. *Langenbecks Arch Chir* 1996; **381**: 7-9 [PMID: 8717168 DOI: 10.1007/BF00184248]

26 **Guidi G**, Catalano O, Rotondo A. Spontaneous rupture of a hepatic angiomyolipoma: CT findings and literature review. *Eur Radiol* 1997; **7**: 335-337 [PMID: 9087353 DOI: 10.1007/s003300050162]

27 **Tsui WM**, Colombari R, Portmann BC, Bonetti F, Thung SN, Ferrell LD, Nakanuma Y, Snover DC, Bioulac-Sage P, Dhillon AP. Hepatic angiomyolipoma: a clinicopathologic study of 30 cases and delineation of unusual morphologic variants. *Am J Surg Pathol* 1999; **23**: 34-48 [PMID: 9888702 DOI: 10.1097/00000478-199901000-00004]

28 **Zhou YM**, Li B, Xu F, Wang B, Li DQ, Zhang XF, Liu P, Yang JM. Clinical features of hepatic angiomyolipoma. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 284-287 [PMID: 18522883]

29 **Occhionorelli S**, Dellachiesa L, Stano R, Cappellari L, Tartarini D, Severi S, Palini GM, Pansini GC, Vasquez G. Spontaneous rupture of a hepatic epithelioid angiomyolipoma: damage control surgery. A case report. *G Chir* 2013; **34**: 320-322 [PMID: 24342160]

30 **Aoki H**, Arata T, Morihiro T, Kanaya N, Takeda S, Sui K, Shigeyasu K, Katsuda K, Tanakaya K, Takeuchi H. Spontaneous rupture of a hepatic angiomyolipoma: Report of a case. *Clin J Gastroenterol* 2014; **7**: 429-433 [PMID: 26184024 DOI: 10.1007/s12328-014-0517-z]

31 **Tajima S**, Suzuki A, Suzumura K. Ruptured hepatic epithelioid angiomyolipoma: a case report and literature review. *Case Rep Oncol* 2014; **7**: 369-375 [PMID: 24987358 DOI: 10.1159/000363690]

32 **Kai K**, Miyosh A, Aishima S, Wakiyama K, Nakashita S, Iwane S, Azama S, Irie H, Noshiro H. Granulomatous reaction in hepatic inflammatory angiomyolipoma after chemoembolization and spontaneous rupture. *World J Gastroenterol* 2015; **21**: 9675-9682 [PMID: 26327777 DOI: 10.3748/wjg.v21.i32.9675]

33 **Kim SH**, Kang TW, Lim K, Joh HS, Kang J, Sinn DH. A case of ruptured hepatic angiomyolipoma in a young male. *Clin Mol Hepatol* 2017; **23**: 179-183 [PMID: 28449573 DOI: 10.3350/cmh.2016.0027]

34 **Klompenhouwer AJ**, Dwarkasing RS, Doukas M, Pellegrino S, Vilgrain V, Paradis V, Soubrane O, Beane JD, Geller DA, Nalesnik MA, Tripke V, Lang H, Schmelzle M, Pratschke J, Schöning W, Beal E, Sun S, Pawlik TM, de Man RA, Ijzermans JNM. Hepatic angiomyolipoma: an international multicenter analysis on diagnosis, management and outcome. *HPB (Oxford)* 2020; **22**: 622-629 [PMID: 31619346 DOI: 10.1016/j.hpb.2019.09.004]

35 **Yang X**, Lei C, Qiu Y, Shen S, Lu C, Yan L, Wang W. Selecting a suitable surgical treatment for hepatic angiomyolipoma: a retrospective analysis of 92 cases. *ANZ J Surg* 2018; **88**: E664-E669 [PMID: 29241297 DOI: 10.1111/ans.14323]

36 **Pontis A**, Piras B, Meloni A, De Lisa A, Melis GB, Angioni S. Rupture of renal angiomyolipoma in pregnancy. *J Obstet Gynaecol* 2013; **33**: 628-629 [PMID: 23919867 DOI: 10.3109/01443615.2013.810201]

37 **Yamakado K**, Tanaka N, Nakagawa T, Kobayashi S, Yanagawa M, Takeda K. Renal angiomyolipoma: relationships between tumor size, aneurysm formation, and rupture. *Radiology* 2002; **225**: 78-82 [PMID: 12354988 DOI: 10.1148/radiol.2251011477]

38 **Chang Z**, Zhang JM, Ying JQ, Ge YP. Characteristics and treatment strategy of hepatic angiomyolipoma: a series of 94 patients collected from four institutions. *J Gastrointestin Liver Dis* 2011; **20**: 65-69 [PMID: 21451800 DOI: 10.1007/s11749-010-0230-2]

39 **Eble JN**. Angiomyolipoma of kidney. *Semin Diagn Pathol* 1998; **15**: 21-40 [PMID: 9503504]

40 **Kamimura K**, Nomoto M, Aoyagi Y. Hepatic angiomyolipoma: diagnostic findings and management. *Int J Hepatol* 2012; **2012**: 410781 [PMID: 23320180 DOI: 10.1155/2012/410781]

41 **Osborne JP**, Fryer A, Webb D. Epidemiology of tuberous sclerosis. *Ann N Y Acad Sci* 1991; **615**: 125-127 [PMID: 2039137 DOI: 10.1111/j.1749-6632.1991.tb37754.x]

42 **Black ME**, Hedgire SS, Camposano S, Paul E, Harisinghani M, Thiele EA. Hepatic manifestations of tuberous sclerosis complex: a genotypic and phenotypic analysis. *Clin Genet* 2012; **82**: 552-557 [PMID: 22251200 DOI: 10.1111/j.1399-0004.2012.01845.x]

43 **Orlova KA**, Crino PB. The tuberous sclerosis complex. *Ann N Y Acad Sci* 2010; **1184**: 87-105 [PMID: 20146692 DOI: 10.1111/j.1749-6632.2009.05117.x]

44 **Fricke BL**, Donnelly LF, Casper KA, Bissler JJ. Frequency and imaging appearance of hepatic angiomyolipomas in pediatric and adult patients with tuberous sclerosis. *AJR Am J Roentgenol* 2004; **182**: 1027-1030 [PMID: 15039181 DOI: 10.2214/ajr.182.4.1821027]

45 **Folpe AL**, Kwiatkowski DJ. Perivascular epithelioid cell neoplasms: pathology and pathogenesis. *Hum Pathol* 2010; **41**: 1-15 [PMID: 19604538 DOI: 10.1016/j.humpath.2009.05.011]

46 **Folpe AL**, Mentzel T, Lehr HA, Fisher C, Balzer BL, Weiss SW. Perivascular epithelioid cell neoplasms of soft tissue and gynecologic origin: a clinicopathologic study of 26 cases and review of the literature. *Am J Surg Pathol* 2005; **29**: 1558-1575 [PMID: 16327428 DOI: 10.1097/01.pas.0000173232.22117.37]

47 **Roach ES**, Gomez MR, Northrup H. Tuberous sclerosis complex consensus conference: revised clinical diagnostic criteria. *J Child Neurol* 1998; **13**: 624-628 [PMID: 9881533 DOI: 10.1177/088307389801301206]

48 **Aydin H**, Magi-Galluzzi C, Lane BR, Sercia L, Lopez JI, Rini BI, Zhou M. Renal angiomyolipoma: clinicopathologic study of 194 cases with emphasis on the epithelioid histology and tuberous sclerosis association. *Am J Surg Pathol* 2009; **33**: 289-297 [PMID: 18852677 DOI: 10.1097/PAS.0b013e31817ed7a6]

49 **Mao JX**, Yuan H, Sun KY, Liu C, Fu H, Ding GS, Guo WY, Teng F. Pooled analysis of hepatic inflammatory angiomyolipoma. *Clin Res Hepatol Gastroenterol* 2020; **44**: e145-e151 [PMID: 32482543 DOI: 10.1016/j.clinre.2020.04.005]

50 **Bonetti F**, Pea M, Martignoni G, Zamboni G. PEC and sugar. *Am J Surg Pathol* 1992; **16**: 307-308 [PMID: 1599021 DOI: 10.1097/00000478-199203000-00013]

51 **Hornick JL**, Fletcher CD. Sclerosing PEComa: clinicopathologic analysis of a distinctive variant with a predilection for the retroperitoneum. *Am J Surg Pathol* 2008; **32**: 493-501 [PMID: 18223480 DOI: 10.1097/PAS.0b013e318161dc34]

52 **Liu J**, Zhang CW, Hong DF, Tao R, Chen Y, Shang MJ, Zhang YH. Primary hepatic epithelioid angiomyolipoma: A malignant potential tumor which should be recognized. *World J Gastroenterol* 2016; **22**: 4908-4917 [PMID: 27239117 DOI: 10.3748/wjg.v22.i20.4908]

53 **Yeh CN**, Lee KF, Chen MF. Immunohistochemical study of hepatic angiomyolipoma. *Hepatogastroenterology* 2005; **52**: 1151-1153 [PMID: 16001650]

54 **L'Hostis H**, Deminiere C, Ferriere JM, Coindre JM. Renal angiomyolipoma: a clinicopathologic, immunohistochemical, and follow-up study of 46 cases. *Am J Surg Pathol* 1999; **23**: 1011-1020 [PMID: 10478660 DOI: 10.1097/00000478-199909000-00003]

55 **Ohmori T**, Arita N, Uraga N, Tabei R, Yamamoto M, Kataoka M, Hamamoto K. Giant hepatic angiomyolipoma. *Histopathology* 1989; **15**: 540-543 [PMID: 2599515 DOI: 10.1111/j.1365-2559.1989.tb01615.x]

56 **Kumasaka S**, Arisaka Y, Tokue A, Higuchi T, Nakajima T, Tsushima Y. A case of multiple hepatic angiomyolipomas with high (18) F-fluorodeoxyglucose uptake. *BMC Med Imaging* 2014; **14**: 17 [PMID: 24885757 DOI: 10.1186/1471-2342-14-17]

57 **Dumortier J**, Guillaud O, Walter T, Ber CE, Partensky C, Boillot O, Scoazec JY. Liver transplantation for multiple angiomyolipomas complicating tuberous sclerosis complex. *Gastroenterol Clin Biol* 2010; **34**: 494-498 [PMID: 20674202 DOI: 10.1016/j.gcb.2010.06.005]

58 **Vagefi PA**, Eilers H, Hiniker A, Freise CE. Liver transplantation for giant hepatic angiomyolipoma. *Liver Transpl* 2011; **17**: 985-986 [PMID: 21462294 DOI: 10.1002/lt.22310]

59 **Bissler JJ**, Kingswood JC, Radzikowska E, Zonnenberg BA, Frost M, Belousova E, Sauter M, Nonomura N, Brakemeier S, de Vries PJ, Whittemore VH, Chen D, Sahmoud T, Shah G, Lincy J, Lebwohl D, Budde K. Everolimus for angiomyolipoma associated with tuberous sclerosis complex or sporadic lymphangioleiomyomatosis (EXIST-2): a multicentre, randomised, double-blind, placebo-controlled trial. *Lancet* 2013; **381**: 817-824 [PMID: 23312829 DOI: 10.1016/S0140-6736(12)61767-X]

60 **Bissler JJ**, Kingswood JC, Radzikowska E, Zonnenberg BA, Frost M, Belousova E, Sauter M,

Nonomura N, Brakemeier S, de Vries PJ, Berkowitz N, Miao S, Segal S, Peyrard S, Budde K. Everolimus for renal angiomyolipoma in patients with tuberous sclerosis complex or sporadic lymphangioleiomyomatosis: extension of a randomized controlled trial. *Nephrol Dial Transplant* 2016; **31**: 111-119 [PMID: 26156073 DOI: 10.1093/ndt/gfv249]

61 **Franz DN**. Everolimus in the treatment of subependymal giant cell astrocytomas, angiomyolipomas, and pulmonary and skin lesions associated with tuberous sclerosis complex. *Biologics* 2013; **7**: 211-221 [PMID: 24143074 DOI: 10.2147/BTT.S25095]

62 **Li M**, Zhou Y, Chen C, Yang T, Zhou S, Chen S, Wu Y, Cui Y. Efficacy and safety of mTOR inhibitors (rapamycin and its analogues) for tuberous sclerosis complex: a meta-analysis. *Orphanet J Rare Dis* 2019; **14**: 39 [PMID: 30760308 DOI: 10.1186/s13023-019-1012-x]

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MINIREVIEWS

Risk of hepatitis B virus reactivation in patients with autoimmune diseases undergoing non-tumor necrosis factor-targeted biologics

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Abstract

Hepatitis B virus reactivation (HBVr) can occur in patients treated with immunosuppressive medications. Risk stratification for HBVr based on hepatitis B virus (HBV) serology and viral load is an important strategy to determine appropriate HBV monitoring and antiviral prophylaxis use. Recent advances in the understanding of pathophysiology of autoimmune diseases have led the development of cytokine-targeted therapies. Tumor necrosis factor (TNF)-α inhibitors have been widely used for patients with inflammatory bowel disease, psoriasis, and rheumatic diseases. Further, the clinical benefits of interleukin (IL)-12/23, IL-17, or Janus kinases inhibitors have been demonstrated in these patients. It is well known that TNF-α inhibitor use can lead to HBVr, however, the risk of HBVr in patients undergoing non-TNF-targeted biologics have not been fully understood. In this review, we discuss the risk of HBVr in patients treated with non-TNF-targeted biologics, and immunological mechanisms of these medications causing HBVr.

Key Words: Hepatitis B virus; Autoimmune diseases; Biological therapy; Interleukin-23; Interleukin-17; Janus kinases

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Core Tip: Although the risk of hepatitis B virus reactivation (HBVr) in patients undergoing non-tumor necrosis factor (TNF)-targeted biologics have not been fully understood, some previous studies showed that the risk of HBVr in patients with non-TNF-targeted biologics might be higher than that in patients with TNF-α inhibitors. While patients with chronic hepatitis B virus (HBV) should receive antiviral

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prophylaxis when they start non-TNF-targeted biologics, antiviral prophylaxis may be a favorable strategy rather than the pre-emptive strategy in patients with resolved HBV. Large-scale studies are needed to ascertain the differential risk of HBVr between patients with TNF-α inhibitors and non-TNF-targeted biologics.

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INTRODUCTION

Hepatitis B virus reactivation (HBVr) can occur in patients treated with immunosuppressive therapy and chemotherapy. In the current era of biologics, physicians need to understand the risk of HBVr in patients with autoimmune diseases undergoing anti-cytokine therapies.

The following three components are important for the development of HBVr: (1) The host immune response; (2) The covalently closed circular DNA of the viral genome of HBV (cccDNA); and (3) The use of immunosuppressive drugs[1]. HBV infection induces a series of innate[2] and adaptive[3] immune responses[1]. The host immune responses against hepatitis B virus (HBV) infection recruit adaptive cytotoxic T (Tc) cells to induce both cytolytic-dependent and -independent antiviral effects. In the cytolytic-independent effect, interferons (IFN) play an important role to suppress the HBV replication. To produce neutralizing antibodies to clear circulating HBV, B cells are also recruited to limit the viral spread of HBV (Figure 1)[4]. However, even when clinical resolution of HBV infection is achieved, it does not mean complete elimination of HBV-DNA because cccDNA can persist in the nucleus of hepatocytes and it can be a source of HBVr when immunosuppressive medications are used.

Tumor necrosis factor (TNF)-α is a key cytokine not only in the pathogenesis of autoimmune diseases but also in the host immune reactions against HBV infection. TNF-α is synthesized by macrophages and T cells and induce the production of a variety of inflammatory cytokines, suppressing viral replication[5]. TNF-α is also necessary for the proliferation of HBV-specific Tc cells that are essential for suppression of HBV replication[6]. Hence, TNF-α inhibitors (*e.g.*, infliximab, adalimumab, and etanercept) can inhibit the anti-HBV immune response, leading to HBV replication[7]. Indeed, the pooled prevalence of HBVr in patients with autoimmune diseases undergoing TNF-α inhibitors was reported to be 4.2% (95%CI: 1.4%-8.2%)[8].

The signaling pathways involving interleukin (IL)-12/23, IL-17, and Janus kinases (JAKs) have been highlighted as novel specific therapeutic targets for autoimmune diseases. A recent multicenter observational study for patients with psoriasis showed that HBVr was significantly more common among patients receiving anti-TNF-α therapies than IL-17 inhibitors[9]. However, there is still limited data in understanding the risk of HBVr in patients who are treated with biologics which inhibit such specific inflammatory pathways. In the present article, we aimed to review previous literatures which assessed the risk of HBVr in patients treated with non-TNF-targeted biologics and discuss how each medication can influence the development of HBVr.

DEFINITIONS OF HBV INFECTION AND REACTIVATION

The professional societies in the United States [American Association for the Study of Liver Diseases (AASLD); American Gastroenterological Association (AGA)], Europe [European Association for the Study of the Liver (EASL)] and Asia [Asian Pacific Association for the Study of the Liver (APASL)] have published guidelines to assist providers with HBVr management[10-13]. In this review article, we divide patients into 2 risk groups which is consistent with the professional society guidelines[10-13] when assessing practical management of HBVr.

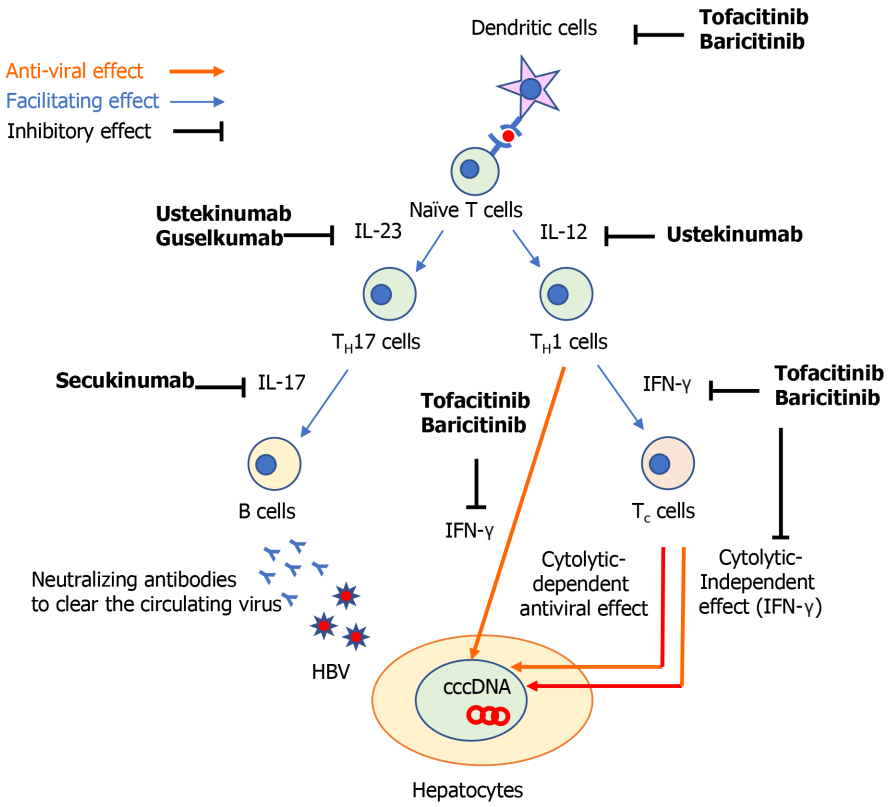


Figure 1 The possible immunological mechanism to explain how non-tumor necrosis factor-targeted biologics can induce the development of hepatitis B reactivation. cccDNA: Covalently closed circular DNA; HBV: Hepatitis B virus; IFN: Interferon; IL: Interleukin; T_H17 cells: IL-17 producing T helper cells; T_H1 cells: T helper 1 cells; Tc cells: Cytotoxic T cells.

Chronic HBV

Chronic HBV (CHB) [*i.e.*, Hepatitis B surface antigen (HBsAg)-positive and antibody to hepatitis B core antigen (anti-HBc)-positive] which includes patients with chronic active [serum HBV DNA ≥ 2000 IU/mL and normal or elevated serum alanine transaminase (ALT)] or inactive (serum HBV DNA < 2000 IU/mL and normal ALT) HBV infection.

Resolved HBV

Resolved HBV (*i.e.*, HBsAg-negative and anti-HBc-positive). Of note, there is insufficient evidence to support the use of anti-HBs titers as a decision aid when making a recommendation regarding prophylaxis[11].

There are subtle differences in the definition of HBVr among the professional society guidelines, however, the general concept is the same[10-13]. In patients with CHB, HBVr is defined by a rise in HBV DNA above baseline. In patients with resolved HBV, HBVr is defined by either the appearance of HBV DNA in the blood or conversion to the HBsAg+ state (*i.e.*, seroreversion). The heterogeneity observed in HBVr definition is also reflected in the existing studies on HBVr. The majority of studies included the following parameters: (1) An acute rise in HBV-DNA levels compared with baseline; (2) Elevated levels of serum aminotransferases; and (3) Seroreversion[1]. In this review article, we followed the criteria of HBVr described in each article.

THE MANAGEMENT OF PATIENTS WITH HBV INFECTION UNDERGOING IMMUNOSUPPRESSION

Risk stratification

Patients with CHB have an increased risk of HBVr when undergoing immunosuppressive therapy compared to patients with resolved HBV. For example, among patients treated with TNF-α inhibitors there is an estimated 5-fold increased risk of

HBVr in patients with CHB compared to patients with resolved HBV (15.4% *vs* 3.0% risk of HBVr)[8]. Further, another study showed that the pooled rate of HBVr without antiviral prophylaxis was 15.6% (95%CI: 2.3-35.7) in patients with CHB who were treated with TNF-α inhibitors. In patients with resolved HBV, the pooled rates of HBVr without antiviral prophylaxis in patients who were treated with TNF-α inhibitors and non-TNF-targeted biologics were 1.4% (95%CI: 0.5%-2.6%) and 6.1% (95%CI: 0.0%-16.6%), respectively[14]. Each of the 4 professional societal guidelines recommend testing HBV serology on all candidates for immunosuppressive therapy or chemotherapy to enable appropriate risk stratification (*i.e.* CHB *vs* resolved HBV)[10-13].

Next, the degree of expected iatrogenic immunosuppression should be assessed. Hematopoietic stem cell transplant (HSCT) recipients and B cell–depleting therapies (*e.g.*, rituximab) are high-potency regimens and confer the highest risk of HBVr[10-13]. The AGA guidelines ascertain that anthracyclines (*e.g.*, doxorubicin) and moderate- to high-dose corticosteroids (CS) (*i.e.*, ≥ 10 mg of daily prednisone or equivalent for ≥ 4 wk) confer higher risk than other immunosuppressants[11].

Therapeutic prophylaxis

CHB: In general, the professional societal guidelines recommend HBV prophylaxis, typically entecavir or tenofovir, for all candidates for immunosuppression who have CHB, apart from patients treated with traditional immunosuppressive agents (*e.g.*, thiopurines, methotrexate), intra-articular CSs, or oral CSs ≤ 1 wk[10-13]. The AGA risk stratify this cohort of patients into moderate (1%-10%) and high risk (> 10%) groups for HBVr[11]. Antiviral prophylaxis should be started before and continued after cessation of immunosuppression, generally 12 to 18 mo if high-potency therapies are used and 6 to 12 mo for other therapies[10-13].

Resolved HBV: Guidelines largely agree that resolved HBV patients on high-potency immunosuppression (HSCT recipients and B cell–depleting therapies) should receive HBVr prophylaxis, with the AGA placing this group of patients in the high risk HBVr group (> 10%)[10-13].

For resolved HBV patients not on a high-potency regimen, the guidelines are more dissimilar. AGA recommend prophylaxis for resolved HBV patients at moderate risk (1%-10%) of HBVr, which include patients treated with TNF-α inhibitors, other cytokine or integrin inhibitors, tyrosine kinase inhibitors, moderate- or high-dose CSs for ≥ 4 wk and anthracycline derivatives. In contrast, AASLD, EASL and APASL recommend a pre-emptive therapy for this patient cohort, not prophylaxis, whereby serial lab monitoring (HBV DNA, HBsAg) is performed at 1- to 3-mo intervals on therapy and up to 12 mo after cessation of immunosuppression with on-demand antiviral therapy if needed[10,12,13]. Given that HBsAg seroreversion can lead to fatal acute hepatitis, antiviral therapy should be started immediately, independently of ALT level[11]. Of note, both EASL and APASL recommend treating resolved HBV patients similarly to HBsAg-positive patients if baseline serum HBV-DNA is positive[12,13].

AGA classify resolved HBV patients who are treated with traditional immunosuppressive agents (*e.g.*, thiopurines, methotrexate), low-dose CSs ≥ 4 wk, intra-articular CSs, or any dose of oral CSs for ≤ 1 wk, as low-risk (< 1%) for HBVr and do not recommend prophylaxis, similar to the other society guidelines[10-13].

The risk of HBVr in patients who are treated with non-TNF-targeted biologics

Given the paucity of data on the HBVr risk among patients treated with non-TNF-targeted biologics, we reviewed the existing literature on the risk of HBVr in patients with autoimmune diseases who received non-TNF-targeted biologics and summarized the findings in Tables 1-3. A majority of articles focused on patients with CHB or resolved infection. According to the AGA guideline, CHB and resolved HBV patients treated with non-TNF-targeted therapies are categorized into the moderate-risk HBVr group and therapeutic prophylaxis is recommended. However, AASLD, EASL and APASL recommend serial monitoring among resolved HBV (if HBV DNA is negative) with pre-emptive prophylaxis if HBVr is observed. Therefore, it is important to determine the precise HBVr with non-TNF-targeted biologics to ascertain if a strategy of monitoring/pre-emptive may be too lax, and perhaps a uniform strategy of prophylaxis may be more optimal as recommended by the AGA. Given that patients with resolved infection should be treated similarly to those with CHB patients if their serum HBV-DNA tests are positive at baseline[12,13], we present their baseline HBV-DNA in Tables 1-3.

Table 1 The risk of hepatitis B virus reactivation in patients treated with interleukin-12/23 or interleukin-23 inhibitors

Ref.	Number of HBV patients	HBV status	Disease	Drugs	Prophylaxis	Follow-up	HBV reactivation
Ting *et al*[27], 2018	54	(1) 10 CHB; and (2) 44 resolved HBV. HBV-DNA at baseline (-)	Psoriasis	Ustekinumab	Yes: 2 patients with CHB	24 mo	(1) 2 patients with CHB without prophylaxis. (no hepatitis); and (2) 1 patient with resolved HBV (mild hepatitis)
Solay *et al*[5], 2018	29	29 resolved HBV. HBV-DNA at baseline (-)	Psoriasis/HS/AS/RA/CD	Ustekinumab (*n* = 7)	NA	22 wk	1 patient with psoriasis without prophylaxis (no data regarding hepatitis)
Sanz-Bueno *et al*[68], 2015	20	20 resolved HBV. HBV-DNA at baseline (-) but viral load was assessed in 7 of 20 patients	Psoriasis	Ustekinumab (*n* = 6)	No	40 mo	0
Chiu *et al*[28], 2013	14	(1) 11 CHB; and (2) 3 resolved HBV. HBV-DNA at baseline was not available	Psoriasis	Ustekinumab	Yes: 4 patients with CHB	10 mo	(1) 2 patients with CHB without prophylaxis (No hepatitis); and (2) 0
Navarro *et al*[69], 2013	5	5 CHB	Psoriasis	Ustekinumab (*n* = 1)	Yes	25 mo	0
Hayashi *et al*[70], 2014	5	5 resolved HBV. HBV-DNA at baseline was not available	Psoriasis	Ustekinumab	No	52 wk	0
Koskinas *et al*[41], 2013	1	Resolved HBV. HBV-DNA at baseline was not available	Psoriasis	Ustekinumab	No	16 mo	1 with hepatitis (ALT 65 IU/mL)
Steglich *et al*[71], 2014	1	Resolved HBV. HBV-DNA at baseline (-)	Psoriasis	Ustekinumab	Yes	36 mo	0
Duncan *et al*[43], 2019	1	Resolved HBV. HBV-DNA at baseline was not available	Palmoplantar Psoriasis	Guselkumab	No	12 mo	0

If a study included both of patients with chronic hepatitis B virus (HBV) and those with resolved HBV, we labeled the former with (1) and the latter with (2) in the column with HBV status. HBV: Hepatitis B virus; AS: Ankylosing spondylitis; CD: Crohn's disease; CHB: Chronic HBV; HS: Hidradenitis suppurativa; RA: Rheumatoid arthritis; NA: Not available; ALT: Alanine transaminase.

IL-12/23 INHIBITORS

Mechanism of HBV reactivation

The cytokine IL-12 contributes to the differentiation of naïve T cells to T helper 1 (T_H1) cells and IL-23 maintains and expand IL-17 producing T helper (T_H17) cells (Figure 1) [15]. These two cytokines play a central role to regulate T cell-mediated immune responses, which are dysregulated in various autoimmune diseases including psoriasis and Crohn's disease (CD)[15,16]. The clinical benefit of IL-12 and IL-23 inhibition has been demonstrated in psoriasis, CD, and ulcerative colitis by ustekinumab[17-19], which is an antibody against p40, the common subunit of IL-12 and IL-23. IL-12 plays an important role in achieving sustained control of HBV replication. IL-12 can promote cell-mediated immunity by facilitating the production of IFN-γ production by T_H1 cells, resulting in the inhibition of HBV replication[20,21] and the induction of antiviral effects of HBV-specific Tc cells[22,23]. Indeed, patients with CHB who were treated with recombinant human IL-12 exhibited a high proportion of HBV clearance in a dose-dependent manner[24] and the addition of IL-12 to lamivudine enhanced T cell reactivity to HBV and IFN-γ production[25]. Furthermore, patients with CHB responding to IFN-α treatment were shown to have higher IL-12 and IFN-γ expression levels during the treatment[26]. These findings suggest that ustekinumab might theoretically increase the risk of HBVr.

Table 2 The risk of hepatitis B virus reactivation in patients with interleukin-17 inhibitors

Ref.	Number of HBV patients	HBV status	Disease	Drugs	Prophylaxis	Follow-up	HBV reactivation
Chiu *et al*[52], 2018	49	(1) 25 CHB; and (2) 24 resolved HBV. HBV-DNA at baseline (-) in 11 patients with resolved HBV	Psoriasis	Secukinumab	Yes: 3 patients with CHB	3 mo	(1) 6 patients with CHB without prophylaxis. (no hepatitis); and (2) 1 patient with resolved HBV with positive viral load at baseline (no hepatitis)
Moneva-Leniz *et al*[72], 2020	4	(1) 2 CHB; and (2) 2 resolved HBV. HBV-DNA at baseline (-)	Psoriasis/palmoplantar psoriasis	Secukinumab	Yes: 1 patient with CHB and 1 patient with resolved HBV	20 mo	(1) 0; and (2) 0
Feaster *et al*[73], 2018	1	A carrier of congenital HBV infection[1]	Psoriasis and PsA	Secukinumab	No	24 mo	0
Bevans *et al*[74], 2018	1	Seropositive hepatitis[1]	Palmoplantar psoriasis and AS	Secukinumab	No	14 mo	0
Yanagihara *et al*[75], 2017	1	CHB	Psoriasis vulgaris	Secukinumab	Yes	9 mo	0
Peccerillo *et al*[76], 2018	1	Resolved HBV. HBV-DNA at baseline (-)	Psoriasis	Secukinumab	Yes	14 mo	0
Koike *et al*[53], 2019	1	CHB	Psoriasis and PsA	Ixekizumab	Yes	18 mo	0
Lora *et al*[54], 2019	1	Resolved HBV. HBV-DNA at baseline (-)	Psoriasis	Ixekizumab	Yes	12 mo	0

[1]A diagnosis in an article was used due to lack of data regarding hepatitis B virus (HBV) serology and viral load. If a study included both of patients with chronic HBV and those with resolved HBV, we labeled the former with (1) and the latter with (2) in the column with HBV status. HBV: Hepatitis B virus; AS: Ankylosing spondylitis; CHB: Chronic HBV; PsA: Psoriatic arthritis.

Table 3 The risk of hepatitis B virus reactivation in patients with Janus kinase inhibitors

Ref.	Number of HBV patients	Serology for HBV infectious	Disease	Drugs	Prophylaxis	Follow-up	HBV reactivation
Chen *et al*[66], 2018	81	(1) 6 CHB; and (2) 75 resolved HBV. HBV-DNA at baseline (-) but viral load was assessed in 53 patients with resolved HBV	RA	Tofacitinib	Yes: 2 patients with CHB	3-6 mo	(1) 2 patients with CHB without prophylaxis (1 patient developed hepatitis); and (2) 0
Serling-Boyd *et al*[67], 2021	8	8 resolved HBV. HBV-DNA was assessed in 6 patients, but viral loads were not available	7 RA, 1 PsA	Tofacitinib	Yes: 2 patients	3.1 yr	0
Harigai *et al*[62], 2020	215	215 resolved HBV. HBV-DNA (-) at baseline in 30 patients with resolved HBV who had detectable post-baseline HBV-DNA	RA	Baricitinib	NA	2.7 yr	8 patients with resolved HBV had HBV-DNA ≥ 29 IU/mL (4 patients med the criteria of HBVr in this study, no hepatitis)

If a study included both of patients with chronic hepatitis B virus (HBV) and those with resolved HBV, we labeled the former with (1) and the latter with (2) in the column with HBV status. HBV: Hepatitis B virus; CHB: Chronic HBV; PsA: Psoriatic arthritis; RA: Rheumatoid arthritis; NA: Not available; HBVr: Hepatitis B virus reactivation.

Clinical studies in patients with autoimmune diseases

Several studies have assessed the risk of HBVr in patients treated with ustekinumab (Table 1). A retrospective study showed that no HBVr occurred among 2 patients with CHB taking antiviral prophylaxis after starting ustekinumab, whereas, 2 patients (25%) developed HBVr without hepatitis among 8 patients with CHB without antiviral prophylaxis[27]. Another study demonstrated a 29% rate of HBVr after ustekinumab initiation without antiviral prophylaxis in patients with CHB[28]. Given that patients

with CHB have a high risk of HBVr without antiviral prophylaxis, these patients require antiviral prophylaxis and appropriate monitoring for HBV-DNA and serology tests after initiating ustekinumab treatment.

A retrospective study on 44 patients with resolved HBV who initiated ustekinumab without antiviral prophylaxis found that 1 patient (2.3%) developed HBVr complicated with mild hepatitis[27]. This patient discontinued concurrent methotrexate when reactivation occurred and HBV-DNA became undetectable without antiviral therapy in 6 mo. In another study involving 7 patients with resolved HBV on ustekinuamb, 1 patient (14.3%) developed HBVr. This patient was not on antiviral prophylaxis and started entecavir for treatment of HBVr[5]. These data suggest that there is a certain risk of HBVr in patients with resolved HBV even without detectable HBV-DNA at baseline after starting ustekinumab, suggesting that these patients might need antiviral prophylaxis as is the AGA guidelines preferred option. While the guidelines of AASLD, EASL, APASL recommend pre-emptive therapy if HBV DNA is negative, further studies are warranted in order to understand if these patients require antiviral prophylaxis.

IL-23 INHIBITORS

Mechanism of HBV reactivation

IL-23-specific antagonists, such as tildrakizumab[29,30], risankizumab[31,32], guselkumab[33,34], and brazikumab[35], have been shown to be effective for psoriasis and CD. These medications bind to the p19 subunit on IL-23 and inhibit its interaction with the IL-23 receptors[36]. The potential mechanism of HBVr in patients treated with IL-23 inhibitors is still unclear. Previous studies found that T_H17 cells, which are expanded by IL-23 (Figure 1), increase with the severity of liver damage in patients with CHB[37-39]. An observational, clinical-controlled study also demonstrated that the expression levels of IL-23 and IL-17 were associated with increased possibilities of hepatitis B e-antigen (HBeAg) clearance and HBsAg decline in patients with HBeAg-positive CHB during pegylated IFN therapy[40]. This study also found that high serum IL-23 Levels can predict the response to IFN therapy in patients with HBeAg-positive CHB[40]. Given that T_H17 cells promote the differentiation and function of B cells[41,42], IL-23 might activate the humoral immune response against circulating HBV and play a role to facilitate HBV clearance by IFN therapy (Figure 1). Although this hypothesis suggest that IL-23 inhibitors may abrogate the HBV clearance, it still remains to be elucidated whether these medications contribute to the development of HBVr.

Clinical studies in patients with autoimmune diseases

The data regarding the safety of IL-23 inhibitors in patients with HBV infection is limited. A case report showed that a patient with resolved HBV infection did not develop HBVr 1 year after starting guselkumab (Table 1)[43]. There have been no reported studies focusing on the risk of HBVr in patients treated with other IL-23 inhibitors.

IL-17 INHIBITORS

Mechanism of HBV reactivation

IL-17 is a major effector cytokine of T_H17 cells and mediate host defense mechanisms[44]. Inhibition of IL-17 with secukinumab, ixekizumab, and brodalumab have demonstrated clinical benefits in patients with psoriasis[45-47], psoriatic arthritis[48], and ankylosing spondylitis[49]. T_H17/IL-17 axis is involved in the process of fibrogenesis and increases the expression of proinflammatory cytokines, promoting the recruitment of inflammatory cells in patients with CHB[50]. A previous study showed that Th17 cells were significantly increased in patients with CHB, as well as the expression level of IL-17[51]. They also demonstrated that the suppression of viral replication induced by IFN-α resulted in a decrease in T_H17 cells and IL-17 expression, suggesting that T_H17 cells might play an important role during IFN-α treatment to eliminate HBV[51]. As we described above, T_H17 cells also facilitate B cells[41,42] and would enhance the humoral response to clear circulating HBV (Figure 1). These findings implicate that T_H17/IL-17 axis might be associated with HBV clearance and its inhibition may increase the risk of HBVr.

Clinical studies in patients with autoimmune diseases

A prospective multicenter study on 22 patients with CHB with no antiviral prophylaxis after starting secukinumab showed that 6 patients (27.3%) developed HBVr (Table 2)[52]. Three patients with HBVr started antiviral treatments and their viral loads decreased rapidly within 3 mo. The remaining three patients with HBVr were followed without antiviral drugs and their viral loads remained low without acute hepatitis. Notably, none of the 3 patients with CHB who received antiviral prophylaxis developed HBVr[52]. Hence, this study reinforced the importance of antiviral prophylaxis in patients with CHB starting treatment with IL-17 inhibitors. This study also included 24 patients with resolved HBV who did not receive antiviral prophylaxis and identified one patient (4.2%) with a positive viral load at baseline who developed HBVr without acute hepatitis[52]. This study re-affirmed the EASL and APASL guidelines which recommend antiviral prophylaxis in patients with resolved HBV if their baseline viral loads are positive.

A case report on a patient with CHB treated with ixekizumab and entecavir simultaneously did not develop HBVr after 18 mo of treatment[53]. Another report showed that a patient with resolved HBV did not experience HBVr during follow-up (Table 2)[54]. Given that data regarding the risk of HBVr in patients treated with ixekizumab or brodalumab are still limited, further studies with larger sample sizes are warranted.

JAK INHIBITORS

Mechanism of HBV reactivation

JAKs bind to type I and II cytokine receptors and transmit extracellular cytokine signals to activate various signal transducers and activators of transcription, which drive the proinflammatory machinery of the cellular immune response[55]. The clinical benefit of JAK inhibitors has been demonstrated in patients with rheumatoid arthritis[56,57], psoriatic arthritis[58,59], and ulcerative colitis[60,61]. Important signaling pathways in host-defense include innate antiviral responses *via* IFN-α/β mediated by JAK1-tyrosine kinase 2 complexes, and IFN-γ mediated by JAK1-JAK2 complexes[55]. Hence, JAK inhibitors might counteract the suppressive effects of IFN on viral replication[62,63]. Further, dendritic cells and effective T cell lineages including T_H cells and Tc cells play important roles to defense against HBV-infection (Figure 1)[64]. A previous study demonstrated that a JAK inhibitor can block the differentiation and function of dendritic cells, leading to impaired T cell activation (Figure 1)[65]. Thus, it was suggested that JAK inhibitors might negatively interact with the defense mechanism against HBV infection. Further studies investigating how JAK inhibitors influence the development of HBVr are warranted.

Clinical studies in patients with autoimmune diseases

A retrospective cohort study including 6 patients with CHB showed that 2 out of 4 patients (50%) without antiviral prophylaxis developed HBVr after starting tofacitinib. One patient had an elevated ALT level and started entecavir, resulting in declines in HBV-DNA and ALT levels. Another patient started entecavir and did not develop acute hepatitis. Both patients continued tofacitinib after the development of HBVr. Meanwhile, 2 patients with CHB who received antiviral prophylaxis did not develop HBVr after initiating tofacitinib. Further, in this study, none of 75 patients with resolved HBV received antiviral prophylaxis and no HBVr was observed in this group[66]. Another study also demonstrated that patients with resolved HBV did not develop HBVr after starting tofacitinib (Table 3)[67].

A study assessing data which was integrated from four phase 3 trials of baricitinib in patients showed that, among 215 patients with resolved HBV, 8 patients (3.7%) had a single quantifiable result of HBV-DNA viral load (HBV-DNA level ≥ 29 IU/mL) after initiating baricitinib. Among these 8 patients, 4 patients met the definition of HBVr (HBV-DNA ≥ 100 IU/mL), but no patients developed hepatitis. HBV-DNA at baseline was assessed in 6 patients and all examined patients did not have detectable HBV-DNA level. Antiviral therapy was not used in 5 of 8 patients[62].

All these findings suggest that patients with CHB should receive antiviral prophylaxis when they start JAK inhibitors. As for patients with resolved HBV infection, given that HBVr was occasionally reported even if their HBV-DNA levels were not detected at baseline, an appropriate consultation with hepatologists is necessary. There has been limited data regarding the risk of HBVr in patients with autoimmune diseases who are treated with other JAK inhibitors (*e.g.*, upadacitinib,

filgotinib, peficitinib).

CONCLUSION

In summary, considering antiviral prophylaxis with an appropriate risk stratification is necessary when we start non-TNF-targeted biologics for patients with autoimmune diseases. The frequencies of HBVr without antiviral prophylaxis in patients with CHB on IL-12/23, IL-17, and JAK inhibitors are up to 29%, 27%, and 50%, respectively. A meta-analysis demonstrated that the pooled rate of HBVr without antiviral prophylaxis was 15.6% (95%CI: 2.3-35.7) in patients with CHB who were treated with TNF-α inhibitors, suggesting that non-TNF-targeted biologics, particularly JAK inhibitors, may have a higher risk of HBVr compared with TNF-α inhibitors. Given that no patients who received antiviral prophylaxis developed HBVr, HBVr is preventable with antiviral therapy in patients with CHB on non-TNF-targeted biologics. As all of professional societies recommended in their guidelines, patients with CHB should receive antiviral prophylaxis when they start non-TNF-targeted biologics. In patients with resolved HBV, the rates of HBVr without antiviral prophylaxis in patients on IL-12/23, IL-17, and JAK inhibitors are up to 2.3%, 4.2%, and 0%, respectively. The meta-analysis showed that the pooled rates of HBVr without antiviral prophylaxis in patients who were treated with TNF-α inhibitors and non-TNF-targeted biologics were 1.4% (95%CI: 0.5%-2.6%) and 6.1% (95%CI: 0.0%-16.6%), respectively[14]. These data supported that the risk of HBVr in patients treated with non-TNF-targeted biologics might be higher than that in patients with TNF-α inhibitors even if their HBV status is resolved HBV. According to the AGA guideline, patients with resolved HBV who are treated with non-TNF-targeted biologics are categorized into the moderate risk group and antiviral prophylaxis are recommended for this patient cohort[11]. However, as stated previously, AASLD, EASL and APASL recommend the pre-emptive therapeutic strategy for this cohort, although APASL and EASL do include the caveat of potentially using HBV DNA assessment to aid decision-making[10,12,13]. Given the higher risk of HBVr with non-TNF-targeted biologics compared with TNF-α inhibitors, antiviral prophylaxis may be a favorable strategy rather than the pre-emptive strategy to prevent HBVr in patients with resolved HBV. Large-scale studies are needed to ascertain the differential risk of HBVr between patients with TNF-α inhibitors and non-TNF-targeted biologics and to stratify the risk of HBVr by the type of non-TNF-targeted biologics. While HBsAg seroreversion can lead to fatal acute hepatitis, a consultation with hepatologists or infectious disease specialists is recommended.

REFERENCES

1 **Gentile G**, Antonelli G. HBV Reactivation in Patients Undergoing Hematopoietic Stem Cell Transplantation: A Narrative Review. *Viruses* 2019; **11** [PMID: 31717647 DOI: 10.3390/v11111049]

2 **Maini MK**, Gehring AJ. The role of innate immunity in the immunopathology and treatment of HBV infection. *J Hepatol* 2016; **64**: S60-S70 [PMID: 27084038 DOI: 10.1016/j.jhep.2016.01.028]

3 **Bertoletti A**, Ferrari C. Adaptive immunity in HBV infection. *J Hepatol* 2016; **64**: S71-S83 [PMID: 27084039 DOI: 10.1016/j.jhep.2016.01.026]

4 **Guidotti LG**, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006; **1**: 23-61 [PMID: 18039107 DOI: 10.1146/annurev.pathol.1.110304.100230]

5 **Solay AH**, Acar A, Eser F, Kuşcu F, Tütüncü EE, Kul G, Şentürk GÇ, Gürbüz Y. Reactivation rates in patients using biological agents, with resolved HBV infection or isolated anti-HBc IgG positivity. *Turk J Gastroenterol* 2018; **29**: 561-565 [PMID: 30260778 DOI: 10.5152/tjg.2018.18032]

6 **Kasahara S**, Ando K, Saito K, Sekikawa K, Ito H, Ishikawa T, Ohnishi H, Seishima M, Kakumu S, Moriwaki H. Lack of tumor necrosis factor alpha induces impaired proliferation of hepatitis B virus-specific cytotoxic T lymphocytes. *J Virol* 2003; **77**: 2469-2476 [PMID: 12551985 DOI: 10.1128/jvi.77.4.2469-2476.2003]

7 **Manzano-Alonso ML**, Castellano-Tortajada G. Reactivation of hepatitis B virus infection after cytotoxic chemotherapy or immunosuppressive therapy. *World J Gastroenterol* 2011; **17**: 1531-1537 [PMID: 21472116 DOI: 10.3748/wjg.v17.i12.1531]

8 **Cantini F**, Boccia S, Goletti D, Iannone F, Leoncini E, Panic N, Prignano F, Gaeta GB. HBV Reactivation in Patients Treated with Antitumor Necrosis Factor-Alpha (TNF-α) Agents for Rheumatic and Dermatologic Conditions: A Systematic Review and Meta-Analysis. *Int J Rheumatol* 2014; **2014**: 926836 [PMID: 25114684 DOI: 10.1155/2014/926836]

9 **Chiu HY**, Chiu YM, Chang Liao NF, Chi CC, Tsai TF, Hsieh CY, Hsieh TY, Lai KL, Chiu TM, Wu NL, Hui RC, Lee CN, Wang TS, Chen PH, Yang CC, Huang YH. Predictors of hepatitis B and C

virus reactivation in patients with psoriasis treated with biological agent: A nine-year multicenter cohort study. *J Am Acad Dermatol* 2019 [PMID: 31821860 DOI: 10.1016/j.jaad.2019.12.001]

10 **Terrault NA**, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM, Brown RS Jr, Bzowej NH, Wong JB. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. *Hepatology* 2018; **67**: 1560-1599 [PMID: 29405329 DOI: 10.1002/hep.29800]

11 **Reddy KR**, Beavers KL, Hammond SP, Lim JK, Falck-Ytter YT; American Gastroenterological Association Institute. American Gastroenterological Association Institute guideline on the prevention and treatment of hepatitis B virus reactivation during immunosuppressive drug therapy. *Gastroenterology* 2015; **148**: 215-9; quiz e16 [PMID: 25447850 DOI: 10.1053/j.gastro.2014.10.039]

12 **European Association for the Study of the Liver**. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]

13 **Sarin SK**, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS, Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int* 2016; **10**: 1-98 [PMID: 26563120 DOI: 10.1007/s12072-015-9675-4]

14 **Lin TC**, Yoshida K, Tedeschi SK, de Abreu MM, Hashemi N, Solomon DH. Risk of Hepatitis B Virus Reactivation in Patients With Inflammatory Arthritis Receiving Disease-Modifying Antirheumatic Drugs: A Systematic Review and Meta-Analysis. *Arthritis Care Res (Hoboken)* 2018; **70**: 724-731 [PMID: 28834412 DOI: 10.1002/acr.23346]

15 **Yen D**, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA, Rennick D. IL-23 is essential for T cell-mediated colitis and promotes inflammation *via* IL-17 and IL-6. *J Clin Invest* 2006; **116**: 1310-1316 [PMID: 16670770 DOI: 10.1172/JCI21404]

16 **Lee E**, Trepicchio WL, Oestreicher JL, Pittman D, Wang F, Chamian F, Dhodapkar M, Krueger JG. Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *J Exp Med* 2004; **199**: 125-130 [PMID: 14707118 DOI: 10.1084/jem.20030451]

17 **Papp KA**, Langley RG, Lebwohl M, Krueger GG, Szapary P, Yeilding N, Guzzo C, Hsu MC, Wang Y, Li S, Dooley LT, Reich K; PHOENIX 2 study investigators. Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 52-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 2). *Lancet* 2008; **371**: 1675-1684 [PMID: 18486740 DOI: 10.1016/S0140-6736(08)60726-6]

18 **Sandborn WJ**, Gasink C, Gao LL, Blank MA, Johanns J, Guzzo C, Sands BE, Hanauer SB, Targan S, Rutgeerts P, Ghosh S, de Villiers WJ, Panaccione R, Greenberg G, Schreiber S, Lichtiger S, Feagan BG; CERTIFI Study Group. Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *N Engl J Med* 2012; **367**: 1519-1528 [PMID: 23075178 DOI: 10.1056/NEJMoa1203572]

19 **Sands BE**, Sandborn WJ, Panaccione R, O'Brien CD, Zhang H, Johanns J, Adedokun OJ, Li K, Peyrin-Biroulet L, Van Assche G, Danese S, Targan S, Abreu MT, Hisamatsu T, Szapary P, Marano C; UNIFI Study Group. Ustekinumab as Induction and Maintenance Therapy for Ulcerative Colitis. *N Engl J Med* 2019; **381**: 1201-1214 [PMID: 31553833 DOI: 10.1056/NEJMoa1900750]

20 **Zeuzem S**, Carreño V. Interleukin-12 in the treatment of chronic hepatitis B and C. *Antiviral Res* 2001; **52**: 181-188 [PMID: 11672828 DOI: 10.1016/s0166-3542(01)00183-8]

21 **Cavanaugh VJ**, Guidotti LG, Chisari FV. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. *J Virol* 1997; **71**: 3236-3243 [PMID: 9060687 DOI: 10.1128/JVI.71.4.3236-3243.1997]

22 **Guidotti LG**. The role of cytotoxic T cells and cytokines in the control of hepatitis B virus infection. *Vaccine* 2002; **20** Suppl 4: A80-A82 [PMID: 12477433 DOI: 10.1016/s0264-410x(02)00392-4]

23 **McClary H**, Koch R, Chisari FV, Guidotti LG. Relative sensitivity of hepatitis B virus and other hepatotropic viruses to the antiviral effects of cytokines. *J Virol* 2000; **74**: 2255-2264 [PMID: 10666256 DOI: 10.1128/jvi.74.5.2255-2264.2000]

24 **Carreño V**, Zeuzem S, Hopf U, Marcellin P, Cooksley WG, Fevery J, Diago M, Reddy R, Peters M, Rittweger K, Rakhit A, Pardo M. A phase I/II study of recombinant human interleukin-12 in patients with chronic hepatitis B. *J Hepatol* 2000; **32**: 317-324 [PMID: 10707873 DOI: 10.1016/s0168-8278(00)80078-1]

25 **Rigopoulou EI**, Suri D, Chokshi S, Mullerova I, Rice S, Tedder RS, Williams R, Naoumov NV. Lamivudine plus interleukin-12 combination therapy in chronic hepatitis B: antiviral and immunological activity. *Hepatology* 2005; **42**: 1028-1036 [PMID: 16250037 DOI: 10.1002/hep.20888]

26 **Rossol S**, Marinos G, Carucci P, Singer MV, Williams R, Naoumov NV. Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. *J Clin Invest* 1997; **99**: 3025-3033 [PMID: 9185527 DOI: 10.1172/JCI119498]

27 **Ting SW**, Chen YC, Huang YH. Risk of Hepatitis B Reactivation in Patients with Psoriasis on Ustekinumab. *Clin Drug Investig* 2018; **38**: 873-880 [PMID: 29968197 DOI: 10.1007/s40261-018-0671-z]

28 **Chiu HY**, Chen CH, Wu MS, Cheng YP, Tsai TF. The safety profile of ustekinumab in the treatment of patients with psoriasis and concurrent hepatitis B or C. *Br J Dermatol* 2013; **169**: 1295-1303

[PMID: 23746170 DOI: 10.1111/bjd.12461]
29 **Reich K**, Papp KA, Blauvelt A, Tyring SK, Sinclair R, Thaçi D, Nograles K, Mehta A, Cichanowitz N, Li Q, Liu K, La Rosa C, Green S, Kimball AB. Tildrakizumab *vs* placebo or etanercept for chronic plaque psoriasis (reSURFACE 1 and reSURFACE 2): results from two randomised controlled, phase 3 trials. *Lancet* 2017; **390**: 276-288 [PMID: 28596043 DOI: 10.1016/S0140-6736(17)31279-5]
30 **Papp K**, Thaçi D, Reich K, Riedl E, Langley RG, Krueger JG, Gottlieb AB, Nakagawa H, Bowman EP, Mehta A, Li Q, Zhou Y, Shames R. Tildrakizumab (MK-3222), an anti-interleukin-23p19 monoclonal antibody, improves psoriasis in a phase IIb randomized placebo-controlled trial. *Br J Dermatol* 2015; **173**: 930-939 [PMID: 26042589 DOI: 10.1111/bjd.13932]
31 **Gordon KB**, Strober B, Lebwohl M, Augustin M, Blauvelt A, Poulin Y, Papp KA, Sofen H, Puig L, Foley P, Ohtsuki M, Flack M, Geng Z, Gu Y, Valdes JM, Thompson EHZ, Bachelez H. Efficacy and safety of risankizumab in moderate-to-severe plaque psoriasis (UltIMMa-1 and UltIMMa-2): results from two double-blind, randomised, placebo-controlled and ustekinumab-controlled phase 3 trials. *Lancet* 2018; **392**: 650-661 [PMID: 30097359 DOI: 10.1016/S0140-6736(18)31713-6]
32 **Feagan BG**, Sandborn WJ, D'Haens G, Panés J, Kaser A, Ferrante M, Louis E, Franchimont D, Dewit O, Seidler U, Kim KJ, Neurath MF, Schreiber S, Scholl P, Pamulapati C, Lalovic B, Visvanathan S, Padula SJ, Herichova I, Soaita A, Hall DB, Böcher WO. Induction therapy with the selective interleukin-23 inhibitor risankizumab in patients with moderate-to-severe Crohn's disease: a randomised, double-blind, placebo-controlled phase 2 study. *Lancet* 2017; **389**: 1699-1709 [PMID: 28411872 DOI: 10.1016/S0140-6736(17)30570-6]
33 **Gordon KB**, Duffin KC, Bissonnette R, Prinz JC, Wasfi Y, Li S, Shen YK, Szapary P, Randazzo B, Reich K. A Phase 2 Trial of Guselkumab *vs* Adalimumab for Plaque Psoriasis. *N Engl J Med* 2015; **373**: 136-144 [PMID: 26154787 DOI: 10.1056/NEJMoa1501646]
34 **Reich K**, Armstrong AW, Foley P, Song M, Wasfi Y, Randazzo B, Li S, Shen YK, Gordon KB. Efficacy and safety of guselkumab, an anti-interleukin-23 monoclonal antibody, compared with adalimumab for the treatment of patients with moderate to severe psoriasis with randomized withdrawal and retreatment: Results from the phase III, double-blind, placebo- and active comparator-controlled VOYAGE 2 trial. *J Am Acad Dermatol* 2017; **76**: 418-431 [PMID: 28057361 DOI: 10.1016/j.jaad.2016.11.042]
35 **Sands BE**, Chen J, Feagan BG, Penney M, Rees WA, Danese S, Higgins PDR, Newbold P, Faggioni R, Patra K, Li J, Klekotka P, Morehouse C, Pulkstenis E, Drappa J, van der Merwe R, Gasser RA Jr. Efficacy and Safety of MEDI2070, an Antibody Against Interleukin 23, in Patients With Moderate to Severe Crohn's Disease: A Phase 2a Study. *Gastroenterology* 2017; **153**: 77-86. e6 [PMID: 28390867 DOI: 10.1053/j.gastro.2017.03.049]
36 **Frieder J**, Kivelevitch D, Haugh I, Watson I, Menter A. Anti-IL-23 and Anti-IL-17 Biologic Agents for the Treatment of Immune-Mediated Inflammatory Conditions. *Clin Pharmacol Ther* 2018; **103**: 88-101 [PMID: 28960267 DOI: 10.1002/cpt.893]
37 **Zhang JY**, Zhang Z, Lin F, Zou ZS, Xu RN, Jin L, Fu JL, Shi F, Shi M, Wang HF, Wang FS. Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. *Hepatology* 2010; **51**: 81-91 [PMID: 19842207 DOI: 10.1002/hep.23273]
38 **Xia L**, Tian D, Huang W, Zhu H, Wang J, Zhang Y, Hu H, Nie Y, Fan D, Wu K. Upregulation of IL-23 expression in patients with chronic hepatitis B is mediated by the HBx/ERK/NF-κB pathway. *J Immunol* 2012; **188**: 753-764 [PMID: 22174449 DOI: 10.4049/jimmunol.1101652]
39 **Ge J**, Wang K, Meng QH, Qi ZX, Meng FL, Fan YC. Implication of Th17 and Th1 cells in patients with chronic active hepatitis B. *J Clin Immunol* 2010; **30**: 60-67 [PMID: 19756987 DOI: 10.1007/s10875-009-9328-2]
40 **Yu C**, Gong X, Yang Q, Lian J, Xu K, Ruan B, Li L. The serum IL-23 Level predicts the response to pegylated interferon therapy in patients with chronic hepatitis B. *Liver Int* 2015; **35**: 1549-1556 [PMID: 25312687 DOI: 10.1111/liv.12701]
41 **Koskinas J**, Tampaki M, Doumba PP, Rallis E. Hepatitis B virus reactivation during therapy with ustekinumab for psoriasis in a hepatitis B surface-antigen-negative anti-HBs-positive patient. *Br J Dermatol* 2013; **168**: 679-680 [PMID: 23121260 DOI: 10.1111/bjd.12120]
42 **Mitsdoerffer M**, Lee Y, Jäger A, Kim HJ, Korn T, Kolls JK, Cantor H, Bettelli E, Kuchroo VK. Proinflammatory T helper type 17 cells are effective B-cell helpers. *Proc Natl Acad Sci USA* 2010; **107**: 14292-14297 [PMID: 20660725 DOI: 10.1073/pnas.1009234107]
43 **Duncan JR**, Orlowski TJ, Elewski BE. Safety of guselkumab in hepatitis B virus infection. *Dermatol Online J* 2019; **25** [PMID: 31735016]
44 **Curtis MM**, Way SS. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. *Immunology* 2009; **126**: 177-185 [PMID: 19125888 DOI: 10.1111/j.1365-2567.2008.03017.x]
45 **Langley RG**, Elewski BE, Lebwohl M, Reich K, Griffiths CE, Papp K, Puig L, Nakagawa H, Spelman L, Sigurgeirsson B, Rivas E, Tsai TF, Wasel N, Tyring S, Salko T, Hampele I, Notter M, Karpov A, Helou S, Papavassilis C; ERASURE Study Group; FIXTURE Study Group. Secukinumab in plaque psoriasis--results of two phase 3 trials. *N Engl J Med* 2014; **371**: 326-338 [PMID: 25007392 DOI: 10.1056/NEJMoa1314258]
46 **Gordon KB**, Blauvelt A, Papp KA, Langley RG, Luger T, Ohtsuki M, Reich K, Amato D, Ball SG, Braun DK, Cameron GS, Erickson J, Konrad RJ, Muram TM, Nickoloff BJ, Osuntokun OO, Secrest RJ, Zhao F, Mallbris L, Leonardi CL; UNCOVER-1 Study Group; UNCOVER-2 Study Group; UNCOVER-3 Study Group. Phase 3 Trials of Ixekizumab in Moderate-to-Severe Plaque Psoriasis. *N*

Engl J Med 2016; **375**: 345-356 [PMID: 27299809 DOI: 10.1056/NEJMoa1512711]

47 **Lebwohl M**, Strober B, Menter A, Gordon K, Weglowska J, Puig L, Papp K, Spelman L, Toth D, Kerdel F, Armstrong AW, Stingl G, Kimball AB, Bachelez H, Wu JJ, Crowley J, Langley RG, Blicharski T, Paul C, Lacour JP, Tyring S, Kircik L, Chimenti S, Callis Duffin K, Bagel J, Koo J, Aras G, Li J, Song W, Milmont CE, Shi Y, Erondu N, Klekotka P, Kotzin B, Nirula A. Phase 3 Studies Comparing Brodalumab with Ustekinumab in Psoriasis. *N Engl J Med* 2015; **373**: 1318-1328 [PMID: 26422722 DOI: 10.1056/NEJMoa1503824]

48 **Nash P**, Kirkham B, Okada M, Rahman P, Combe B, Burmester GR, Adams DH, Kerr L, Lee C, Shuler CL, Genovese M; SPIRIT-P2 Study Group. Ixekizumab for the treatment of patients with active psoriatic arthritis and an inadequate response to tumour necrosis factor inhibitors: results from the 24-week randomised, double-blind, placebo-controlled period of the SPIRIT-P2 phase 3 trial. *Lancet* 2017; **389**: 2317-2327 [PMID: 28551073 DOI: 10.1016/S0140-6736(17)31429-0]

49 **Baeten D**, Sieper J, Braun J, Baraliakos X, Dougados M, Emery P, Deodhar A, Porter B, Martin R, Andersson M, Mpofu S, Richards HB; MEASURE 1 Study Group; MEASURE 2 Study Group. Secukinumab, an Interleukin-17A Inhibitor, in Ankylosing Spondylitis. *N Engl J Med* 2015; **373**: 2534-2548 [PMID: 26699169 DOI: 10.1056/NEJMoa1505066]

50 **Paquissi FC**. Immunity and Fibrogenesis: The Role of Th17/IL-17 Axis in HBV and HCV-induced Chronic Hepatitis and Progression to Cirrhosis. *Front Immunol* 2017; **8**: 1195 [PMID: 29033929 DOI: 10.3389/fimmu.2017.01195]

51 **Feng H**, Yin J, Han YP, Zhou XY, Chen S, Yang L, Yan JR, Zhang GX. Sustained Changes of Treg and Th17 Cells During Interferon-α Therapy in Patients with Chronic Hepatitis B. *Viral Immunol* 2015; **28**: 412-417 [PMID: 26266573 DOI: 10.1089/vim.2015.0024]

52 **Chiu HY**, Hui RC, Huang YH, Huang RY, Chen KL, Tsai YC, Lai PJ, Wang TS, Tsai TF. Safety Profile of Secukinumab in Treatment of Patients with Psoriasis and Concurrent Hepatitis B or C: A Multicentric Prospective Cohort Study. *Acta Derm Venereol* 2018; **98**: 829-834 [PMID: 29972221 DOI: 10.2340/00015555-2989]

53 **Koike Y**, Fujiki Y, Higuchi M, Fukuchi R, Kuwatsuka S, Murota H. An interleukin-17 inhibitor successfully treated a complicated psoriasis and psoriatic arthritis patient with hepatitis B virus infection and end-stage kidney disease on hemodialysis. *JAAD Case Rep* 2019; **5**: 150-152 [PMID: 30733983 DOI: 10.1016/j.jdcr.2018.11.016]

54 **Lora V**, Graceffa D, De Felice C, Morrone A, Bonifati C. Treatment of severe psoriasis with ixekizumab in a liver transplant recipient with concomitant hepatitis B virus infection. *Dermatol Ther* 2019; **32**: e12909 [PMID: 30964590 DOI: 10.1111/dth.12909]

55 **Winthrop KL**. The emerging safety profile of JAK inhibitors in rheumatic disease. *Nat Rev Rheumatol* 2017; **13**: 234-243 [PMID: 28250461 DOI: 10.1038/nrrheum.2017.23]

56 **Burmester GR**, Blanco R, Charles-Schoeman C, Wollenhaupt J, Zerbini C, Benda B, Gruben D, Wallenstein G, Krishnaswami S, Zwillich SH, Koncz T, Soma K, Bradley J, Mebus C; ORAL Step investigators. Tofacitinib (CP-690,550) in combination with methotrexate in patients with active rheumatoid arthritis with an inadequate response to tumour necrosis factor inhibitors: a randomised phase 3 trial. *Lancet* 2013; **381**: 451-460 [PMID: 23294500 DOI: 10.1016/S0140-6736(12)61424-X]

57 **Kremer J**, Li ZG, Hall S, Fleischmann R, Genovese M, Martin-Mola E, Isaacs JD, Gruben D, Wallenstein G, Krishnaswami S, Zwillich SH, Koncz T, Riese R, Bradley J. Tofacitinib in combination with nonbiologic disease-modifying antirheumatic drugs in patients with active rheumatoid arthritis: a randomized trial. *Ann Intern Med* 2013; **159**: 253-261 [PMID: 24026258 DOI: 10.7326/0003-4819-159-4-201308200-00006]

58 **Gladman D**, Rigby W, Azevedo VF, Behrens F, Blanco R, Kaszuba A, Kudlacz E, Wang C, Menon S, Hendrikx T, Kanik KS. Tofacitinib for Psoriatic Arthritis in Patients with an Inadequate Response to TNF Inhibitors. *N Engl J Med* 2017; **377**: 1525-1536 [PMID: 29045207 DOI: 10.1056/NEJMoa1615977]

59 **Mease P**, Hall S, FitzGerald O, van der Heijde D, Merola JF, Avila-Zapata F, Cieślak D, Graham D, Wang C, Menon S, Hendrikx T, Kanik KS. Tofacitinib or Adalimumab *vs* Placebo for Psoriatic Arthritis. *N Engl J Med* 2017; **377**: 1537-1550 [PMID: 29045212 DOI: 10.1056/NEJMoa1615975]

60 **Sandborn WJ**, Su C, Sands BE, D'Haens GR, Vermeire S, Schreiber S, Danese S, Feagan BG, Reinisch W, Niezychowski W, Friedman G, Lawendy N, Yu D, Woodworth D, Mukherjee A, Zhang H, Healey P, Panés J; OCTAVE Induction 1, OCTAVE Induction 2, and OCTAVE Sustain Investigators. Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis. *N Engl J Med* 2017; **376**: 1723-1736 [PMID: 28467869 DOI: 10.1056/NEJMoa1606910]

61 **Sandborn WJ**, Ghosh S, Panes J, Vranic I, Su C, Rousell S, Niezychowski W; Study A3921063 Investigators. Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. *N Engl J Med* 2012; **367**: 616-624 [PMID: 22894574 DOI: 10.1056/NEJMoa1112168]

62 **Harigai M**, Winthrop K, Takeuchi T, Hsieh TY, Chen YM, Smolen JS, Burmester G, Walls C, Wu WS, Dickson C, Liao R, Genovese MC. Evaluation of hepatitis B virus in clinical trials of baricitinib in rheumatoid arthritis. *RMD Open* 2020; **6** [PMID: 32098857 DOI: 10.1136/rmdopen-2019-001095]

63 **Kirito K**, Sakamoto M, Enomoto N. Elevation of the Hepatitis B Virus DNA during the Treatment of Polycythemia Vera with the JAK Kinase Inhibitor Ruxolitinib. *Intern Med* 2016; **55**: 1341-1344 [PMID: 27181544 DOI: 10.2169/internalmedicine.55.5529]

64 **Rehermann B**, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 2005; **5**: 215-229 [PMID: 15738952 DOI: 10.1038/nri1573]

65 **Heine A**, Held SA, Daecke SN, Wallner S, Yajnanarayana SP, Kurts C, Wolf D, Brossart P. The

JAK-inhibitor ruxolitinib impairs dendritic cell function *in vitro* and in vivo. *Blood* 2013; **122**: 1192-1202 [PMID: 23770777 DOI: 10.1182/blood-2013-03-484642]

66 **Chen YM**, Huang WN, Wu YD, Lin CT, Chen YH, Chen DY, Hsieh TY. Reactivation of hepatitis B virus infection in patients with rheumatoid arthritis receiving tofacitinib: a real-world study. *Ann Rheum Dis* 2018; **77**: 780-782 [PMID: 28663308 DOI: 10.1136/annrheumdis-2017-211322]

67 **Serling-Boyd N**, Mohareb AM, Kim AY, Hyle EP, Wallace ZS. The use of tocilizumab and tofacitinib in patients with resolved hepatitis B infection: a case series. *Ann Rheum Dis* 2021; **80**: 274-276 [PMID: 32732241 DOI: 10.1136/annrheumdis-2020-218289]

68 **Sanz-Bueno J**, Vanaclocha F, García-Doval I, Torrado R, Carretero G, Daudén E, Patricia Ruiz-Genao D, Alsina-Gibert MM, Pérez-Zafrilla B, Pérez-Rial G, Rivera R; members of the BIOBADADERM group. Risk of Reactivation of Hepatitis B Virus Infection in Psoriasis Patients Treated With Biologics: A Retrospective Analysis of 20 Cases From the BIOBADADERM Database. *Actas Dermosifiliogr* 2015; **106**: 477-482 [PMID: 25776200 DOI: 10.1016/j.ad.2015.01.010]

69 **Navarro R**, Vilarrasa E, Herranz P, Puig L, Bordas X, Carrascosa JM, Taberner R, Ferrán M, García-Bustinduy M, Romero-Maté A, Pedragosa R, García-Diez A, Daudén E. Safety and effectiveness of ustekinumab and antitumour necrosis factor therapy in patients with psoriasis and chronic viral hepatitis B or C: a retrospective, multicentre study in a clinical setting. *Br J Dermatol* 2013; **168**: 609-616 [PMID: 22985451 DOI: 10.1111/bjd.12045]

70 **Hayashi M**, Umezawa Y, Fukuchi O, Ito T, Saeki H, Nakagawa H. Efficacy and safety of ustekinumab treatment in elderly patients with psoriasis. *J Dermatol* 2014; **41**: 974-980 [PMID: 25346301 DOI: 10.1111/1346-8138.12653]

71 **Steglich RB**, Meneghello LP, Carvalho AV, Cheinquer H, Muller FM, Reginatto FP. The use of ustekinumab in a patient with severe psoriasis and positive HBV serology. *An Bras Dermatol* 2014; **89**: 652-654 [PMID: 25054756 DOI: 10.1590/abd1806-4841.20143013]

72 **Moneva-Leniz LM**, Sahuquillo-Torralba A, Vila-Payeras A, Mateu-Puchades A. Risk of Hepatitis B Virus Reactivation in Patients on Secukinumab for Psoriasis: A Series of 4 Cases. *Actas Dermosifiliogr* 2020; **111**: 613-614 [PMID: 32589963 DOI: 10.1016/j.ad.2019.02.022]

73 **Feaster B**, Cline A, Feldman SR. Secukinumab for psoriasis in a patient with hepatitis B. *Dermatol Online J* 2018; **24** [PMID: 30677838]

74 **Bevans SL**, Mayo TT, Elewski BE. Safety of secukinumab in hepatitis B virus. *J Eur Acad Dermatol Venereol* 2018; **32**: e120-e121 [PMID: 28960490 DOI: 10.1111/jdv.14608]

75 **Yanagihara S**, Sugita K, Yoshida Y, Tsuruta D, Yamamoto O. Psoriasis vulgaris in a hepatitis B virus carrier successfully treated with secukinumab and entecavir combination therapy. *Eur J Dermatol* 2017; **27**: 185-186 [PMID: 27965188 DOI: 10.1684/ejd.2016.2939]

76 **Peccerillo F**, Odorici G, Pellacani G, Conti A. Secukinumab: A positive outcome in a patient with severe psoriasis and HBV-HCV co-infection. *Dermatol Ther* 2018; **31**: e12601 [PMID: 29633448 DOI: 10.1111/dth.12601]

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MINIREVIEWS

Burden of venous thromboembolism in patients with pancreatic cancer

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Abstract

Pancreatic cancer (PC) is a devastating malignancy with fewer than 10% of patients being alive at 5 years after diagnosis. Venous thromboembolism (VTE) occurs in approximatively 20% of patients with PC, resulting in increased morbidity, mortality and significant health care costs. The management of VTE is particularly challenging in these frail patients. Adequate selection of the most appropriate anticoagulant for each individual patient according to the current international guidelines is warranted for overcoming treatment challenges. The International Initiative on Thrombosis and Cancer multi-language web-based mobile application (downloadable for free at www.itaccme.com) has been developed to help clinicians in decision making in the most complex situations. In this narrative review, we will discuss the contemporary epidemiology and burden of VTE in PC patients, the performances and limitations of current risk assessment models to predict the risk of VTE, as well as evidence from recent clinical trials for the primary prophylaxis and treatment of cancer-associated VTE that support updated clinical practice guidelines.

Key Words: Pancreatic cancer; Venous thromboembolism; Low-molecular weight heparin; Direct oral anticoagulant; Multi-language mobile application; Risk-assessment models; Thromboprophylaxis

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Core Tip: Venous thromboembolism (VTE) is a common but potentially life-threatening complication in patients with Pancreatic cancer (PC). There is an urgent need to raise awareness on this underrecognized issue. This review discusses the incidence and risk factors of VTE in PC patients, and the results from recent clinical trials for the primary prophylaxis and treatment of VTE in cancer patients supporting the most recent clinical practice guidelines.

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INTRODUCTION

Pancreatic cancer (PC) is a devastating disease with fewer than 10% of patients being alive at 5 years[1]. Its prevalence continues to increase worldwide[2]. In most cases, there is no effective treatment. Given its dismal prognosis[3], there is an urgent need to improve patient quality of life by integrating best supportive care[4,5].

Venous thromboembolism (VTE) is a frequent but still underrecognized complication in PC patients[6]. According to a recent large population-based cohort study[7], the incidence of PC-associated VTE has doubled from 1997 to 2017, due to increase in PC prevalence, improved survival, advanced age of PC patients, and better detection of incidental VTE with the routine use of computed tomography scans. Primary thromboprophylaxis is a supportive care with a well-documented clinical benefit, which remains unfortunately underused nowadays. Since 2013, the International Initiative on Thrombosis and Cancer (ITAC), a multidisciplinary group of experts from across the globe committed to improve the management of patients with cancer-associated thrombosis through dissemination of educational initiatives to health professionals, strives to raise awareness on this important issue[8].

Anticoagulation therapy is the mainstay of the VTE prevention and treatment, but its management is particularly challenging for the treating physicians in these patients who already suffer from multiple co-morbidities such as renal failure, hepatic failure, thrombocytopenia, and who undergo complex cancer treatment protocols[9,10].

Herein, we discuss the most recent data on the incidence and risk factors of VTE in PC patients, as well as evidence from recent clinical trials of low-molecular-weight heparin (LMWH) and direct oral anticoagulants (DOAC) for the primary prophylaxis and treatment of cancer-associated VTE that support current clinical practices guidelines (CPGs)[8,11,12].

EPIDEMIOLOGY OF VTE IN PC AND IMPACT ON SURVIVAL

Cancer has been demonstrated to be an independent major risk factor for VTE[13]. The extent of this risk mainly depends on cancer type and disease stage. Among all cancer types, PC carries the highest risk for VTE[7,14]. In retrospective cohorts of PC patients, the reported incidence of VTE varies broadly from 5% to 57%[15-33], depending on the study population, the duration of follow-up, the definition of VTE and the methods used for diagnosing VTE.

Due to their large sample sizes, multicenter prospective design, and systematic follow-up, phase 3 randomized control trials (RCTs) conducted in PC patients are expected to provide reliable data on the true incidence of VTE. However, a recent systematic review and meta-analysis of chemotherapeutic and thromboprophylaxis RCTs conducted in PC patients highlighted that VTE events were underreported in chemotherapeutic RCTs[6]. The pooled rate of VTE in chemotherapy studies (n = 13, 5694 patients) was 5.9% [95% confidence interval (CI): 3.9-9.0; I^2 = 94%] and significantly lower than the corresponding 16.5% (95%CI: 11.7-23.3; P < 0.001) reported in thromboprophylaxis studies (n = 9, 631 patients, I^2 = 69%). Importantly, 30 eligible chemotherapy RCTs (n = 9000 patients) were excluded from this meta-analysis because they did not report VTE as adverse events[6], which reveals quite clearly a lack of awareness on the burden of VTE among oncologists.

The incidence and risk factors for VTE was recently assessed in a large prospective multicenter cohort of patients with newly diagnosed PC[34], providing real-life contemporary estimates. In this study, 152 out of 731 (20.79%) patients developed a VTE event, with a median time from PC diagnosis to VTE of 4 mo. In competing-risk analysis, the cumulative rates of VTE were approximatively 13% and 20% at 6 mo and 1 year, respectively[34].

The most common VTE events occurring in PC patients are deep vein thrombosis (DVT) and pulmonary embolism (PE)[35], but incidental PE and incidental visceral vein thrombosis (VVT) are increasingly diagnosed, accounting now for approximatively 50% of all reported VTE events[23,30,34,36]. In the BACAP-VTE study[34], DVT, PE, VVT, and combined events were observed in 26%, 17%, 30% and 21% of patients, respectively. Overall, 46% of VTE events were symptomatic and 54% of them were asymptomatic[34].

Early retrospective studies reported no association between VTE and overall survival (OS) in PC patients[21,27]. However, all patients included in these studies had metastatic disease with a short life expectancy. By contrast, later studies reported that the onset of VTE was associated with a poorer prognosis. In a retrospective cohort of 227 patients with unresectable PC, VTE during the course of chemotherapy was associated with a 2.5-fold decrease in progression-free survival (PFS) and a 1.6-fold risk decrease in OS[17]. Similarly, in a small cohort of 135 PC patients, the onset of VTE was significantly associated with increased mortality[23]. Importantly, survival was significantly improved in patients with VTE receiving anticoagulant therapy compared to those who did not receive anticoagulant therapy [hazard ratio (HR) 0.30, 95%CI: 0.12-0.74, $P = 0.009$][23]. Retrospective studies focusing on incidental VTE in PC patients also reported an association between VVT and mortality[36,37]. Similarly, in a prospective cohort of 731 newly diagnosed PC, patients who developed asymptomatic or symptomatic VTE during follow-up had significantly shorter PFS (HR 1.74; 95%CI: 1.19-2.54; $P = 0.004$) and OS (HR 2.02; 95%CI: 1.57-2.60; $P < 0.001$) compared to those who did not developed VTE[34].

RISK FACTORS FOR VTE AND RISK STRATIFICATION IN PC PATIENTS

Several studies have demonstrated that the most important risk factor for VTE in PC patients is the presence of a metastatic disease[16,18,27,31,34,38,39]. In a recent retrospective cohort of 165 PC patients, metastatic disease was associated with a 4.8-fold increase in the risk for VTE; 41 out of 51 patients who developed VTE had metastasis at diagnosis[39]. Similarly, in the BACAP-VTE study[34], metastatic tumors were associated with a 2.5-fold increased risk for VTE compared to non-metastatic tumors.

Major abdominal surgery is also an important risk factor for VTE in PC patients. In an early observational study of 1915 patients with exocrine PC, 127 out of 383 (33.1%) patients requiring pancreatic surgery developed postsurgical VTE[22]. Similarly, 31 out of 209 (14.8%) patients requiring pancreatic surgery developed postsurgical VTE in a large retrospective study of 1,115 conducted in East Asian population[27].

Chemotherapy increases the risk of VTE in cancer patients[40]. Nevertheless, as recently highlighted by Chiasakul *et al*[6], the rates of VTE were underreported in PC chemotherapy RCTs and data on the respective risk of various chemotherapy regimens remain scarce. In recent retrospective or prospective cohorts of PC patients, the rate of VTE did not differ between those receiving gemcitabine-based chemotherapy and those receiving FOLFIRINOX[30,34]. In the subgroup of 273 PC patients included in the CASSINI trial[41], the rates of VTE did not differ between patients treated with 5-fluorouracil-based regimen *vs* gemcitabine-based regimen.

Systematic screening of VTE is not recommended in daily clinical practice. However, all PC patients should receive verbal and written information on the risk factors for VTE, as well as on the signs and symptoms of VTE to promote self-diagnosis and reporting of VTE symptoms.

Over the past ten years, many efforts have been made to develop risk assessment models (RAM) aiming to select cancer patients at highest risk for VTE, and therefore expected to have the best benefit from thromboprophylaxis. However, none of these RAM was designed to specifically assess this risk in PC patients.

The Caprini score is the most widely RAM to assess the risk of VTE in patients undergoing surgery. It has been validated in several types of cancers[42]. However, this model was unable to identify patients at highest risk for VTE in a retrospective cohort of 426 PC patients undergoing preoperative treatment followed by surgical

resection[43].

Furthermore, the Khorana score[44] is the most widely used RAM to assess the risk of VTE in ambulatory cancer patients. It was developed ten years ago[44]. It assigns 1 to 2 points to 5 simple clinical and laboratory variables (primary tumor site, platelet count ≥ 350 × 10^9/L, hemoglobin concentration ≤ 10 g/dL or use of erythropoiesis-stimulating agents, leukocyte count ≥ 11 × 10^9/L, body mass index (BMI) ≥ 35 kg/m^2. Patients are classified as being at "low-risk" (Khorana score = 0), "intermediate-risk" (Khorana score = 1-2), or "high-risk" (Khorana score ≥ 3). All PC patients are classified as being at intermediate- or high-risk. Unfortunately, this model did not discriminate between these two risk categories, neither in retrospective studies of PC patients undergoing chemotherapy[25,28-30,32,39,45], nor in the large prospective BACAP-VTE study[34], nor in the subgroup of 273 PC patients included in the recent CASSINI trial[41] (Table 1), questioning its relevance in this specific population.

Several modifications to this RAM by the addition of other variables to the model have been proposed. The PROTECHT score[46], which includes treatment with cisplatin or carboplatin-based chemotherapy or gemcitabine was found to perform better than the Khorana score in a retrospective analysis of the PROTECHT study, decreasing the number needed to treat (NTT) from 50 to 17. However, this score has not been externally validated in PC patients. More recently, the ONKOTEV score[47] was developed in a prospective cohort of 843 various cancers patients in Italy and Germany, including 253 patient with gastroenteric cancer. The ONKOTEV score assigns one point to four variables, namely: a Khorana score > 2, a history of previous VTE, a metastatic disease, and a compression of vascular structures by the tumor. The ONKOTEV score demonstrated a significantly higher predictive power compared to the Khorana score in the original development cohort and was recently externally validated in a retrospective single-center cohort of 165 PC patients treated in Portugal with promising results[39]. Ninety-two (55.8%) patients had a metastatic disease at diagnosis and 109 (66.1%) received systemic chemotherapy. At inclusion, 18.2% of patients had an ONKOTEV score of 0, 38.2% of patients had an ONKOTEV score of 1, 33.3% of patients had an ONKOTEV score of 2, and 10.3% of patients had an ONKOTEV score > 2. During a median observation period of 6.3 mo, 51 out 165 (30.9%) PC patients developed VTE. The cumulative incidence of VTE was 82.4% in patients with an ONKOTEV > 2 compared to 3.3% in those with an ONKOTEV score of 0[39]. These results suggest that the ONKOTEV score could be of help to better stratify PC patients having the highest risk for VTE but deserve further confirmation in prospective cohorts of ambulatory PC patients.

Integration of relevant biomarkers into current RAMs might improve their ability to predict VTE. Faille *et al*[38] recently assessed the diagnosis performances of several biomarkers to predict VTE in a prospective cohort of 50 PC patients, including Factor VIII, D-dimers, von Willebrand factor, free tissue factor pathway inhibitor, microvesicle-tissue factor (MV-TF) activity and CA 19.9. In multivariate analysis, baseline D-dimers ≥ 2.16 μg/mL (HR 4.9; 95%CI: 1.0-23.1), baseline MV-TF activity 2.37 pg/mL (HR 10.5; 95%CI: 1.5-72.4), and baseline CA 19.9 ≥ 2153 U/mL (HR 9.5; 95%CI: 1.5-60.2) were significantly associated with VTE after adjustment for age and sex, with the best sensitivity and specificity in predicting VTE obtained for CA 19-9[38]. However, these associations were no more significant after adjustment for the presence of metastasis, suggesting once again that the presence of a metastatic disease is the most important risk factor for VTE in PC patients.

The clinical-genetic Thrombo inCode-Oncology (TiC-Onco) score was developed in a prospective cohort of 391 ambulatory patients with various cancers initiating systemic chemotherapy, including 72 (18.5%) patients with PC[48]. Seventy-one out of 391 (18%) patients developed VTE within 6 mo. The prespecified variable selection process selected both clinical variables (tumor site, family history of VTE, BMI ≥ 25 kg/m^2) and genetic variables (germline polymorphisms in the *F5*, *F13* and *SERPINA10* genes) for inclusion in the score. In the derivation cohort, the TiC-Onco score performed better than the Khorana score in predicting VTE at 6 mo (sensitivity 49% *vs* 22%, specificity 81% *vs* 82%, positive predictive value 37% *vs* 22%, and negative predictive value 88% *vs* 82%)[48]. Importantly, patients suffering from PC had higher rates of VTE (40%) than patients with other type of cancers (18%), suggesting that PC has a major impact on the accuracy of the TiC-Onco score. However, this model has not yet been externally validated in a cohort of PC patients.

The CATS/MICA score[49] includes two variables, namely: tumour-site risk category (very high *vs* high and high *vs* low or intermediate) and continuous D-dimer levels. It was developed in the prospective Vienna Cancer and Thrombosis Study (CATS) cohort of 1423 ambulatory cancer patients undergoing chemotherapy, including 118 (8%) patients with PC[49]. During a median follow-up of 6 mo, 80 out of

Table 1 Studies assessing the predictive values of risk assessment models in pancreatic cancer patients

Ref.	Study design	Country	Patients analyzed/included	VTE screening at study entry	RAMs	Number of patients in each group	Study or median observation period	Patients with VTE during the overall follow-up, *n* (%)	Rates of VTE
Pelzer *et al*[45], 2013	Retrospective analysis of theCONKO-004 RCT	Germany	144/312, APC included in the CONKO-004 trial (control arm)	No	Khorana score	Intermediate risk: 55/144 (38.2%); High risk: 89/144 (61.8%)	12 mo	21/144 (14.6%)	At 6 mo: Intermediate risk: 4/55 (7.2%); High risk: 17/89 (19.1%)
Muñoz Martín *et al*[25], 2014	Retrospective	Spain	73/84, ambulatory PC patientsreceiving chemotherapy	No	Khorana score	Intermediate risk: 36/84 (43%); High risk: 48/84 (57%)	2008-2011	30/84 (35.7%)	At 6 mo: Intermediate risk: 4/37 (10.8%); High risk: 10/36 (27.8%)
van Es *et al*[29], 2017	Retrospective	Netherlands	147/178, ambulatory PC patientsstarting chemotherapy	No	Khorana score	Intermediate risk: 101/147 (69%); High risk: 46/147 (31%)	2003-2014	20/147(13.6%)	At 6 mo: Intermediate risk: 9/101 (8.9%); High risk: 4/46 (8.7%)
Kruger *et al*[28], 2017	Retrospective	Germany	111/172, APC patients undergoing palliative chemotherapy	No	Khorana score	Intermediate risk: 69/111 (38%); High risk: 42/111 (62%)	2002-2012	16/111 (14.4%)	At 6 mo: Intermediate risk: 6/69 (8.6%)High risk: 5/42 (11.9%); During the overall observation period; Intermediate risk: 8/69 (11.6%); High risk: 8/42 (19.0%); $P = 0.4$
Berger *et al*[30], 2017	Retrospective	Germany	150, PC patients receiving chemotherapy	No	Khorana score	Intermediate risk: 87/150 (58%); High risk: 63/150 (42%)	2010-2014	37/150 (24.7%)	Unspecified; During the overall observation period: no difference between groups ($P = 0.44$)
Godinho *et al*[39], 2020	Retrospective	Portugal	165 newly diagnosed PC patients	No	Khorana score; Onkotev score	Khorana score: Intermediate risk: 106/165 (64%); High risk: 59/165 (36%). Onkotev score: Score 0: 30/165 (18.2%); Score 1: 63/165 (38.2%); Score 2: 55/165 (33.3%); Score ≥ 3: 17/165 (10.3%)	6.3 mo	51/165 (31%)	During the overall observation period: Khorana score: Intermediate risk: 28/106 (26.4%); High risk: 23/59 (38.9%). Onkotev score: Score 0: 1/30 (< 10%); Score 1: 8/63 (< 10%); Score 2: 28/55 (41.8%); Score ≥ 3: 14/17 (70.6%)
Kim *et al*[32], 2018	Retrospective	Korea	216 metastatic PC patients receiving palliative chemotherapy	No	Khorana score	Intermediate risk: 135/216 (62.5%); High risk: 81/21 (37.5%)	2005-2015	50/216 (23.1%)	During the overall observation period: Intermediate risk: 30/135

									(22.2%); High risk: 20/81 (24.7%); *P* = 0.677
Frere *et al*[34], 2020	Prospective	France	675 newly diagnosed PC patients	Yes, patients excluded if VTE at diagnosis	Khorana score	Intermediate risk: 492/675 (73%); High risk: 183/675 (27%)	2014-2019; 19.3 mo	141/675 (20.8%)	During the total follow-up: Intermediate risk: 108/492 (22%); High risk: 33/183 (18%); *P* = 0.26
Vadhan-Raj *et al*[41], 2020	Retrospective subgroup analysis of the CASSINI RCT	International	138 PC patients undergoing chemotherapy included in the CASSINI trial (control arm)	Yes, patients excluded if VTE at diagnosis	Khorana score	Intermediate risk: 100/138 (72.5%); High risk: 38/138 (27.5%)	6 mo	18/138 (13.0%)	At 6 mo: Intermediate risk: 14/100 (14.0%); High risk: 4/38 (10.5%)

APC: Advanced Pancreatic cancer; PC: Pancreatic cancer; RAM: Risk assessment model; VTE: Venous thromboembolism.

1423 patients (6%) developed VTE. In the CATS cohort, the C-index of the model was 0.66 (95%CI: 0.63-0.67) compared to 0.61 (95%CI: 0.51-0.70) for the Khorana score[49]. The score was then validated in the prospective Multinational Cohort Study to Identify Cancer Patients at High Risk of Venous Thromboembolism (MICA) cohort (n = 832), including 116 (14%) patients with PC[49]. Using this RAM, all PC patients are classified at intermediate or high risk of VTE. Of note, the CATS/MICA score has not yet been externally validated in a cohort of PC patients.

Finally, machine learning methods are increasingly used for the development of prediction models. Two recent studies conducted in various cancer patients[50] or in ovarian cancer patients[51] have demonstrated that such models could improve the prediction of VTE compared to conventional methods.

WHEN SHOULD WE CONSIDER PRIMARY PROPHYLAXIS IN PC PATIENTS?

Surgical PC patients

Prolonged thromboprophylaxis following major abdominal surgery has been shown to decrease the rate of VTE by approximately 50%[52]. Accordingly, all current CPGs recommend using thromboprophylaxis in surgical PC patients[8,11]. In those undergoing laparotomy or laparoscopic surgery without contraindications to LMWH, the highest LMWH prophylactic dose should be used for an extended duration of 4 wk (Grade 1A)[8]. External compression devices alone should be used only in patients with contraindications to anticoagulants (Grade 2B)[8]. Inferior vena cava filters should not be used systematically in this setting (Grade 1A)[8]. The risks of VTE should be balanced by the competing risk of bleeding. Numerous factors such as advanced or metastatic disease, older age, anemia, thrombocytopenia, renal impairment, liver dysfunction, and concomitant anticancer therapies may potentiate the overall bleeding risk and should be taken into account. The careful evaluation of each individual profile is warranted for overcoming management challenges.

Hospitalized PC patients

Acute medical illness and bed rest constitute transient factors increasing the risk of VTE in hospitalized cancer patients. Although there is no large RCT specifically demonstrating the benefit of thromboprophylaxis in cancer inpatients, RCTs conducted in non-cancer inpatients have demonstrated that LMWH improves survival and reduces VTE in general medical patients hospitalized with acute medical conditions, and recommendations for cancer patients have been extrapolated from these RCTs. The ITAC CPGs[8] recommend using LMWH at prophylactic doses or unfractionated heparin (UFH) or Fondaparinux in PC inpatients without contraindications to anticoagulants (Grade 1B)[8]. Due to the lack of data on the efficacy and safety of DOAC in this setting, they should not be used (Best clinical practice)[8].

Ambulatory PC patients

Most cancer patients develop VTE in the outpatient setting[53]. The net clinical benefit of primary thromboprophylaxis in advanced PC patients has been firmly established in two pivotal RCTs[54,55] which specifically addressed the efficacy and safety of LMWH in this setting (Table 2). Based on the results of these two trials, the ITAC CPGs recommend using primary thromboprophylaxis with LMWH in ambulatory advanced PC patients receiving chemotherapy with a Grade 1B evidence level since 2013[8,56,57].

The FRAGEM trial randomized 123 advanced PC patients to receive gemcitabine plus weight-adjusted therapeutic doses of dalteparin for 12 wk or gemcitabine alone[54]. The coprimary endpoints were the rate of symptomatic or incidentally diagnosed VTE events during the 12-wk anticoagulation period and the rate of symptomatic or incidentally diagnosed VTE events during the overall follow-up period. The rate of VTE was significantly lower in the dalteparin arm (3.4% *vs* 23% in the control arm, risk ratio 0.145, 95%CI: 0.035-0.612, $P = 0.002$), resulting in a NNT of 6 patients to prevent 1 VTE event. No VTE-related deaths occurred in the dalteparin arm compared to 5 (8.3%) VTE-related deaths in the control arm. The rates of major bleeding did not differ between the 2 arms and were lower than 3%, with only 2 patients experiencing a major bleeding requiring anticoagulation discontinuation. Of note, patients in the dalteparin arm experienced more minor bleeding such as skin bruising or epistaxis (9% *vs* 3% in the gemcitabine alone arm)[54]. There was no difference in PFS or OS between the two arms.

The PROSPECT-CONKO 004 trial randomized 312 advanced PC patients to receive supra-prophylactic doses of enoxaparin during the first 3 mo of chemotherapy or chemotherapy alone[55]. Unlike in FRAGEM, incidental VTE events were excluded from the analysis. The cumulative incidence rate of symptomatic VTE within the first 3 mo was 1.3% in the enoxaparin arm compared to 10.2% in the control arm (HR 0.12, 95%CI: 0.03-0.52), resulting in a NNT of 11 patients to prevent 1 VTE event. The rates of major bleeding events were similar in both arms. PFS and OS did not differ between the 2 arms[55].

Two additional phase III double-blinded placebo-controlled trials (the PROTECHT [58] and the SAVE-ONCO studies[59]) evaluated the efficacy and safety of primary thromboprophylaxis with prophylactic doses of other LMWH in ambulatory cancer patients receiving chemotherapy. In the PROTECHT study ($n = 1150$)[58], while nadroparin reduced the rate of VTE from 3.9% to 2.0% ($P = 0.02$) without difference in major bleeding in the overall population, the rates of VTE did not differ between the two arms in the subgroup of 53 PC patients ($P = 0.755$). In the SAVE-ONCO study ($n = 3221$)[59] the rate of VTE was 1.2% in the semuloparin arm compared to 3.4% in the placebo arm (HR 0.36, 95%CI: 0.21-0.60; $P < 0.001$) in the overall population, without difference in major bleeding (HR 1.05, 95%CI: 0.55-1.99). The absolute VTE risk reduction with semuloparin appeared to be much higher in the subgroup of 254 PC patients. The magnitude of the VTE risk reduction was similar to that obtained with therapeutic doses of dalteparin in the FRAGEM study[54] or with supra-prophylactic doses of enoxaparin in the PROSPECT-CONKO 004 study[55].

More recently, two randomized placebo-controlled trials assessed the efficacy and safety of primary thromboprophylaxis with prophylactic doses of DOACs (apixaban 2.5 mg twice daily for up to 6 mo in the AVERT trial[60]; rivaroxaban 10 mg once daily for up to 6 mo in the CASSINI trial[61]) in cancer patients with a Khorana score ≥ 2 undergoing chemotherapy. Results from a subgroup of PC patients were reported only for the CASSINI trial[41]. Among the 273 PC patients included in this prespecified subgroup analysis, 214 (78%) had a locally advanced or metastatic PC and 271 (99.3%) were receiving cytotoxic chemotherapy (fluorouracil-based in 47.6% of cases and gemcitabine-based in 44.7% of cases). Rivaroxaban did not significantly reduce the rates of the primary efficacy endpoint of symptomatic DVT, asymptomatic proximal DVT, any PE and VTE-related death within the 6 mo observation period (absolute difference of 3.4%, P = not significant). However, most of VTE events occurred after discontinuation of rivaroxaban (61.5%) compared to placebo (22.2%). During the intervention period, rivaroxaban significantly reduced the rates of the primary efficacy endpoint from 10.1% to 3.7% (absolute difference of 6.4%, HR 0.35, 95%CI: 0.13-0.97, $P = 0.034$), resulting in a NTT of 16 patients to prevent 1 event. Importantly, 2 out of 5 events in the rivaroxaban arm and 5 out of 14 events in the placebo arm were asymptomatic lower-extremity proximal DVT diagnosed by ultrasound screening during the follow-up, leading to overestimate the rates of VTE in both arms. The rates of major bleeding and all-cause mortality did not differ between the two arms[41].

Table 2 Studies assessing the clinical benefit of anticoagulants for the prevention of venous thromboembolism in ambulatory pancreatic cancer patients

	PROTECHT	SAVE ONCO	FRAGEM	CONKO-0004	CASSINI
	Agnelli *et al*[58], 2009	Agnelli *et al*[59], 2012	Maraveyas *et al*[54], 2012	Pelzer *et al*[55], 2015	Khorana *et al*[61], 2019 and Vadhan-Raj *et al*[41], 2020
Population	Ambulatory patients > 18 yr on chemotherapy with metastatic or locally advanced lung, gastrointestinal, breast, ovarian, or head and neck cancer	Patients with metastatic or locally advanced lung, pancreatic, gastric, colorectal, bladder, and ovarian cancer beginning to receive a course of chemotherapy	Patients aged 18 yr or older; Histologically/cytologically confirmed advanced or metastatic pancreatic cancer; KPS: 60-100	Patients with histologically proven advanced pancreatic cancer were randomly assigned to ambulant first-line chemotherapy	Adult ambulatory patients with various cancers initiating a new systemic regimen and at increased risk for VTE (defined as Khorana score ≥ 2)
Study design	Randomized, placebo-controlled, double-blind, multicenter study	Randomized, placebo-controlled, double-blind, multicenter study	Randomized, controlled Phase 2b study	Prospective, open label, randomized, multicenter and group-sequential 2b trial	Double-blind, randomized, placebo-controlled, parallel-group, multicenter study
Intervention	Arm A: nadroparin 3800 IU/d; Arm B: placebo; For duration of chemotherapy (up to 4 mo maximum)	Arm A: Semuloparin, 20 mg/d; Arm B: placebo; For duration of chemotherapy (median: 3.5 mo)	Arm A: Gemcitabine + Dalteparin 200 IU/kg s.c., o.d., for 4 wk, followed by a step-down regimen to 150 IU/kg for a further 8 wk); Arm B: Gemcitabine alone; For up to 12 wk	Arm A: Enoxaparin 1 mg/kg per day; Arm B: No enoxaparin	Arm A: rivaroxaban 10 mg o.d. up to day 180; Arm B: placebo up to day 180
Number of patients analyzed	Overall population: Arm A: 769 patients; Arm B: 381 patients. PC subgroup: Arm A: 36 patients; Arm B: 17 patients	Overall population: Arm A: 1608 patients; Arm B: 1604 patients. PC subgroup: Arm A: 126 patients; Arm B: 128 patients	Arm A: 59 patients; Arm B: 62 patients	Arm A: 160 patients; Arm B: 152 patients	Overall population: Arm A: 420 patients; Arm B: 404 patients. PC patients: Arm A: 135 patients; Arm B: 138 patients
Follow-up	120 d	3 mo	3 mo	3 mo	6 mo
Thromboembolic endpoint events	Overall population: Arm A: 11/769 (1.4%); Arm B: 11/381 (2.9%); $P = 0.02$. PC subgroup: Arm A: 3/36 (8.3%); Arm B: 1/17 (5.9%); $P = 0.755$	Overall population: Arm A:20/1608 (1.2%); Arm B: 55/1064 (1.2%); HR 0.36 (95%CI: 0.21-0.60); $P < 0.001$. PC subgroup: Arm A: 3/126 (2.4%); Arm B: 14/128 (10.9%); HR 0.22 (95%CI: 0.06-0.76); $P = 0.015$. At 3 mo: Arm A: 2/59 (3%); Arm B: 14/62 (23%); RR 0.145 (95%CI: 0.035-0.612); $P = 0.002$	At 3 mo: Arm A: 2/160 (1.25%); Arm B: 15/152 (9.8%); HR 0.12 (95%CI: 0.03-0.52); $P = 0.001$. Entire study: Arm A: 7/59 (12%); Arm B: 17/62 (28%); RR 0.419 (95%CI: 0.187-0.935); $P = 0.039$	Cumulative incidence rates: Arm A: 6.4%; Arm B: 15.1%; HR 0.40 (95%CI: 0.19-0.83); $P = 0.01$	Overall population: Up-to-day-180 observation period: Arm A: 25/420 (5.95%); Arm B: 37/421 (8.79%); HR 0.66 (95%CI: 0.40-1.09); $P = 0.101$; NNT =3 5. Intervention period: Arm A: 11/420 (2.62%); Arm B: 27/421 (6.41%); HR 0.40 (95%CI: 0.20-0.80); $P = 0.007$; NNT = 26. PC subgroup: Up-to-day-180 observation period: Arm A: 13/135 (9.6%); Arm B: 18/138 (13.0%); HR 0.70 (95%CI: 0.34-1.43); $P = 0.329$. Intervention period: Arm A: 5/135 (3.7%); Arm B: 14/138 (10.1%); HR 0.35 (95%CI: 0.130-0.96); $P = 0.043$; NNT = 16
Bleeding	Overall population: Major bleeding: Arm A: 5/769 (0.7%); Arm B: 0/381; $P = 0.18$. Minor bleeding: Arm A: 57/769 (7.4%); Arm B: 30/381 (7.9%); P = not significant. PC subgroup: P = not significant	Overall population: Major bleeding: Arm A: 19/1589 (1.2%); Arm B: 18/1583 (1.1%); OR 1.05 (95%CI: 0.55-2.04). CRNMB: Arm A: 26/1589 (2.8%); Arm B: 14/1583 (0.9%); OR 1.86 (95%CI: 0.98-3.68). PC subgroup: P = not significant	ISTH severe: Arm A: 2/59 (3%); Arm B: 2/62 (3%). ISTH non severe: Arm A: 5/59 (9%); Arm B: 2/62 (3%)	Major bleeding: Arm A: 8.3%; Arm B: 6.9%; HR 1.23 (95%CI: 0.54-2.79); $P = 0.63$	Overall population: Major bleeding: Arm A: 8/405 (1.98%); Arm B: 4/404 (0.99%); HR 1.96 (95%CI: 0.59-6.49) $P = 0.265$; NNH = 101. CRNMB: Arm A: 2.72%; Arm B: 1.98%; HR 1.96 (95%CI: 0.59-6.49); $P = 0.265$; NNH = 101. PC subgroup: Major bleeding: Arm A: 2/130 (1.5%); Arm B: 3/131(2.3%); HR 0.67 (95%CI: 0.11-3.99); $P = 0.654$. CRNMB: Arm A: 3/131(2.3%); Arm B: 2/130 (1.5%); HR 2.47 (95%CI: 0.48-12.72); $P = 0.264$
Survival	Overall population: Arm A: 33/769	Not significant	Arm A: 8.7 mo; Arm B: 9.7 mo	Arm A: 8.2 mo; Arm B: 8.51 mo;	Overall population: All-cause mortality: Arm A: 20.0%;

	(4·3%); Arm B: 16/381 (4.2%); *P* = not significant. PC subgroup: not significant	HR 1.01 (95%CI: 0.87-1.38); *P* = 0.44	Arm B: 23.8%; HR 0.83 (95%CI 0.62-1.11); *P* = 0.213. PC subgroup: Arm A: 34/135 (25.2%); Arm B: 33/138 (23.9%)

CI: Confidence interval; CRNMB: Clinically relevant non-major bleeding; HR: Hazard ratio; ISTH: International society of thrombosis and haemostasis; KPS: Karnofsky performance status; o.d.: Once daily; NTT: Number needed to treat; OR: Odds ratio; PC: Pancreatic cancer; RR: Risk ratio.

Recently, a systematic review and meta-analysis aggregated the data from the 1003 PC patients enrolled in the 5 above-mentioned RCTs[62]. Primary thromboprophylaxis was estimated to significantly reduce the risk of symptomatic VTE by approximately 69%, resulting in a NTT of 11.9 to prevent one VTE event, without increase in the risk of major bleeding. Sensitivity analyzes showed that primary prophylaxis with LMWH or DOAC, and prophylactic doses or supra-prophylactic doses of anticoagulants reduced the risk of VTE with the same magnitude.

In light of the results from the AVERT and CASSINI trials, the ITAC[8] and ASCO[11] CPGs now recommend thromboprophylaxis with apixaban or rivaroxaban in cancer outpatients undergoing chemotherapy having a Khorana score ≥ 2, no bleeding risk and no drug-drug interactions (Grade 1B)[8]. Since the Khorana score assigns + 2 points for PC, thromboprophylaxis with DOAC or LMWH may be now offered in all ambulatory PC patients. Decisions to initiate thromboprophylaxis should be made based on a multidisciplinary patient-centered approach, after close discussion with the patient.

Nevertheless, primary thromboprophylaxis has not been yet widely adopted in PC outpatients, mainly due to fear of bleeding in otherwise frail subjects and inherent costs for such therapy.

HOW TO TREAT VTE IN PC PATIENTS?

A step-based adapted approach

For many years, monotherapy with LMWH has been the standard of care to treat cancer-associated VTE, based on the results of 5 landmark RCTs comparing LMWH to vitamin K antagonists[63-67]. However, positive results from 4 recent RCTs comparing DOAC to LMWH monotherapy for the treatment of cancer-associated thrombosis [68-71] (Table 3) prompted current updated CPGs to include DOACs as a new first-line option in selected patients, but not all[8,11].

The ability to now use oral-only anticoagulation strategies, precluding the need for long-term daily injection and dose adjustment, may seem appealing but adds to the complexity of decision making. Appropriate selection of anticoagulants appears more than ever as a critical element of high-quality care for cancer patients with VTE, and numerous factors must be taken into consideration when choosing one anticoagulant rather than the other[72]. A personalized approach is warranted.

The ITAC CPGs recommend using LMWH for the initial and long-term treatment of established VTE when creatinine clearance is ≥ 30 mL per min (Grade 1B)[8]. For patients without risk of gastrointestinal or genitourinary bleeding, rivaroxaban (in the first 10 d) or edoxaban (started after at least 5 d of parenteral anticoagulation) can also be used (Grade 1B)[8]. UFH provides an alternative option when LMWH or DOACs are contraindicated, or not available (Grade 2C)[8]. Anticoagulation should be continued for at least 6 mo (Grade 1A) or indefinitely while cancer is active or treated [8].

LMWH are the preferred option in patients with VVT due their short half-life and possible dose reduction in case of esophageal varices.

Briefly, DOAC are a reasonable option in ambulatory PC patients with DVT or PE with an intact upper gastrointestinal tract, without nausea or vomiting, with a low risk of bleeding, with a platelet count > 50000/mm^3, with a creatinine clearance > 30 mL/min, without severe hepatic impairment and for whom no surgical intervention is planned. They should not be used in patients with creatinine clearance < 30 mL/min, luminal gastrointestinal lesion, platelet count < 50000/mm^3, high bleeding risk, recent or planned surgery, or potential drug-drug interactions[73,74].

A step-based adapted approach (Figure 1), incorporating tumor type, careful examination of bleeding risk, potential drug–drug interactions, and patient preferences, has been proposed by several authors[73,74]. The multi-language web-

Table 3 Randomized trials assessing the efficacy and safety of direct oral anticoagulants in cancer patients with venous thromboembolism

	HOKUSAI-CANCER VTE[68] (*n* = 1050)		SELECT-D[69] (*n* = 406)		ADAM-VTE[70] (*n* = 300)		CARAVAGGIO[71] (*n* = 1155)	
	Edoxaban	Dalteparin	Rivaroxaban	Dalteparin	Apixaban	Dalteparin	Apixaban	Dalteparin
Dose	LMWH × 5 d, then 60 mg OD	200 IU/kg × 1 mo, then 150 U/kg daily	15 mg BID × 3 wk, then 20 mg OD × 6mo	200 IU/kg × 1 mo, then 150 U/kg daily	10 mg BID × 7 d, then 5 mg BID × 6 mo	200 IU/kg × 1 mo, then 150 U/kg daily	10 mg BID × 7 d, then 5 mg BID × 6 mo	200 IU/kg × 1 mo, then 150 U/kg daily
Patients	Patients with active cancer and symptomatic or incidental popliteal, femoral or iliac or IVC DVT, symptomatic or incidental PE		Patients with active cancer and symptomatic DVT, symptomatic PE, or incidental PE		Active cancer patients with acute DVT (including upper extremity), PE, splanchnic or cerebral vein thrombosis		Patients with active or recent cancer and acute DVT or PE	
PrimaryEndpoint	Composite of recurrent VTE/major bleeding at 12 mo		VTE recurrence over 6 mo		Primary safety: Major bleeding at 6mo; secondary efficacy: VTE at 6 mo		Efficacy: Recurrent VTE at 6 mo; Safety: Major bleeding at 6 mo	
Follow-up	12 mo		6 mo		6 mo		6 mo	
Recurrent VTE (%)	41/525 (7.9)	59/525 (11.3)	8/203 (4)	18/203 (11)	1/145 (0.7)	9/142 (6.3)	32/576 (5.6)	46/579 (7.9)
HR (95%CI) for recurrent VTE	0.71 (0.48-1.06), *P* = 0.006		0.43 (0.19-0.99)		0.099 (0.013-0.780), *P* = 0.03		0.63 (0.37-1.07, *P* < 0.001)	
Major bleeding (%)	36/525 (6.9)	21/525 (4.0)	11/203 (4)	6/203 (6)	0/145 (0)	2/142 (1.4)	22/576 (3.8)	23/579 (4)
HR (CI) for major bleeding	1.77 (1.03-3.04)		1.83 (0.68-4.96)		Not estimable		0.82 (0.40-1.69, *P* = 0.6)	
CRNMB (%)	76/525 (14.6)	58/525 (11.1)	25/203 (12.3)	7/203 (3.4)	9/145 (6.2)	7/142 (4.9)	52/576 (9)	35/579 (6.0)
HR (95%CI) for CRNMB	1.38 (0.98-1.94)		3.76 (1.63-8.69)		0.931 (0.43-2.02), *P* = 0.88		1.42 (0.88-2.30)	

DVT: Deep vein thrombosis; PE: Pulmonary embolism; VTE: Venous thromboembolism; CI: Confidence interval; CRNMB: Clinically relevant non-major bleeding; HR: Hazard ratio; LMWH: Low-molecular-weight heparin.

based mobile application developed by the ITAC (downloadable for free at www.itaccme.com) based on such decision-tree algorithms is paramount to help clinicians in decision making[8].

Patients should be actively involved in treatment decisions and those treated with anticoagulants should be educated on the rationale for their treatment, the potential treatment safety concerns, and the risk of drug-drug interactions to ensure optimal adherence and treatment outcomes.

Incidental VTE is associated with high risks of recurrent VTE and VTE-related mortality[75-77] and should be treated as symptomatic VTE[8].

CONCLUSION

VTE is a common and potentially life-threatening complication in PC patients. Strict adherence to current evidence-based guidelines and dedicated patient education programs are warranted to optimize both the primary thromboprophylaxis and the treatment of VTE in PC patients. Clinical innovative tools, such as the multi-language web-based mobile application developed by the ITAC (downloadable for free at www.itaccme.com) will be paramount to assist clinicians in rigorously implementing updated CPGs and further decrease the burden of VTE in PC patients.

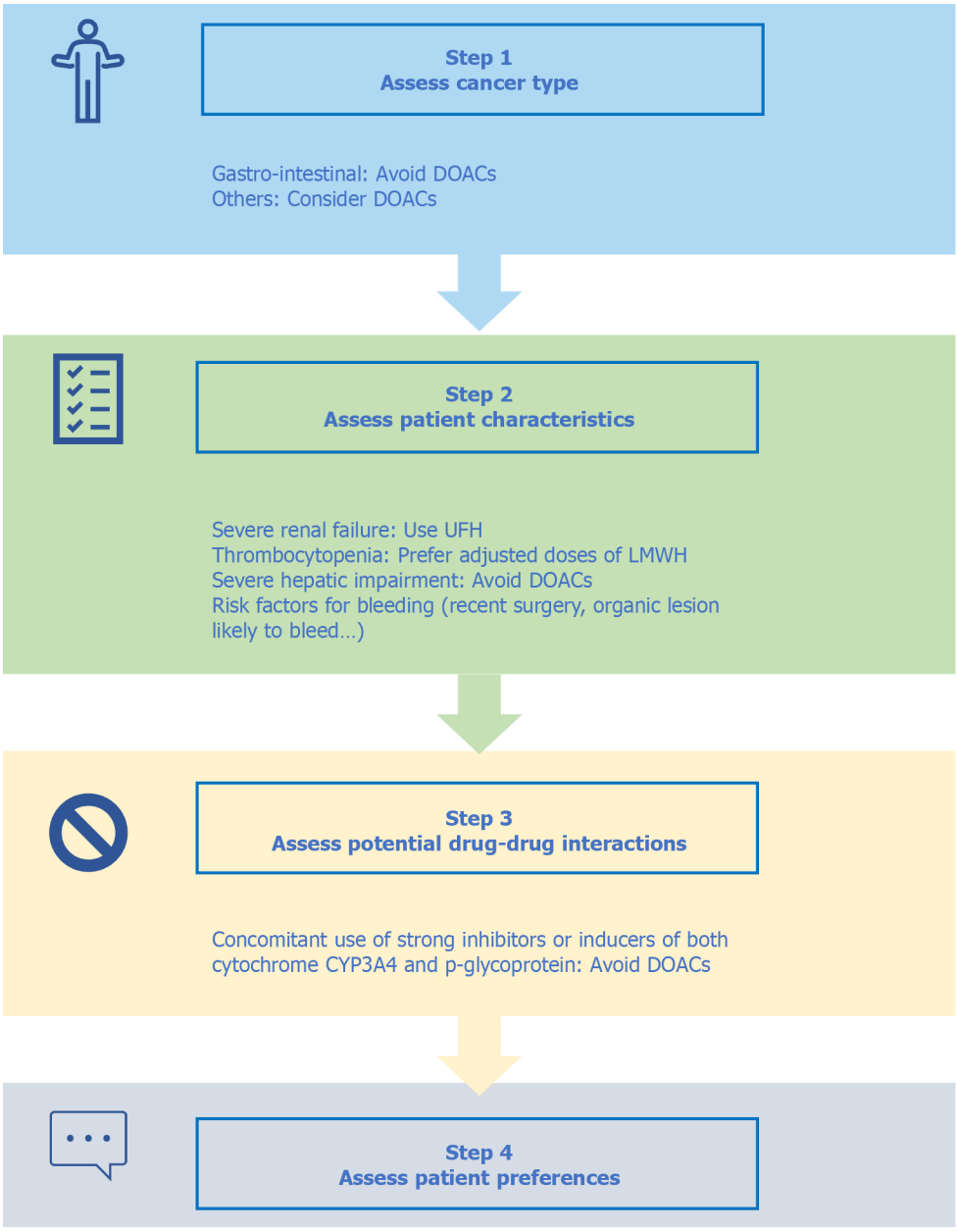


Figure 1 Four step adapted approach for the treatment of cancer-associated venous thromboembolism. DOACs: Direct oral anticoagulants; UFH: Unfractionated heparin; LMWH: Low-molecular-weight heparin; CYP3A4: Cytochrome P450 3A4.

REFERENCES

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

2 **Rahib L**, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; **74**: 2913-2921 [PMID: 24840647 DOI: 10.1158/0008-5472.CAN-14-0155]

3 **Lambert A**, Schwarz L, Borbath I, Henry A, Van Laethem JL, Malka D, Ducreux M, Conroy T. An update on treatment options for pancreatic adenocarcinoma. *Ther Adv Med Oncol* 2019; **11**: 1758835919875568 [PMID: 31598142 DOI: 10.1177/1758835919875568]

4 **Moffat GT**, Epstein AS, O'Reilly EM. Pancreatic cancer-A disease in need: Optimizing and integrating supportive care. *Cancer* 2019; **125**: 3927-3935 [PMID: 31381149 DOI: 10.1002/cncr.32423]

5 **Farge D**, Bournet B, Conroy T, Vicaut E, Rak J, Zogoulous G, Barkun J, Ouaissi M, Buscail L, Frere C. Primary Thromboprophylaxis in Pancreatic Cancer Patients: Why Clinical Practice Guidelines Should Be Implemented. *Cancers (Basel)* 2020; **12** [PMID: 32155940 DOI: 10.3390/cancers12030618]

6 **Chiasakul T**, Patell R, Maraveyas A, Carrier M, Zwicker JI. Discordant reporting of VTE in

pancreatic cancer: A systematic review and meta-analysis of thromboprophylaxis *vs* chemotherapeutic trials. *J Thromb Haemost* 2021; **19**: 489-501 [PMID: 33174368 DOI: 10.1111/jth.15175]

7 **Mulder FI**, Horváth-Puhó E, van Es N, van Laarhoven HWM, Pedersen L, Moik F, Ay C, Büller HR, Sørensen HT. Venous thromboembolism in cancer patients: a population-based cohort study. *Blood* 2021; **137**: 1959-1969 [PMID: 33171494 DOI: 10.1182/blood.2020007338]

8 **Farge D**, Frere C, Connors JM, Ay C, Khorana AA, Munoz A, Brenner B, Kakkar A, Rafii H, Solymoss S, Brilhante D, Monreal M, Bounameaux H, Pabinger I, Douketis J; International Initiative on Thrombosis and Cancer (ITAC) advisory panel. 2019 international clinical practice guidelines for the treatment and prophylaxis of venous thromboembolism in patients with cancer. *Lancet Oncol* 2019; **20**: e566-e581 [PMID: 31492632 DOI: 10.1016/S1470-2045(19)30336-5]

9 **Prandoni P**, Lensing AW, Piccioli A, Bernardi E, Simioni P, Girolami B, Marchiori A, Sabbion P, Prins MH, Noventa F, Girolami A. Recurrent venous thromboembolism and bleeding complications during anticoagulant treatment in patients with cancer and venous thrombosis. *Blood* 2002; **100**: 3484-3488 [PMID: 12393647 DOI: 10.1182/blood-2002-01-0108]

10 **Abdulla A**, Davis WM, Ratnaweera N, Szefer E, Ballantyne Scott B, Lee AYY. A Meta-Analysis of Case Fatality Rates of Recurrent Venous Thromboembolism and Major Bleeding in Patients with Cancer. *Thromb Haemost* 2020; **120**: 702-713 [PMID: 32289865 DOI: 10.1055/s-0040-1708481]

11 **Key NS**, Khorana AA, Kuderer NM, Bohlke K, Lee AYY, Arcelus JI, Wong SL, Balaban EP, Flowers CR, Francis CW, Gates LE, Kakkar AK, Levine MN, Liebman HA, Tempero MA, Lyman GH, Falanga A. Venous Thromboembolism Prophylaxis and Treatment in Patients With Cancer: ASCO Clinical Practice Guideline Update. *J Clin Oncol* 2020; **38**: 496-520 [PMID: 31381464 DOI: 10.1200/JCO.19.01461]

12 **National Comprehensive Cancer Network**. NCCN guideline on cancer-associated venous thromboembolic disease. Version 1. 2020. [cited 3 January 2021]. In: National Comprehensive Cancer Network [Internet]. Available from: https://www.nccn.org/professionals/physician_gls/pdf/vte.pdf

13 **Levitan N**, Dowlati A, Remick SC, Tahsildar HI, Sivinski LD, Beyth R, Rimm AA. Rates of initial and recurrent thromboembolic disease among patients with malignancy *vs* those without malignancy. Risk analysis using Medicare claims data. *Medicine (Baltimore)* 1999; **78**: 285-291

14 **Horsted F**, West J, Grainge MJ. Risk of venous thromboembolism in patients with cancer: a systematic review and meta-analysis. *PLoS Med* 2012; **9**: e1001275 [PMID: 22859911 DOI: 10.1371/journal.pmed.1001275]

15 **Sproul EE**. Carcinoma and Venous Thrombosis: The Frequency of Association of Carcinoma in the Body or Tail of the Pancreas with Multiple Venous Thrombosis. *Am J Cancer* 1938; **34**: 566-585

16 **Blom JW**, Osanto S, Rosendaal FR. High risk of venous thrombosis in patients with pancreatic cancer: a cohort study of 202 patients. *Eur J Cancer* 2006; **42**: 410-414 [PMID: 16321518 DOI: 10.1016/j.ejca.2005.09.013]

17 **Mandalà M**, Reni M, Cascinu S, Barni S, Floriani I, Cereda S, Berardi R, Mosconi S, Torri V, Labianca R. Venous thromboembolism predicts poor prognosis in irresectable pancreatic cancer patients. *Ann Oncol* 2007; **18**: 1660-1665 [PMID: 17660490 DOI: 10.1093/annonc/mdm284]

18 **Mitry E**, Taleb-Fayad R, Deschamps A, Mansencal N, Lepère C, Declety G, Lièvre A, Vaillant JN, Lesur G, Cramer E, Dubourg O, Rougier P. Risk of venous thrombosis in patients with pancreatic adenocarcinoma. *Gastroenterol Clin Biol* 2007; **31**: 1139-1142 [PMID: 18176374 DOI: 10.1016/s0399-8320(07)78352-5]

19 **Oh SY**, Kim JH, Lee KW, Bang SM, Hwang JH, Oh D, Lee JS. Venous thromboembolism in patients with pancreatic adenocarcinoma: lower incidence in Asian ethnicity. *Thromb Res* 2008; **122**: 485-490 [PMID: 18234292 DOI: 10.1016/j.thromres.2007.12.015]

20 **Poruk KE**, Firpo MA, Huerter LM, Scaife CL, Emerson LL, Boucher KM, Jones KA, Mulvihill SJ. Serum platelet factor 4 is an independent predictor of survival and venous thromboembolism in patients with pancreatic adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 2605-2610 [PMID: 20729288 DOI: 10.1158/1055-9965.EPI-10-0178]

21 **Shaib W**, Deng Y, Zilterman D, Lundberg B, Saif MW. Assessing risk and mortality of venous thromboembolism in pancreatic cancer patients. *Anticancer Res* 2010; **30**: 4261-4264 [PMID: 21036750]

22 **Epstein AS**, Soff GA, Capanu M, Crosbie C, Shah MA, Kelsen DP, Denton B, Gardos S, O'Reilly EM. Analysis of incidence and clinical outcomes in patients with thromboembolic events and invasive exocrine pancreatic cancer. *Cancer* 2012; **118**: 3053-3061 [PMID: 21989534 DOI: 10.1002/cncr.26600]

23 **Menapace LA**, Peterson DR, Berry A, Sousou T, Khorana AA. Symptomatic and incidental thromboembolism are both associated with mortality in pancreatic cancer. *Thromb Haemost* 2011; **106**: 371-378 [PMID: 21713322 DOI: 10.1160/TH10-12-0789]

24 **Afsar CU**, Gunaldi M, Kum P, Sahin B, Erkisi M, Kara IO, Paydas S, Duman BB, Ercolak V, Karaca F, Uyeturk U, Guner SI. Pancreatic carcinoma, thrombosis and mean platelet volume: single center experience from the southeast region of Turkey. *Asian Pac J Cancer Prev* 2014; **15**: 9143-9146 [PMID: 25422192 DOI: 10.7314/apjcp.2014.15.21.9143]

25 **Muñoz Martín AJ**, García Alfonso P, Rupérez Blanco AB, Pérez Ramírez S, Blanco Codesido M, Martín Jiménez M. Incidence of venous thromboembolism (VTE) in ambulatory pancreatic cancer patients receiving chemotherapy and analysis of Khorana's predictive model. *Clin Transl Oncol* 2014; **16**: 927-930 [PMID: 24643701 DOI: 10.1007/s12094-014-1165-y]

26 **Krepline AN**, Christians KK, George B, Ritch PS, Erickson BA, Tolat P, Evans DB, Tsai S. Venous thromboembolism prophylaxis during neoadjuvant therapy for resectable and borderline resectable pancreatic cancer-Is it indicated? *J Surg Oncol* 2016; **114**: 581-586 [PMID: 27760280 DOI: 10.1002/jso.24361]

27 **Lee JC**, Ro YS, Cho J, Park Y, Lee JH, Hwang JH, Choi HJ, Lee S. Characteristics of Venous Thromboembolism in Pancreatic Adenocarcinoma in East Asian Ethnics: A Large Population-Based Observational Study. *Medicine (Baltimore)* 2016; **95**: e3472 [PMID: 27124043 DOI: 10.1097/MD.0000000000003472]

28 **Kruger S**, Haas M, Burkl C, Goehring P, Kleespies A, Roeder F, Gallmeier E, Ormanns S, Westphalen CB, Heinemann V, Rank A, Boeck S. Incidence, outcome and risk stratification tools for venous thromboembolism in advanced pancreatic cancer - A retrospective cohort study. *Thromb Res* 2017; **157**: 9-15 [PMID: 28675831 DOI: 10.1016/j.thromres.2017.06.021]

29 **van Es N**, Franke VF, Middeldorp S, Wilmink JW, Büller HR. The Khorana score for the prediction of venous thromboembolism in patients with pancreatic cancer. *Thromb Res* 2017; **150**: 30-32 [PMID: 28002757 DOI: 10.1016/j.thromres.2016.12.013]

30 **Berger AK**, Singh HM, Werft W, Muckenhuber A, Sprick MR, Trumpp A, Weichert W, Jäger D, Springfeld C. High prevalence of incidental and symptomatic venous thromboembolic events in patients with advanced pancreatic cancer under palliative chemotherapy: A retrospective cohort study. *Pancreatology* 2017; **17**: 629-634 [PMID: 28462862 DOI: 10.1016/j.pan.2017.04.012]

31 **Chen JS**, Hung CY, Chang H, Liu CT, Chen YY, Lu CH, Chang PH, Hung YS, Chou WC. Venous Thromboembolism in Asian Patients with Pancreatic Cancer Following Palliative Chemotherapy: Low Incidence but a Negative Prognosticator for Those with Early Onset. *Cancers (Basel)* 2018; **10** [PMID: 30544670 DOI: 10.3390/cancers10120501]

32 **Kim JS**, Kang EJ, Kim DS, Choi YJ, Lee SY, Kim HJ, Seo HY, Kim JS. Early venous thromboembolism at the beginning of palliative chemotherapy is a poor prognostic factor in patients with metastatic pancreatic cancer: a retrospective study. *BMC Cancer* 2018; **18**: 1260 [PMID: 30558603 DOI: 10.1186/s12885-018-5154-3]

33 **Ouaissi M**, Frasconi C, Mege D, Panicot-Dubois L, Boiron L, Dahan L, Debourdeau P, Dubois C, Farge D, Sielezneff I. Impact of venous thromboembolism on the natural history of pancreatic adenocarcinoma. *Hepatobiliary Pancreat Dis Int* 2015; **14**: 436-442 [PMID: 26256090 DOI: 10.1016/s1499-3872(15)60397-6]

34 **Frere C**, Bournet B, Gourgou S, Fraisse J, Canivet C, Connors JM, Buscail L, Farge D; BACAP Consortium. Incidence of Venous Thromboembolism in Patients With Newly Diagnosed Pancreatic Cancer and Factors Associated With Outcomes. *Gastroenterology* 2020; **158**: 1346-1358. e4 [PMID: 31843588 DOI: 10.1053/j.gastro.2019.12.009]

35 **Khorana AA**, Fine RL. Pancreatic cancer and thromboembolic disease. *Lancet Oncol* 2004; **5**: 655-663 [PMID: 15522652 DOI: 10.1016/S1470-2045(04)01606-7]

36 **Mier-Hicks A**, Raj M, Do RK, Yu KH, Lowery MA, Varghese A, O'Reilly EM. Incidence, Management, and Implications of Visceral Thrombosis in Pancreatic Ductal Adenocarcinoma. *Clin Colorectal Cancer* 2018; **17**: 121-128 [PMID: 29477452 DOI: 10.1016/j.clcc.2018.01.008]

37 **Afzal A**, Suhong L, Gage BF, Schoen MW, Carson K, Thomas T, Sanfilippo K. Splanchnic vein thrombosis predicts worse survival in patients with advanced pancreatic cancer. *Thromb Res* 2020; **185**: 125-131 [PMID: 31812026 DOI: 10.1016/j.thromres.2019.11.023]

38 **Faille D**, Bourrienne MC, de Raucourt E, de Chaisemartin L, Granger V, Lacroix R, Panicot-Dubois L, Hammel P, Lévy P, Ruszniewski P, Ajzenberg N, Rebours V. Biomarkers for the risk of thrombosis in pancreatic adenocarcinoma are related to cancer process. *Oncotarget* 2018; **9**: 26453-26465 [PMID: 29899870 DOI: 10.18632/oncotarget.25458]

39 **Godinho J**, Casa-Nova M, Moreira-Pinto J, Simões P, Paralta Branco F, Leal-Costa L, Faria A, Lopes F, Teixeira JA, Passos-Coelho JL. ONKOTEV Score as a Predictive Tool for Thromboembolic Events in Pancreatic Cancer-A Retrospective Analysis. *Oncologist* 2020; **25**: e284-e290 [PMID: 32043787 DOI: 10.1634/theoncologist.2019-0510]

40 **Heit JA**. Epidemiology of venous thromboembolism. *Nat Rev Cardiol* 2015; **12**: 464-474 [PMID: 26076949 DOI: 10.1038/nrcardio.2015.83]

41 **Vadhan-Raj S**, McNamara MG, Venerito M, Riess H, O'Reilly EM, Overman MJ, Zhou X, Vijapurkar U, Kaul S, Wildgoose P, Khorana AA. Rivaroxaban thromboprophylaxis in ambulatory patients with pancreatic cancer: Results from a pre-specified subgroup analysis of the randomized CASSINI study. *Cancer Med* 2020; **9**: 6196-6204 [PMID: 32663379 DOI: 10.1002/cam4.3269]

42 **Cronin M**, Dengler N, Krauss ES, Segal A, Wei N, Daly M, Mota F, Caprini JA. Completion of the Updated Caprini Risk Assessment Model (2013 Version). *Clin Appl Thromb Hemost* 2019; **25**: 1076029619838052 [PMID: 30939900 DOI: 10.1177/1076029619838052]

43 **Boone BA**, Zenati MS, Rieser C, Hamad A, Al-Abbas A, Zureikat AH, Hogg ME, Neal MD, Zeh HJ 3rd. Risk of Venous Thromboembolism for Patients with Pancreatic Ductal Adenocarcinoma Undergoing Preoperative Chemotherapy Followed by Surgical Resection. *Ann Surg Oncol* 2019; **26**: 1503-1511 [PMID: 30652227 DOI: 10.1245/s10434-018-07148-z]

44 **Khorana AA**, Kuderer NM, Culakova E, Lyman GH, Francis CW. Development and validation of a predictive model for chemotherapy-associated thrombosis. *Blood* 2008; **111**: 4902-4907 [PMID: 18216292 DOI: 10.1182/blood-2007-10-116327]

45 **Pelzer U**, Sinn M, Stieler J, Riess H. [Primary pharmacological prevention of thromboembolic events in ambulatory patients with advanced pancreatic cancer treated with chemotherapy? *Dtsch Med*

Wochenschr 2013; **138**: 2084-2088 [PMID: 24085361 DOI: 10.1055/s-0033-1349608]
46 **Verso M**, Agnelli G, Barni S, Gasparini G, LaBianca R. A modified Khorana risk assessment score for venous thromboembolism in cancer patients receiving chemotherapy: the Protecht score. *Intern Emerg Med* 2012; **7**: 291-292 [PMID: 22547369 DOI: 10.1007/s11739-012-0784-y]
47 **Cella CA**, Di Minno G, Carlomagno C, Arcopinto M, Cerbone AM, Matano E, Tufano A, Lordick F, De Simone B, Muehlberg KS, Bruzzese D, Attademo L, Arturo C, Sodano M, Moretto R, La Fata E, De Placido S. Preventing Venous Thromboembolism in Ambulatory Cancer Patients: The ONKOTEV Study. *Oncologist* 2017; **22**: 601-608 [PMID: 28424324 DOI: 10.1634/theoncologist.2016-0246]
48 **Muñoz Martín AJ**, Ortega I, Font C, Pachón V, Castellón V, Martínez-Marín V, Salgado M, Martínez E, Calzas J, Rupérez A, Souto JC, Martín M, Salas E, Soria JM. Multivariable clinical-genetic risk model for predicting venous thromboembolic events in patients with cancer. *Br J Cancer* 2018; **118**: 1056-1061 [PMID: 29588512 DOI: 10.1038/s41416-018-0027-8]
49 **Pabinger I**, van Es N, Heinze G, Posch F, Riedl J, Reitter EM, Di Nisio M, Cesarman-Maus G, Kraaijpoel N, Zielinski CC, Büller HR, Ay C. A clinical prediction model for cancer-associated venous thromboembolism: a development and validation study in two independent prospective cohorts. *Lancet Haematol* 2018; **5**: e289-e298 [PMID: 29885940 DOI: 10.1016/S2352-3026(18)30063-2]
50 **Ferroni P**, Zanzotto FM, Scarpato N, Riondino S, Guadagni F, Roselli M. Validation of a Machine Learning Approach for Venous Thromboembolism Risk Prediction in Oncology. *Dis Markers* 2017; **2017**: 8781379 [PMID: 29104344 DOI: 10.1155/2017/8781379]
51 **Fresard ME**, Erices R, Bravo ML, Cuello M, Owen GI, Ibanez C, Rodriguez-Fernandez M. Multi-Objective Optimization for Personalized Prediction of Venous Thromboembolism in Ovarian Cancer Patients. *IEEE J Biomed Health Inform* 2020; **24**: 1500-1508 [PMID: 31562113 DOI: 10.1109/JBHI.2019.2943499]
52 **Felder S**, Rasmussen MS, King R, Sklow B, Kwaan M, Madoff R, Jensen C. Prolonged thromboprophylaxis with low molecular weight heparin for abdominal or pelvic surgery. *Cochrane Database Syst Rev* 2019; **8**: CD004318 [PMID: 31449321 DOI: 10.1002/14651858.CD004318.pub5]
53 **Spencer FA**, Lessard D, Emery C, Reed G, Goldberg RJ. Venous thromboembolism in the outpatient setting. *Arch Intern Med* 2007; **167**: 1471-1475 [PMID: 17646600 DOI: 10.1001/archinte.167.14.1471]
54 **Maraveyas A**, Waters J, Roy R, Fyfe D, Propper D, Lofts F, Sgouros J, Gardiner E, Wedgwood K, Ettelaie C, Bozas G. Gemcitabine *vs* gemcitabine plus dalteparin thromboprophylaxis in pancreatic cancer. *Eur J Cancer* 2012; **48**: 1283-1292 [PMID: 22100906 DOI: 10.1016/j.ejca.2011.10.017]
55 **Pelzer U**, Opitz B, Deutschinoff G, Stauch M, Reitzig PC, Hahnfeld S, Müller L, Grunewald M, Stieler JM, Sinn M, Denecke T, Bischoff S, Oettle H, Dörken B, Riess H. Efficacy of Prophylactic Low-Molecular Weight Heparin for Ambulatory Patients With Advanced Pancreatic Cancer: Outcomes From the CONKO-004 Trial. *J Clin Oncol* 2015; **33**: 2028-2034 [PMID: 25987694 DOI: 10.1200/JCO.2014.55.1481]
56 **Farge D**, Debourdeau P, Beckers M, Baglin C, Bauersachs RM, Brenner B, Brilhante D, Falanga A, Gerotzafias GT, Haim N, Kakkar AK, Khorana AA, Lecumberri R, Mandala M, Marty M, Monreal M, Mousa SA, Noble S, Pabinger I, Prandoni P, Prins MH, Qari MH, Streiff MB, Syrigos K, Bounameaux H, Büller HR. International clinical practice guidelines for the treatment and prophylaxis of venous thromboembolism in patients with cancer. *J Thromb Haemost* 2013; **11**: 56-70 [PMID: 23217107 DOI: 10.1111/jth.12070]
57 **Farge D**, Bounameaux H, Brenner B, Cajfinger F, Debourdeau P, Khorana AA, Pabinger I, Solymoss S, Douketis J, Kakkar A. International clinical practice guidelines including guidance for direct oral anticoagulants in the treatment and prophylaxis of venous thromboembolism in patients with cancer. *Lancet Oncol* 2016; **17**: e452-e466 [PMID: 27733271 DOI: 10.1016/S1470-2045(16)30369-2]
58 **Agnelli G**, Gussoni G, Bianchini C, Verso M, Mandalà M, Cavanna L, Barni S, Labianca R, Buzzi F, Scambia G, Passalacqua R, Ricci S, Gasparini G, Lorusso V, Bonizzoni E, Tonato M; PROTECHT Investigators. Nadroparin for the prevention of thromboembolic events in ambulatory patients with metastatic or locally advanced solid cancer receiving chemotherapy: a randomised, placebo-controlled, double-blind study. *Lancet Oncol* 2009; **10**: 943-949 [PMID: 19726226 DOI: 10.1016/S1470-2045(09)70232-3]
59 **Agnelli G**, George DJ, Kakkar AK, Fisher W, Lassen MR, Mismetti P, Mouret P, Chaudhari U, Lawson F, Turpie AG; SAVE-ONCO Investigators. Semuloparin for thromboprophylaxis in patients receiving chemotherapy for cancer. *N Engl J Med* 2012; **366**: 601-609 [PMID: 22335737 DOI: 10.1056/NEJMoa1108898]
60 **Carrier M**, Abou-Nassar K, Mallick R, Tagalakis V, Shivakumar S, Schattner A, Kuruvilla P, Hill D, Spadafora S, Marquis K, Trinkaus M, Tomiak A, Lee AYY, Gross PL, Lazo-Langner A, El-Maraghi R, Goss G, Le Gal G, Stewart D, Ramsay T, Rodger M, Witham D, Wells PS; AVERT Investigators. Apixaban to Prevent Venous Thromboembolism in Patients with Cancer. *N Engl J Med* 2019; **380**: 711-719 [PMID: 30511879 DOI: 10.1056/NEJMoa1814468]
61 **Khorana AA**, Soff GA, Kakkar AK, Vadhan-Raj S, Riess H, Wun T, Streiff MB, Garcia DA, Liebman HA, Belani CP, O'Reilly EM, Patel JN, Yimer HA, Wildgoose P, Burton P, Vijapurkar U, Kaul S, Eikelboom J, McBane R, Bauer KA, Kuderer NM, Lyman GH; CASSINI Investigators. Rivaroxaban for Thromboprophylaxis in High-Risk Ambulatory Patients with Cancer. *N Engl J Med* 2019; **380**: 720-728 [PMID: 30786186 DOI: 10.1056/NEJMoa1814630]

62 **Frere C**, Crichi B, Bournet B, Canivet C, Abdallah NA, Buscail L, Farge D. Primary Thromboprophylaxis in Ambulatory Pancreatic Cancer Patients Receiving Chemotherapy: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Cancers (Basel)* 2020; **12** [PMID: 32722064 DOI: 10.3390/cancers12082028]

63 **Meyer G**, Marjanovic Z, Valcke J, Lorcerie B, Gruel Y, Solal-Celigny P, Le Maignan C, Extra JM, Cottu P, Farge D. Comparison of low-molecular-weight heparin and warfarin for the secondary prevention of venous thromboembolism in patients with cancer: a randomized controlled study. *Arch Intern Med* 2002; **162**: 1729-1735 [PMID: 12153376 DOI: 10.1001/archinte.162.15.1729]

64 **Lee AY**, Levine MN, Baker RI, Bowden C, Kakkar AK, Prins M, Rickles FR, Julian JA, Haley S, Kovacs MJ, Gent M; Randomized Comparison of Low-Molecular-Weight Heparin vs Oral Anticoagulant Therapy for the Prevention of Recurrent Venous Thromboembolism in Patients with Cancer (CLOT) Investigators. Low-molecular-weight heparin *vs* a coumarin for the prevention of recurrent venous thromboembolism in patients with cancer. *N Engl J Med* 2003; **349**: 146-153 [PMID: 12853587 DOI: 10.1056/NEJMoa025313]

65 **Deitcher SR**, Kessler CM, Merli G, Rigas JR, Lyons RM, Fareed J; ONCENOX Investigators. Secondary prevention of venous thromboembolic events in patients with active cancer: enoxaparin alone *vs* initial enoxaparin followed by warfarin for a 180-day period. *Clin Appl Thromb Hemost* 2006; **12**: 389-396 [PMID: 17000884 DOI: 10.1177/1076029606293692]

66 **Hull RD**, Pineo GF, Brant RF, Mah AF, Burke N, Dear R, Wong T, Cook R, Solymoss S, Poon MC, Raskob G; LITE Trial Investigators. Long-term low-molecular-weight heparin *vs* usual care in proximal-vein thrombosis patients with cancer. *Am J Med* 2006; **119**: 1062-1072 [PMID: 17145251 DOI: 10.1016/j.amjmed.2006.02.022]

67 **Lee AYY**, Kamphuisen PW, Meyer G, Bauersachs R, Janas MS, Jarner MF, Khorana AA; CATCH Investigators. Tinzaparin *vs* Warfarin for Treatment of Acute Venous Thromboembolism in Patients With Active Cancer: A Randomized Clinical Trial. *JAMA* 2015; **314**: 677-686 [PMID: 26284719 DOI: 10.1001/jama.2015.9243]

68 **Raskob GE**, van Es N, Verhamme P, Carrier M, Di Nisio M, Garcia D, Grosso MA, Kakkar AK, Kovacs MJ, Mercuri MF, Meyer G, Segers A, Shi M, Wang TF, Yeo E, Zhang G, Zwicker JI, Weitz JI, Büller HR; Hokusai VTE Cancer Investigators. Edoxaban for the Treatment of Cancer-Associated Venous Thromboembolism. *N Engl J Med* 2018; **378**: 615-624 [PMID: 29231094 DOI: 10.1056/NEJMoa1711948]

69 **Young AM**, Marshall A, Thirlwall J, Chapman O, Lokare A, Hill C, Hale D, Dunn JA, Lyman GH, Hutchinson C, MacCallum P, Kakkar A, Hobbs FDR, Petrou S, Dale J, Poole CJ, Maraveyas A, Levine M. Comparison of an Oral Factor Xa Inhibitor With Low Molecular Weight Heparin in Patients With Cancer With Venous Thromboembolism: Results of a Randomized Trial (SELECT-D). *J Clin Oncol* 2018; **36**: 2017-2023 [PMID: 29746227 DOI: 10.1200/JCO.2018.78.8034]

70 **McBane RD 2nd**, Wysokinski WE, Le-Rademacher JG, Zemla T, Ashrani A, Tafur A, Perepu U, Anderson D, Gundabolu K, Kuzma C, Perez Botero J, Leon Ferre RA, Henkin S, Lenz CJ, Houghton DE, Vishnu P, Loprinzi CL. Apixaban and dalteparin in active malignancy-associated venous thromboembolism: The ADAM VTE trial. *J Thromb Haemost* 2020; **18**: 411-421 [PMID: 31630479 DOI: 10.1111/jth.14662]

71 **Agnelli G**, Becattini C, Meyer G, Muñoz A, Huisman MV, Connors JM, Cohen A, Bauersachs R, Brenner B, Torbicki A, Sueiro MR, Lambert C, Gussoni G, Campanini M, Fontanella A, Vescovo G, Verso M; Caravaggio Investigators. Apixaban for the Treatment of Venous Thromboembolism Associated with Cancer. *N Engl J Med* 2020; **382**: 1599-1607 [PMID: 32223112 DOI: 10.1056/NEJMoa1915103]

72 **Frere C**, Benzidia I, Marjanovic Z, Farge D. Recent Advances in the Management of Cancer-Associated Thrombosis: New Hopes but New Challenges. *Cancers (Basel)* 2019; **11** [PMID: 30634638 DOI: 10.3390/cancers11010071]

73 **Carrier M**, Wang TF. Direct oral anticoagulants and cancer-associated VTE: good for all, or just some? *Blood* 2020; **136**: 669-673 [PMID: 32575112 DOI: 10.1182/blood.2019004177]

74 **O'Connell C**, Escalante CP, Goldhaber SZ, McBane R, Connors JM, Raskob GE. Treatment of Cancer-Associated Venous Thromboembolism with Low-Molecular-Weight Heparin or Direct Oral Anticoagulants: Patient Selection, Controversies, and Caveats. *Oncologist* 2021; **26**: e8-e16 [PMID: 33275319 DOI: 10.1002/onco.13584]

75 **den Exter PL**, Hooijer J, Dekkers OM, Huisman MV. Risk of recurrent venous thromboembolism and mortality in patients with cancer incidentally diagnosed with pulmonary embolism: a comparison with symptomatic patients. *J Clin Oncol* 2011; **29**: 2405-2409 [PMID: 21555690 DOI: 10.1200/JCO.2010.34.0984]

76 **van der Hulle T**, den Exter PL, Planquette B, Meyer G, Soler S, Monreal M, Jiménez D, Portillo AK, O'Connell C, Liebman HA, Shteinberg M, Adir Y, Tiseo M, Bersanelli M, Abdel-Razeq HN, Mansour AH, Donnelly OG, Radhakrishna G, Ramasamy S, Bozas G, Maraveyas A, Shinagare AB, Hatabu H, Nishino M, Huisman MV, Klok FA. Risk of recurrent venous thromboembolism and major hemorrhage in cancer-associated incidental pulmonary embolism among treated and untreated patients: a pooled analysis of 926 patients. *J Thromb Haemost* 2016; **14**: 105-113 [PMID: 26469193 DOI: 10.1111/jth.13172]

77 **Kraaijpoel N**, Bleker SM, Meyer G, Mahé I, Muñoz A, Bertoletti L, Bartels-Rutten A, Beyer-Westendorf J, Porreca E, Boulon C, van Es N, Iosub DI, Couturaud F, Biosca M, Lerede T, Lacroix P, Maraveyas A, Aggarwal A, Girard P, Büller HR, Di Nisio M; UPE investigators. Treatment and

Long-Term Clinical Outcomes of Incidental Pulmonary Embolism in Patients With Cancer: An International Prospective Cohort Study. *J Clin Oncol* 2019; **37**: 1713-1720 [PMID: 31116676 DOI: 10.1200/JCO.18.01977]

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MINIREVIEWS

Pathophysiological mechanisms underlying gastrointestinal symptoms in patients with COVID-19

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Abstract

Gastrointestinal (GI) symptoms, such as diarrhea, abdominal pain, vomiting, and anorexia, are frequently observed in patients with coronavirus disease 2019 (COVID-19). However, the pathophysiological mechanisms connecting these GI symptoms to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections remain elusive. Previous studies indicate that the entry of SARS-CoV-2 into intestinal cells leads to downregulation of angiotensin converting enzyme 2 (ACE2) receptors resulting in impaired barrier function. While intestinal ACE2 functions as a chaperone for the amino acid transporter B0AT1, the B0AT1/ACE2 complex within the intestinal epithelium acts as a regulator of gut microbiota composition and function. Alternations to the B0AT1/ACE2 complex lead to microbial dysbiosis through increased local and systemic immune responses. Previous studies have also suggested that altered serotonin metabolism may be the underlying cause of GI disorders involving diarrhea. The findings of elevated plasma serotonin levels and high fecal calprotectin in COVID-19 patients with diarrhea indicate that the viral infection evokes a systemic inflammatory response that specifically involves the GI. Interestingly, the elevated proinflammatory cytokines correlate with elevated serotonin and fecal calprotectin levels further supporting the evidence of GI inflammation, a hallmark of functional GI disorders. Moreover, the finding that rectal swabs of COVID-19 patients remain positive for SARS-CoV-2 even after the nasopharynx clears the virus, suggests that viral replication and shedding from the GI tract may be more robust than that of the respiratory tract, further indicating fecal-oral transmission as another important route of viral spread. This review summarized the evidence for pathophysiological mechanisms (impaired barrier function, gut inflammation, altered serotonin metabolism and gut microbiota dysbiosis) underlying the GI symptoms in patients with COVID-19.

Key Words: COVID-19; Gastrointestinal symptoms; Gut microbiota dysbiosis; Impaired

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Core Tip: Since the declaration of the pandemic on March 11, 2020 by the World Health Organization, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has quickly become a global health threat. In addition to respiratory symptoms, gastrointestinal (GI) symptoms have been widely observed in coronavirus disease 2019 (COVID-19) patients. Here, we have summarized the GI symptoms seen in COVID-19 patients that have been reported in nineteen studies and recapitulated potential mechanisms that are responsible for the GI symptoms in COVID-19 patients. This biochemical understanding may assist in new therapeutic approaches.

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INTRODUCTION

Since December 2019, an acute respiratory infection, referred to as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), caused by the novel coronavirus has rapidly spread worldwide[1-3]. In the United States alone, 198589 deaths (60.3/100000) have been reported due to the coronavirus pandemic from February 13, 2020 to September 19, 2020[4].

Based on next-generation sequencing data from patient samples, SARS-CoV-2 is closely associated with two bat-derived SARS-like coronaviruses, bat-SL-CoVZC45 and bat-SL-CoVZXC21 (88% identity)[5]. The binding of the SARS-CoV-2 spike protein to the host receptor, angiotensin-converting enzyme 2 (ACE2), is critical for viral invasion[6]. This viral infection may be asymptomatic or cause symptoms, such as fever, cough, headache, and myalgia[7-9]. Interestingly, up to 40% of coronavirus disease 2019 (COVID-19) patients experience gastrointestinal (GI) symptoms, including diarrhea, anorexia, nausea, vomiting, and abdominal pain (Table 1). In order to provide appropriate medical care to COVID-19 patients, it is necessary to explore pathophysiological mechanisms underlying their GI symptoms.

In this review, we summarized the studies that describe the various GI symptoms in COVID-19 patients and highlighted the likely underlying pathophysiological mechanisms. These insights offer potential new therapeutic approaches for containment of the global inflammatory response. Furthermore, we also shed light on the importance of the altered gut microbiota profile in the possible pathogenesis of COVID-19.

CLINICAL PRESENTATION OF COVID-19 PATIENTS WITH GI SYMPTOMS

The clinical severity of COVID-19 patients may be stratified into three grades: Placid, ordinary, and grave cases. The incubation period of SARS-CoV-2 ranges from 1-14 d, but is more commonly 3-7 d. The typical clinical presentation of SARS-CoV-2 consists of fever, fatigue, dry cough, and shortness of breath. Other common symptoms involve congestion and rhinorrhea, pharyngalgia, myalgias, and diarrhea. In grave cases, the infection culminates in acute respiratory distress syndrome, which is associated with a high degree of mortality. Although the majority of symptomatic SARS-CoV-2 cases present with pulmonary symptoms, extra-pulmonary symptoms are also common, and several case studies have described the presence of digestive symptoms in the SARS-CoV-2 infection.

We identified and analyzed the GI symptoms of COVID-19 patients reported in nineteen published papers, which included diarrhea, nausea, abdominal pain, vomiting, anorexia, and bleeding (Table 1). Out of the nineteen papers, thirteen were

Table 1 Clinical presentation of gastrointestinal symptoms among coronavirus disease 2019 patients, *n* (%)

Ref.	Number of patients	Diarrhea	Anorexia	Nausea	Vomiting	Abdominal pain	GI Bleeding
Ha *et al*[12]	80	10 (12.5)	-	6 (7.5)	2 (2.5)	7 (8.8)	1 (1.3)
Jin *et al*[10]	651	53 (8.1)	-	10 (1.5)	11 (1.7)	-	-
Lin *et al*[11]	95	23 (24.2)	17 (17.9)	17 (17.9)	4 (4.2)	2 (2.1)[1]	2 (2.1)[2]
Zhang *et al*[65]	139	18 (12.9)	17 (12.2)	24 (17.3)	7 (5)	8 (5.8)	-
Wang *et al*[66]	138	14 (10.1)	55 (39.9)	14 (10.1)	5 (3.6)	3 (2.2)	-
Chen *et al*[67]	99	2(2)	-	1 (1)[3]			
Chen *et al*[68]	9	2 (22.2)	-	-	-	-	-
Young *et al*[13]	18	3 (16.7)	-	-	-	-	-
Chang *et al*[69]	13	1 (7.7)	-	-	-	-	-
Liu *et al*[70]	137	11 (8)	-	-	-	-	-
Pan *et al*[71]	204	35 (17.2)	81 (39.7)	-	4 (2)	2 (1)	-
Wang *et al*[72]	69	10 (14.5)	7 (10.1)	-	3 (4.3)	-	-
Yang *et al*[73]	52	-	-	-	2 (3.8)	-	-
Spiteri *et al*[74]	38	1 (2.6)	-	1 (2.6)	-	-	-
Han *et al*[75]	206	67 (32.5)	32 (15.5)	-	24 (11.7)	9 (4.4)	-
Nobel *et al*[76]	278	56 (20.1)	-	63 (22.7)[3]			
Zhou *et al*[77]	254	46 (18.1)	-	21 (8.3)	15 (5.9)	3 (1.2)	-
Cholankeril *et al*[78]	116	12 (10.3)	22 (25.3)	12 (10.3)	5 (4.3)	10 (8.8)	-
Redd *et al*[79]	318	107 (33.7)	110 (34.8)	84 (26.4)	49 (15.4)	46 (14.5)	2 (0.63)
Total	2914	471 (16.2)	341 (11.7)	253 (8.7)	131 (4.5)	90 (3.1)	5 (0.2)

[1]Epigastric discomfort.
[2]Upper gastrointestinal hemorrhage.
[3]Nausea and vomiting. GI: Gastrointestinal.

from China, four were from the United States, one was from Singapore, and one was from Europe. Among GI symptoms, diarrhea was the most prevalent, accounting for 2% to 33.7% of all patients. The median duration period of diarrhea in COVID-19 patients was 4 d with a range of 1 d to 9 d[10]. Other frequently reported GI symptoms were anorexia (341/2914, 11.7%), nausea (253/2914, 8.7%), vomiting (131/2914, 4.5%), abdominal pain (90/2914, 3.1%) and bleeding (5/2914, 0.2%). GI symptoms were more frequently reported during hospitalization than at the time of admission[11]. We have also recently reported a strong correlation between diarrhea and the severity of the disease[12]. These data suggest that GI symptoms should be included in the assessment of the disease severity in COVID-19.

Previously, it was shown that RNA from SARS-CoV-2 was found in fecal samples (four out of eight patients) regardless of the presence of diarrhea[13]. Furthermore, another study demonstrated that SARS-CoV-2 RNA was found in the feces of 22/42 (52.4%) COVID-19 patients with GI symptoms. Among 23 COVID-19 patients without GI symptoms, SARS-CoV-2 RNA was found in the feces of 9 (39.1%) patients[11]. Although the clinical relevance of SARS-CoV-2 RNA in fecal material remains unclear, understanding the biochemical mechanisms behind the SARS-CoV-2 mediated induction of GI symptoms is important to gain further understanding of the pathophysiology of COVID-19. Therefore, we described potential mechanisms, by which GI symptoms might occur in COVID-19 patients and proposed new therapeutic strategies to modulate the global inflammatory response.

PATHOPHYSIOLOGICAL MECHANISMS FOR GI SYMTOMS IN COVID-19

Intestinal ACE2 receptor mediated impaired barrier function

ACE2 has emerged as a critical regulator of the renin angiotensin system (RAS) by metabolizing angiotensin (Ang) II into the beneficial peptide Ang 1-7[14]. ACE2 has also been identified as the key receptor for SARS-CoV and SARS-CoV-2[15]. Spike protein exposure led to increased Ang II and pulmonary edema, which was mediated by angiotensin II type I receptor (AT1R)[16]. Given the similarities between the SARS-CoV and SARS-CoV-2 spike proteins, a similar mechanism of spike-mediated ACE2 down-regulation most likely underlies tissue damage in COVID-19 by skewing the RAS[16].

The pathophysiology of GI symptoms in COVID-19 remains poorly understood. However, evidence points to a role of ACE2 cell-surface receptors and SARS-CoV-2 induced inflammatory processes in the GI tract[17]. A vital structural protein of SARS-CoV-2 is the spike glycoprotein (S). It consists of two functional units, S1 and S2, that bind to the host cell ACE2 receptor by membrane fusion, replicates through replication-transcription complexes, and promotes proliferation by interfering with and suppressing the host's immune response[18]. SARS-CoV-2 is highly concentrated in air droplets exhaled by infected subjects and inhalation of these particles by a noninfected individual may lead to infection of the recipient's respiratory tract *via* ACE2 receptors. The respiratory tract is one of the primary sites of viral entry. Interestingly, ACE2 receptors are also highly expressed in the digestive tract making it another potential route of SARS-CoV-2 infection[19]. In the gut, ACE2 has a completely different function independent of RAS. ACE2 stabilizes neutral amino acid transporters, such as B0AT1 and loss of ACE2 compromises intestinal uptake of certain dietary amino acids, such as tryptophan[20]. Because tryptophan plays an important role in immunity, ACE2 knockout mice exhibited altered gut microbiota and developed more severe dextran sulfate sodium–induced colitis compared with wild-type control mice[21]. These studies implicated ACE2 in SARS-CoV-2 infections in the gut.

ACE2 plays a major role in amino acid transport in the intestinal epithelium, a mechanism linked to the production of antimicrobial peptides, which suggests its role in intestinal barrier maintenance and gut microbiota equilibrium[22]. ACE2 controls expression of B0AT1 in the intestine, which is the primary apical membrane transporter in the intestine that permits Na^+ coupled uptake of neutral amino acids, such as tryptophan[23]. Notably, B0AT1 substrates, such as tryptophan and glutamine, signal to downregulate lymphoid pro-inflammatory cytokines, maintain the integrity of intestinal tight junctions, activate the release of antimicrobial peptides, and modulate mucosal cell autophagy as defense mechanisms[23]. Altered B0AT1 expression mediated through ACE2 in COVID-19 may be a major contributor to the leaky gut. Thus, it is possible that SARS-CoV-2 mediated disruption of the gut barrier could lead to a systemic elevation of bacterial lipopolysaccharide and peptidoglycan, further worsening GI inflammation. For instance, one study showed that the spike protein of SARS-CoV-2 (S1 subunit) interacted with the ACE2 complex and the tryptophan amino acid transporter B0AT1[24]. Furthermore, downregulated intestinal ACE2-B0AT1 cell surface expression led to a series of downstream sequelae to promote a leaky gut as well as gut microbiota dysbiosis[23,24]. Therefore, ACE2 mediated impaired barrier function in combination with microbial dysbiosis may contribute to the cytokine storm seen in patients severely ill with COVID-19 and may also be responsible for their GI symptoms.

Gut inflammation in COVID-19 patients with diarrhea

Fecal calprotectin (FC) has evolved as a reliable fecal biomarker allowing detection of intestinal inflammation in inflammatory bowel disease (IBD) and infectious colitis[25]. Previous studies have shown that COVID-19 patients with diarrhea without IBD had high FC compared to patients without diarrhea, indicating that the infection evokes a significant intestinal inflammatory process[25]. Furthermore, FC levels correlated significantly with the pro-inflammatory interleukin - 6 (IL-6) serum concentrations, and a murine study showed that deficiency of ACE2 results in highly increased susceptibility to intestinal inflammation induced by epithelial damage[21]. Collectively, the aforementioned studies highlighted that GI inflammation was overrepresented in patients with COVID-19 that also had functional GI disorders (FGIDs) or post-infection (PI) GI disorders.

Alterations in serotonin metabolism in COVID-19 patients

We have reported that plasma serotonin (5-hydroxytrytamine, 5-HT) levels were elevated in COVID-19 patients with diarrhea[12]. 5-HT is a hormone and neurotransmitter that has a monoamine structure. 5-HT synthesis begins with the amino acid L-tryptophan, which is converted to 5-hydroxytryptophan (5-HTP) *via* the rate-limiting enzyme tryptophan hydroxylase (TPH). 5-HTP is then rapidly decarboxylated by aromatic L-amino acid decarboxylase to produce 5-HT[26,27]. 5-HT either circulates in our body or is absorbed by the cells that express serotonin reuptake transporter to act or decompose, resulting in 5-hydroxyindoleacetic acid (5-HIAA)[28]. TPH is an enzyme specifically found in 5-HT producing cells, and there are two different isoforms, TPH1 and TPH2[29,30]. TPH1 dependent 5-HT synthesis occurs in enterochromaffin (EC) cells in GI tract, while TPH2 is involved in 5-HT synthesis in the central nervous system and enteric nervous system[31,32].

Since 95% of total 5-HT is produced by EC cells in GI tract, 5-HT has been widely studied for GI functions, especially in GI motility. Many studies have demonstrated that 5-HT is important for colonic peristaltic reflexes and GI transit[33-35]. Moreover, altered 5-HT levels are closely associated with irritable bowel syndrome (IBS), and it has been shown that platelet-depleted plasma 5-HT levels are increased in IBS patients with diarrhea[36]. Therefore, approaches to target 5-HT signaling have been proposed as a way to alleviate GI dysmotility. A total of seven classes of 5-HT receptors have been identified, and it is well-known that 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 are expressed in the GI tract to influence gut motor function[37]. 5-HT_3 antagonists are especially effective in treating IBS with diarrhea[38,39] and 5-HT_4 agonists are effective in treating IBS with constipation[40,41].

Previously, we have reported that plasma 5-HT levels are increased in COVID-19 patients and are directly correlated to the severity of COVID-19 symptoms. Moreover, COVID-19 patients with diarrhea had increased plasma 5-HT and a lower ratio of plasma 5-HIAA/5-HT levels compared to healthy subjects or COVID-19 patients without diarrhea[12]. These data suggest that 5-HT is not being broken down into 5-HIAA, and 5-HT remains in some COVID-19 patients' for a longer duration, resulting in GI symptoms such as diarrhea. Thus, regulating the amount of 5-HT might be a therapeutic modality for COVID-19 patients with diarrhea.

Gut microbiota dysbiosis in COVID-19 patients

From ancient times, viral infectious diseases have been plaguing mankind through a wide-range of clinical manifestations. Moreover, scientific annals depict the occurrence of life-threatening viral diseases that are enumerated as epidemics and pandemics[42]. Examples include: The flu, polio, Ebola, acquired immune deficiency syndrome, and the very recent COVID-19. In 2020 the past several months, COVID-19 has reached pandemic status, exposing the world to iminent danger. Previously, two other similar viral infections including the Middle East respiratory syndrome virus and SARS-CoV have been reported[43]. SARS-CoV-2 is an enveloped virus in the *Coronaviridae* family. They harbor single stranded RNA as their genetic material that has positive polarity. Some studies published during the recent pandemic of COVID-19 have provided insight into parameters pertaining to the transmission, susceptibility, clinical presentation, and laboratory findings of this potential pathogen[44,45]. Although respiratory droplets and contact are the prime route of transmission for SARS-CoV2, there have been some instances where prolonged exposure to aerosols with high concentrations of the virus may facilitate transmission. Symptoms and severity of COVID-19 differ from patient to patient[46]. In general, humans of all ages are susceptible. However, individuals with an attenuated immune response including elderly, infants, children below 6 years old, patients with underlying diseases (transplants, cancers, diabetes, asthma, heart ailment, and other peril maladies) are at higher risk.

To inject their genetic material into the host, SARS-CoV-2 pierces the pulmonary epithelial cells of the lower respiratory tract thereby commandeering the host's cellular machinery[47]. Moreover, this process is enhanced by the spike (S) protein that interacts with ACE2[47,48]. Thus, the importance of the gut and its microbiome cannot be underestimated. The knowledge in gut research has augmented with a plethora of scientific annals that point towards the role of gut microbes in many degenerative and infectious diseases[49]. Gut dysbiosis has been reported in patients with COVID-19 with enrichment of pathogens and depletion of beneficial commensals[17]. An inverse correlation between the abundance of *Faecalibacterium prausnitzii* (*F. prausnitzii*) and disease severity has been observed. *F. prausnitzii* has anti-inflammatory properties, and its depletion has been related to IBS[17]. Another study showed the gut

microbiome composition was significantly altered in patients with COVID-19 compared with non-COVID-19 individuals irrespective of whether patients had received medication[50]. Several gut commensals with known immunomodulatory potential such as *F. prausnitzii*, *Eubacterium rectale* and Bifidobacteria were underrepresented in patients and remained hampered in samples collected up to 30 d after disease resolution[17,51]. Moreover, this perturbed composition exhibited stratification with disease severity concordant with elevated concentrations of inflammatory cytokines and blood markers such as C-reactive protein, lactate dehydrogenase, aspartate aminotransferase and gamma-glutamyl transferase[17]. The depletion of several bacterial species in the COVID-19 cohort was linked to increased concentrations of tumor necrosis factor-alpha, C-X-C motif chemokine ligand 10, C-C motif chemokine ligand 2 and IL-10. These studies highlighted the need to understand how gut microorganisms are involved in inflammation and COVID-19 pathogenesis[50].

Another study found a signature of active gut viral infection in a subset of patients with COVID-19 even in the absence of GI symptoms, suggesting a 'quiescent' GI infection of SARS-CoV-2[52]. The transcriptional activity of viral infection and replication persisted in the gut even after respiratory clearance of SARS-CoV-2. Fecal samples with a signature of high SARS-CoV-2 infectivity harbored a higher abundance of opportunistic pathogens, for instance, *Morganella morganii, Collinsella aerofaciens*, *Streptococcus infantis*, and *Collinsella tanakaei* and an enhanced capacity for the biosynthesis of nucleotides and amino acids, along with carbohydrate metabolism, whereas fecal samples with a signature of no SARS-CoV-2 infection had a higher abundance of short-chain fatty acid producing bacteria, for instance, *Bacteroides stercoris*, *Parabacteroides merdae*, *Lachnospiraceae bacterium*, and *Alistipes onderdonkii*[52]. This study provided evidence for active and prolonged 'quiescent' GI infection even in the absence of GI manifestations and after recovery from respiratory infection of SARS-CoV-2. The gut microbiota of patients with active SARS-CoV-2 GI infection was characterized by enrichment of opportunistic pathogens; loss of salutary bacteria and increased functional capacity for nucleotides, along with increased amino acid biosynthesis and carbohydrate metabolism[52].

In addition, bacterial groups belonging to the genus Bacteroides, known to downregulate the ACE2 expression in the murine colon, inversely correlated with fecal SARS-CoV-2 nucleic acid loads. Similarly, SARS-CoV-2 infection of GI epithelial cells has been associated with: (1) Lamina propria infiltration of plasma cells and lymphocytes, and edema in the stomach, duodenum, and rectum; (2) Increased levels of FC; (3) Higher fecal levels of IL-8 and lower levels of the anti-inflammatory IL-10 when compared with uninfected controls[53]; (4) SARS-CoV-2-specific IgA and limited inflammatory cytokines were also present in the stool of patients with acute COVID-19; and (5) Gut microbiota dysbiosis. Interestingly, gut microbiota dysbiosis persisted after the resolution of SARS-CoV-2 infection, suggesting that microbiota perturbation may contribute to the persistence of gut dysfunction and symptoms even after the infection has subsided. Indeed, the persistent microbial dysbiosis may contribute to maintaining a chronic state of low-grade GI inflammation, increased intestinal permeability, increased sensory perception, and bile acid malabsorption, which have all been previously associated with symptoms of GI motility disorders.

Post-COVID-19 functional GI disorders

Evidence supports the development of FGIDs after a bout of viral, bacterial, or protozoal gastroenteritis or after resolution of an acute flareup of GI inflammatory diseases such as IBD[54]. Individual susceptibility to these so-called postinfectious functional gastrointestinal disorders (PI-FGIDs) involves genetic predisposition and the presence of pre-existing psychological disturbances such as anxiety and/or depression[55,56]. PI-FGIDs have also been associated with dysregulation of gut motility, visceral hypersensitivity, microbial dysbiosis, intestinal barrier dysfunction, bile acid malabsorption, and alterations in serotonin metabolism[54,57]. Current data suggest that the resolution of the SARS-CoV-2 infection may lead to persistent GI dysfunction resembling certain aspects of PI-FGIDs[17]. Transient non-specific gut inflammation is the common trigger for long-lasting symptoms of FGIDs, regardless of the initiating event (*i.e.*, viral, parasitic, bacterial, after resolution of IBD flares)[58].

SARS-CoV-2 in stool: Suggesting fecal-oral transmission

Evidence of fecal shedding of viral RNA further supports viral replication in the digestive tract and potentially a fecal-oral route of transmission. Studies showed that more than one-half of COVID-19 patients tested positive for fecal SARS-CoV-2 RNA[59]. One study in a group of pediatric patients infected with SARS-CoV-2 had positive rectal swabs for SARS-CoV-2, even after the nasopharynx was cleared of the

virus, suggesting that viral shedding from the digestive tract might be more prolonged than that from the respiratory tract[60]. Another study showed that SARS-CoV-2 can infect the enterocytes of bats in an organoid culture system of bat intestinal epithelium [61]. One study indicated that infection by SARS-CoV-2 led to an altered fecal microbiome during hospitalization[62]. The authors showed enrichment of opportunistic pathogens and depletion of commensals during SARS-CoV-2 infection. *Coprobacillus*, *Clostridium ramosum*, and *Clostridum mathewayi* were found more commonly in patients with severe COVID-19. In contrast, the presence of *F. prausnitzii* was correlated with milder disease. Gut microbial dysbiosis persisted in the majority of COVID-19 patients in spite of clearance of the virus, suggesting that exposure to SARS-CoV-2 might be associated with more long-lasting deleterious effects to the healthy gut microbiome [23,62]. These studies support the possibility for SARS-CoV-2 fecal-oral route of transmission. Therefore, from both clinical and public health standpoints, it is critical to fully understand all routes of transmission of SARS-CoV-2. If high levels of infectious viruses are present in the intestinal lumen of infected patients, especially in asymptomatic patients, this poses risks during endoscopy and colonoscopy to gastroenterologists, endoscopy personnel and other patients. For the general public, infectious viral particles in the feces shed by infected individuals, if aerosolized, have great implications in confined environments such as cruise ships, hospitals, individual households, and densely populated housing, such as those in regions with poor sanitation[19].

CONCLUSION

GI symptoms are overrepresented in patients with COVID-19. A proportion of patients affected by COVID-19 may develop PI-FGIDs based on the following pathophysiological mechanisms: Intestinal barrier dysfunction, chronic low-grade intestinal inflammation, altered serotonin metabolism, and gut microbiota dysbiosis. The question of whether gut inflammation is associated with gut microbiota dysbiosis in patients, which may have a central role in the COVID-19 disease progression warrants further investigation. However, there is mounting evidence that gut microorganisms are linked to GI inflammatory diseases, which highlights the urgent need to understand the specific roles of gut microorganisms that are responsible for the immune dysfunction and systemic inflammation in COVID-19.

The abundance of SARS-CoV-2 viral RNA in stool and the stability of the virus in the environment suggest that fecal contamination may be an important modality for the spread among human hosts. Fecal sources may lead to viral transmission, especially when aerosols are generated. The significance of GI involvement in COVID-19 patients requires attention in clinical practices, such as incorporation of rectal swab testing before discharging patients, as well as the importance of personal protective equipment in the endoscopy setting. These precautions will be imperative in our battle against COVID-19[63].

Considering the critical role of the ACE2 receptor in the pathogenesis of COVID-19 and the potential impact on severity of symptoms in some patients, several therapeutic approaches have been evaluated such as a soluble form of ACE2 (rhACE2), ACE2 blockers, TMPRSS2 inhibitors, and Ang 1-7 receptor agonists. Some of these therapeutic approaches appeared to show promising results and are currently in clinical trials. Another strategy to manage COVID-19 might be to restore the microbiota during the dysbiosis through prebiotic and/or probiotic interventions and dietary nutritional supplementation[64].

This review sheds light on the studies that formulate the pathophysiological mechanisms (impaired barrier function, gut inflammation, altered serotonin metabolism and gut microbiota dysbiosis) underlying GI symptoms in patients with COVID-19 (Figure 1). To the best of our knowledge we are the first to propose altered serotonin metabolism in the pathogenesis of COVID-19 associated with diarrhea. This novel insight of serotonin metabolism might be a key player underpinning GI symptoms and severity in patients with COVID-19 as altered serotonin signaling modulates the majority of pathological mechanisms in patients with FGIDs. Therapeutic modalities regulating serotonin signaling might offer potential treatment options in a subset of COVID-19 patients. Furthermore, we highlighted the important concept of post-SARS-CoV-2-FGIDs, which warrants future studies to dissect persistent GI symptoms after the clearance of SARS-CoV-2 infection. Scientists and clinicians should be aware of this new clinical scenario, and additional studies will be needed to further characterize and uncover the pathophysiological mechanisms of this

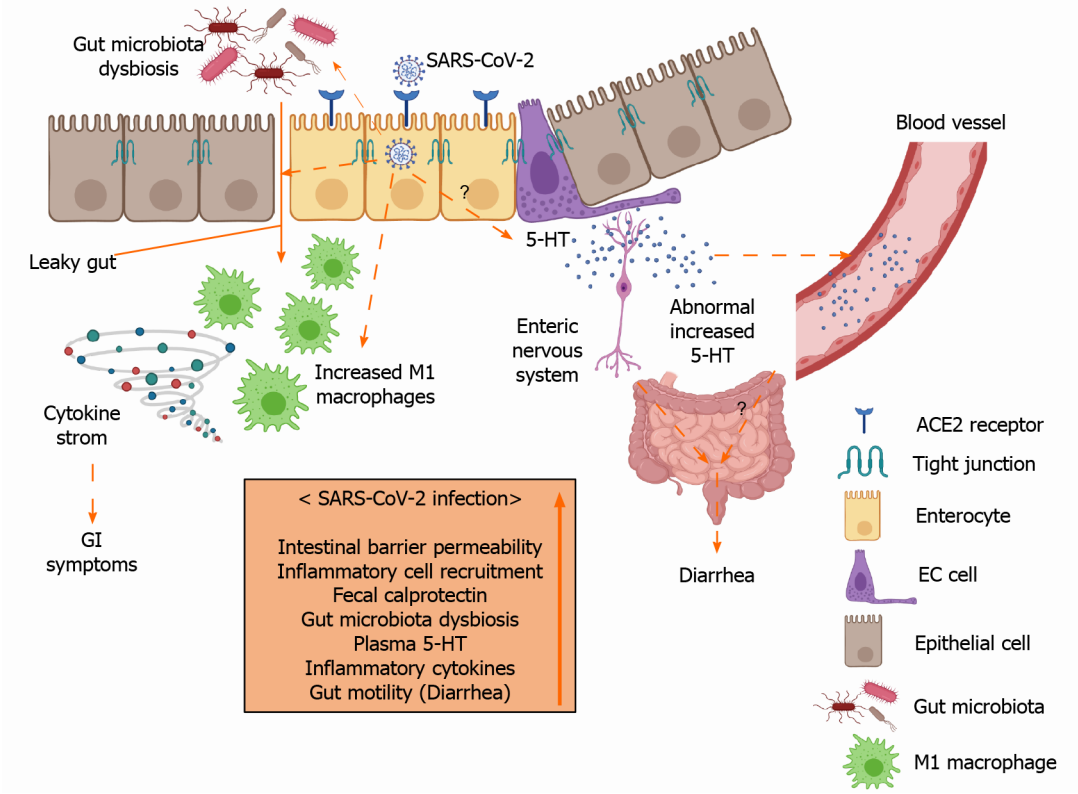


Figure 1 A simplified diagram of the potential pathological mechanisms for gastrointestinal symptoms associated with severe acute respiratory syndrome coronavirus 2 infection. The figure was created with BioRender.com. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; 5-HT: 5-hydroxytrytamine; EC cell: Enterochromaffin; ACE2 cell: Angiotensin converting enzyme 2.

phenomenon. Furthermore, studies are warranted to elucidate the following: (1) the cause-and-effect relationship between changes in relative abundance of gut bacteria and COVID-19; (2) the possibility that the microbiota plays a role in illness severity; and (3) the relationship between the host's immune response (regulatory T cell response) to SARS-CoV-2 resulting in a high or low cytokine storm.

REFERENCES

1 **Hui DS**, I Azhar E, Madani TA, Ntoumi F, Kock R, Dar O, Ippolito G, Mchugh TD, Memish ZA, Drosten C, Zumla A, Petersen E. The continuing 2019-nCoV epidemic threat of novel coronaviruses to global health - The latest 2019 novel coronavirus outbreak in Wuhan, China. *Int J Infect Dis* 2020; **91**: 264-266 [PMID: 31953166 DOI: 10.1016/j.ijid.2020.01.009]

2 **Lu H**, Stratton CW, Tang YW. Outbreak of pneumonia of unknown etiology in Wuhan, China: The mystery and the miracle. *J Med Virol* 2020; **92**: 401-402 [PMID: 31950516 DOI: 10.1002/jmv.25678]

3 **Bogoch II**, Watts A, Thomas-Bachli A, Huber C, Kraemer MUG, Khan K. Potential for global spread of a novel coronavirus from China. *J Travel Med* 2020; **27**: taaa011 [PMID: 31985790 DOI: 10.1093/jtm/taaa011]

4 **Bilinski A**, Emanuel EJ. COVID-19 and Excess All-Cause Mortality in the US and 18 Comparison Countries. *JAMA* 2020; **324**: 2100-2102 [PMID: 33044514 DOI: 10.1001/jama.2020.20717]

5 **Lu R**, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 2020; **395**: 565-574 [PMID: 32007145 DOI: 10.1016/S0140-6736(20)30251-8]

6 **Wan Y**, Shang J, Graham R, Baric RS, Li F. Receptor Recognition by the Novel Coronavirus from Wuhan: an Analysis Based on Decade-Long Structural Studies of SARS Coronavirus. *J Virol* 2020; 94: e00127-20 [PMID: 31996437 DOI: 10.1128/JVI.00127-20]

7 **Dhama K**, Khan S, Tiwari R, Sircar S, Bhat S, Malik YS, Singh KP, Chaicumpa W, Bonilla-Aldana DK, Rodriguez-Morales AJ. Coronavirus Disease 2019-COVID-19. *Clin Microbiol Rev* 2020; **33**: e00028-20 [PMID: 32580969 DOI: 10.1128/CMR.00028-20]

8 **Vabret N**, Britton GJ, Gruber C, Hegde S, Kim J, Kuksin M, Levantovsky R, Malle L, Moreira A,

Park MD, Pia L, Risson E, Saffern M, Salomé B, Esai Selvan M, Spindler MP, Tan J, van der Heide V, Gregory JK, Alexandropoulos K, Bhardwaj N, Brown BD, Greenbaum B, Gümüş ZH, Homann D, Horowitz A, Kamphorst AO, Curotto de Lafaille MA, Mehandru S, Merad M, Samstein RM; Sinai Immunology Review Project. Immunology of COVID-19: Current State of the Science. *Immunity* 2020; **52**: 910-941 [PMID: 32505227 DOI: 10.1016/j.immuni.2020.05.002]

9 **Machhi J**, Herskovitz J, Senan AM, Dutta D, Nath B, Oleynikov MD, Blomberg WR, Meigs DD, Hasan M, Patel M, Kline P, Chang RC, Chang L, Gendelman HE, Kevadiya BD. The Natural History, Pathobiology, and Clinical Manifestations of SARS-CoV-2 Infections. *J Neuroimmune Pharmacol* 2020; **15**: 359-386 [PMID: 32696264 DOI: 10.1007/s11481-020-09944-5]

10 **Jin X**, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, Hao SR, Jia HY, Cai H, Zhang XL, Yu GD, Xu KJ, Wang XY, Gu JQ, Zhang SY, Ye CY, Jin CL, Lu YF, Yu X, Yu XP, Huang JR, Xu KL, Ni Q, Yu CB, Zhu B, Li YT, Liu J, Zhao H, Zhang X, Yu L, Guo YZ, Su JW, Tao JJ, Lang GJ, Wu XX, Wu WR, Qv TT, Xiang DR, Yi P, Shi D, Chen Y, Ren Y, Qiu YQ, Li LJ, Sheng J, Yang Y. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. *Gut* 2020; **69**: 1002-1009 [PMID: 32213556 DOI: 10.1136/gutjnl-2020-320926]

11 **Lin L**, Jiang X, Zhang Z, Huang S, Fang Z, Gu Z, Gao L, Shi H, Mai L, Liu Y, Lin X, Lai R, Yan Z, Li X, Shan H. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. *Gut* 2020; **69**: 997-1001 [PMID: 32241899 DOI: 10.1136/gutjnl-2020-321013]

12 **Ha S**, Jin B, Clemmensen B, Park P, Mahboob S, Gladwill V, Lovely FM, Gottfried-Blackmore A, Habtezion A, Verma S, Ro S. Serotonin is elevated in COVID-19-associated diarrhoea. *Gut* 2021; epub ahead of print [PMID: 33402416 DOI: 10.1136/gutjnl-2020-323542]

13 **Young BE**, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, Tan TY, Ng TY, Cui L, Said Z, Kurupatham L, Chen MI, Chan M, Vasoo S, Wang LF, Tan BH, Lin RTP, Lee VJM, Leo YS, Lye DC; Singapore 2019 Novel Coronavirus Outbreak Research Team. Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore. *JAMA* 2020; **323**: 1488-1494 [PMID: 32125362 DOI: 10.1001/jama.2020.3204]

14 **Gheblawi M**, Wang K, Viveiros A, Nguyen Q, Zhong JC, Turner AJ, Raizada MK, Grant MB, Oudit GY. Angiotensin-Converting Enzyme 2: SARS-CoV-2 Receptor and Regulator of the Renin-Angiotensin System: Celebrating the 20th Anniversary of the Discovery of ACE2. *Circ Res* 2020; **126**: 1456-1474 [PMID: 32264791 DOI: 10.1161/CIRCRESAHA.120.317015]

15 **Zhang H**, Penninger JM, Li Y, Zhong N, Slutsky AS. Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. *Intensive Care Med* 2020; **46**: 586-590 [PMID: 32125455 DOI: 10.1007/s00134-020-05985-9]

16 **Ni W**, Yang X, Yang D, Bao J, Li R, Xiao Y, Hou C, Wang H, Liu J, Xu Y, Cao Z, Gao Z. Role of angiotensin-converting enzyme 2 (ACE2) in COVID-19. *Crit Care* 2020; **24**: 422 [PMID: 32660650 DOI: 10.1186/s13054-020-03120-0]

17 **Schmulson M**, Ghoshal UC, Barbara G. Managing the Inevitable Surge of Post-COVID-19 Functional Gastrointestinal Disorders. *Am J Gastroenterol* 2021; **116**: 4-7 [PMID: 33273261 DOI: 10.14309/ajg.0000000000001062]

18 **Xu X**, Chen P, Wang J, Feng J, Zhou H, Li X, Zhong W, Hao P. Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. *Sci China Life Sci* 2020; **63**: 457-460 [PMID: 32009228 DOI: 10.1007/s11427-020-1637-5]

19 **Ding S**, Liang TJ. Is SARS-CoV-2 Also an Enteric Pathogen With Potential Fecal-Oral Transmission? *Gastroenterology* 2020; **159**: 53-61 [PMID: 32353371 DOI: 10.1053/j.gastro.2020.04.052]

20 **Camargo SMR**, Vuille-Dit-Bille RN, Meier CF, Verrey F. ACE2 and gut amino acid transport. *Clin Sci (Lond)* 2020; **134**: 2823-2833 [PMID: 33140827 DOI: 10.1042/CS20200477]

21 **Hashimoto T**, Perlot T, Rehman A, Trichereau J, Ishiguro H, Paolino M, Sigl V, Hanada T, Hanada R, Lipinski S, Wild B, Camargo SM, Singer D, Richter A, Kuba K, Fukamizu A, Schreiber S, Clevers H, Verrey F, Rosenstiel P, Penninger JM. ACE2 Links amino acid malnutrition to microbial ecology and intestinal inflammation. *Nature* 2012; **487**: 477-481 [PMID: 22837003 DOI: 10.1038/nature11228]

22 **Perlot T**, Penninger JM. ACE2 - from the renin-angiotensin system to gut microbiota and malnutrition. *Microbes Infect* 2013; **15**: 866-873 [PMID: 23962453 DOI: 10.1016/j.micinf.2013.08.003]

23 **Penninger JM**, Grant MB, Sung JJY. The Role of Angiotensin Converting Enzyme 2 in Modulating Gut Microbiota, Intestinal Inflammation, and Coronavirus Infection. *Gastroenterology* 2021; **160**: 39-46 [PMID: 33130103 DOI: 10.1053/j.gastro.2020.07.067]

24 **Yan R**, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science* 2020; **367**: 1444-1448 [PMID: 32132184 DOI: 10.1126/science.abb2762]

25 **Effenberger M**, Grabherr F, Mayr L, Schwaerzler J, Nairz M, Seifert M, Hilbe R, Seiwald S, Scholl-Buergi S, Fritsche G, Bellmann-Weiler R, Weiss G, Müller T, Adolph TE, Tilg H. Faecal calprotectin indicates intestinal inflammation in COVID-19. *Gut* 2020; **69**: 1543-1544 [PMID: 32312790 DOI: 10.1136/gutjnl-2020-321388]

26 **Boadle-Biber MC**. Regulation of serotonin synthesis. *Prog Biophys Mol Biol* 1993; **60**: 1-15 [PMID: 8480026 DOI: 10.1016/0079-6107(93)90009-9]

27 **Höglund E**, Øverli Ø, Winberg S. Tryptophan Metabolic Pathways and Brain Serotonergic Activity: A Comparative Review. *Front Endocrinol (Lausanne)* 2019; **10**: 158 [PMID: 31024440 DOI: 10.3389/fendo.2019.00158]

28 **Bian X**, Patel B, Dai X, Galligan JJ, Swain G. High mucosal serotonin availability in neonatal guinea pig ileum is associated with low serotonin transporter expression. *Gastroenterology* 2007; **132**: 2438-2447 [PMID: 17570217 DOI: 10.1053/j.gastro.2007.03.103]

29 **Côté F**, Thévenot E, Fligny C, Fromes Y, Darmon M, Ripoche MA, Bayard E, Hanoun N, Saurini F, Lechat P, Dandolo L, Hamon M, Mallet J, Vodjdani G. Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function. *Proc Natl Acad Sci USA* 2003; **100**: 13525-13530 [PMID: 14597720 DOI: 10.1073/pnas.2233056100]

30 **Walther DJ**, Peter JU, Bashammakh S, Hörtnagl H, Voits M, Fink H, Bader M. Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science* 2003; **299**: 76 [PMID: 12511643 DOI: 10.1126/science.1078197]

31 **Bellono NW**, Bayrer JR, Leitch DB, Castro J, Zhang C, O'Donnell TA, Brierley SM, Ingraham HA, Julius D. Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. *Cell* 2017; **170**: 185-198. e16 [PMID: 28648659 DOI: 10.1016/j.cell.2017.05.034]

32 **Israelyan N**, Del Colle A, Li Z, Park Y, Xing A, Jacobsen JPR, Luna RA, Jensen DD, Madra M, Saurman V, Rahim R, Latorre R, Law K, Carson W, Bunnett NW, Caron MG, Margolis KG. Effects of Serotonin and Slow-Release 5-Hydroxytryptophan on Gastrointestinal Motility in a Mouse Model of Depression. *Gastroenterology* 2019; **157**: 507-521. e4 [PMID: 31071306 DOI: 10.1053/j.gastro.2019.04.022]

33 **Li Z**, Chalazonitis A, Huang YY, Mann JJ, Margolis KG, Yang QM, Kim DO, Côté F, Mallet J, Gershon MD. Essential roles of enteric neuronal serotonin in gastrointestinal motility and the development/survival of enteric dopaminergic neurons. *J Neurosci* 2011; **31**: 8998-9009 [PMID: 21677183 DOI: 10.1523/JNEUROSCI.6684-10.2011]

34 **Heredia DJ**, Gershon MD, Koh SD, Corrigan RD, Okamoto T, Smith TK. Important role of mucosal serotonin in colonic propulsion and peristaltic reflexes: *in vitro* analyses in mice lacking tryptophan hydroxylase 1. *J Physiol* 2013; **591**: 5939-5957 [PMID: 24127620 DOI: 10.1113/jphysiol.2013.256230]

35 **Heredia DJ**, Dickson EJ, Bayguinov PO, Hennig GW, Smith TK. Localized release of serotonin (5-hydroxytryptamine) by a fecal pellet regulates migrating motor complexes in murine colon. *Gastroenterology* 2009; **136**: 1328-1338 [PMID: 19138686 DOI: 10.1053/j.gastro.2008.12.010]

36 **Atkinson W**, Lockhart S, Whorwell PJ, Keevil B, Houghton LA. Altered 5-hydroxytryptamine signaling in patients with constipation- and diarrhea-predominant irritable bowel syndrome. *Gastroenterology* 2006; **130**: 34-43 [PMID: 16401466 DOI: 10.1053/j.gastro.2005.09.031]

37 **De Ponti F**. Pharmacology of serotonin: what a clinician should know. *Gut* 2004; **53**: 1520-1535 [PMID: 15361507 DOI: 10.1136/gut.2003.035568]

38 **Gershon MD**, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology* 2007; **132**: 397-414 [PMID: 17241888 DOI: 10.1053/j.gastro.2006.11.002]

39 **Fayyaz M**, Lackner JM. Serotonin receptor modulators in the treatment of irritable bowel syndrome. *Ther Clin Risk Manag* 2008; **4**: 41-48 [PMID: 18728719 DOI: 10.2147/tcrm.s140]

40 **Sullivan KL**, Staffetti JF, Hauser RA, Dunne PB, Zesiewicz TA. Tegaserod (Zelnorm) for the treatment of constipation in Parkinson's disease. *Mov Disord* 2006; **21**: 115-116 [PMID: 16142776 DOI: 10.1002/mds.20666]

41 **Di Palma JA**, Cleveland MV, McGowan J, Herrera JL. A randomized, multicenter comparison of polyethylene glycol laxative and tegaserod in treatment of patients with chronic constipation. *Am J Gastroenterol* 2007; **102**: 1964-1971 [PMID: 17573794 DOI: 10.1111/j.1572-0241.2007.01365.x]

42 **Bloom DE**, Cadarette D. Infectious Disease Threats in the Twenty-First Century: Strengthening the Global Response. *Front Immunol* 2019; **10**: 549 [PMID: 30984169 DOI: 10.3389/fimmu.2019.00549]

43 **Elfiky AA**. SARS-CoV-2 Spike-Heat Shock Protein A5 (GRP78) Recognition may be Related to the Immersed Human Coronaviruses. *Front Pharmacol* 2020; **11**: 577467 [PMID: 33362542 DOI: 10.3389/fphar.2020.577467]

44 **Kakodkar P**, Kaka N, Baig MN. A Comprehensive Literature Review on the Clinical Presentation, and Management of the Pandemic Coronavirus Disease 2019 (COVID-19). *Cureus* 2020; **12**: e7560 [PMID: 32269893 DOI: 10.7759/cureus.7560]

45 **Voto C**, Berkner P, Brenner C. Overview of the Pathogenesis and Treatment of SARS-CoV-2 for Clinicians: A Comprehensive Literature Review. *Cureus* 2020; **12**: e10357 [PMID: 33062480 DOI: 10.7759/cureus.10357]

46 **Shah SJ**, Barish PN, Prasad PA, Kistler A, Neff N, Kamm J, Li LM, Chiu CY, Babik JM, Fang MC, Abe-Jones Y, Alipanah N, Alvarez FN, Botvinnik OB, Castaneda G; CZB CLIAhub Consortium, Dadasovich RM, Davis J, Deng X, DeRisi JL, Detweiler AM, Federman S, Haliburton J, Hao S, Kerkhoff AD, Kumar GR, Malcolm KB, Mann SA, Martinez S, Mary RK, Mick E, Mwakibete L, Najafi N, Peluso MJ, Phelps M, Pisco AO, Ratnasiri K, Rubio LA, Sellas A, Sherwood KD, Sheu J, Spottiswoode N, Tan M, Yu G, Kangelaris KN, Langelier C. Clinical features, diagnostics, and outcomes of patients presenting with acute respiratory illness: A retrospective cohort study of patients with and without COVID-19. *EClinicalMedicine* 2020; **27**: 100518 [PMID: 32864588 DOI: 10.1016/j.eclinm.2020.100518]

47 **Xu J**, Xu X, Jiang L, Dua K, Hansbro PM, Liu G. SARS-CoV-2 induces transcriptional signatures in

human lung epithelial cells that promote lung fibrosis. *Respir Res* 2020; **21**: 182 [PMID: 32664949 DOI: 10.1186/s12931-020-01445-6]

48 **Carvalho T**. Extrapulmonary SARS-CoV-2 manifestations. *Nat Med* 2020; **26**: 1806 [PMID: 33288941 DOI: 10.1038/s41591-020-01162-z]

49 **Villapol S**. Gastrointestinal symptoms associated with COVID-19: impact on the gut microbiome. *Transl Res* 2020; **226**: 57-69 [PMID: 32827705 DOI: 10.1016/j.trsl.2020.08.004]

50 **Yeoh YK**, Zuo T, Lui GC, Zhang F, Liu Q, Li AY, Chung AC, Cheung CP, Tso EY, Fung KS, Chan V, Ling L, Joynt G, Hui DS, Chow KM, Ng SSS, Li TC, Ng RW, Yip TC, Wong GL, Chan FK, Wong CK, Chan PK, Ng SC. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut* 2021; **70**: 698-706 [PMID: 33431578 DOI: 10.1136/gutjnl-2020-323020]

51 **Lordan C**, Thapa D, Ross RP, Cotter PD. Potential for enriching next-generation health-promoting gut bacteria through prebiotics and other dietary components. *Gut Microbes* 2020; **11**: 1-20 [PMID: 31116628 DOI: 10.1080/19490976.2019.1613124]

52 **Zuo T**, Liu Q, Zhang F, Lui GC, Tso EY, Yeoh YK, Chen Z, Boon SS, Chan FK, Chan PK, Ng SC. Depicting SARS-CoV-2 faecal viral activity in association with gut microbiota composition in patients with COVID-19. *Gut* 2021; **70**: 276-284 [PMID: 32690600 DOI: 10.1136/gutjnl-2020-322294]

53 **Britton GJ**, Chen-Liaw A, Cossarini F, Livanos AE, Spindler MP, Plitt T, Eggers J, Mogno I, Gonzalez-Reiche A, Siu S, Tankelevich M, Grinspan L, Dixon RE, Jha D, Martinez-Delgado G, Amanat F, Hoagland DA, tenOever B, Dubinsky MC, Merad M, van Bakel H, Krammer F, Bongers G, Mehandru S, Faith JJ. SARS-CoV-2-specific IgA and limited inflammatory cytokines are present in the stool of select patients with acute COVID-19. 2020 Preprint. Available from: medRxiv [PMID: 32909002 DOI: 10.1101/2020.09.03.20183947]

54 **Barbara G**, Grover M, Bercik P, Corsetti M, Ghoshal UC, Ohman L, Rajilić-Stojanović M. Rome Foundation Working Team Report on Post-Infection Irritable Bowel Syndrome. *Gastroenterology* 2019; **156**: 46-58. e7 [PMID: 30009817 DOI: 10.1053/j.gastro.2018.07.011]

55 **Barbara G**, Cremon C, Stanghellini V. Inflammatory bowel disease and irritable bowel syndrome: similarities and differences. *Curr Opin Gastroenterol* 2014; **30**: 352-358 [PMID: 24811054 DOI: 10.1097/MOG.0000000000000070]

56 **Marshall JK**, Thabane M, Borgaonkar MR, James C. Postinfectious irritable bowel syndrome after a food-borne outbreak of acute gastroenteritis attributed to a viral pathogen. *Clin Gastroenterol Hepatol* 2007; **5**: 457-460 [PMID: 17289440 DOI: 10.1016/j.cgh.2006.11.025]

57 **Ghoshal UC**, Rahman MM. Post-infection irritable bowel syndrome in the tropical and subtropical regions: Vibrio cholerae is a new cause of this well-known condition. *Indian J Gastroenterol* 2019; **38**: 87-94 [PMID: 31073702 DOI: 10.1007/s12664-019-00959-2]

58 **Barbara G**, Feinle-Bisset C, Ghoshal UC, Quigley EM, Santos J, Vanner S, Vergnolle N, Zoetendal EG. The Intestinal Microenvironment and Functional Gastrointestinal Disorders. *Gastroenterology* 2016; **150**: 1305-1318 [PMID: 27144620 DOI: 10.1053/j.gastro.2016.02.028]

59 **Gupta S**, Parker J, Smits S, Underwood J, Dolwani S. Persistent viral shedding of SARS-CoV-2 in faeces - a rapid review. *Colorectal Dis* 2020; **22**: 611-620 [PMID: 32418307 DOI: 10.1111/codi.15138]

60 **Donà D**, Minotti C, Costenaro P, Da Dalt L, Giaquinto C. Fecal-Oral Transmission of SARS-CoV-2 In Children: is it Time to Change Our Approach? *Pediatr Infect Dis J* 2020; **39**: e133-e134 [PMID: 32304466 DOI: 10.1097/INF.0000000000002704]

61 **Zhou J**, Li C, Liu X, Chiu MC, Zhao X, Wang D, Wei Y, Lee A, Zhang AJ, Chu H, Cai JP, Yip CC, Chan IH, Wong KK, Tsang OT, Chan KH, Chan JF, To KK, Chen H, Yuen KY. Infection of bat and human intestinal organoids by SARS-CoV-2. *Nat Med* 2020; **26**: 1077-1083 [PMID: 32405028 DOI: 10.1038/s41591-020-0912-6]

62 **Zuo T**, Zhang F, Lui GCY, Yeoh YK, Li AYL, Zhan H, Wan Y, Chung ACK, Cheung CP, Chen N, Lai CKC, Chen Z, Tso EYK, Fung KSC, Chan V, Ling L, Joynt G, Hui DSC, Chan FKL, Chan PKS, Ng SC. Alterations in Gut Microbiota of Patients With COVID-19 During Time of Hospitalization. *Gastroenterology* 2020; **159**: 944-955. e8 [PMID: 32442562 DOI: 10.1053/j.gastro.2020.05.048]

63 **Goh KL**, Chuah KH. COVID-19 and the digestive system: More than just a "flu". *JGH Open* 2020; **4**: 318-319 [PMID: 32514430 DOI: 10.1002/jgh3.12364]

64 **Viana SD**, Nunes S, Reis F. ACE2 imbalance as a key player for the poor outcomes in COVID-19 patients with age-related comorbidities - Role of gut microbiota dysbiosis. *Ageing Res Rev* 2020; **62**: 101123 [PMID: 32683039 DOI: 10.1016/j.arr.2020.101123]

65 **Zhang JJ**, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, Akdis CA, Gao YD. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy* 2020; **75**: 1730-1741 [PMID: 32077115 DOI: 10.1111/all.14238]

66 **Wang D**, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 2020; **323**: 1061-1069 [PMID: 32031570 DOI: 10.1001/jama.2020.1585]

67 **Chen N**, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020; **395**: 507-513 [PMID: 32007143 DOI: 10.1016/S0140-6736(20)30211-7]

68 **Chen Q**, Quan B, Li X, Gao G, Zheng W, Zhang J, Zhang Z, Liu C, Li L, Wang C, Zhang G, Li J, Dai Y, Yang J, Han W. A report of clinical diagnosis and treatment of nine cases of coronavirus disease 2019. *J Med Virol* 2020; **92**: 683-687 [PMID: 32162699 DOI: 10.1002/jmv.25755]

69 **Chang**, Lin M, Wei L, Xie L, Zhu G, Dela Cruz CS, Sharma L. Epidemiologic and Clinical Characteristics of Novel Coronavirus Infections Involving 13 Patients Outside Wuhan, China. *JAMA* 2020; **323**: 1092-1093 [PMID: 32031568 DOI: 10.1001/jama.2020.1623]

70 **Liu K**, Fang YY, Deng Y, Liu W, Wang MF, Ma JP, Xiao W, Wang YN, Zhong MH, Li CH, Li GC, Liu HG. Clinical characteristics of novel coronavirus cases in tertiary hospitals in Hubei Province. *Chin Med J (Engl)* 2020; **133**: 1025-1031 [PMID: 32044814 DOI: 10.1097/CM9.0000000000000744]

71 **Pan L**, Mu M, Yang P, Sun Y, Wang R, Yan J, Li P, Hu B, Wang J, Hu C, Jin Y, Niu X, Ping R, Du Y, Li T, Xu G, Hu Q, Tu L. Clinical Characteristics of COVID-19 Patients With Digestive Symptoms in Hubei, China: A Descriptive, Cross-Sectional, Multicenter Study. *Am J Gastroenterol* 2020; **115**: 766-773 [PMID: 32287140 DOI: 10.14309/ajg.0000000000000620]

72 **Wang Z**, Yang B, Li Q, Wen L, Zhang R. Clinical Features of 69 Cases With Coronavirus Disease 2019 in Wuhan, China. *Clin Infect Dis* 2020; **71**: 769-777 [PMID: 32176772 DOI: 10.1093/cid/ciaa272]

73 **Yang X**, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, Zou X, Yuan S, Shang Y. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med* 2020; **8**: 475-481 [PMID: 32105632 DOI: 10.1016/S2213-2600(20)30079-5]

74 **Spiteri G**, Fielding J, Diercke M, Campese C, Enouf V, Gaymard A, Bella A, Sognamiglio P, Sierra Moros MJ, Riutort AN, Demina YV, Mahieu R, Broas M, Bengnér M, Buda S, Schilling J, Filleul L, Lepoutre A, Saura C, Mailles A, Levy-Bruhl D, Coignard B, Bernard-Stoecklin S, Behillil S, van der Werf S, Valette M, Lina B, Riccardo F, Nicastri E, Casas I, Larrauri A, Salom Castell M, Pozo F, Maksyutov RA, Martin C, Van Ranst M, Bossuyt N, Siira L, Sane J, Tegmark-Wisell K, Palmérus M, Broberg EK, Beauté J, Jorgensen P, Bundle N, Pereyaslov D, Adlhoch C, Pukkila J, Pebody R, Olsen S, Ciancio BC. First cases of coronavirus disease 2019 (COVID-19) in the WHO European Region, 24 January to 21 February 2020. *Euro Surveill* 2020; **25** [PMID: 32156327 DOI: 10.2807/1560-7917.ES.2020.25.9.2000178]

75 **Han C**, Duan C, Zhang S, Spiegel B, Shi H, Wang W, Zhang L, Lin R, Liu J, Ding Z, Hou X. Digestive Symptoms in COVID-19 Patients With Mild Disease Severity: Clinical Presentation, Stool Viral RNA Testing, and Outcomes. *Am J Gastroenterol* 2020; **115**: 916-923 [PMID: 32301761 DOI: 10.14309/ajg.0000000000000664]

76 **Nobel YR**, Phipps M, Zucker J, Lebwohl B, Wang TC, Sobieszczyk ME, Freedberg DE. Gastrointestinal Symptoms and Coronavirus Disease 2019: A Case-Control Study From the United States. *Gastroenterology* 2020; **159**: 373-375. e2 [PMID: 32294477 DOI: 10.1053/j.gastro.2020.04.017]

77 **Zhou Z**, Zhao N, Shu Y, Han S, Chen B, Shu X. Effect of Gastrointestinal Symptoms in Patients With COVID-19. *Gastroenterology* 2020; **158**: 2294-2297 [PMID: 32199880 DOI: 10.1053/j.gastro.2020.03.020]

78 **Cholankeril G**, Podboy A, Aivaliotis VI, Tarlow B, Pham EA, Spencer SP, Kim D, Hsing A, Ahmed A. High Prevalence of Concurrent Gastrointestinal Manifestations in Patients With Severe Acute Respiratory Syndrome Coronavirus 2: Early Experience From California. *Gastroenterology* 2020; **159**: 775-777 [PMID: 32283101 DOI: 10.1053/j.gastro.2020.04.008]

79 **Redd WD**, Zhou JC, Hathorn KE, McCarty TR, Bazarbashi AN, Thompson CC, Shen L, Chan WW. Prevalence and Characteristics of Gastrointestinal Symptoms in Patients With Severe Acute Respiratory Syndrome Coronavirus 2 Infection in the United States: A Multicenter Cohort Study. *Gastroenterology* 2020; **159**: 765-767. e2 [PMID: 32333911 DOI: 10.1053/j.gastro.2020.04.045]

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ORIGINAL ARTICLE

Retrospective Cohort Study

Risk factors and prognostic value of acute severe lower gastrointestinal bleeding in Crohn's disease

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Institutional review board statement: The study was

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Abstract

BACKGROUND

Acute severe lower gastrointestinal bleeding (LGIB) is an uncommon but challenging complication of Crohn's disease (CD).

AIM

To identify the predictors of acute severe LGIB and to evaluate the impact of acute severe LGIB on the subsequent clinical course in CD patients.

METHODS

A retrospective inception cohort study was conducted in 75 CD patients with acute severe LGIB and 1359 CD patients without acute severe LGIB who were diagnosed between February 1991 and November 2019 at Asan Medical Center, a tertiary university hospital in Korea. Multivariable analysis with Cox proportional hazard regression was performed to identify the risk factors for acute severe LGIB. A matched analysis using 72 patients with bleeding and 267 matched patients without within the cohort was also conducted to investigate whether acute severe LGIB is a predictor of clinical outcomes of CD.

RESULTS

Multivariable Cox regression analysis revealed that early use of thiopurines [hazard ratio (HR): 0.23, 95% confidence interval (CI): 0.12-0.48; $P < 0.001$] and female sex (HR: 0.51, 95%CI: 0.27-0.94; $P = 0.031$) were significantly associated

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with a lower risk of acute severe LGIB. The cumulative risks of behavioral progression and intestinal resection were not significantly different between the two matched groups (P = 0.139 and P = 0.769, respectively). The hospitalization rate was higher in the bleeding group than in the matched non-bleeding group (22.1/100 *vs* 13.2/100 patient-years; P = 0.012). However, if hospitalizations due to bleeding episodes were excluded from the analysis, the hospitalization rate was not significantly different between the bleeding group and the matched non-bleeding group (14.5/100 *vs* 13.2/100 patient-years; P = 0.631).

CONCLUSION
Early use of thiopurines may reduce the risk of acute severe LGIB. History of acute severe LGIB may not have a significant prognostic value in patients with CD.

Key Words: Gastrointestinal hemorrhage; Lower gastrointestinal tract; Crohn's disease; Risk factors; Cohort studies; Clinical course

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Core Tip: A retrospective cohort study was conducted to identify the predictors of acute severe lower gastrointestinal bleeding (LGIB) in Crohn's disease (CD) and the impact of acute severe LGIB on the subsequent clinical course thereof. In multivariable analysis, early use of thiopurines and female sex were associated with a lower risk of acute severe LGIB. Moreover, matched analyses within the cohort demonstrated that the risks of behavioral progression, intestinal resection, and hospitalization due to non-bleeding causes did not significantly differ according to bleeding, which suggests that a history of acute severe LGIB may not have a significant prognostic value in CD.

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INTRODUCTION

Acute severe lower gastrointestinal bleeding (LGIB) is an uncommon manifestation that occurs in 0.6%-6.0% of patients with Crohn's disease (CD); however, it is a challenging problem with high rates of recurrence (21.4%-41.4%)[1-5], surgery (7.1%-39.7%)[1-4], and mortality (0%-8.2%)[2,3]. Despite this clinical importance, acute severe LGIB in CD has not been well-studied. Two studies have evaluated the risk factors for acute severe LGIB[1,3]; however, because the patient populations in these studies were not inception cohorts, it is still unknown what characteristics at the time of CD diagnosis predispose a patient to acute severe LGIB. Moreover, no previous studies have evaluated the impact of acute severe LGIB on the subsequent clinical course of CD. Therefore, by using a well-defined hospital-based inception cohort, we aimed to identify the risk factors for acute severe LGIB in CD and to investigate whether a history of acute severe LGIB is a predictor of a worse clinical course for CD.

MATERIALS AND METHODS

Patients

Between February 1991 and November 2019, a total of 4010 patients with CD were registered at the Inflammatory Bowel Disease (IBD) Center of Asan Medical Center, a tertiary university hospital in Seoul, Korea. Of these 4010 patients, 1437 had been first diagnosed and/or first treated with CD at Asan Medical Center. Among them, 78

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patients experienced acute severe gastrointestinal (GI) bleeding and 1359 patients did not. Of the 78 patients, 75 with acute severe LGIB were finally selected for this study after those with postoperative bleeding (n = 1), upper GI bleeding (n = 1), or anal bleeding (n = 1) were excluded.

To identify the risk factors for acute severe LGIB, we performed a retrospective cohort study in the 75 patients with acute severe LGIB and 1359 patients without acute severe LGIB. In addition, to investigate whether acute severe LGIB is a predictor of a worse clinical course of CD, a matched cohort analysis was performed by matching patients without acute severe LGIB to those with acute severe LGIB at a ratio of 4:1 in terms of sex, age at CD diagnosis (± 5 years), calendar year of CD diagnosis (± 5 years), and disease location and behavior at CD diagnosis. According to the matching conditions, a total of 72 patients with acute severe LGIB were matched to 267 patients without. The selection process for our study population is shown as a flowchart (Figure 1). The study protocol was approved by the Institutional Review Board of Asan Medical Center.

Data collection

The patients' demographic and clinical information were retrieved from the Asan IBD Registry, which has been prospectively maintained since 1997. Information missing in the IBD registry was obtained by reviewing the medical records. To identify the risk factors for acute severe LGIB, we retrieved data on sex, date of birth, date of CD diagnosis, date of acute severe LGIB, disease location and behavior at diagnosis, smoking status at diagnosis, early use of medications including corticosteroids, thiopurines, and anti-tumor necrosis factor (TNF) agents, diagnostic modalities for identification of bleeding site, identified bleeding site, and date of final follow-up. In addition, to investigate whether acute severe LGIB is a predictor of a worse clinical course of CD, we additionally retrieved data on medications (*i.e.*, corticosteroids, thiopurines, and anti-TNF agents), progression of disease behavior, intestinal resection, and hospitalization.

Definitions and classifications

CD was diagnosed using standard clinical, radiological, endoscopic, and histopathological criteria[6]. Acute severe LGIB was defined as acute overt rectal bleeding that resulted in (1) an abrupt decrease in the hemoglobin level to < 9 g/dL or at least 2 g/dL below the baseline; and/or (2) transfusion of at least two units of blood within 24 h[1]. The ligament of Treitz was regarded as the anatomic landmark separating LGIB from upper GI bleeding. Postoperative bleeding was defined as bleeding that occurred within 1 mo after intestinal surgery. The lesion found was defined as the bleeding site when it showed active bleeding or adherent blood clot[2]. Early use of corticosteroids was defined as the initiation of treatment within 3 mo of diagnosis[7]; early use of thiopurines or anti-TNF agents was defined as the initiation of therapy within 6 mo of diagnosis[8,9] and at least 6 mo before the first intestinal resection and acute severe LGIB episode[7,10]. In the matched analysis, each matched patient in the non-bleeding group was given an index date that corresponded to the date of acute severe LGIB in the matched patient in the bleeding group, such that the time interval between CD diagnosis and the index date of each non-bleeding patient was equal to that between CD diagnosis and the acute severe LGIB date for the matched bleeding patient. Disease location and behavior were defined according to the Montreal classification[11]. Behavioral progression was defined as the development of stricturing or penetrating disease in patients who had non-stricturing, non-penetrating disease at the start of follow-up. Hospitalization was defined as care in a hospital setting for ≥ 3 d for flare-ups or complications of CD[12]. Hospitalizations only for disease evaluation or due to conditions unrelated to CD were excluded. Index hospitalization was defined as the first hospitalization for acute severe LGIB.

Statistical analyses

Categorical variables are expressed as numbers with percentages, and continuous variables are expressed as medians with interquartile ranges (IQRs). The Chi-squared test or Fisher's exact test was used to compare categorical variables, as appropriate, and the t-test was used to compare continuous variables. Multivariable analysis with Cox proportional hazard regression was performed to identify the risk factors for acute severe LGIB and to calculate their hazard ratios (HRs) and 95% confidence intervals (CIs). All variables with P values < 0.2 in the univariate analysis were included in the multivariable analysis, and backward elimination was performed. In the matched analyses, the balance in the distribution of baseline characteristics

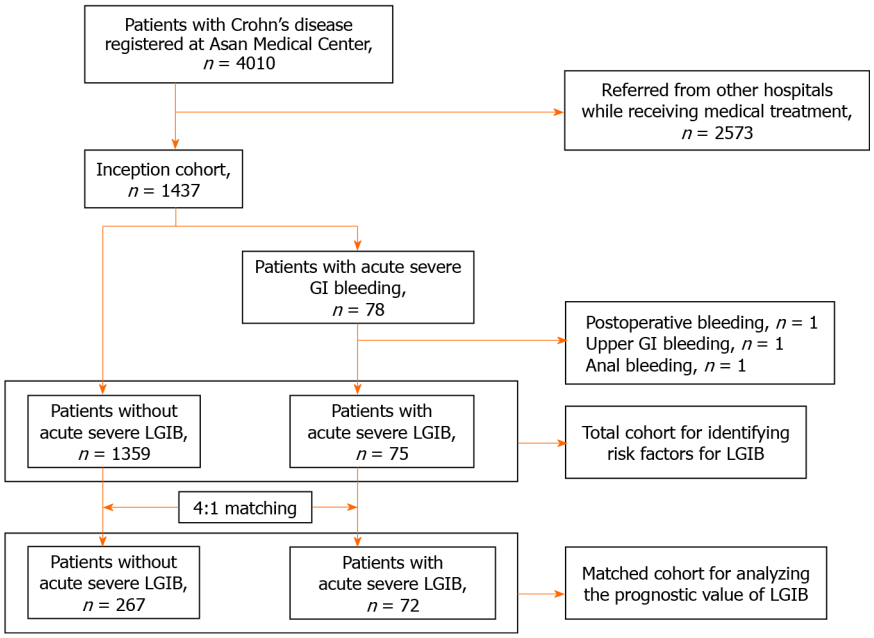


Figure 1 Flowchart of study patients. GI: Gastrointestinal; LGIB: Lower gastrointestinal bleeding.

between the matched groups was quantified using the standardized mean difference (SMD). An SMD < 0.1 was regarded to indicate a fair balance of confounders between the matched groups[13]. Disease courses such as medication use, behavioral progression, and intestinal resection before the bleeding/index date were compared between the matched groups using conditional logistic regression analysis. Cumulative risks of medication use, behavioral progression, and intestinal resection after the bleeding/index date were calculated using the Kaplan-Meier method, and the values were compared between the matched groups using the log-rank test. Hospitalization rates *per* 100 patient-years of follow-up were calculated in both the bleeding group and the non-bleeding group, and the relative rates and associated CIs were estimated using Poisson regression with generalized estimating equation. *P* values < 0.05 were considered statistically significant. Statistical evaluations were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, United States) and R version 3.6.2 for Windows (R Foundation for Statistical Computing, Vienna, Austria). The statistical methods of this study were reviewed by Kim YJ from the Department of Clinical Epidemiology and Biostatistics at Asan Medical Center, University of Ulsan College of Medicine.

RESULTS

Characteristics of patients and bleeding episodes

During a median follow-up of 84.8 mo (IQR 43.2-141.8), 75 (5.2%) of 1437 patients developed the first episode of acute severe LGIB. The cumulative risks of bleeding at 1, 5, 10, and 20 years after CD diagnosis were 3.0%, 4.4%, 5.9%, and 11.6%, respectively. Sixty-three of the 75 patients were men, yielding a male-to-female ratio of 5.2:1. The median age at the first bleeding episode was 27.0 years (IQR 21-34), and the median duration of CD at the first bleeding episode was 7.7 mo (IQR 0-42.1). After excluding 26 patients who were first diagnosed with CD at the time of bleeding, the median duration of disease at the first bleeding episode in the other 49 patients was 31.0 mo (IQR 8.0-66.0).

Of the 75 patients with acute severe LGIB, bleeding sites were identified in 19 (25.3%) patients through ileocolonoscopy, computed tomography, angiography, bleeding scan, and surgery. Capsule endoscopy or double-balloon enteroscopy was not used in the evaluation of acute severe LGIB. In the patients as a whole, the sites of bleeding were the jejunum in 2, the ileum in 12, and the colon in 5. Ileocolonoscopy was performed in 66 patients and revealed bleeding sites in 7 (10.6%) patients (colon, *n* = 5; terminal ileum, *n* = 2). Computed tomography was performed in 55 patients and revealed bleeding sites in 6 (10.9%) patients (jejunum, *n* = 2; ileum, *n* = 4). Mesenteric

angiography was performed in 14 patients and identified bleeding sites in the ileum in 4 (28.5%) patients. Radionuclide bleeding scan was performed in 22 patients and identified bleeding sites in the ileum in 4 (18.2%) patients. Surgery was performed to control bleeding in 4 patients and revealed ileal ulcers with adherent blood clots in 2 (50%) patients.

Risk factors for acute severe LGIB

Table 1 shows the baseline demographic and clinical characteristics according to the presence of acute severe LGIB. The proportions of male patients and patients with ileal disease at diagnosis were significantly higher in the bleeding group (P = 0.045 and P = 0.002, respectively), whereas the proportion of patients with early use of thiopurines was significantly higher in the non-bleeding group (P < 0.001). There were no significant differences between the two groups in terms of age at diagnosis, disease behavior at diagnosis, smoking status at diagnosis, early use of corticosteroids, and early use of anti-TNF agents.

Multivariable Cox regression analysis revealed that female sex (HR: 0.51, 95%CI: 0.27-0.94; P = 0.031) and early use of thiopurines (HR: 0.23, 95%CI: 0.12-0.48; P < 0.001) were significantly associated with a lower risk of acute severe LGIB (Table 2). In addition, patients with ileal disease at diagnosis showed a trend toward a higher risk of bleeding compared with those with colonic disease at diagnosis (HR: 6.58, 95%CI: 0.90-48.12; P = 0.063).

Impact of acute severe LGIB on the clinical course of CD

In the matched analysis between patients in the non-bleeding group and the bleeding group, the baseline characteristics including age at diagnosis, sex, disease location at diagnosis, and disease behavior at diagnosis were well-balanced between the matched groups (Table 3). The median duration of follow-up from CD diagnosis to the bleeding/index date was 7.1 mo (IQR 0.9-38.6) among 72 patients with acute severe LGIB and 8.7 mo (IQR 0.01-38.6) among 267 matched patients without (P = 0.423). During the follow-up period before the bleeding/index date, there were no significant differences in the rates of medication use, behavioral progression, intestinal resection, and hospitalization between the two matched groups (Tables 4 and 5).

The median duration of follow-up from the bleeding/index date to the last follow-up was 105.2 mo (IQR 50.7-135.3) in the bleeding group and 84.4 mo (IQR 46.7-144.2) in the matched non-bleeding group (P = 0.766). During the follow-up period after the bleeding/index date, the cumulative risks of receiving corticosteroids and thiopurines were not significantly different between the two matched groups (P = 0.068 and 0.248, respectively; Figure 2A and B). In contrast, the cumulative risk of receiving anti-TNF agents was significantly higher in the bleeding group (P = 0.035; Figure 2C). The cumulative risk of behavioral progression did not significantly differ between the two matched groups (P = 0.139; Figure 3A). Intestinal resection was performed in 13 (18.1%) of the 72 patients in the bleeding group and 53 (19.9%) of the 267 patients in the matched non-bleeding group (P = 0.86). Four (30.8%) of the 13 patients who underwent intestinal resection in the bleeding group underwent surgery due to acute severe LGIB. The cumulative risks of intestinal resection did not significantly differ between the two matched groups (P = 0.769; Figure 3B). The hospitalization rate after the bleeding/index date was significantly higher in the bleeding group than in the matched non-bleeding group (22.1/100 *vs* 13.2/100 patient-years; P = 0.012), even when the index hospitalization was excluded from the analysis. However, if all hospitalizations due to bleeding episodes were excluded from the analysis, the hospitalization rate did not significantly differ between the bleeding group and the matched non-bleeding group (14.5/100 *vs* 13.2/100 patient-years; P = 0.631; Table 5).

DISCUSSION

In this study, we investigated the risk factors for acute severe LGIB in CD and the impact of acute severe LGIB on the subsequent clinical course of CD by using a well-defined hospital-based inception cohort. To our knowledge, this is the first study that evaluated the prognostic value of acute severe LGIB in CD. Our results demonstrated that early use of thiopurines and female sex were negatively associated with the risk of bleeding and that there were no significant differences in the clinical course of CD including the rates of medication use (*e.g.*, corticosteroids and thiopurines), behavioral progression, intestinal resection, and hospitalization due to non-bleeding causes between patients who experienced acute severe LGIB and those who did not.

Table 1 Demographic and clinical characteristics of patients with Crohn's disease according to the presence of acute severe lower gastrointestinal bleeding

	Bleeding group (*n* = 75)	Non-bleeding group (*n* = 1359)	*P* value
Age at diagnosis, years (median, IQR)	23.6 (18.9-31.9)	23.2 (18.6-33.5)	0.952
Sex, male, *n* (%)	63 (84.0)	1000 (73.6)	0.045
Disease location at diagnosis, *n* (%)			0.002
Ileum	34 (45.3)	377 (27.7)	
Colon	1 (1.3)	81 (6.0)	
Ileocolon	40 (53.4)	901 (66.3)	
Upper GI modifier at diagnosis, *n* (%)	15 (20)	355 (26.1)	0.238
Disease behavior at diagnosis, *n* (%)			0.157
Non-stricturing, non-penetrating	59 (78.6)	1012 (74.5)	
Stricturing	11 (14.7)	155 (11.4)	
Penetrating	5 (6.7)	192 (14.1)	
Perianal modifier at diagnosis, *n* (%)	32 (42.7)	686 (50.5)	0.204
Smoking status at diagnosis, *n* (%)			0.540
Never smokers	51 (68.0)	874 (64.3)	
Ex-smokers	3 (4.0)	99 (7.3)	
Current smokers	21 (28.0)	386 (28.4)	
Early use of medications, *n* (%)			
Corticosteroids	21 (28.0)	399 (29.4)	0.840
Thiopurines	10 (13.3)	649 (47.8)	< 0.001
Anti-TNF agents	1 (1.3)	43 (3.2)	0.560

IQR: Interquartile range; GI: Gastrointestinal; TNF: Tumor necrosis factor.

However, the use of anti-TNF agents was more common in the bleeding group.

To date, only two studies have investigated the risk factors of acute severe LGIB in CD[1,3], with Kim *et al*[1] reporting that the use of thiopurines was associated with a lower risk of bleeding[1] and Li *et al*[3] confirming this result[3]. Li *et al*[3] also reported that left colon involvement and a history of bleeding were associated with a higher risk of bleeding. Similar to the results of previous studies, our present study demonstrated that early use of thiopurines was associated with a lower risk of bleeding. Thiopurines are effective for maintaining remission in patients with CD[14,15], and some studies have also reported that early use of thiopurines is associated with a lower risk of intestinal resection in patients with CD, albeit there are some disagreements in the literature[7,10,16]. Given the results of these previous studies, it can be assumed that early use of thiopurines can lower the risk of bleeding in patients with CD.

Another risk factor for bleeding in our study was male sex. In most population-based cohort studies, sex was not associated with the risk for surgery in patients with CD[7,17,18]. As a result, sex has not been considered a prognostic factor of CD[19]. However, sex differences in the phenotypic characteristics and clinical course of CD are increasingly being recognized[20]. Mazor *et al*[21] reported that only male sex was independently associated with complicated diseases such as stricturing disease, penetrating disease, perianal disease, and abdominal surgery[21]. Some studies have also shown that male sex was significantly associated with a higher risk for surgery[22-24]. These results collectively suggest that male sex may be a real risk factor for bleeding. Further studies are required to confirm whether male sex is an independent risk factor for bleeding in patients with CD.

In the present study, patients with ileal disease at diagnosis had a higher risk of bleeding compared with those with colonic disease at diagnosis, although the result did not reach statistical significance. This is in line with the results of previous studies

Table 2 Risk factors of acute severe lower gastrointestinal bleeding in patients with Crohn's disease

	Univariate analysis		Multivariable analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age at diagnosis	1.01 (0.98-1.03)	0.663		
Sex				
Male	Reference		Reference	
Female	0.51 (0.28-0.95)	0.035	0.51 (0.27-0.94)	0.031
Disease location at diagnosis				
Colon	Reference		Reference	
Ileum	7.56 (1.04-55.26)	0.046	6.58 (0.90-48.12)	0.063
Ileocolon	3.60 (0.50-26.22)	0.206	3.86 (0.53-28.09)	0.182
Upper GI modifier at diagnosis				
No	Reference			
Yes	0.78 (0.45-1.38)	0.401		
Disease behavior at diagnosis				
Non-stricturing, non-penetrating	Reference			
Stricturing	1.10 (0.58-2.10)	0.763		
Penetrating	0.45 (0.18-1.13)	0.067		
Perianal modifier at diagnosis				
No	Reference		Reference	
Yes	0.71 (0.45-1.12)	0.142	0.74 (0.42-1.19)	0.209
Smoking status at diagnosis				
Never smokers	Reference			
Ex-smokers	0.59 (0.18-1.88)	0.368		
Current smokers	0.93 (0.56-1.55)	0.783		
Early use of medications				
Corticosteroids	0.94 (0.57-1.58)	0.840		
Thiopurines	0.17 (0.09-0.34)	0.001	0.23 (0.12-0.48)	< 0.001
Anti-TNF agents	0.41 (0.12-3.21)	0.560		

HR: Hazard ratio; CI: Confidence interval; GI: Gastrointestinal; TNF: Tumor necrosis factor.

that ileal disease is a predictor of complicated behavior[25] and surgery[17,26,27], whereas colonic disease is a predictor of a milder disease course[17,28]. However, other studies reported contrasting results in that bleeding was more common in patients with colonic involvement than in those with isolated small bowel disease[2,29]. These conflicting results warrant further targeted investigation.

The impact of acute severe LGIB on the subsequent clinical course of CD has not been investigated to date. To investigate this issue after controlling for potential confounders, we performed a matched cohort study. In our matched analyses, the cumulative risks of behavioral progression and intestinal resection after the bleeding/index date did not significantly differ between the bleeding group and the matched non-bleeding group. In particular, although 31% of the patients in the bleeding group who underwent intestinal resection received surgery due to bleeding, the overall rate of intestinal resection in the bleeding group was not significantly higher than that in the matched non-bleeding group. Moreover, when we excluded the hospitalizations due to bleeding episodes from the analysis, the hospitalization rate was not significantly different between the two matched groups. These results suggest that a history of acute severe LGIB may not have a significant prognostic value in patients with CD. However, a definite conclusion on this issue cannot be made

Table 3 Comparison of the baseline parameters between patients with acute severe lower gastrointestinal bleeding and matched patients without

	Before matching			After matching		
	Bleeding group (*n* = 75)	Non-bleeding group (*n* = 1359)	SMD	Bleeding group (*n* = 72)	Non-bleeding group (*n* = 267)	SMD
Age at diagnosis, years (median, IQR)	23 (18-32)	23 (18-33)	0.009	22 (18-31.5)	23 (18-30)	0.061
Male, *n* (%)	63 (84.0)	1000 (73.6)	0.257	63 (87.5)	234 (87.6)	0.004
Disease location at diagnosis, *n* (%)			0.391			0.020
Ileum	34 (45.3)	377 (27.7)		31 (43.1)	111 (41.6)	
Colon	1 (1.3)	81 (6.0)		1 (1.4)	4 (1.5)	
Ileocolon	40 (53.3)	901 (66.3)		40 (55.6)	152 (56.9)	
Disease behavior at diagnosis, *n* (%)			0.301			0.030
Non-stricturing, non-penetrating	59 (78.7)	1012 (74.5)		58 (80.6)	219 (82.0)	
Stricturing	11 (14.7)	155 (11.4)		9 (12.5)	32 (12.0)	
Penetrating	5 (6.7)	192 (14.1)		5 (6.9)	16 (6.0)	

SMD: Standardized mean difference; IQR: Interquartile range.

Table 4 Comparison of the outcome parameters between patients with acute severe lower gastrointestinal bleeding and matched patients without during the period before the bleeding/index date

	Bleeding group (*n* = 72)	Non-bleeding group (*n* = 267)	Matched OR (95%CI)	*P* value
Use of medications, *n* (%)				
Corticosteroids	19/72 (26.4)	78/267 (29.2)	0.82 (0.43-1.57)	0.55
Thiopurines	27/72 (37.5)	108/267 (40.4)	0.74 (0.36-1.51)	0.40
Anti-TNF agents	6/72 (8.3)	27/267 (10.1)	0.68 (0.24-1.91)	0.47
Behavioral progression, *n* (%)	10/58 (17.2)	21/219 (9.6)	1.96 (0.87-4.44)	0.10
Intestinal resection, *n* (%)	13/72 (18.1)	30/267 (11.2)	1.83 (0.73-4.58)	0.20

OR: Odds ratio; CI: Confidence interval; TNF: Tumor necrosis factor.

because anti-TNF agents were more commonly used in the bleeding group than in the matched non-bleeding group. In addition, the rate of all-cause hospitalization was higher in the bleeding group than in the matched non-bleeding group; this is an expected result considering the high recurrence rates (21.4%-41.4%) of acute severe LGIB reported in previous studies[1-5]. A higher need for anti-TNF agents and a higher rate of hospitalization in the bleeding group indicate that a history of acute severe LGIB may have some prognostic value. Regardless of whether acute severe LGIB in CD is a poor prognostic factor, it is a common practice to use anti-TNF agents to lower the risk of rebleeding in CD patients who develop acute severe LGIB[1,5,30-32], and major disease outcomes including behavioral progression and intestinal resection in the bleeding group are comparable with those in the non-bleeding group.

The strength of our present study is that we used a well-defined inception cohort, which enabled us to identify the patients' demographic and clinical characteristics at the time of or at an early stage of CD diagnosis that could predict the occurrence of acute severe LGIB. However, our study has some limitations. First, although our results suggested that male sex was associated with a higher risk of bleeding, we cannot exclude the possibility that this difference was derived from non-biological

Table 5 Comparison of the hospitalization rate between patients with acute severe lower gastrointestinal bleeding and matched patients without

Hospitalization rate (*per* 100 patient-years)	Bleeding group (*n* = 72)	Non-bleeding group (*n* = 267)	Hospitalization rate ratio (95%CI)	*P* value
Before bleeding/index date (95%CI)	21.0 (14.5-30.5)	23.6 (19.0-29.2)	0.89 (0.58-1.37)	0.599
After bleeding/index date (95%CI)				
Excluded: Index hospitalization	22.1 (16.5-29.4)	13.2 (11.2-15.5)	1.67 (1.20-2.33)	0.012
Excluded: Hospitalizations due to bleeding	14.5 (10.3-20.4)	13.2 (11.2-15.5)	1.10 (0.75-1.60)	0.631

CI: Confidence interval.

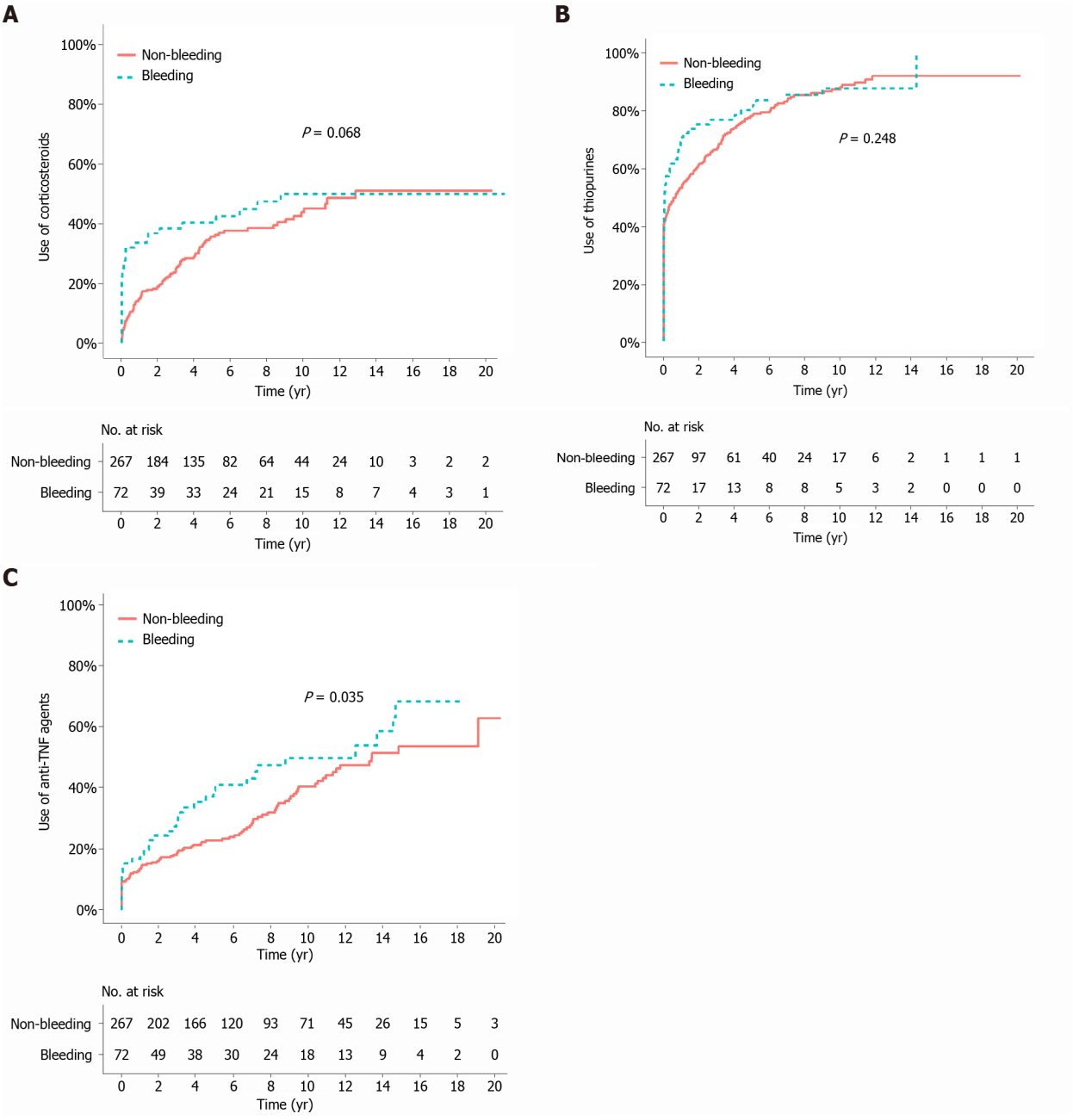


Figure 2 Cumulative risks of receiving medications after the bleeding/index date according to bleeding. A: Corticosteroids (*P* = 0.068); B: Thiopurines (*P* = 0.248); C: Anti-tumor necrosis factor agents (*P* = 0.035). TNF: Tumor necrosis factor.

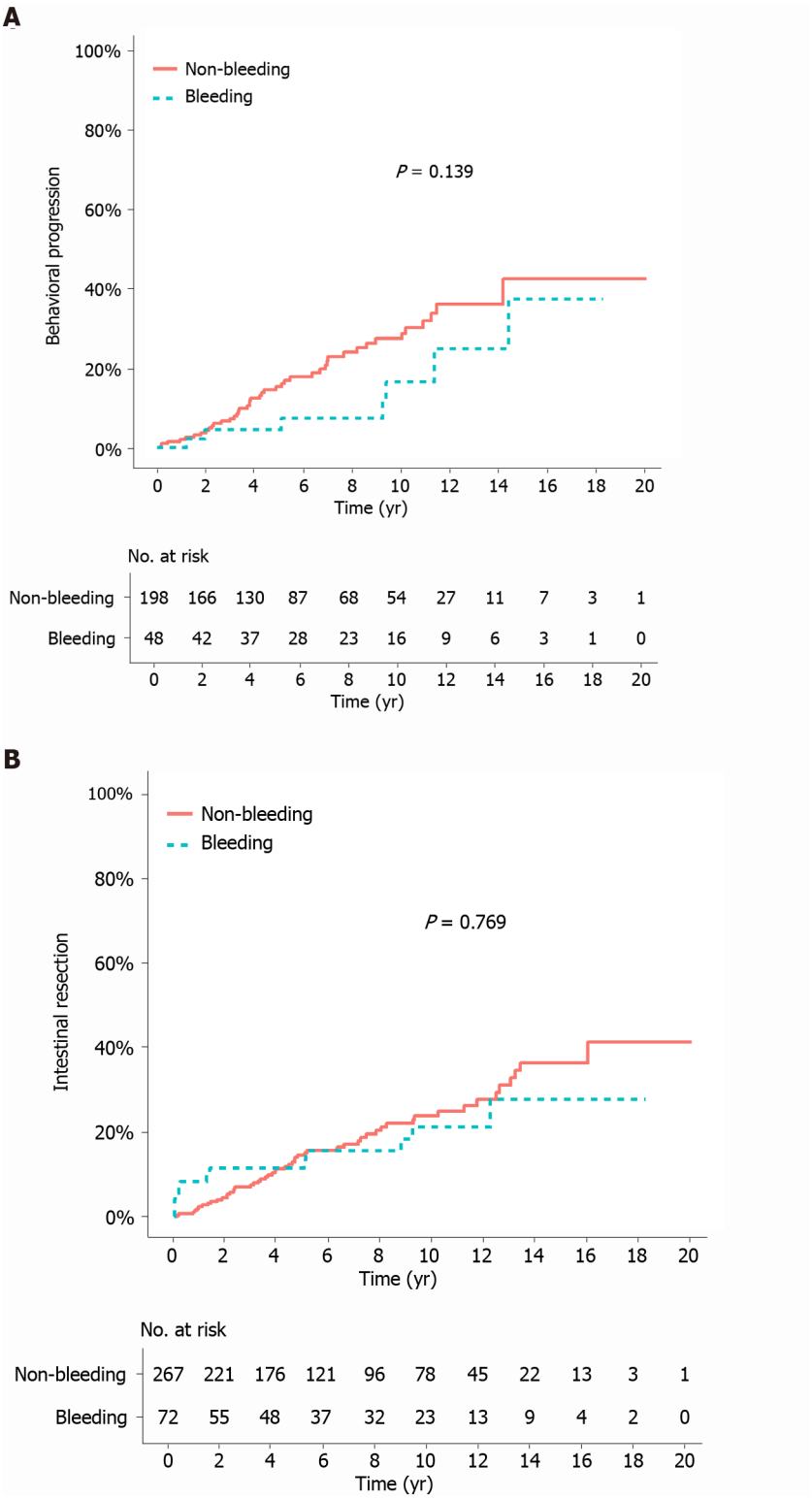


Figure 3 Cumulative risks of behavioral progression and intestinal resection after the bleeding/index date according to bleeding. A: Behavioral progression (P = 0.139); B: Intestinal resection (P = 0.769).

causes such as sex differences in access to health care or adherence to therapy in patients with IBD[33-35]. Second, our result that male sex is a risk factor for bleeding may not be generalized in Western patients considering the sex-related differences between Asian and Western patients with CD. For example, the male predominance in the incidence of CD is evident in Asian populations but not in Western populations [36].

CONCLUSION

Our results suggest that early use of thiopurines may reduce the risk of acute severe LGIB. In addition, a history of acute severe LGIB may not have a significant impact on the subsequent clinical course of patients with CD in terms of behavioral progression, intestinal resection, and hospitalization due to non-bleeding causes. Further studies are needed to determine whether our results on the prognostic value of acute severe LGIB were biased by the differential use of anti-TNF agents between patients with acute severe LGIB and those without.

ARTICLE HIGHLIGHTS

Research background

Acute severe lower gastrointestinal bleeding (LGIB) is an uncommon but challenging complication of Crohn's disease (CD).

Research motivation

Acute severe LGIB in CD has not been well-studied.

Research objectives

To identify the predictors of acute severe LGIB and to evaluate the impact of acute severe LGIB on the subsequent clinical course in patients with CD.

Research methods

A hospital-based retrospective inception cohort study was conducted. Multivariable analysis with Cox proportional hazard regression was performed to identify the predictors of acute severe LGIB. A matched analysis within the cohort was also conducted to investigate whether acute severe LGIB is a predictor of clinical outcomes of CD. Disease courses were compared using conditional logistic regression analysis.

Research results

Early use of thiopurines and female sex were associated with a decreased risk of acute severe LGIB. The risks of behavioral progression, intestinal resection, and hospitalization due to non-bleeding causes did not significantly differ between the bleeding group and the matched non-bleeding group.

Research conclusions

Early use of thiopurines may reduce the risk of acute severe LGIB. History of acute severe LGIB may not have a significant prognostic value in patients with CD.

Research perspectives

Further studies are needed to investigate whether our findings on the possible null prognostic value of acute severe LGIB in CD was biased by the differential use of anti-tumor necrosis factor agents between patients with acute severe LGIB and those without.

REFERENCES

1 **Kim KJ**, Han BJ, Yang SK, Na SY, Park SK, Boo SJ, Park SH, Yang DH, Park JH, Jeong KW, Ye BD, Byeon JS, Myung SJ, Kim JH. Risk factors and outcome of acute severe lower gastrointestinal bleeding in Crohn's disease. *Dig Liver Dis* 2012; **44**: 723-728 [PMID: 22497905 DOI: 10.1016/j.dld.2012.03.010]

2 **Belaiche J**, Louis E, D'Haens G, Cabooter M, Naegels S, De Vos M, Fontaine F, Schurmans P, Baert F, De Reuck M, Fiasse R, Holvoet J, Schmit A, Van Outryve M. Acute lower gastrointestinal bleeding in Crohn's disease: characteristics of a unique series of 34 patients. Belgian IBD Research Group. *Am J Gastroenterol* 1999; **94**: 2177-2181 [PMID: 10445546 DOI: 10.1111/j.1572-0241.1999.01291.x]

3 **Li G**, Ren J, Wang G, Wu Q, Gu G, Ren H, Liu S, Hong Z, Li R, Li Y, Guo K, Wu X, Li J. Prevalence and risk factors of acute lower gastrointestinal bleeding in Crohn disease. *Medicine (Baltimore)* 2015; **94**: e804 [PMID: 25984665 DOI: 10.1097/MD.0000000000000804]

4 **Pardi DS**, Loftus EV Jr, Tremaine WJ, Sandborn WJ, Alexander GL, Balm RK, Gostout CJ. Acute major gastrointestinal hemorrhage in inflammatory bowel disease. *Gastrointest Endosc* 1999; **49**:

153-157 [PMID: 9925691 DOI: 10.1016/s0016-5107(99)70479-7]
5 **Lee S**, Ye BD, Park SH, Lee KJ, Kim AY, Lee JS, Kim HJ, Yang SK. Diagnostic Value of Computed Tomography in Crohn's Disease Patients Presenting with Acute Severe Lower Gastrointestinal Bleeding. *Korean J Radiol* 2018; **19**: 1089-1098 [PMID: 30386140 DOI: 10.3348/kjr.2018.19.6.1089]
6 **Lennard-Jones JE**. Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl* 1989; **170**: 2-6; discussion 16 [PMID: 2617184 DOI: 10.3109/00365528909091339]
7 **Ramadas AV**, Gunesh S, Thomas GA, Williams GT, Hawthorne AB. Natural history of Crohn's disease in a population-based cohort from Cardiff (1986-2003): a study of changes in medical treatment and surgical resection rates. *Gut* 2010; **59**: 1200-1206 [PMID: 20650924 DOI: 10.1136/gut.2009.202101]
8 **Cosnes J**, Bourrier A, Laharie D, Nahon S, Bouhnik Y, Carbonnel F, Allez M, Dupas JL, Reimund JM, Savoye G, Jouet P, Moreau J, Mary JY, Colombel JF; Groupe d'Etude Thérapeutique des Affections Inflammatoires du Tube Digestif (GETAID). Early administration of azathioprine *vs* conventional management of Crohn's Disease: a randomized controlled trial. *Gastroenterology* 2013; **145**: 758-65. quiz e14-5 [PMID: 23644079 DOI: 10.1053/j.gastro.2013.04.048]
9 **Burisch J**, Kiudelis G, Kupcinskas L, Kievit HAL, Andersen KW, Andersen V, Salupere R, Pedersen N, Kjeldsen J, D'Incà R, Valpiani D, Schwartz D, Odes S, Olsen J, Nielsen KR, Vegh Z, Lakatos PL, Toca A, Turcan S, Katsanos KH, Christodoulou DK, Fumery M, Gower-Rousseau C, Zammit SC, Ellul P, Eriksson C, Halfvarson J, Magro FJ, Duricova D, Bortlik M, Fernandez A, Hernández V, Myers S, Sebastian S, Oksanen P, Collin P, Goldis A, Misra R, Arebi N, Kaimakliotis IP, Nikuina I, Belousova E, Brinar M, Cukovic-Cavka S, Langholz E, Munkholm P; Epi-IBD group. Natural disease course of Crohn's disease during the first 5 years after diagnosis in a European population-based inception cohort: an Epi-IBD study. *Gut* 2019; **68**: 423-433 [PMID: 29363534 DOI: 10.1136/gutjnl-2017-315568]
10 **Lakatos PL**, Golovics PA, David G, Pandur T, Erdelyi Z, Horvath A, Mester G, Balogh M, Szipocs I, Molnar C, Komaromi E, Veres G, Lovasz BD, Szathmari M, Kiss LS, Lakatos L. Has there been a change in the natural history of Crohn's disease? *Am J Gastroenterol* 2012; **107**: 579-588 [PMID: 22233693 DOI: 10.1038/ajg.2011.448]
11 **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus EV Jr, Peña AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5A-36A [PMID: 16151544 DOI: 10.1155/2005/269076]
12 **Yamamoto-Furusho JK**, Al Harbi O, Armuzzi A, Chan W, Ponce de Leon E, Qian J, Shapina M, Toruner M, Tu CH, Ye BD, Guennec M, Sison C, Demuth D, Fadeeva O, Khan QMR. Incidence of suboptimal response to tumor necrosis factor antagonist therapy in inflammatory bowel disease in newly industrialised countries: The EXPLORE study. *Dig Liver Dis* 2020; **52**: 869-877 [PMID: 32563721 DOI: 10.1016/j.dld.2020.05.031]
13 **Austin PC**. Using the standardized difference to compare the prevalence of a binary variable between two groups in observational research. *Commun Stat Simul Comput* 2009; **38**: 1228-1234 [DOI: 10.1080/03610910902859574]
14 **Maeda T**, Sakuraba H, Hiraga H, Yoshida S, Kakuta Y, Kikuchi H, Kawaguchi S, Hasui K, Tatsuta T, Chinda D, Mikami T, Fukuda S. Long-term efficacy and tolerability of dose-adjusted thiopurine treatment in maintaining remission in inflammatory bowel disease patients with NUDT15 heterozygosity. *Intest Res* 2021 [PMID: 33472343 DOI: 10.5217/ir.2020.00133]
15 **Chande N**, Patton PH, Tsoulis DJ, Thomas BS, MacDonald JK. Azathioprine or 6-mercaptopurine for maintenance of remission in Crohn's disease. *Cochrane Database Syst Rev* 2015; CD000067 [PMID: 26517527 DOI: 10.1002/14651858.CD000067.pub3]
16 **Park SH**, Yang SK, Park SK, Kim JW, Yang DH, Jung KW, Kim KJ, Ye BD, Byeon JS, Myung SJ, Yoon YS, Yu CS, Kim JH. Long-term prognosis of crohn's disease and its temporal change between 1981 and 2012: a hospital-based cohort study from Korea. *Inflamm Bowel Dis* 2014; **20**: 488-494 [PMID: 24412992 DOI: 10.1097/01.MIB.0000441203.56196.46]
17 **Solberg IC**, Vatn MH, Høie O, Stray N, Sauar J, Jahnsen J, Moum B, Lygren I; IBSEN Study Group. Clinical course in Crohn's disease: results of a Norwegian population-based ten-year follow-up study. *Clin Gastroenterol Hepatol* 2007; **5**: 1430-1438 [PMID: 18054751 DOI: 10.1016/j.cgh.2007.09.002]
18 **Bernell O**, Lapidus A, Hellers G. Risk factors for surgery and postoperative recurrence in Crohn's disease. *Ann Surg* 2000; **231**: 38-45 [PMID: 10636100 DOI: 10.1097/00000658-200001000-00006]
19 **Torres J**, Caprioli F, Katsanos KH, Lobatón T, Micic D, Zerôncio M, Van Assche G, Lee JC, Lindsay JO, Rubin DT, Panaccione R, Colombel JF. Predicting Outcomes to Optimize Disease Management in Inflammatory Bowel Diseases. *J Crohns Colitis* 2016; **10**: 1385-1394 [PMID: 27282402 DOI: 10.1093/ecco-jcc/jjw116]
20 **Rustgi SD**, Kayal M, Shah SC. Sex-based differences in inflammatory bowel diseases: a review. *Therap Adv Gastroenterol* 2020; **13**: 1756284820915043 [PMID: 32523620 DOI: 10.1177/1756284820915043]
21 **Mazor Y**, Maza I, Kaufman E, Ben-Horin S, Karban A, Chowers Y, Eliakim R. Prediction of disease complication occurrence in Crohn's disease using phenotype and genotype parameters at diagnosis. *J Crohns Colitis* 2011; **5**: 592-597 [PMID: 22115380 DOI: 10.1016/j.crohns.2011.06.002]
22 **Severs M**, Spekhorst LM, Mangen MJ, Dijkstra G, Löwenberg M, Hoentjen F, van der Meulen-de

Jong AE, Pierik M, Ponsioen CY, Bouma G, van der Woude JC, van der Valk ME, Romberg-Camps MJL, Clemens CHM, van de Meeberg P, Mahmmod N, Jansen J, Jharap B, Weersma RK, Oldenburg B, Festen EAM, Fidder HH. Sex-Related Differences in Patients With Inflammatory Bowel Disease: Results of 2 Prospective Cohort Studies. *Inflamm Bowel Dis* 2018; **24**: 1298-1306 [PMID: 29688413 DOI: 10.1093/ibd/izy004]

23 **Peyrin-Biroulet L**, Harmsen WS, Tremaine WJ, Zinsmeister AR, Sandborn WJ, Loftus EV Jr. Surgery in a population-based cohort of Crohn's disease from Olmsted County, Minnesota (1970-2004). *Am J Gastroenterol* 2012; **107**: 1693-1701 [PMID: 22945286 DOI: 10.1038/ajg.2012.298]

24 **Hofer B**, Böttger T, Hernandez-Richter T, Seifert JK, Junginger T. The impact of clinical types of disease manifestation on the risk of early postoperative recurrence in Crohn's disease. *Hepatogastroenterology* 2001; **48**: 152-155 [PMID: 11268954]

25 **Thia KT**, Sandborn WJ, Harmsen WS, Zinsmeister AR, Loftus EV Jr. Risk factors associated with progression to intestinal complications of Crohn's disease in a population-based cohort. *Gastroenterology* 2010; **139**: 1147-1155 [PMID: 20637205 DOI: 10.1053/j.gastro.2010.06.070]

26 **Magro F**, Portela F, Lago P, Ramos de Deus J, Vieira A, Peixe P, Cremers I, Cotter J, Cravo M, Tavares L, Reis J, Gonçalves R, Lopes H, Caldeira P, Ministro P, Carvalho L, Azevedo L, da Costa-Pereira A; GEDII. Crohn's disease in a southern European country: Montreal classification and clinical activity. *Inflamm Bowel Dis* 2009; **15**: 1343-1350 [PMID: 19235885 DOI: 10.1002/ibd.20901]

27 **Watanabe K**. Clinical management for small bowel of Crohn's disease in the treat-to-target era: now is the time to optimize treatment based on the dominant lesion. *Intest Res* 2020; **18**: 347-354 [PMID: 33131231 DOI: 10.5217/ir.2020.00032]

28 **Sands BE**, Arsenault JE, Rosen MJ, Alsahli M, Bailen L, Banks P, Bensen S, Bousvaros A, Cave D, Cooley JS, Cooper HL, Edwards ST, Farrell RJ, Griffin MJ, Hay DW, John A, Lidofsky S, Olans LB, Peppercorn MA, Rothstein RI, Roy MA, Saletta MJ, Shah SA, Warner AS, Wolf JL, Vecchio J, Winter HS, Zawacki JK. Risk of early surgery for Crohn's disease: implications for early treatment strategies. *Am J Gastroenterol* 2003; **98**: 2712-2718 [PMID: 14687822 DOI: 10.1111/j.1572-0241.2003.08674.x]

29 **Robert JR**, Sachar DB, Greenstein AJ. Severe gastrointestinal hemorrhage in Crohn's disease. *Ann Surg* 1991; **213**: 207-211 [PMID: 1998401 DOI: 10.1097/00000658-199103000-00004]

30 **Tsujikawa T**, Nezu R, Andoh A, Saotome T, Araki Y, Ishizuka Y, Sasaki M, Koyama S, Fujiyama Y. Infliximab as a possible treatment for the hemorrhagic type of Crohn's disease. *J Gastroenterol* 2004; **39**: 284-287 [PMID: 15065007 DOI: 10.1007/s00535-003-1290-9]

31 **Belaiche J**, Louis E. Severe lower gastrointestinal bleeding in Crohn's disease: successful control with infliximab. *Am J Gastroenterol* 2002; **97**: 3210-3211 [PMID: 12492221 DOI: 10.1111/j.1572-0241.2002.07143.x]

32 **Meyer MM**, Levine EJ. Acute hemorrhagic Crohn's disease controlled with infliximab. *Inflamm Bowel Dis* 2009; **15**: 1456-1457 [PMID: 19107774 DOI: 10.1002/ibd.20840]

33 **Zelante A**, De Giorgi A, Borgoni R, Trevisani L, Gallerani M. Adherence to medical treatment in inflammatory bowel disease patients. *Minerva Gastroenterol Dietol* 2014; **60**: 269-274 [PMID: 25384805]

34 **Lopez A**, Billioud V, Peyrin-Biroulet C, Peyrin-Biroulet L. Adherence to anti-TNF therapy in inflammatory bowel diseases: a systematic review. *Inflamm Bowel Dis* 2013; **19**: 1528-1533 [PMID: 23518810 DOI: 10.1097/MIB.0b013e31828132cb]

35 **Blumenstein I**, Bock H, Zosel C, Dignass AU, Hartmann F, Zeuzem S, Stein JM, Schroeder O. [Are there gender-related differences in the therapeutic management of patients suffering from inflammatory bowel disease? *Z Gastroenterol* 2009; **47**: 1045-1051 [PMID: 19809954 DOI: 10.1055/s-0028-1109647]

36 **Shah SC**, Khalili H, Chen CY, Ahn HS, Ng SC, Burisch J, Colombel JF. Sex-based differences in the incidence of inflammatory bowel diseases-pooled analysis of population-based studies from the Asia-Pacific region. *Aliment Pharmacol Ther* 2019; **49**: 904-911 [PMID: 30773656 DOI: 10.1111/apt.15178]

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ORIGINAL ARTICLE

Retrospective Study

Changes in the nutritional status of nine vitamins in patients with esophageal cancer during chemotherapy

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Abstract

BACKGROUND

Many studies have investigated the relationships between vitamins and esophageal cancer (EC). Most of these studies focused on the roles of vitamins in the prevention and treatment of EC, and few studies have examined the changes in vitamin nutritional status and their influencing factors before and after chemotherapy for EC. Chemotherapy may have a considerable effect on EC patients' vitamin levels and hematological indicators.

AIM

To research the nutritional status of multiple vitamins in EC patients during chemotherapy and to assess its clinical significance.

METHODS

EC patients admitted to our center from July 2017 to September 2020 were enrolled in this study. Serum concentrations of nine vitamins (A, D, E, B_9, B_{12}, B_1, C, B_2 and B_6), hemoglobin, total protein, albumin, blood calcium, blood phosphorus concentrations and body mass index (BMI) were measured in all EC patients. The changes in nine vitamins, hematological indicators and BMI were compared before and after two cycles of chemotherapy. The possible influential factors were analyzed.

RESULTS

In total, 203 EC patients receiving chemotherapy were enrolled in this study. Varying degrees of vitamin A, D, C and B_2 deficiency and weight loss were found in these patients, and the proportions of vitamin B_2 and vitamin C deficiencies increased significantly after chemotherapy (both $P < 0.05$). Serum concentrations

of vitamins A, C, B_2 and B_6 and BMI before and after chemotherapy were statistically significant (all $P < 0.05$). Multivariate analysis showed that vitamin A levels significantly differed between male and female EC patients, whereas vitamin D concentration significantly differed in EC patients in different stages (all $P < 0.05$). Correlations were observed between the changes in serum concentrations of vitamin A and C before and after two cycles chemotherapy and the change in BMI ($P < 0.05$). Hemoglobin, total protein, serum albumin and blood calcium concentrations significantly decreased in EC patients after chemotherapy (all $P < 0.05$), while the blood phosphorus level significantly increased after chemotherapy ($P < 0.05$). Using the difference in vitamin concentrations as the independent variables and the difference in BMI as the dependent variable, logistic regression analysis revealed statistically significant differences for vitamin A, vitamin D and vitamin C ($F = 5.082$, $P = 0.002$).

CONCLUSION

Vitamin A, D, C and B_2 were mainly deficient in patients with EC during chemotherapy. Multivitamin supplementation may help to improve the nutritional status, chemotherapy tolerance and efficacy.

Key Words: Esophageal cancer; Chemotherapy; Vitamins; Nutritional status; Body mass index

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Core Tip: This retrospective study investigated the changes in serum vitamins before and after chemotherapy for esophageal cancer. Vitamin deficiencies are common in esophageal cancer patients during chemotherapy and may be associated with the change in body mass index. There were correlations between the changes in vitamin A and C concentrations and the change in body mass index during chemotherapy. Vitamin A level after chemotherapy showed a significant difference between males and females, and the vitamin D level after chemotherapy showed a significant difference among different stages. Vitamin supplementation may reduce the adverse effects of chemotherapy and improve the nutritional status.

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INTRODUCTION

Esophageal cancer (EC) is the ninth most commonly diagnosed cancer and the sixth leading cause of cancer death worldwide[1]. The incidence and mortality rates of EC are highly heterogeneous in terms of gender, histological type, geographic distribution and race[2]. The morbidity and mortality of EC in China are higher than the global averages[3]. Chemoradiotherapy remains the mainstay of treatment for patients with advanced EC. The most common complications during chemoradiotherapy in EC patients include weight loss, malnutrition, bone marrow suppression, electrolyte disturbances, hypoproteinemia and decreased quality of life[4-7]. Multiple vitamins are involved in the pathogenesis, progression and prognosis of tumors and are closely related to the tumor microenvironment. Vitamin testing is valuable in tumor patients as it can identify whether there is a specific vitamin deficiency and/or justify vitamin therapy. Most clinical studies have shown that vitamin nutritional status varies greatly in tumors of different systems[8]. Although many studies have investigated the relationships between vitamins B, A, D and C with EC, most of these studies focused on the roles of vitamins in the prevention and treatment of EC. Few studies have examined the changes in vitamin nutritional status and their influencing factors before and after chemotherapy for EC.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Many vitamins have antitumor effects and are closely related to the occurrence, progression, prognosis and recovery of tumors. Vitamin supplementation can lower the risk of tumorigenesis and prevent abnormal DNA methylation changes in tumor cells[9]. With the advances in nutritional therapy in China, nutritional screenings, assessments and interventions have increasingly been applied in tumor patients. The standardized nutritional therapies involve three major macronutrients including carbohydrates, proteins and fats and have specific requirements on micronutrients. Nutritional therapy for tumor patients provides nutrients and energy and focuses on the metabolism-regulating roles of nutrients[10]. Vitamin supplementation is a common nutritional therapy for tumor patients in clinical practice. Serum vitamin levels vary among patients with different tumors. Zhang *et al*[11] analyzed the vitamin nutritional status of approximately 1000 hospitalized tumor patients and found that vitamin B1 concentrations were low in patients with digestive system tumors such as EC and gastric cancer. A study on postoperative nutritional deficiencies in patients with EC or gastric cancer revealed that the incidence of ferritin, folic acid, vitamin B_{12} and vitamin D deficiencies was 42.86%, 9.52%, 6.35% and 36.67%, respectively, and the vitamin levels were significantly improved after nutritional interventions[12]. In another study[13], most patients with advanced tumors had vitamin (particularly vitamins D, B_6 and C) deficiency symptoms during palliative care, and further analysis revealed a correlation between the degree of vitamin deficiency and clinical discomfort in these patients. Most vitamins were found to be negatively associated with the risk of colorectal and gastric cancer in addition to EC in an observational study, yet interventional treatment failed to demonstrate a clear preventive effect in these malignancies [14].

In the current study, we analyzed the effects of chemotherapeutic drugs on EC patients' vitamin levels and hematological indicators by detecting the changes in nine vitamins and hematological indicators (*e.g.*, hemoglobin, total protein, serum albumin and electrolytes) before and after chemotherapy, with an attempt to provide evidence for clinical vitamin supplementation and nutritional therapies. Our findings may be valuable for the implementation of tailored nutritional interventions, which will help to reduce chemotherapy-related complications, alleviate treatment resistance and improve chemotherapy efficacy.

MATERIALS AND METHODS

Subjects

In total, 203 EC patients (181 men and 22 women) aged 37-78 yrs (mean: 60.03 ± 7.95 years) who were receiving chemotherapy in our center between July 2017 and September 2020 were enrolled in this study. The vast majority of subjects had esophageal squamous cell carcinoma (n = 192, 94.58%), and 166 patients (81.77%) were in stage III-IV (Table 1). The inclusion criteria were as follows: (1) pathologically confirmed esophageal malignancies, with indications for chemotherapy, regardless of gender; (2) aged 18-80 years; (3) an expected survival of > 6 mo; and (4) a Karnofsky performance status score of ≥ 70 points. The exclusion criteria included: (1) contraindications to chemotherapy; (2) coexisting tumors in other systems; (3) coexisting primary blood diseases or endocrine diseases; (4) coexisting cardiopulmonary dysfunction; and (5) coexisting psychiatric disorders.

Methods

Determination of vitamin concentrations and hematological indicators: Vitamin levels were measured using electrochemiluminescence with an LK3000VI vitamin detector (Tianjin Lanbiao Electronic Technology Development Co., Ltd., Tianjin, China) pre- and postchemotherapy in EC patients. The normal thresholds were as follows: hemoglobin: 137-179 g/L in males and 116-155 g/L in females; total protein: 55-88 g/L; serum albumin: 35-50 g/L; blood calcium: 2.09-2.54 mmol/L; blood phosphorus: 0.89-1.60 mmol/L; vitamin A: 0.52-2.20 mmol/L; vitamin D: 25-200 nmol/L; vitamin E: 10-15 mg/mL; vitamin B_9: 6.8-36.3 nmol/L; vitamin B_{12}: 200-900 pg/mL; vitamin B_1: 50-150 nmol/L; vitamin C: 34-114 mmol/L; vitamin B_2: 4.26-18.42 mg/L; and vitamin B_6: 14.6-72.9 nmol/L.

Chemotherapy regimens: Of these 203 EC patients, 168 were treated with induction chemotherapy, 15 with postoperative adjuvant chemotherapy and 20 with postrelapse chemotherapy. The specific regimens were as follows: (1) squamous cell carcinoma: paclitaxel + platinum; (2) adenocarcinoma: oxaliplatin + fluorouracil; and (3) small cell

Table 1 Baseline data of esophageal cancer patients (*n* = 203)

Clinical features	*n*	(%)
Gender		
Males	181	89.16
Females	22	10.84
Age		
< 60 yr	94	46.31
≥ 60 yr	109	53.69
Tumor stage		
II	16	7.88
III	98	48.28
IV	68	33.50
Uncertain	21	10.34
Pathologic type		
Squamous cell carcinoma	192	94.58
Nonsquamous cell carcinoma	11	5.42
Treatment		
Induction chemotherapy	168	82.76
Postoperative adjuvant chemotherapy	15	7.39
Postrelapse chemotherapy	20	9.85
Primary tumor site		
Cervical	14	6.90
Upper thoracic	28	13.79
Middle thoracic	77	37.93
Lower thoracic	84	41.38

carcinoma: etoposide + platinum. All of these regimens were repeated every 3 wk.

Statistical methods

SPSS 24.0 statistical software was used for analyzing the data. Normally distributed data were expressed as mean ± SD, and intergroup comparisons were based on the paired-sample *t*-test. Non-normally distributed data were expressed as quartiles and medians, and the rank sum test was applied for comparisons between two groups. The Spearman method was used to test for correlations between the changes in multiple vitamin serum concentrations (the independent variables) and body mass index (BMI) (the dependent variable) during chemotherapy using. P value < 0.05 was regarded as statistically significant.

RESULTS

Variation in BMI and hematological parameters in EC patients during chemotherapy

BMI declined after chemotherapy in 133 cases (65.52%). The number of patients with anemia increased from 119 before chemotherapy to 182 after chemotherapy. The differences in these proportions were statistically significant (P < 0.05) (Table 2).

Vitamin levels in EC patients pre- and postchemotherapy

Vitamin A, C, B_2 and B_6 concentrations significantly differed before and after chemotherapy (all P < 0.05) (Table 3).

Table 2 Changes in body mass index and hematological indicators in esophageal cancer patients before and after chemotherapy [*n* (%)]

Item	Before chemotherapy	After chemotherapy	χ^2/*Z*	*P* value
Body mass index (kg/m^2)			-2.646	0.008
< 18.5	25 (12.32)	22 (10.84)		
18.5-23.9	103 (50.74)	123 (60.59)		
> 23.9	75 (36.95)	58 (28.57)		
Hemoglobin (g/L)			55.710	< 0.001
Decreased	119 (58.62)	182 (89.66)		
Normal	84 (41.38)	21 (10.34)		
Serum albumin (g/L)			2.717	0.099
Decreased	34 (16.75)	47 (23.15)		
Normal	169 (83.25)	156 (76.85)		
Serum calcium (mmol/L)			4.780	0.029
Decreased	21 (10.34)	36 (17.73)		
Normal	182 (89.66)	167 (82.27)		
Blood phosphorus (mmol/L)			2.382	0.123
Decreased	23 (11.33)	13 (6.40)		
Normal	180 (88.67)	190 (93.60)		

Table 3 Vitamin concentrations in esophageal cancer patients before and after chemotherapy

Item	Before chemotherapy (Q1-Q3) median	After chemotherapy (Q1-Q3) median	*Z*	*P* value
Vitamin A (μmol/L)	(0.561-0.980) 0.741	(0.528-0.858) 0.678	-3.465	0.001
Vitamin D (nmol/L)	(33.618-49.939) 41.383	(33.235-46.473) 38.832	-1.599	0.110
Vitamin E (μg/mL)	(10.898-11.673) 11.267	(10.905-11.867) 11.298	-1.678	0.093
Vitamin B_9 (nmol/L)	(14.529-23.014) 18.718	(13.859-22.037) 17.539	-1.578	0.115
Vitamin B_{12} (pg/mL)	(491.023-598.303) 557.219	(491.112-590.338) 546.789	-0.326	0.745
Vitamin B_1 (nmol/L)	(75.056-97.909) 84.070	(75.826-93.322) 83.095	-1.477	0.140
Vitamin C (μmol/L)	(34.324-38.543) 36.075	(33.299-37.849) 35.259	-3.824	< 0.001
Vitamin B_2 (μg/L)	(4.182-5.050) 4.501	(4.105-4.761) 4.284	-2.631	0.009
Vitamin B_6 (nmol/L)	(29.702-33.645) 31.747	(28.875-32.868) 31.363	-2.351	0.019

Q1-Q3: Quartiles 1-3.

Vitamin deficiencies in EC patients undergoing chemotherapy

Deficiencies of vitamin A, D, C and B_2 were detected pre- and postchemotherapy, and the proportion of each of these four vitamin deficiencies increased after chemotherapy. In particular the proportions of vitamin C and B_2 deficiencies (24.46% *vs* 38.85%) increased significantly (both $P < 0.05$) (Table 4). Three EC patients had excessively high vitamin A concentrations before chemotherapy, which decreased to normal levels in 2 cases and to vitamin A deficiency in 1 case after chemotherapy. None of the other five vitamin (E, B_9, B_{12}, B_1, B_6) deficiencies were detected during chemotherapy.

Changes in hematological indicators in EC patients pre- and postchemotherapy

Hemoglobin, total protein, serum albumin and blood calcium concentrations significantly decreased, and blood phosphorus concentrations significantly increased after chemotherapy (all $P < 0.05$) (Table 5).

Table 4 Vitamin deficiencies in esophageal cancer patients before and after chemotherapy, *n* (%)

Item	Before chemotherapy		After chemotherapy		χ^2	*P* value
	Normal	Deficiency	Normal	Deficiency		
Vitamin A (μmol/L)	163 (81.50)	37 (18.50)	158 (79.00)	42 (21.00)	0.485	0.486
Vitamin D (nmol/L)	194 (95.57)	9 (4.43)	191 (94.09)	12 (5.91)	0.235	0.629
Vitamin C (μmol/L)	155 (76.35)	48 (23.65)	133 (65.52)	70 (34.48)	6.682	0.010
Vitamin B_2 (μg/L)	139 (68.47)	64 (31.53)	108 (53.20)	95 (46.80)	9.677	0.002

Table 5 Changes in hematological indicators in esophageal cancer patients before and after chemotherapy

Item	Before chemotherapy (mean ± SD)	After chemotherapy (mean ± SD)	*t*	*P* value
Hemoglobin (g/L)	130.070 ± 16.484	115.010 ± 14.584	15.342	< 0.001
Total protein (g/L)	66.572 ± 5.726	64.499 ± 5.528	5.032	< 0.001
Serum albumin (g/L)	38.880 ± 4.138	37.546 ± 3.719	4.355	< 0.001
Blood calcium (mmol/L)	2.240 ± 0.128	2.207 ± 0.132	2.835	0.005
Blood phosphorus (mmol/L)	1.130 ± 0.200	1.180 ± 0.184	-2.818	0.005

SD: Standard deviation.

Factors affecting vitamins and hematological indicators in EC patients

After adjustment for covariates of each indicator before chemotherapy, vitamin A levels after chemotherapy showed a significant difference between males and females. Vitamin D levels after chemotherapy showed a significant difference among different tumor grades (both $P < 0.05$). No statistically significant differences were shown for the other baseline data (all $P > 0.05$) (Table 6).

Correlation between vitamin concentrations and BMI during chemotherapy in EC patients

There were correlations between the changes in serum vitamin A and C concentrations and the change in BMI before and after chemotherapy ($P < 0.05$) (Table 7).

Regression analysis of vitamins and BMI in EC patients

Using the difference in vitamin concentrations as the independent variables and the difference in BMI as the dependent variable, logistic regression analysis revealed statistically significant differences for three vitamins ($F = 5.082$, $P = 0.002$) (Table 8).

DISCUSSION

It was found that EC patients had different degrees of vitamin deficiency during chemotherapy, and the hemoglobin, total protein, serum albumin concentration and blood calcium concentration significantly decreased after chemotherapy. In addition, the proportions of patients with weight loss, anemia and hypoproteinemia also significantly increased. Our subjects were most deficient in vitamin B_2 and vitamin C followed by vitamins A and D.

In the current study, EC patients undergoing chemotherapy were most deficient in vitamin B_2 (31.53% and 46.80% before and after chemotherapy, respectively), and vitamin B_2 concentration significantly decreased after chemotherapy ($P < 0.05$). Vitamin B_1, B_9, B_{12} and B_6 deficiency or excess was not found in any of our EC patients. A comparison of vitamin levels before and after chemotherapy suggested that vitamin B_6 concentrations decreased significantly after chemotherapy but were still within the normal range. It has been reported that B vitamin intake is correlated with the risk of EC. Appropriate supplementation with vitamins B_6 and B_9 (also known as folate) can reduce the risk, while higher intake of vitamin B_{12} may increase the risk[15,16]. A study of residents in Yanting County, Sichuan Province, a high-incidence area of EC in

Table 6 Factors affecting various indicators in esophageal cancer patients during chemotherapy

Item	Gender (Males/females)		Age (≥ 60 yr/< 60 yr)		Tumor grades (grades II/III/IV)		Tumor location (cervical/upper, middle, lower thoracic)	
	F	*P* value	F	*P* value	F	*P* value	F	*P* value
Body mass index	0.014	0.904	0.042	0.837	0.672	0.512	0.718	0.542
Hemoglobin	0.044	0.834	0.757	0.385	0.747	0.475	0.195	0.900
Serum albumin	0.707	0.402	3.387	0.067	0.210	0.811	2.554	0.057
Vitamin A	5.407	0.021	0.059	0.809	0.151	0.860	0.692	0.558
Vitamin D	0.594	0.442	0.326	0.568	6.899	0.001	0.324	0.808
Vitamin C	3.774	0.053	0.537	0.464	2.633	0.075	2.261	0.083
Vitamin B_2	0.178	0.674	0.863	0.354	0.381	0.684	2.273	0.081

The descriptive statistics of each indicator before and after chemotherapy are shown in Tables 3 and 5.

Table 7 Relationships between the differences in vitamins before and after chemotherapy and the difference in body mass index before and after chemotherapy in esophageal cancer patients

Difference in vitamin concentration	Difference in BMI	
	r	*P* value
Vitamin A	0.240	0.001
Vitamin D	-0.080	0.259
Vitamin E	-0.095	0.177
Vitamin B_9	-0.016	0.824
Vitamin B_{12}	-0.053	0.449
Vitamin B_1	0.021	0.771
Vitamin C	0.188	0.007
Vitamin B_2	-0.102	0.149
Vitamin B_6	-0.078	0.269

BMI: Body mass index.

Table 8 Regression analysis of the relationships between the concentrations of various vitamins and body mass index in 203 esophageal cancer patients

Independent variables (differences)	Dependent variable (difference in BMI)		
	B (coefficient)	*t*	*P* value
Constant	0.365	4.978	< 0.001
Vitamin A	0.304	1.877	0.062
Vitamin D	-0.009	-2.355	0.020
Vitamin C	0.026	1.793	0.074

BMI: Body mass index.

China, found that riboflavin intake was markedly deficient, and riboflavin supplements in high-risk groups reduced the incidence of EC[17]. In a multicenter study in China, whole blood riboflavin was tested in 764 EC patients (and in controls), and the analysis revealed that whole blood riboflavin levels were not significantly correlated with the prevalence of EC. However, high whole blood riboflavin level was more

favorable for the survival of elderly EC patients aged 50-70 yrs[18]. Therefore, B vitamin supplementation in EC patients undergoing chemotherapy is beneficial to improve vitamin nutritional status and reduce complications.

In our research, the proportion of vitamin C deficiency cases was 23.65% and 34.48%, before and after chemotherapy, respectively. The difference was statistically significant, and vitamin C was the most deficient vitamin after chemotherapy. Thus, chemotherapy may have a considerable effect on vitamin C levels in EC patients. A meta-analysis showed a negative correlation between dietary vitamin C intake and EC risk and concluded that a high level of vitamin C may prevent EC[19]. Vitamin C supplementation was found to downregulate nuclear factor kappa B activity and significantly decrease proinflammatory cytokine levels in EC patients treated with neoadjuvant radiotherapy[20].

In the current study, vitamin A and D deficiencies were found in EC patients both before and after chemotherapy, and their proportions increased following chemotherapy. However, the changes in the proportions were not statistically significant after chemotherapy. Further stratified analysis of the baseline data showed that the change in vitamin A concentration during chemotherapy might be associated with gender, while vitamin D might differ among different tumor stages. Vitamins A and D are mainly derived from food sources. EC itself can affect the intake and absorption of food, and chemotherapy drugs further damage the nutritional status of patients, resulting in more significant vitamin deficiencies in patients after treatment. In the current study, we further analyzed the difference between vitamin concentrations and BMI in EC patients before and after chemotherapy and found that vitamins A and C were significantly correlated with the change in BMI. Regression analysis of multiple vitamins and BMI revealed a statistically significant overall model for three vitamins (A, D and C). Therefore, body weight, vitamin levels and hematological indicators interact with each other during chemotherapy in EC patients, and the supplementation of macronutrients and micronutrients are equally important. Developing holistic care is the key to nutritional therapy for tumor patients.

There were different degrees of vitamin A and D deficiencies in EC patients treated with chemotherapy, and a higher proportion of vitamin A deficiency than vitamin D deficiency and no vitamin E deficiency was detected during our observation. A meta-analysis suggested that vitamin A levels were negatively correlated with EC risk[21], and further studies are needed to confirm whether it affects the prognosis of EC patients. Vitamin D has been found to inhibit tumor cell proliferation, induce cell differentiation, promote apoptosis and suppress angiogenesis[22]. A meta-analysis did not observe an association between vitamin D levels and the development of esophageal lesions[23]. Another study reported that appropriate vitamin D supplementation in postoperative EC patients improved quality of life and disease-free survival, and further multivariate analysis found that vitamin D supplementation was an independent prognostic factor for disease-free survival but was not associated with overall survival[24].

This study had some limitations: (1) the sample size was not large due to the single center retrospective design of the study; (2) a control group was not included, and it is unclear whether vitamin supplementation is beneficial in EC patients undergoing chemotherapy; (3) the results might be biased due to different disease states and chemotherapy regimens; and (4) some patients might have received targeted therapy or immunotherapy during chemotherapy, which may have had an impact on the study results. In addition, no data on response rate or overall survival were included in our analysis, and the relationship between vitamin nutritional status and prognosis in EC patients requires further investigation in multicenter prospective studies.

CONCLUSION

Vitamin deficiencies (mainly vitamins A, D, C and B_2 deficiencies) are common in EC patients during chemotherapy and may be associated with the change in BMI. In addition, chemotherapy drugs decrease hematological indicators such as hemoglobin and albumin. Appropriate nutritional interventions and vitamin supplementation can reduce the adverse effects of chemotherapy, improve overall nutritional status of patients and improve drug tolerability and quality of life.

ARTICLE HIGHLIGHTS

Research background

Few studies have examined the changes in vitamin nutritional status and their influencing factors during chemotherapy for esophageal cancer (EC). Most vitamins were found to be negatively associated with the risk of colorectal and gastric cancer in addition to EC, yet interventional treatment failed to demonstrate a clear preventive effect in these malignancies. In our study, the effects of chemotherapeutic drugs on EC patients' vitamin levels and hematological indicators were analyzed.

Research motivation

We analyzed the effects of chemotherapeutic drugs on EC patients' vitamin levels and hematological indicators by detecting the changes in nine vitamins and hematological indicators before and after chemotherapy, with an attempt to provide evidence for vitamin supplementation. Many oncologists believe that vitamin testing is valuable in tumor patients as it can identify whether there is a specific vitamin deficiency and/or justify vitamin therapy. Our findings may be valuable for the implementation of tailored nutritional interventions.

Research objectives

To explore multiple vitamin levels and the possible influential factors in EC patients treated with chemotherapy. Varying degrees of vitamin deficiency and weight loss were found in these patients. Vitamin supplementation may reduce the adverse effects of chemotherapy.

Research methods

Vitamin nutritional status was measured using the electrochemiluminescence method with an LK3000VI vitamin detector before and after two cycles of chemotherapy in EC patients. Statistical analysis was performed using the SPSS 24.0 software package. The latent correlations between multiple vitamin levels (the independent variables) and body mass index (the dependent variable) during chemotherapy were analyzed using the Spearman method.

Research results

Varying degrees of vitamin A, D, C and B_2 deficiency and weight loss were found in EC patients. Statistically significant differences were shown in vitamins A, C, B_2 and B_6 levels and body mass index before and after chemotherapy. Multivariate analysis showed that vitamin A levels significantly differed between male and female EC patients, whereas vitamin D concentrations significantly differed in EC patients in different stages. Correlations were observed between the changes in serum vitamin A and C levels pre- and postchemotherapy and the variation in body mass index.

Research conclusions

Varying degrees of vitamin deficiency and weight loss were found in EC patients undergoing chemotherapy. Vitamin supplementation may help to improve the nutritional status, chemotherapy tolerance and efficacy. To detect the concentrations of vitamins is valuable for EC patients.

Research perspectives

A multicenter prospective study should be performed to reveal the suitable vitamin replenishment programs and potential effects on treatment outcomes and the adverse effects of chemotherapy in EC patients. Thus, randomized control studies and intervention are needed to verify our finding.

REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Chen R**, Zheng RS, Zhang SW, Zeng HM, Wang SM, Sun KX, Gu XY, Wei WW, He J. [Analysis of incidence and mortality of esophageal cancer in China, 2015]. *Zhonghua Yu Fang Yi Xue Za Zhi* 2019; **53**: 1094-1097 [PMID: 31683393 DOI: 10.3760/cma.j.issn.0253-9624.2019.11.004]

3 **Malhotra GK**, Yanala U, Ravipati A, Follet M, Vijayakumar M, Are C. Global trends in esophageal

cancer. *J Surg Oncol* 2017; **115**: 564-579 [PMID: 28320055 DOI: 10.1002/jso.24592]
4 **Ohnuma H**, Sato Y, Hayasaka N, Matsuno T, Fujita C, Sato M, Osuga T, Hirakawa M, Miyanishi K, Sagawa T, Fujikawa K, Ohi M, Okagawa Y, Tsuji Y, Hirayama M, Ito T, Nobuoka T, Takemasa I, Kobune M, Kato J. Neoadjuvant chemotherapy with docetaxel, nedaplatin, and fluorouracil for resectable esophageal cancer: A phase II study. *Cancer Sci* 2018; **109**: 3554-3563 [PMID: 30137686 DOI: 10.1111/cas.13772]
5 **Ma L**, Luo GY, Ren YF, Qiu B, Yang H, Xie CX, Liu SR, Liu SL, Chen ZL, Li Q, Fu JH, Liu MZ, Hu YH, Ye WF, Liu H. Concurrent chemoradiotherapy combined with enteral nutrition support: a radical treatment strategy for esophageal squamous cell carcinoma patients with malignant fistulae. *Chin J Cancer* 2017; **36**: 8 [PMID: 28077159 DOI: 10.1186/s40880-016-0171-6]
6 **Verzicco I**, Regolisti G, Quaini F, Bocchi P, Brusasco I, Ferrari M, Passeri G, Cannone V, Coghi P, Fiaccadori E, Vignali A, Volpi R, Cabassi A. Electrolyte Disorders Induced by Antineoplastic Drugs. *Front Oncol* 2020; **10**: 779 [PMID: 32509580 DOI: 10.3389/fonc.2020.00779]
7 **Smyth EC**, Lagergren J, Fitzgerald RC, Lordick F, Shah MA, Lagergren P, Cunningham D. Oesophageal cancer. *Nat Rev Dis Primers* 2017; **3**: 17048 [PMID: 28748917 DOI: 10.1038/nrdp.2017.48]
8 **Jain A**, Tiwari A, Verma A, Jain SK. Vitamins for Cancer Prevention and Treatment: An Insight. *Curr Mol Med* 2017; **17**: 321-340 [PMID: 29210648 DOI: 10.2174/1566524018666171205113329]
9 **Nasir A**, Bullo MMH, Ahmed Z, Imtiaz A, Yaqoob E, Jadoon M, Ahmed H, Afreen A, Yaqoob S. Nutrigenomics: Epigenetics and cancer prevention: A comprehensive review. *Crit Rev Food Sci Nutr* 2020; **60**: 1375-1387 [PMID: 30729798 DOI: 10.1080/10408398.2019.1571480]
10 **Shi HP**. Cancer is a metabolic disease. *Zhongliu Daixie Yu Yingyang Dianzi Zazhi* 2018; **5**: 111-116 [DOI: 10.16689/j.cnki.cn11-9349/r.2018.02.001]
11 **Zhang XS**, Liu ZH, Peng Y, Yang XY, Zhang Y, Xu Q, Liu YH. Analysis of vitamin nutritional status in hospitalized cancer patients. *Zhongguo Zhongliu Linchuang Yu Kangfu* 2018; **25**: 1448-1451 [DOI: 10.13455/j.cnki.cjcor.2018.12.11]
12 **Veeralakshmanan P**, Tham JC, Wright A, Bolter M, Wadhawan H, Humphreys LM, Sanders G, Wheatley T, Berrisford RJ, Ariyarathenam A. Nutritional deficiency post esophageal and gastric cancer surgery: A quality improvement study. *Ann Med Surg (Lond)* 2020; **56**: 19-22 [PMID: 32566222 DOI: 10.1016/j.amsu.2020.05.032]
13 **Zhang X**. Analysis of vitamin deficiency in palliative care cancer patients. *Heilongjiang Yixue* 2020; **44**: 437-438
14 **Masri OA**, Chalhoub JM, Sharara AI. Role of vitamins in gastrointestinal diseases. *World J Gastroenterol* 2015; **21**: 5191-5209 [PMID: 25954093 DOI: 10.3748/wjg.v21.i17.5191]
15 **Ma JL**, Zhao Y, Guo CY, Hu HT, Zheng L, Zhao EJ, Li HL. Dietary vitamin B intake and the risk of esophageal cancer: a meta-analysis. *Cancer Manag Res* 2018; **10**: 5395-5410 [PMID: 30464635 DOI: 10.2147/CMAR.S168413]
16 **Qiang Y**, Li Q, Xin Y, Fang X, Tian Y, Ma J, Wang J, Wang Q, Zhang R, Wang F. Intake of Dietary One-Carbon Metabolism-Related B Vitamins and the Risk of Esophageal Cancer: A Dose-Response Meta-Analysis. *Nutrients* 2018; **10** [PMID: 29954131 DOI: 10.3390/nu10070835]
17 **Li JB**, Zou XN, Wang HY, Tao DM, Qiao YL, Gu YK, Zheng SF. Effect of riboflavin-fortified-salt intervention on esophageal preancerous lesions among population with high risk in Yanting County. *Zhongguo Zhongliu Fangzhi Zazhi* 2009; **16**: 325-328 [DOI: 10.16073/j.cnki.cjcpt.2009.05.002]
18 **Li SS**, Tan HZ, Xu YW, Wu ZY, Wu JY, Zhao XK, Wang LD, Long L, Li EM, Xu LY, Zhang JJ. [The association between the whole blood riboflavin level and the occurrence, development and prognosis of esophageal squamous cell carcinoma]. *Zhonghua Yu Fang Yi Xue Za Zhi* 2019; **53**: 1124-1129 [PMID: 31683399 DOI: 10.3760/cma.j.issn.0253-9624.2019.11.010]
19 **Bo Y**, Lu Y, Zhao Y, Zhao E, Yuan L, Lu W, Cui L, Lu Q. Association between dietary vitamin C intake and risk of esophageal cancer: A dose-response meta-analysis. *Int J Cancer* 2016; **138**: 1843-1850 [PMID: 26355388 DOI: 10.1002/ijc.29838]
20 **Abdel-Latif MMM**, Babar M, Kelleher D, Reynolds JV. A pilot study of the impact of Vitamin C supplementation with neoadjuvant chemoradiation on regulators of inflammation and carcinogenesis in esophageal cancer patients. *J Cancer Res Ther* 2019; **15**: 185-191 [PMID: 30880777 DOI: 10.4103/jcrt.JCRT_763_16]
21 **Li K**, Zhang B. The association of dietary β-carotene and vitamin A intake on the risk of esophageal cancer: a meta-analysis. *Rev Esp Enferm Dig* 2020; **112**: 620-626 [PMID: 32543872 DOI: 10.17235/reed.2020.6699/2019]
22 **Rouphael C**, Kamal A, Sanaka MR, Thota PN. Vitamin D in esophageal cancer: Is there a role for chemoprevention? *World J Gastrointest Oncol* 2018; **10**: 23-30 [PMID: 29375745 DOI: 10.4251/wjgo.v10.i1.23]
23 **Zgaga L**, O'Sullivan F, Cantwell MM, Murray LJ, Thota PN, Coleman HG. Markers of Vitamin D Exposure and Esophageal Cancer Risk: A Systematic Review and Meta-analysis. *Cancer Epidemiol Biomarkers Prev* 2016; **25**: 877-886 [PMID: 27030602 DOI: 10.1158/1055-9965.EPI-15-1162]
24 **Wang L**, Wang C, Wang J, Huang X, Cheng Y. Longitudinal, observational study on associations between postoperative nutritional vitamin D supplementation and clinical outcomes in esophageal cancer patients undergoing esophagectomy. *Sci Rep* 2016; **6**: 38962 [PMID: 27958342 DOI: 10.1038/srep38962]

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ORIGINAL ARTICLE

Observational Study

Effects of sepsis and its treatment measures on intestinal flora structure in critical care patients

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Abstract

BACKGROUND

Sepsis is a common disease in intensive care units, with high morbidity and mortality. Intestinal microecology plays a vital part in the development and progression of this disease, possibly because sepsis and its treatment cause specific changes in the composition of the intestinal flora.

AIM

To investigate the characteristics of intestinal flora disturbance in sepsis patients treated with antibiotics.

METHODS

In this prospective comparative study, we enrolled ten patients with sepsis (sepsis group), hospitalized in the Department of Critical Care Medicine of the General Hospital, Ningxia Medical University, China (a class IIIa general hospital) from February 2017 to June 2017; ten patients without sepsis hospitalized in the same period (non-sepsis group) and ten healthy individuals (control group) were also enrolled. Fecal samples collected from the three groups were subjected to *16S rRNA* gene sequencing and the intestinal flora diversity, structure, and composition were determined. Additionally, the dynamics of the intestinal flora diversity, structure, and composition in sepsis patients were investigated *via 16S rRNA* gene sequencing of samples collected 0 d, 3 d, and 7 d after admittance to the intensive care unit. Correlations between the serum levels of procalcitonin, endotoxin, diamine oxidase, and D-lactic acid and the intestinal flora composition

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of sepsis patients were also investigated.

RESULTS

Compared with the healthy control group, sepsis and non-sepsis patients showed reduced intestinal flora α-diversity and a distinct flora structure, with Firmicutes as the dominant phylum, and significantly decreased proportions of Bacteroidetes, as well as *Prevotella* and *Lachnospira*, among other genera. Of note, the proportion of *Enterococcus* was significantly increased in the intestinal tract of sepsis patients. Interestingly, the α-diversity in the sepsis group decreased gradually, from days 1 to 7 of treatment. However, pairwise comparisons showed that both the diversity and structure of the intestinal flora were not significantly different considering the three different time points studied. Curiously, the serum levels of procalcitonin, endotoxin, diamine oxidase, and D-lactic acid in sepsis patients correlated with the prevalence of various bacterial genera. For example, the prevalence of *Ruminococcus* was positively correlated with serum procalcitonin, endotoxins, and diamine oxidase; similarly, the prevalence of *Roseburia* was positively correlated with serum procalcitonin, endotoxins, and D-lactic acid.

CONCLUSION

Sepsis patients in intensive care units show dysbiosis, lasting for at least 1 wk.

Key Words: Sepsis; Intestinal flora; *16S rRNA* gene sequencing; Dynamic changes; Intestinal barrier index; Procalcitonin

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Core Tip: As the largest reservoir of bacteria and endotoxins in the body, the intestinal tract is regarded as the "engine" of sepsis and multiple organ dysfunction. Through *16S rRNA* gene sequencing, we observed that intestinal flora disturbance occurs in sepsis patients. Notably, here, we revealed for the first time the intestinal flora dynamic changes in sepsis patients during treatment. We found that the abundance of some intestinal bacteria in sepsis patients significantly correlated with infection- and intestinal barrier-related clinical indicators. These findings add to the understanding of the intestinal flora in sepsis, providing a basis for the reversal of dysbiosis.

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INTRODUCTION

Sepsis refers to the life-threatening organ dysfunction caused by imbalanced responses to infection[1]. As the largest reservoir of bacteria and endotoxins in the body, the intestinal tract is regarded as the "engine" of sepsis and multiple organ dysfunction syndrome. Under normal circumstances, the intestinal flora plays beneficial roles in the context of human physiology and immunity[2-3], existing in a homeostatic state important for the maintenance of human health. However, in the context of critical illness, homeostasis is interrupted, leading to abnormal changes in the types, quantities, proportions, and locations of microorganisms in the intestinal tract[4], thus increasing susceptibility to sepsis. In fact, studies have shown that there is a dose–response relationship between the degree of intestinal microecological disturbance and the incidence of subsequent severe sepsis[5]. Therefore, an improved understanding of the status and degree of intestinal flora disturbances in sepsis patients is of great significance; for instance, the establishment of strong intestinal microecology-based biomarkers will allow for the accurate prediction of prognosis of sepsis patients and the consequent adoption of adequate treatment measures. However, there are few reports on the sepsis-related intestinal flora and its dynamic

changes.

High-throughput *16S rRNA* gene sequencing allows for a comprehensive understanding of the intestinal flora structure, and it is the standard technology used for human intestinal flora analysis[6]. In this study, the diversity and composition of the intestinal flora in sepsis patients, non-sepsis patients, and healthy individuals were analyzed and compared. The dynamic changes in the intestinal flora α-diversity and structure throughout a 1 wk period [1, 3, and 7 d after admittance to the intensive care unit (ICU)] were also investigated to disclose the transformation of the intestinal flora in the context of antibiotic treatment. In addition, the potential relationships between the intestinal flora imbalance and clinical indicators in sepsis were investigated *via* correlation analysis.

MATERIALS AND METHODS

General information

This is a prospective observational study, including ten sepsis patients (sepsis group) admitted to the ICU of the General Hospital, Ningxia Medical University, China from February 2017 to June 2017, ten patients without sepsis (non-sepsis group) admitted to the same ICU during the same time period, and ten local healthy individuals (control group). The inclusion criteria for sepsis patients were as follows: 18-75 years old; sepsis patients meeting the latest definition of sepsis in "Sepsis-3.0" issued by the Society of Critical Care Medicine (SCCM) in 2016[7]; and estimated time of more than 2 d spent in the ICU after enrollment. The inclusion criteria for the non-sepsis patients were as follows: 18-75 years old; and patients admitted to the ICU at the same period owing to diseases other than sepsis, such as multiple injuries and high-risk operations. Of note, we tried to match the age, underlying disease, and surgical site with those in patients in the sepsis group. Because there was no definite infection, the non-sepsis group was not given antibiotics before the fecal samples were collected. The exclusion criteria were as follows: Not meeting the inclusion criteria; perianal infection; patients subjected to enterostomy; and patients with chronic gastrointestinal diseases. Additionally, the requirements for the healthy control group were as follows: Matched age with the aforementioned two groups of patients; good health; no history of chronic or metabolic diseases (*e.g.*, hypertension, coronary heart disease, diabetes, hepatitis, and hyperthyroidism); no history of digestive tract diseases or digestive tract surgery; and not using antibiotics, probiotics, enteral nutrients, or other drugs within 3 mo before enrollment. Informed consent was obtained from all patients or their immediate family members, and this study was approved by the hospital ethics committee (ethics approval number: 2016-258).

Research methods

The general clinical data of patients in the sepsis and non-sepsis groups were recorded, including age, main diagnosis, operation type (abdominal cavity organ operation and non-abdominal cavity organ operation), clinical infection site, pathogenic bacterial agent (based on qualitative culture), and use of antibiotics (Tables 1-3). The acute physiology and chronic health (APACHE II) score and sequential organ failure assessment (SOFA) score of patients on the day of ICU admission were recorded (Table 1). The primary infection site, surgical site, and use of antibiotics in the sepsis group were also recorded (Table 2), as were the positive results of blood, urine, sputum, and other body fluids collected from patients with sepsis (Table 3). Additionally, venous blood samples were collected from patients with sepsis on days 1, 3, and 7 after admission to the ICU, and procalcitonin (PCT) was detected by immunochromatography [PCT-Q detection card produced by BRAHMS GmbH in Germany, provided by Thermo Fisher Scientific (China) Co., Ltd.], while the levels of serum D-lactic acid (D-Lac), bacterial endotoxin (endotoxin), and diamine oxidase (DAO) were determined using the JY-DLT intestinal barrier function biochemical index analysis system (Beijing Zhongsheng Jinyu Diagnosis Technology Co., Ltd., China.) within 0.5 h after blood collection.

Collection of stool samples and sequencing

Stool samples were collected from sepsis patients on days 1, 3, and 7 after admission to the ICU, from non-sepsis patients on day 1 after admission to the ICU, and from healthy controls. Samples from patients were taken from a deep part of the fresh stool of patients by using a sampling spoon, and the stool samples were quickly placed in a special stool sample box. The stool samples of healthy control subjects were collected

Table 1 Comparison of the general information among the three groups of individuals

Indicator	Control group (*n* = 10)	Non-sepsis group (*n* = 10)	Sepsis group (*n* = 10)	*H*/*F*/χ^2/*Z*/*t* value	*P* value
Age (yr, mean ± SD)	55 (48.75, 55.25)	59 (50.00, 74.00)[a]	63.50 (44.75, 76.25)[a,b]	2.05	0.36
BMI (kg/m^2, mean ± S)	23.95 ± 2.04	27.19 ± 4.40[a]	23.91 ± 4.09[a,b]	2.64	0.09
Underlying disease (cases)				0.84	0.65
Multiple injuries		3	4		
Malignant tumor		5	3		
Other diseases		2	3		
Type of operation (cases)				0.00	1.00
Surgery on abdominal hollow viscera	-	5	5		
Surgery on non-abdominal hollow viscera	-	5	5		
APACHE II score on day 1 [points, *M* (P_{25}, P_{75})]	-	10.00 (7.00, 17.00)	19.00 (15.25, 21.50)	−2.40	0.02
SOFA score on day 1 (points, mean ± SD)	-	3.30 ± 1.42	10.80 ± 2.97	−7.20	0.00

APACHE II score: Acute Physiologic Assessment and Chronic Health Evaluation II Scoring System; SOFA score: Sequential Organ Failure Assessment Score; BMI: Body mass index.
[a]$P < 0.05$.
[b]$P < 0.01$.

Table 2 General clinical information of the patients in the sepsis group

Patient No.	Primary infection site	Surgical site	Categories of antibiotics used within 7 d after admission to the ICU
S1	Lungs	-	Broad-spectrum penicillins
S2	Abdominal cavity	Hollow viscera	Carbapenems + glycopeptides
S3	Lungs	Hollow viscera	Carbapenems + oxazolidinones
S4	Lungs	Joint	Broad-spectrum penicillins + carbapenems
S5	Abdomen	Hollow viscera	Broad-spectrum penicillins + glycopeptides
S6	Lungs	-	Broad-spectrum penicillins + tetracyclines
S7	Lungs	-	Carbapenems + broad-spectrum penicillins
S8	Abdominal cavity	-	Carbapenems
S9	Abdominal cavity	Hollow viscera	Carbapenems
S10	Abdominal cavity	Hollow viscera	Carbapenems + glycopeptides

Note: S*n* represents the sepsis patient number *n* in the sepsis group; *e.g.*, S1 represents sepsis patient No. 1. ICU: Intensive care unit.

in a special stool sample box after normal defecation. Then, the small stool boxes filled with samples were sealed, labeled, placed in a liquid nitrogen tank, and transferred to a -80 °C freezer for storage.

Each stool sample was added to 790 μL of lysis buffer [4 M guanidine thiocyanate, 250 μL; 10% N-lauroyl sarcosine, 40 μL; 5% N-lauroyl sarcosine-0.1 M phosphate buffer (pH 8.0), 500 μL] together with 1 g glass beads (0.1 mm, Biospec Products, Inc., United States). After sufficient vortex-based homogenization, bead beating was performed for 10 min at full speed. The subsequent extraction was carried out according to the instructions of the extraction kit manufacturer (E.Z.N.A.®Stool DNA Kit, Omega Bio-tek, Inc., GA). The V3–V4 region of the *16S rRNA* gene was amplified with the primers 341F/805R (341F: 5′-CCTACGGGNGGCWGCAG-3′; 805R: 5′-GACTACHVGGGTATCTAATCC-3′), and the PCR products were sequenced on an Illumina MiSeq 2*300 bp platform. The raw sequencing data and accompanying information are available in the Sequence Read Archive database under the accession

Table 3 Pathogenic bacteria isolated in the sepsis group

Pathogenic bacteria	Cultured sample	Patient No.	Corresponding sample collection time (day *n* after admission to the ICU)
Acinetobacter baumannii	Sputum	S5, S6, S8	4, 13, 23
Stenotrophomonas maltophilia	Sputum	S6, S7	1, 8
Enterococcus	Sputum	S7	23
Escherichia coli	Blood	S7	1

S*n* represents the sepsis patient number *n* in the sepsis group; *e.g.*, S5 represents sepsis patient No. 5. In the table, sputum and blood samples from patients in the sepsis group were subjected to qualitative cultures, and bacterial colonization and contamination were ruled out. ICU: Intensive care unit.

number PRJNA691455.

Bioinformatics and statistical analysis

The clinical data were processed and analyzed using SPSS 19.0 software. All data were first tested for normality and homogeneity of variance. Data conforming to a normal distribution and homogeneity of variance are expressed as the mean ± SD, and the *t*-test or analysis of variance were used for statistical analysis. Data not conforming to a normal distribution are expressed as medians (P_{25}, P_{75}), and the rank-sum test was used for statistical analysis. The χ^2 test was used to compare enumeration data, and $P < 0.05$ was considered statistically significant.

USEARCH 8.0 was used to process the raw sequencing data, and operational taxonomic units (OTUs) were classified according to 97% sequence similarity. The OTU representative sequences were compared against the SILVA database (SSU123; http://www.arb-silva.de) with a 70% confidence threshold, and the taxonomic status of each *16S rDNA* sequence was obtained. The α-diversity of each sample was evaluated using the Shannon–Wiener and Simpson's diversity indexes, and the significance of differences was tested using either the nonparametric Mann–Whitney *U* test or the Kruskal–Wallis rank-sum test. Principal coordinates analysis (PCoA) and the calculation of the linear discriminant analysis effect size (LEfSe) were performed using the R software package (http://www.R-project.org/); heatmaps were also obtained using the R software. Spearman correlation analysis was also carried out to assess the relationships between relevant parameters.

RESULTS

Characteristics of the participants

A total of ten sepsis patients, ten non-sepsis patients, and ten healthy individuals were included in this study. Table 1 shows that there were no significant differences with respect to age or body mass index among the groups, as revealed by variance analysis ($P > 0.05$). The underlying diseases of the patients in the sepsis and non-sepsis groups were divided into three categories: Multiple injuries, malignant tumors, and other diseases (including benign tumors and intestinal obstruction). Of note, there were no significant differences in the underlying diseases and operation types between the sepsis and non-sepsis groups as revealed by the χ^2 test ($P > 0.05$), indicating that the groups were matched (and accurate comparisons were possible). The APACHE II and SOFA scores in the sepsis group were significantly higher than those in the non-sepsis group on day 1 of ICU admission ($P < 0.05$), suggesting that the patients in the sepsis group had worse pathology (with acute organ dysfunction) compared with those in the non-sepsis group.

Comparison of intestinal flora structure in healthy individuals and non-sepsis and sepsis patients on day 1 after ICU admittance

A total of 49 fecal samples were included for analysis. Of note, one stool sample in the sepsis group (patient S3, day 3) was missing owing to a problem in specimen collection. After quality control procedures, 629665 high-quality sequences were obtained, and the qualified reads were clustered into 440 species-level OTUs using 97% as the similarity cutoff. In this study, the number of qualified reads of inpatients

in the ICU was generally much lower than that of healthy controls. To understand whether these differences were due to low bacterial numbers, we randomly selected five specimens from each group to quantify the total bacteria in the feces. Regardless of whether bacteria were counted as bacterial copy number per gram fecal samples (mean bacterial copy numbers in sepsis, non-sepsis, and healthy control groups, 5.07E + 11, 1.36E + 12, and 9.27E + 12, respectively) or per nanogram bacterial DNA (9.02E + 06, 3.54E + 07, and 4.90E + 07, respectively), there was a significant overall decreasing trend in both the sepsis and non-sepsis groups relative to that in healthy controls. Therefore, altogether, these results suggest that the low number of qualified reads in some samples in the sepsis and non-sepsis group is secondary to the significantly reduced number of intestinal bacteria, probably because of the use of large amounts of antibiotics in the ICU.

α-diversity: The Mann–Whitney test showed that the α-diversity in the non-sepsis and sepsis groups on day 1 after ICU admittance was significantly lower than that in the control group ($P < 0.05$). Moreover, the diversity of the intestinal flora in sepsis patients was lower than that in non-sepsis patients; however, this difference was not significant (Figure 1 and Table 4).

Overall structural comparison of the intestinal flora: The PCoA plot based on the Bray–Curtis dissimilarity between samples revealed that the flora composition in sepsis patients was similar to that in non-sepsis patients on day 1 after ICU admittance (Figure 2). In line with these results, Adonis analysis showed no significant difference in the composition of the intestinal flora between sepsis and non-sepsis patients on day 1 after ICU admittance ($P > 0.05$). However, the difference in the flora composition between these groups and normal controls was extremely significant (Adonis $P < 0.05$).

Comparison of intestinal flora compositions at the phylum level: The flora composition results revealed that Firmicutes and Bacteroidetes were the dominant phyla in healthy individuals, accounting for more than 70% of the total bacteria. Meanwhile, in sepsis and non-sepsis patients, Firmicutes was the dominant phylum in the intestinal flora (Figure 3A); the proportion of Bacteroidetes decreased significantly compared to that in healthy individuals ($P < 0.05$), and the relative abundance of Proteobacteria increased compared to that in normal controls, although the difference was not significant. Of note, the proportion of Fusobacteria in the intestinal tract of non-sepsis patients was significantly higher than that in both healthy controls and sepsis patients ($P < 0.05$; Figure 3B).

Comparison of intestinal flora compositions at the genus and OTU levels: The results of the Kruskal–Wallis test showed that the proportions of *Prevotella*, *Subdoligranulum*, *Lachnospira*, *Phascolarctobacterium*, *Alloprevotella*, *Megamonas*, and *Parasutterella* in the intestinal tract of patients in the non-sepsis and sepsis groups decreased significantly compared to those in the healthy control group ($P < 0.05$). Furthermore, the abundance of *Enterococcus* was much higher in the intestinal tract of sepsis patients than in non-sepsis patients and healthy controls. In contrast, the abundance of *Fusobacterium*, *Anaerococcus*, and *Peptostreptococcus* was significantly higher in the intestinal tract of non-sepsis patients than in healthy controls and sepsis patients (Figure 4A).

Additionally, as per the heatmap analysis (random forest-based), 64 key OTUs were different among the three groups (Figure 4B). Among these, the relative abundance of OTUs assigned to the genera *Subdoligranulum*, *Alistipes*, *Megamonas*, *Blautia, Bacteroides*, *Dialister*, *Anaerostipes*, *Lachnospira*,*Roseburia*, *Pseudobutyrivibrio, Parasutterella, Prevotella*, *Coprococcus*, *Parabacteroides*, *Paraprevotella*, and *Bifidobacterium* was lower in the non-sepsis and sepsis groups than in the normal control group, whereas the abundance of OTUs assigned to the genera *Peptostreptococcus*, *Enterococcus*, *Thermoanaerobacterium*, and *Anoxybacillus* was decreased in the intestinal microbiota of the normal control group. In addition, the relative abundance of OTUs assigned to the genera *Granulicatella* and *Streptococcus* was lower in the sepsis group than in the non-sepsis group.

Dynamic changes in intestinal flora structure in sepsis patients undergoing treatment

α-diversity: The Mann–Whitney test showed that the α-diversity in the sepsis group decreased significantly within 1 wk (from days 1 to 7 after the initiation of ICU treatment) compared to that in the control group; importantly, these differences were significant ($P < 0.05$). Of note, on days 1, 3, and 7 of treatment, the α-diversity of the intestinal flora decreased gradually, but pairwise comparisons did not show

Table 4 Statistical comparison of the α-diversity indexes of the intestinal flora in the three groups of individuals

α-diversity index	*P* value		
	Control-median *vs* non-sepsis-median	Control-median *vs* sepsis-median	Non-sepsis-median *vs* sepsis-median
Ace	0.003	0.002	0.247
Chao	0.002	0.003	0.393
Shannon	0.023	0.035	0.795
Simpson	0.035	0.023	0.739
Observed OTUs	0.001	0.002	0.623

The Ace and Chao indexes reflect the microbial abundances in samples. Larger values indicate higher microbial abundance. The Shannon and Simpson indexes reflect the microbial diversity of samples. A larger Shannon index indicates a higher diversity, whereas a larger Simpson index indicates a lower diversity. $P < 0.05$ was considered statistically significant. OTUs: Operational taxonomic units.

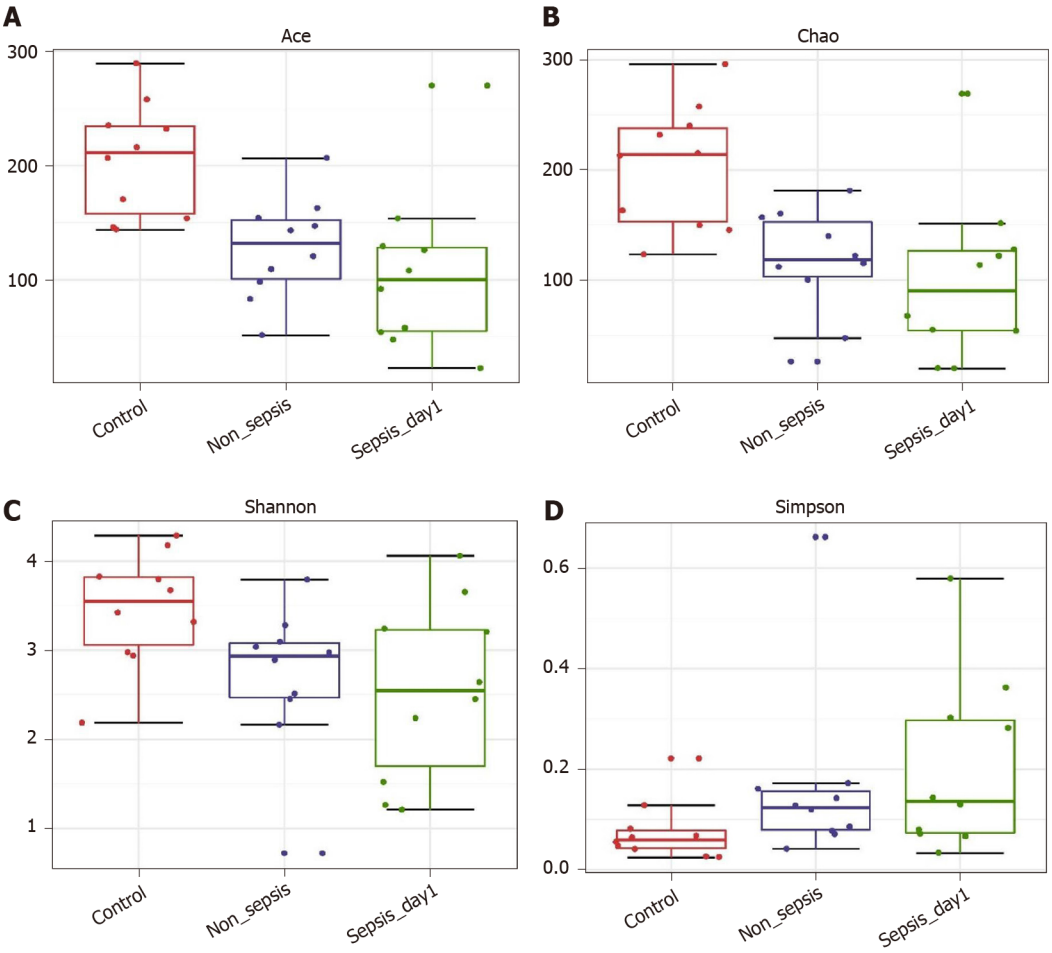


Figure 1 α-diversity indexes of fecal microbiota in normal individuals, non-sepsis patients, and sepsis patients. A: ACE estimator; B: Chao 1 estimator; C: Shannon index; D: Simpson index.

significant differences. This suggests that antibiotics killed many intestinal bacteria, and that the diversity of the intestinal flora not only did not recover but also showed a downward trend when antibiotics were continuously used (Figure 5 and Table 5).

Overall intestinal flora composition changes in sepsis patients within 1 wk after treatment: The PCoA plot based on Bray–Curtis dissimilarities between samples showed that the intestinal flora composition in sepsis patients within 1 wk of treatment (from days 1 to 7 after ICU admittance) was different from that in normal controls (Figure 6); Adonis analysis showed that the difference between sepsis patients

Table 5 Variation in the α-diversity indexes of the intestinal flora in patients with sepsis within 1 wk of treatment (days 1, 3, and 7 after intensive care unit admittance)

α-diversity index	*P* value		
	Day 1 *vs* day 3	Day 1 *vs* day 7	Day 3 *vs* day 7
Ace	0.066	0.121	0.347
Chao	0.205	0.384	0.595
Shannon	0.653	0.307	0.487
Simpson	0.595	0.241	0.347
Observed OTUs	0.177	0.384	0.623

OTUs: Operational taxonomic units.

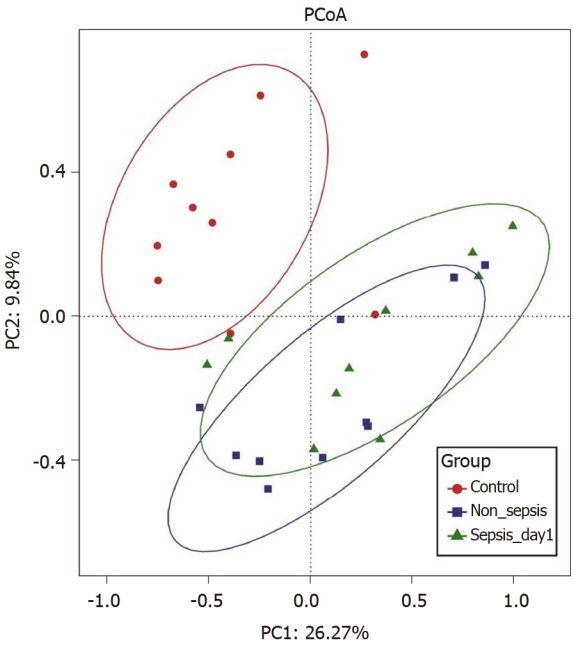


Figure 2 Principal coordinate analysis of intestinal flora among normal individuals, sepsis patients, and non-sepsis patients. Principal coordinate analysis was plotted based on the Bray–Curtis dissimilarity between samples. The ellipses highlight the clustering of the fecal microbiomes according to groups (red: Healthy control group; blue: Non-sepsis group; green: Day 1 of sepsis).

and normal controls was extremely significant ($P < 0.05$). However, the structure of the intestinal flora of sepsis patients did not differ significantly with respect to the different days after ICU admittance in the sepsis group.

Identification of differential bacterial taxa between sepsis patients 1 wk after treatment and normal individuals: To screen for the bacterial genera associated with significant differences in the intestinal flora of sepsis patients within 1 wk of admission to the ICU, we reduced the dimensions *via* the calculation of LEfSe to obtain the linear discriminant analysis scores. Compared to normal controls, in sepsis patients, some harmful bacteria such as *Coprococcus* disappeared from the intestinal flora within 1 wk of ICU treatment, indicating the efficacy of drugs against pathogenic bacteria (Figure 7). At the same time, the abundance of most of the beneficial bacteria, such as *Prevotella* and *Bifidobacterium*, also decreased, indicating that the effect of drug treatment, especially antibiotics, was not limited to harmful bacteria. However, the abundance of some bacteria, such as *Enterococcus* and *Hemophilus*, still increased despite the action of antibiotics, indicating that these bacteria were resistant to the antibiotics used.

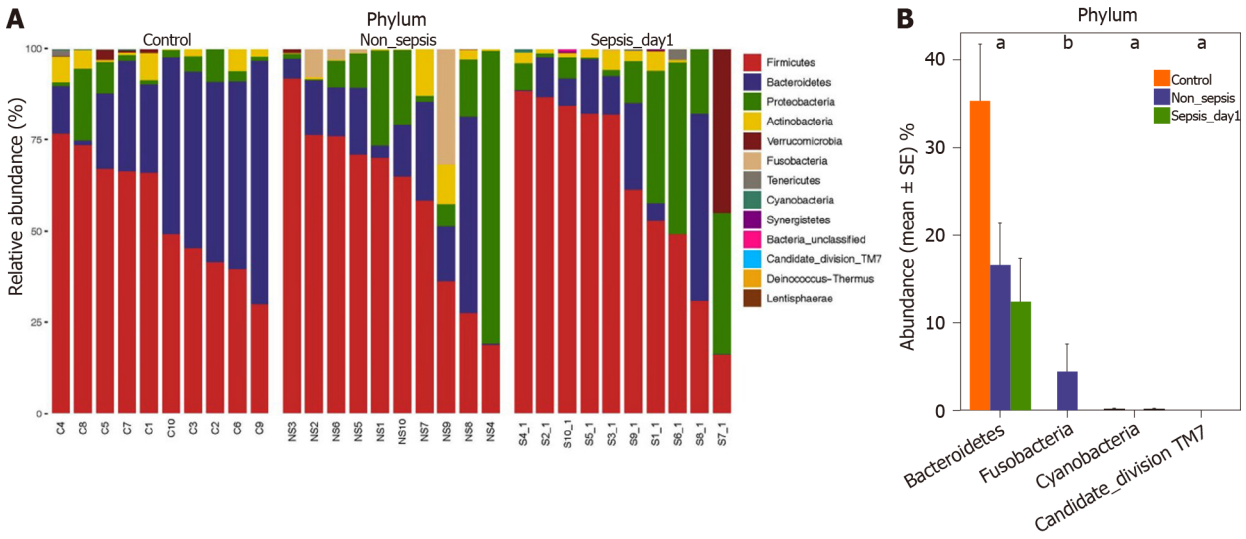


Figure 3 Composition and comparison of the fecal microbiota among normal individuals, sepsis patients, and non-sepsis patients at the phylum level. A: Composition of the fecal microbiota at the phylum level among three groups; B: Comparison of the fecal microbiota at the phylum level among three groups. The average abundance of each phylum is depicted as the mean ± SE. *P* values were calculated using the Kruskal–Wallis rank-sum test: $^{a}P < 0.05$, $^{b}P < 0.01$.

Correlation analysis between clinical indicators and abundance of intestinal bacterial genera in patients with sepsis

There were ten patients in the sepsis group (S1–S10). Venous blood samples were collected on days 1, 3, and 7 after admission to the ICU to determine the levels of permeability-related D-Lac, endotoxin, and DAO and infection-related PCT. In the blood of sepsis patients, the levels of D-Lac, endotoxin, DAO, and PCT were 20.96 ± 11.90 mg/L, 10.65 ± 7.92 U/L, 19.58 ± 17.61 U/L, and 7.23 ± 13.92 ng/mL, respectively.

Based on the Spearman correlation analysis, significant correlations were established between clinical indicators and abundance of intestinal bacterial genera in sepsis patients (Figure 8). The number of days of admission in the ICU negatively correlated with the abundance of *Streptococcus* and *Ruminococcus* ($P < 0.05$). The abundance of *Roseburia* and *Parasutterella* was positively correlated ($P < 0.05$), while that of *Prevotella*, *Lactobacillus*, and *Finegoldia* was negatively correlated with the levels of D-Lac ($P < 0.05$). Additionally, the abundance of *Ruminococcus*, *Roseburia*, *Sutterella*, *Peptostreptococcus*, *Escherichia-Shigella*, *Dorea*, *Bilophila*, *Coprobacter*, *Clostridium_sensu_stricto_1*, *Parabacteroides*, *Bifidobacterium*, *Alistipes*, and *Parasutterella*, as well as some genera belonging to Lachnospiraceae, Christensenellaceae, Ruminococcaceae, and Erysipelotrichaceae, was positively correlated with the levels of PCT ($P < 0.05$). Conversely, the abundance of *Stenotrophomonas* and *Enterococcus* was negatively correlated with the levels of PCT ($P < 0.05$). The abundance of *Peptostreptococcus*, *Roseburia*, *Collinsella*, *Dorea*, and VadinBB60 group norank was also positively correlated with the endotoxin levels ($P < 0.05$), while that of *Dialister*, *Pepto streptococcus*, with the endotoxin levPeptostreptococcus, *Dorea*, *Hemophilus*, *Bifidobacterium*, *Collinsella*, and *Blautia* was positively correlated with the levels of DAO ($P < 0.05$).

DISCUSSION

Characteristics of intestinal flora structure in sepsis patients

Sepsis is associated with high morbidity and mortality worldwide, and its timely identification and treatment are difficult. Therefore, improving the diagnosis and treatment of sepsis is essential from a global health perspective[8]. In February 2016, the SCCM and the European Society of Intensive Care Medicine jointly released the definition of sepsis in "Sepsis 3.0" as follows: "Life-threatening organ dysfunction due to a dysregulated host response to infection"[7]. This definition emphasizes the close relationship between sepsis and organ dysfunction; of note, the intestinal tract is recognized as the "engine" of organ dysfunction. The intestinal flora, as an important component of the intestinal tract, plays major physiological roles in the context of

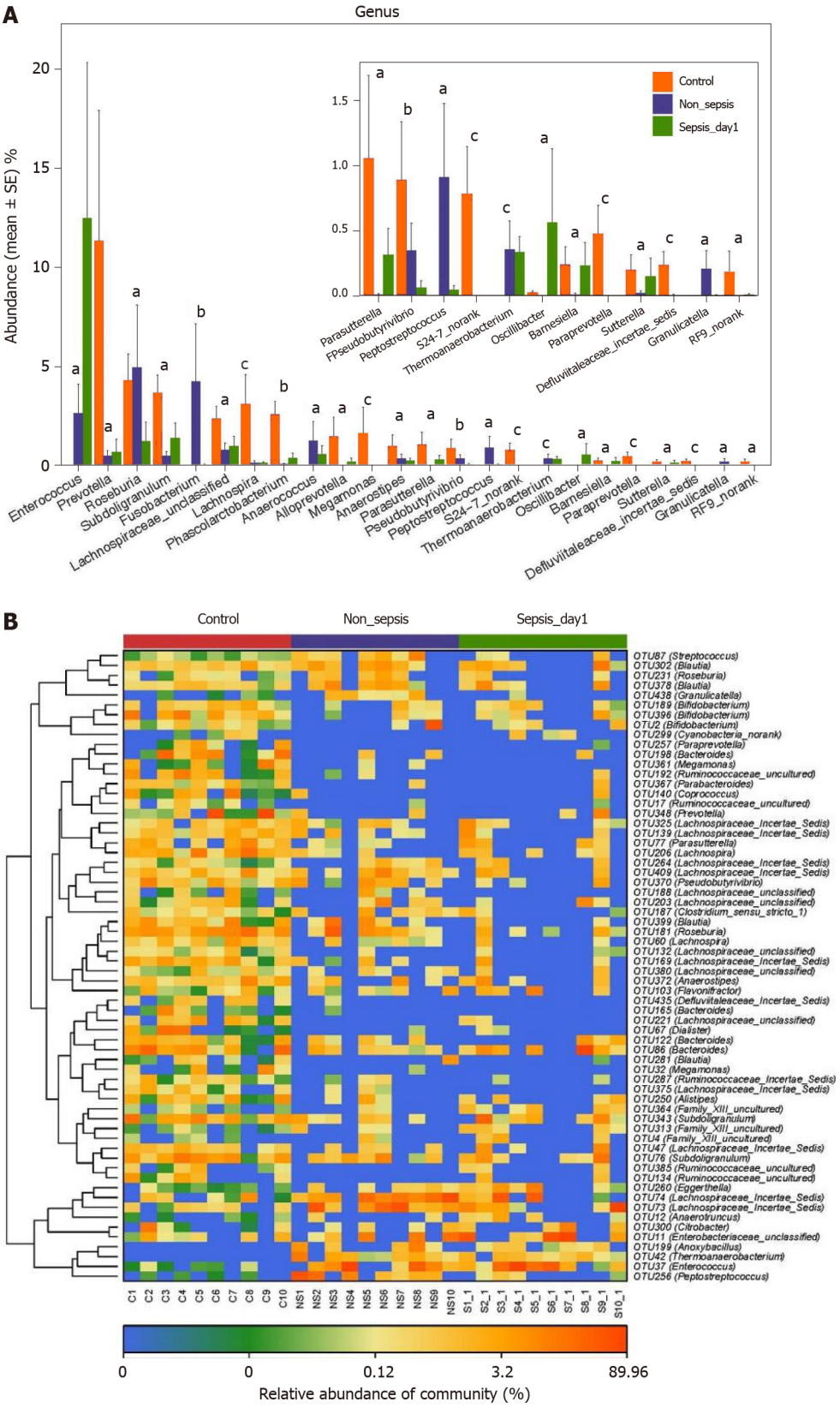


Figure 4 Key phylotypes showing significant differences among normal individuals, sepsis patients, and non-sepsis patients. A: Comparison of the fecal microbiota at the genus level among the three groups. The average abundance of each genus is depicted as the mean ± SE. *P* values were calculated using the Kruskal–Wallis rank-sum test: $^{a}P < 0.05$; $^{b}P < 0.01$, $^{c}P < 0.001$; B: Heatmap representing the relative abundance of differential operational taxonomic units (OTUs) among the three groups. The key OTUs whose relative abundance was above 1%, as per random forest-based models are displayed in the "figure".

biosynthesis and metabolism; however, critical diseases lead to many changes in the diversity of the intestinal flora, causing excessive growth of pathogenic bacteria[9,10].

By comparing the intestinal flora of sepsis patients and healthy controls, we confirmed that there are marked differences in the abundance, distribution, and structure of the intestinal flora in the context of sepsis. In sepsis patients, normal bacteria decreased or disappeared, while the abundance of abnormal bacteria increased sharply. At the phylum level, the abundance of Firmicutes increased significantly, while that of Bacteroidetes decreased significantly in the intestinal flora of sepsis patients *vs* non-septic critically ill patients; of note, the number of bacteria in the phylum Fusobacterium also decreased significantly. At the genus level, in addition to the decrease in the abundance of beneficial symbiotic bacteria such as *Prevotella* and *Lachnospira*, as well as of other genera, including *Fusobacterium* and *Peptostreptococcus*, we found that the abundance of *Enterococcus* increased significantly in the intestinal flora of sepsis patients *vs* non-septic critically ill patients. Therefore, our results suggest that dysbiosis in sepsis shows three major features. First, as the abundance and diversity of the intestinal flora decrease, the structure of the intestinal flora changes, and the differences among individuals are greater[11]. Second, the abundance of dominant obligate anaerobes decreases and that of facultative anaerobes increases[11,12]. Third, the abundance of beneficial symbiotic bacteria decreases, while that of pathogenic bacteria increases, possibly becoming predominant[11,13].

The occurrence of intestinal microecological disorders in sepsis patients is not surprising. First, the physiological state of sepsis patients is completely different from that at homeostasis; specifically, intestinal hypoperfusion and reperfusion injury lead to changes in the intestinal environment and blood supply to the intestinal mucosa, resulting in intestinal mucosal inflammation and, consequently, a series of changes in the intestinal environment, such as increased nitrate concentrations[11] and altered mucosal oxygen gradient[12]. These changes are favorable to the growth of Proteobacteria, leading to the expansion of many clinically familiar pathogenic Gram-negative bacilli, such as *Pseudomonas aeruginosa* and *Escherichia coli*, as well as *Staphylococcus aureus* and *Enterococcus*[14,15]. Second, ICU patients are exposed to various endogenous regulators (such as increased catecholamine production and changes in glucose metabolism) and clinical interventions (such as proton pump inhibitors, opioids, nutritional support, and antibiotics), which affect the living environment of the intestinal flora to varying degrees and, thus, affect the flora structure[16]. Finally, the intestinal mucosa will be damaged and thinned in critically ill patients[17,18]; such mucosal damage leads to the loss of the normal habitat of symbiotic microorganisms, subsequently leading to intestinal microecological disorders.

Dynamic changes in the structure of the intestinal flora of sepsis patients within 1 wk after treatment initiation

Our study suggests that the abundance, distribution, and diversity of the intestinal flora in sepsis patients do not change significantly within 1 wk of ICU admittance. However, in relation to the gut microbiome of healthy subjects, the numbers of most beneficial bacteria, such as *Prevotella* and *Bifidobacterium*, were below the detection limit in sepsis patients, whereas the abundance of infection-related bacteria, such as *Enterococcus* and *Hemophilus*, increased despite the action of antibiotics (with the exception of *Coprococcus*, which decreased in abundance to below the detection limit). These results indicate that patients with intestinal flora disorders associated with sepsis could not recover in a short time, and while the drugs were effective against the bacteria causing infection, they also affected bacteria in the intestines. In fact, the effects of drug treatment, especially antibiotics, on bacteria were not limited to harmful bacteria; the proportion of beneficial intestinal bacteria also decreased. Other studies have also shown that the long-term use of antibiotics changes the normally healthy intestinal flora and leads to the emergence of drug resistance; worrisome enough, the long-term use of antibiotics has the potential to generate organism reservoirs with a multi-drug resistance gene pool[19]. Ma *et al*[20] used rat models with third-degree burns on 30% of the total surface area of the back and quantified/identified the intestinal bacteria after treatment. The results showed that the number of cocci in the gastrointestinal contents of rats increased significantly, and the coccus/bacillus ratio was seriously inverted after treatment with Rocephin. It was considered that broad-spectrum antibiotics destroyed the intestinal microecological balance; of note, the conditional pathogenic intestinal flora showed potential to affect health, disease, and drug action. Our study also revealed that the abundance of *Enterococcus* increased significantly in sepsis patients, which might be related to the use of broad-spectrum antibiotics.

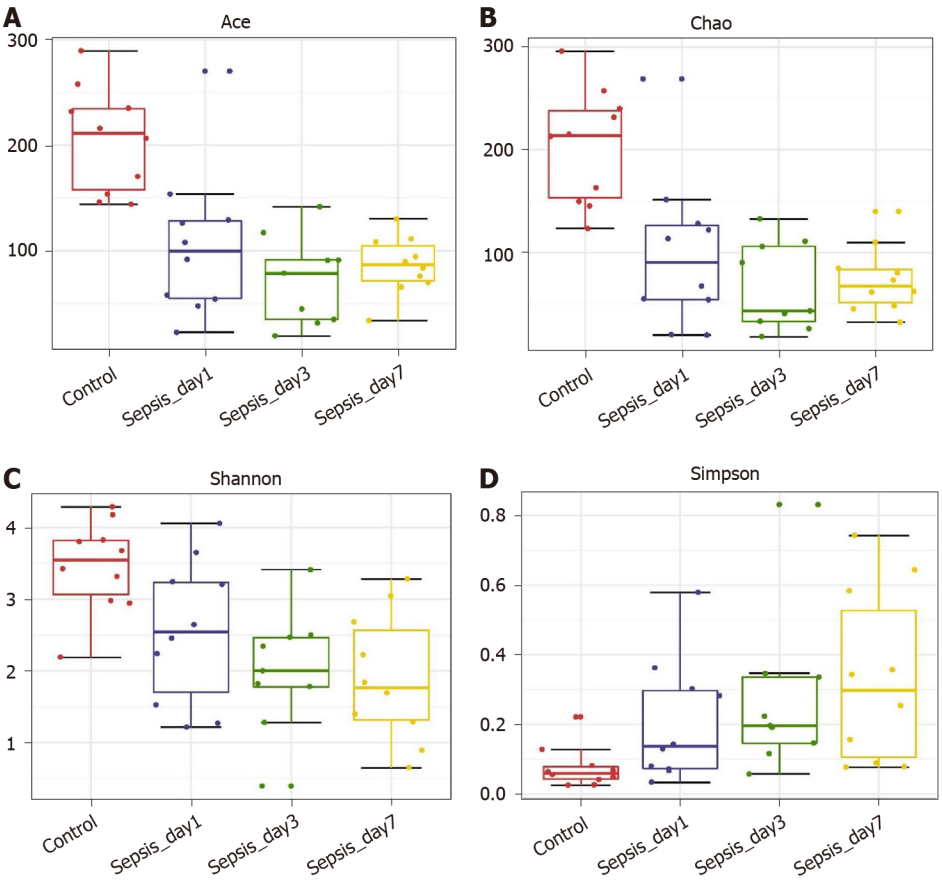


Figure 5 α-diversity indexes of the fecal microbiota in sepsis patients on days 1, 3, and 7 after admission to the intensive care unit *vs* healthy individuals. A: ACE estimator; B: Chao 1 estimator; C: Shannon index; D: Simpson index.

Correlation between the abundance of intestinal bacterial genera and clinical indicators in patients with sepsis

Our study showed a clear correlation between the abundance of intestinal flora components and clinical indicators in sepsis. The clinical indicators used in this study were D-Lac, endotoxin, DAO, and PCT. Under normal circumstances, D-Lac is produced *via* the methylglyoxal metabolism, and its content in the blood is very small. However, when glycolysis results in increases in a large number of gastrointestinal bacteria and the intestinal barrier function is impaired, the content of D-Lac increases sharply. Endotoxins are cell wall components of Gram-negative bacteria that are released only when bacteria are lysed and die. DAO is a highly active intracellular enzyme in the upper villi of the intestinal mucosa of mammals, including humans. Its activity is closely associated with nucleic acid and protein synthesis in mucosal cells. Therefore, the above three indicators can reflect the integrity and damage degree of the intestinal mechanical barrier. Additionally, PCT reflects the active level of the systemic inflammatory response, and the factors affecting its levels include the size and type of the infected organ, the type of pathogenic bacteria, the degree of inflammation, and the state of immune responses. Our study showed that serum PCT, endotoxin, DAO, and D-Lac levels in patients with sepsis were correlated with the abundance of various intestinal bacterial genera. Of note, some of these genera were correlated with multiple clinical indicators at the same time. For example, the abundance of *Peptostreptococcus* and *Dorea* was positively correlated with the serum levels of PCT, endotoxin, and DAO; moreover, the abundance of *Roseburia* was positively correlated with the serum levels of PCT, endotoxin, and D-Lac.

Among these genera, the abundance of *Ruminococcus* had the highest positive correlation with PCT. *Ruminococcus* is an important constituent of the normal intestinal microbiota. A study published in 2017[21] using samples from young Han Chinese individuals revealed changes in the intestinal flora of the Chinese population and showed that the genera *Ruminococcus* and *Fusobacterium*, among others, were relatively highly abundant in obese Chinese individuals, whereas the abundance of *Bacteroides* was greatly reduced. Of note, two *Ruminococcus* species, *Ruminococcus torques* and

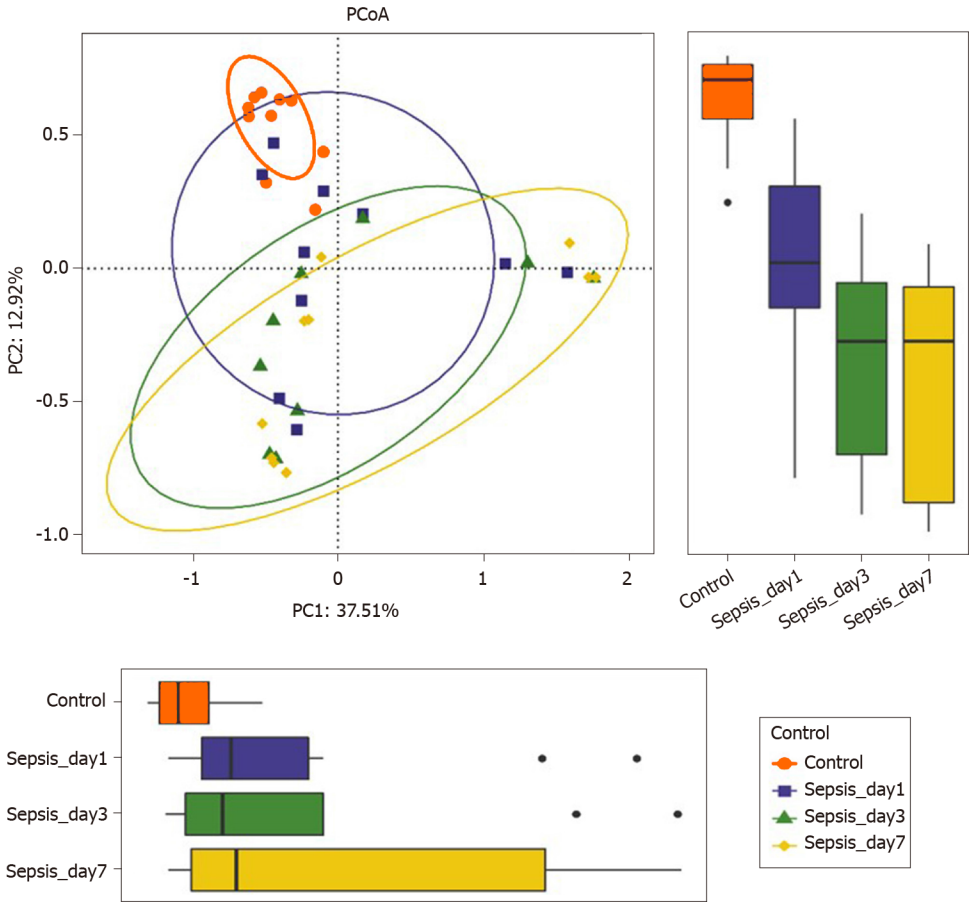


Figure 6 Principal coordinate analysis of the intestinal flora in sepsis patients on days 1, 3, and 7 after admission to the intensive care unit versus healthy individuals. Principal coordinate analysis was plotted based on the Bray–Curtis dissimilarity between samples. The ellipses highlight the clustering of the fecal microbiomes according to the groups (red: Healthy control group; blue: Sepsis patients on day 1; green: Sepsis patients on day 3; yellow: Sepsis patients on day 7).

Ruminococcus gnavus, should be mentioned because they are related to inflammatory bowel disease[22] and metabolic disorder[23]. Importantly, from the significant decrease in the number and abundance of intestinal bacteria, including the decrease in abundance of the phylum Bacteroidetes, the increase in abundance of the phylum Verrucomicrobia, and the increase in abundance of the genus *Ruminococcus* in sepsis patients, we can establish a parallel with the results of the study of obese Chinese individuals. In clinical practice, it is known that obese individuals have more significant characteristics of insulin resistance and dyslipidemia, with a more significant inflammatory phenotype, which are risk factors for cardiovascular diseases, diabetes, osteoporosis, and some cancers among other diseases[24]. Interestingly, patients with sepsis also have clinical manifestations such as insulin resistance, dyslipidemia, and inflammatory responses, which have adverse effects on the severity of illness and prognosis[25]. Therefore, we boldly speculate that the intestinal phenotype of obese patients makes them more susceptible to the complications of sepsis, which might be of great significance for the treatment and prevention of sepsis. Of course, the sample size in this study was small, and thus, it is necessary to conduct larger, multicenter studies to support our findings; moreover, the proposed mechanisms require further validation in animal studies for clarification.

There is a correlation between intestinal flora disorders and intestinal barrier dysfunction in sepsis patients. Our research showed that the abundance of *Roseburia* in the intestinal tract of sepsis patients was definitely related to the levels of serum markers of intestinal barrier function. *Roseburia* is one of the main bacterial genera producing butyrate in the human intestinal flora[26,27]. Butyrate is the main energy source of colonic epithelial cells. Research suggests that the abundance of bacteria producing butyrate in the intestinal tract of critically ill patients decreases or disappears, which leads to a decrease in butyrate production and the apoptosis of intestinal epithelial cells due to "starvation"[26]. Importantly, one study showed that the genus *Roseburia* can improve the intestinal ecosystem, prevent intestinal leakage,

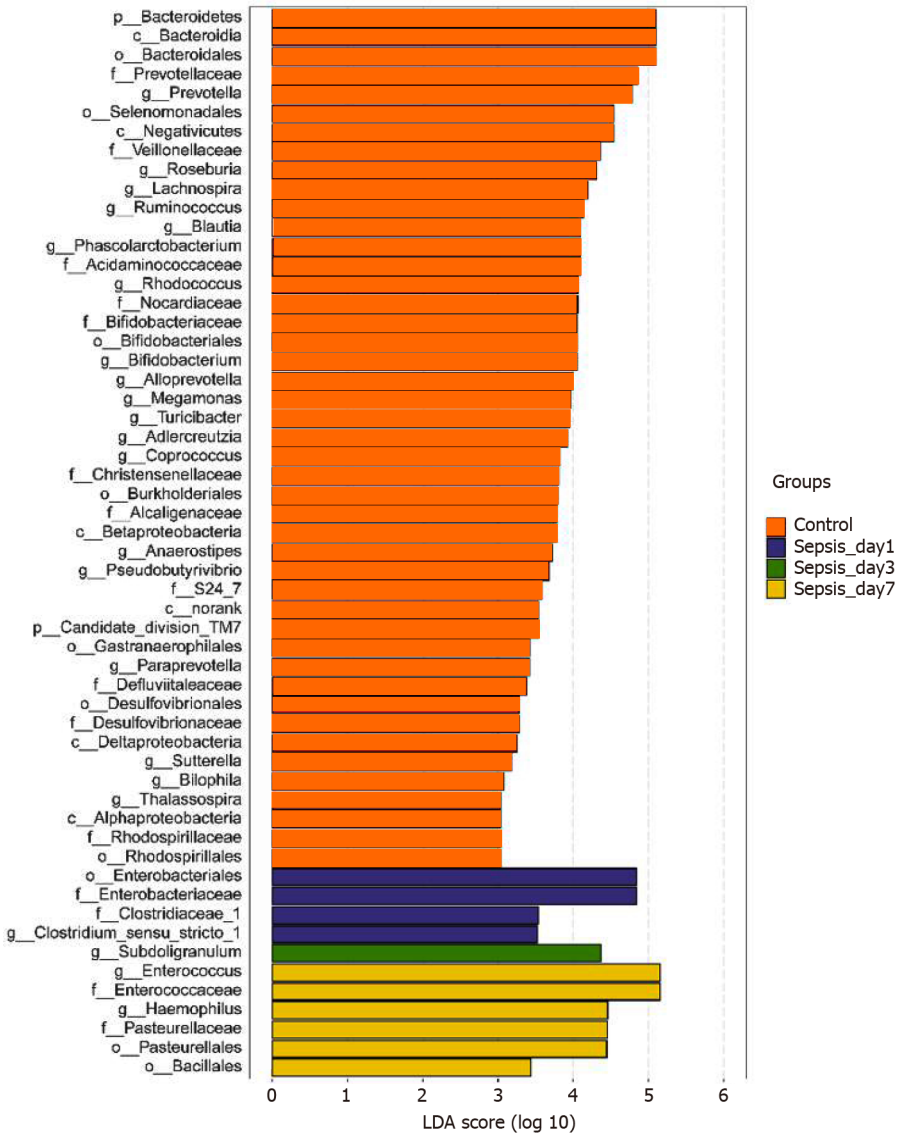


Figure 7 Linear discriminant analysis scores indicating significant differences in the fecal microbiota between the sepsis patients within 1 wk after admission to the intensive care unit and the normal control group. Red: Enriched taxa in the healthy control group; blue: Enriched taxa in sepsis patients on day 1; green: Enriched taxa in sepsis patients on day 3; yellow: Enriched taxa in sepsis patients on day 7.

and reduce the incidence of diabetes[27]. Conversely, other studies have shown that the abundance of *Roseburia* is significantly reduced in patients with Crohn's disease[28] and inflammatory bowel disease[29]. Our study showed that the abundance of intestinal *Roseburia* was positively correlated with serum PCT, endotoxin, and D-Lac levels in sepsis patients, indicating that the intestinal barrier function is more severely damaged and the systemic inflammatory response is more serious with a higher content of *Roseburia*, which seems to contradict the results of existing research. We speculate that systemic inflammatory responses in sepsis patients cause intestinal damage and that the body has a self-regulatory effect on this damage. In addition, a recently published study[30] showed that immune cells and autoantibodies of patients with antiphospholipid syndrome (an autoimmune disease) could cross-react with the mimic epitopes of *Roseburia*; in line with this, *Roseburia* could also cause autoimmune diseases in susceptible mice. Once again, these results suggest that the positive and negative effects of intestinal bacteria are not invariable.

Bacteria can perceive the changes in the host internal environment and then change their own toxic factors to become pathogenic. Under various stimuli such as sepsis, trauma, and burns, a healthy bacterial strain might be transformed into a pathogenic bacterium within a few hours[31]. For example, *Pseudomonas aeruginosa* was injected into the ceca of mice in a sham operation group for culture, and then, the bacteria were collected and implanted into the undamaged abdominal cavity of mice; it was found that no death occurred. However, the same strain was injected into the ceca of mice

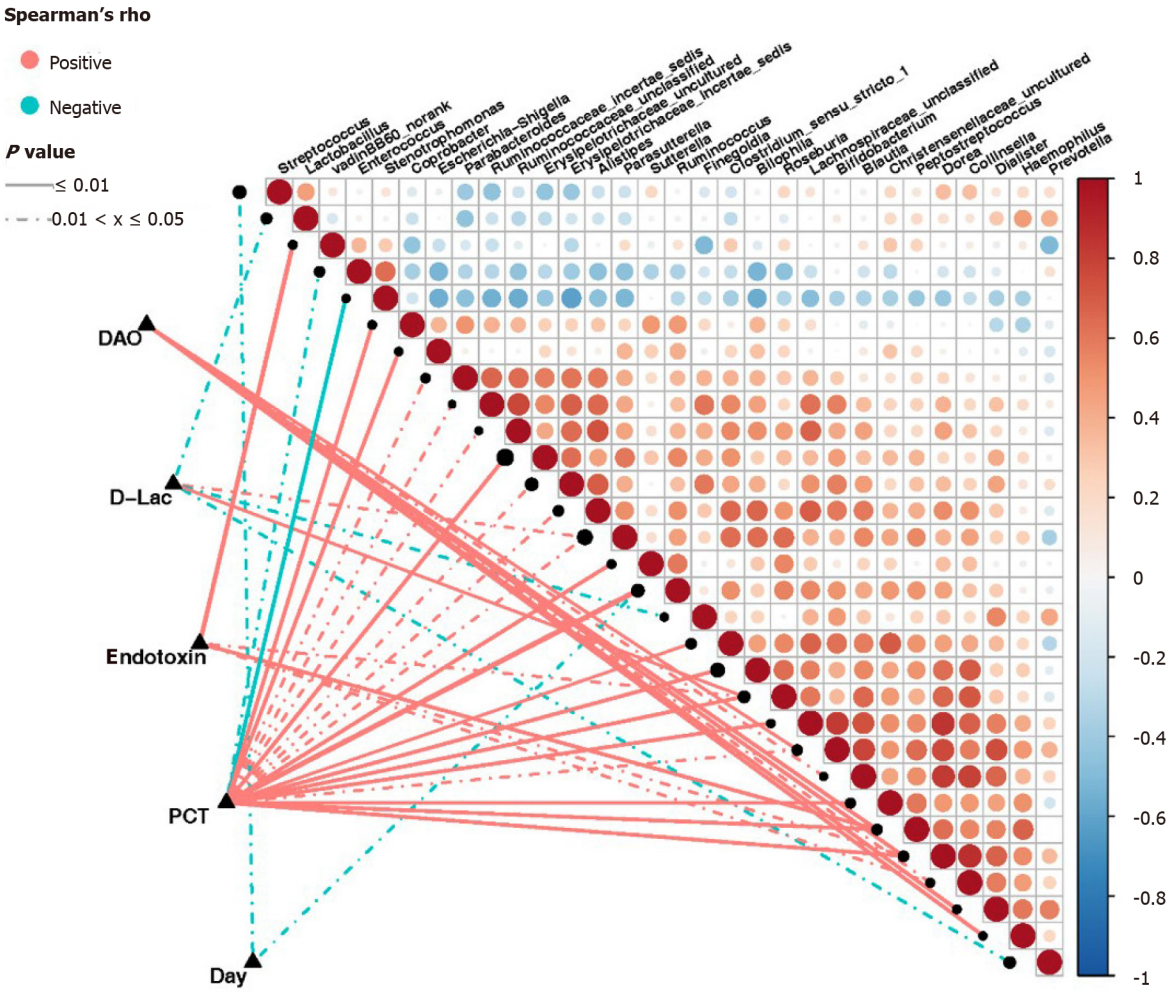


Figure 8 Correlation between clinical indicators and abundance of fecal microbiota in sepsis patients. Heatmap showing partial Spearman's correlation coefficients among 30 genera and clinical indexes. Connecting lines represent the correlation coefficient values above 0.4 (red, positive correlation) or below -0.4 (blue, negative correlation). Solid lines represent $P \leq 0.01$. Dotted lines represent $0.01 < P \leq 0.05$. The intensity of shading in the circles is proportional to the magnitude of the association. In the figure, "day" indicates the collection time of stool samples from sepsis patients after admission to the intensive care unit (ICU) (days 1, 3, and 7 after admission to the ICU). D-Lac: d-lactic acid; PCT: Procalcitonin; DAO: Diamine oxidase.

after 30% hepatectomy for culture, and then were collected and implanted into the undamaged abdominal cavities of mice, which led to their deaths[32]. Although the mechanisms underlying such transformations remain inconclusive, these results suggest that the host environment can not only change the diversity of bacterial species but also change their virulence. Therefore, the beneficial or harmful effects of a specific bacterium are not absolute; they can be influenced by many factors.

CONCLUSION

In this study, we report that sepsis patients in the ICU showed intestinal microecological disorders, lasting for at least 1 wk. Furthermore, intestinal microecological disorder was correlated with inflammation-related and intestinal barrier-related indexes in sepsis. However, this study is not without limitations. The sample size was small, and thus, it is necessary to carry out larger and multicenter studies to support these findings. Additional animal studies might be needed to clarify the related molecular mechanisms.

ARTICLE HIGHLIGHTS

Research background

Sepsis is a common disease in intensive care units, with high morbidity and mortality. Intestinal microecology plays a vital part in the development and progression of this disease, possibly because sepsis and its treatment cause specific changes to the intestinal flora. However, there are few studies on the sepsis-related intestinal flora and its dynamic changes. An improved understanding of the status and degree of intestinal flora disturbances in sepsis patients is of great significance, to allow for the accurate evaluation of the disease condition and prognosis and to optimize the treatment measures.

Research motivation

Studies have shown a dose–response relationship between the degree of intestinal microecological disturbance and the incidence of subsequent severe sepsis. Critical illness leading to abnormal changes in the types, quantities, proportions, and locations of microorganisms in the intestinal flora may, thus, increase susceptibility to sepsis. Therefore, an improved understanding of the status and degree of intestinal flora disturbances in sepsis patients is of great clinical significance.

Research objectives

The main objective of this study was to investigate the characteristics of intestinal flora disturbance in sepsis patients treated with antibiotics.

Research methods

We enrolled ten patients with sepsis admitted to the intensive care unit (ICU), ten patients without sepsis admitted to the ICU in the same period, and ten healthy individuals (sepsis group, non-sepsis group, and control group, respectively). Using *16S rRNA* gene sequencing technology, the fecal samples of the three groups were analyzed, and the intestinal flora diversity, structure, and composition were compared. The fecal samples of sepsis patients on days 1, 3, and 7 after ICU admittance were also analyzed, and the dynamics of the diversity, structure, and composition of the intestinal flora of sepsis patients were compared. Lastly, the serum levels of procalcitonin, endotoxin, diamine oxidase, and D-lactic acid were determined in sepsis patients on days 1, 3, and 7 after ICU admittance and correlated with the abundance of intestinal bacteria.

Research results

Sepsis patients showed a reduced intestinal flora α-diversity and a different flora structure, with Firmicutes as the dominant bacteria, and significantly decreased proportions of Bacteroidetes, as well as *Prevotella*, *Lachnospira*, and other genera. *Enterococcus* was significantly increased in the intestinal tract of sepsis patients. Additionally, from days 1 to 7 of treatment, the α-diversity of the intestinal flora in the sepsis group decreased gradually, although without statistical significance. Of note, some harmful bacteria such as *Coprococcus* disappeared, the abundance of beneficial bacteria such as *Prevotella* and *Bifidobacterium* decreased, while that of *Enterococcus* and other genera increased. Interestingly, the serum levels of procalcitonin, endotoxin, diamine oxidase, and D-lactic acid in sepsis patients correlated with the abundance of various intestinal bacterial genera.

Research conclusions

In this study, we report the characteristics of sepsis intestinal flora disturbance and reveal, for the first time, the dynamic characteristics of the intestinal flora in sepsis patients under antibiotic treatment. Altogether, our results suggest that sepsis patients in the ICU show intestinal microecological disorders, lasting for at least 1 wk. Importantly, we also show that the intestinal microecological disorder in sepsis patients is correlated with inflammation-related and intestinal barrier-related indexes. Of note, the sample size of this study was small, and thus, it is necessary to conduct larger and multicenter studies to support these findings.

Research perspectives

We plan to carry out animal studies to clarify the molecular mechanisms of intestinal flora disturbance in sepsis.

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REFERENCES

1 **Vincent JL**, Opal SM, Marshall JC, Tracey KJ. Sepsis definitions: time for change. *Lancet* 2013; **381**: 774-775 [PMID: 23472921 DOI: 10.1016/S0140-6736(12)61815-7]
2 **O'Hara AM**, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep* 2006; **7**: 688-693 [PMID: 16819463 DOI: 10.1038/sj.embor.7400731]
3 **Clarke G**, Stilling RM, Kennedy PJ, Stanton C, Cryan JF, Dinan TG. Minireview: Gut microbiota: the neglected endocrine organ. *Mol Endocrinol* 2014; **28**: 1221-1238 [PMID: 24892638 DOI: 10.1210/me.2014-1108]
4 **McDonald D**, Ackermann G, Khailova L, Baird C, Heyland D, Kozar R, Lemieux M, Derenski K, King J, Vis-Kampen C, Knight R, Wischmeyer PE. Extreme Dysbiosis of the Microbiome in Critical Illness. *mSphere* 2016; **1** [PMID: 27602409 DOI: 10.1128/mSphere.00199-16]
5 **Prescott HC**, Dickson RP, Rogers MA, Langa KM, Iwashyna TJ. Hospitalization Type and Subsequent Severe Sepsis. *Am J Respir Crit Care Med* 2015; **192**: 581-588 [PMID: 26016947 DOI: 10.1164/rccm.201503-0483OC]
6 **Suau A**, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* 1999; **65**: 4799-4807 [PMID: 10543789 DOI: 10.1128/AEM.65.11.4799-4807.1999]
7 **Bassetti M**, Vena A, Meroi M, Cardozo C, Cuervo G, Giacobbe DR, Salavert M, Merino P, Gioia F, Fernández-Ruiz M, López-Cortés LE, Almirante B, Escolà-Vergé L, Montejo M, Aguilar-Guisado M, Puerta-Alcalde P, Tasias M, Ruiz-Gaitán A, González F, Puig-Asensio M, Marco F, Pemán J, Fortún J, Aguado JM, Soriano A, Carratalá J, Garcia-Vidal C, Valerio M, Sartor A, Bouza E, Muñoz P. Factors associated with the development of septic shock in patients with candidemia: a post hoc analysis from two prospective cohorts. *Crit Care* 2020; **24**: 117 [PMID: 32216822 DOI: 10.1186/s13054-020-2793-y]
8 **Klingensmith NJ**, Coopersmith CM. The Gut as the Motor of Multiple Organ Dysfunction in Critical Illness. *Crit Care Clin* 2016; **32**: 203-212 [PMID: 27016162 DOI: 10.1016/j.ccc.2015.11.004]
9 **Shimizu K**, Ogura H, Goto M, Asahara T, Nomoto K, Morotomi M, Yoshiya K, Matsushima A, Sumi Y, Kuwagata Y, Tanaka H, Shimazu T, Sugimoto H. Altered gut flora and environment in patients with severe SIRS. *J Trauma* 2006; **60**: 126-133 [PMID: 16456446 DOI: 10.1097/01.ta.0000197374.99755.fe]
10 **Lankelma JM**, van Vught LA, Belzer C, Schultz MJ, van der Poll T, de Vos WM, Wiersinga WJ. Critically ill patients demonstrate large interpersonal variation in intestinal microbiota dysregulation: a pilot study. *Intensive Care Med* 2017; **43**: 59-68 [PMID: 27837233 DOI: 10.1007/s00134-016-4613-z]
11 **Winter SE**, Winter MG, Xavier MN, Thiennimitr P, Poon V, Keestra AM, Laughlin RC, Gomez G, Wu J, Lawhon SD, Popova IE, Parikh SJ, Adams LG, Tsolis RM, Stewart VJ, Bäumler AJ. Host-derived nitrate boosts growth of E. coli in the inflamed gut. *Science* 2013; **339**: 708-711 [PMID: 23393266 DOI: 10.1126/science.1232467]
12 **Albenberg L**, Esipova TV, Judge CP, Bittinger K, Chen J, Laughlin A, Grunberg S, Baldassano RN, Lewis JD, Li H, Thom SR, Bushman FD, Vinogradov SA, Wu GD. Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. *Gastroenterology* 2014; **147**: 1055-63. e8 [PMID: 25046162 DOI: 10.1053/j.gastro.2014.07.020]
13 **Lupp C**, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2007; **2**: 119-129 [PMID: 18005726 DOI: 10.1016/j.chom.2007.06.010]
14 **Honda K**, Littman DR. The microbiome in infectious disease and inflammation. *Annu Rev Immunol* 2012; **30**: 759-795 [PMID: 22224764 DOI: 10.1146/annurev-immunol-020711-074937]
15 **Grootjans J**, Lenaerts K, Derikx JP, Matthijsen RA, de Bruïne AP, van Bijnen AA, van Dam RM, Dejong CH, Buurman WA. Human intestinal ischemia-reperfusion-induced inflammation characterized: experiences from a new translational model. *Am J Pathol* 2010; **176**: 2283-2291 [PMID: 20348235 DOI: 10.2353/ajpath.2010.091069]
16 **Haak BW**, Levi M, Wiersinga WJ. Microbiota-targeted therapies on the intensive care unit. *Curr Opin Crit Care* 2017; **23**: 167-174 [PMID: 28092309 DOI: 10.1097/MCC.0000000000000389]
17 **Lu Q**, Xu DZ, Sharpe S, Doucet D, Pisarenko V, Lee M, Deitch EA. The anatomic sites of disruption of the mucus layer directly correlate with areas of trauma/hemorrhagic shock-induced gut injury. *J Trauma* 2011; **70**: 630-635 [PMID: 20664373 DOI: 10.1097/TA.0b013e3181e1221b]
18 **Rupani B**, Caputo FJ, Watkins AC, Vega D, Magnotti LJ, Lu Q, Xu DZ, Deitch EA. Relationship

between disruption of the unstirred mucus layer and intestinal restitution in loss of gut barrier function after trauma hemorrhagic shock. *Surgery* 2007; **141**: 481-489 [PMID: 17383525 DOI: 10.1016/j.surg.2006.10.008]

19 **Jandhyala SM**, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Nageshwar Reddy D. Role of the normal gut microbiota. *World J Gastroenterol* 2015; **21**: 8787-8803 [PMID: 26269668 DOI: 10.3748/wjg.v21.i29.8787]

20 **Ma LQ**, Chen DC, Liu S. Selective action of broad-spectrum antibiotics on intestinal flora in sepsis in rats. *Zhongguo Weizhongbing Jijiuyixue* 2007; **19**: 456-459 [DOI: 10.3760/j.issn:1003-0603.2007.08.003]

21 **Liu R**, Hong J, Xu X, Feng Q, Zhang D, Gu Y, Shi J, Zhao S, Liu W, Wang X, Xia H, Liu Z, Cui B, Liang P, Xi L, Jin J, Ying X, Zhao X, Li W, Jia H, Lan Z, Li F, Wang R, Sun Y, Yang M, Shen Y, Jie Z, Li J, Chen X, Zhong H, Xie H, Zhang Y, Gu W, Deng X, Shen B, Yang H, Xu G, Bi Y, Lai S, Wang J, Qi L, Madsen L, Ning G, Kristiansen K, Wang W. Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention. *Nat Med* 2017; **23**: 859-868 [PMID: 28628112 DOI: 10.1038/nm.4358]

22 **Peterson CT**, Sharma V, Elmén L, Peterson SN. Immune homeostasis, dysbiosis and therapeutic modulation of the gut microbiota. *Clin Exp Immunol* 2015; **179**: 363-377 [PMID: 25345825 DOI: 10.1111/cei.12474]

23 **Le Chatelier E**, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S, Leonard P, Li J, Burgdorf K, Grarup N, Jørgensen T, Brandslund I, Nielsen HB, Juncker AS, Bertalan M, Levenez F, Pons N, Rasmussen S, Sunagawa S, Tap J, Tims S, Zoetendal EG, Brunak S, Clément K, Doré J, Kleerebezem M, Kristiansen K, Renault P, Sicheritz-Ponten T, de Vos WM, Zucker JD, Raes J, Hansen T; MetaHIT consortium, Bork P, Wang J, Ehrlich SD, Pedersen O. Richness of human gut microbiome correlates with metabolic markers. *Nature* 2013; **500**: 541-546 [PMID: 23985870 DOI: 10.1038/nature12506]

24 **Yu E**, Malik VS, Hu FB. Cardiovascular Disease Prevention by Diet Modification. *J Am Coll Cardiol* 2018; **72**: 914-926 [PMID: 30115231 DOI: 10.1016/j.jacc.2018.02.085]

25 **Golucci APBS**, Marson F, Ribeiro AF, Nogueira RJN. Lipid profile associated with the systemic inflammatory response syndrome and sepsis in critically ill patients. *Nutrition* 2018; **55-56**: 7-14 [PMID: 29960160 DOI: 10.1016/j.nut.2018.04.007]

26 **Donohoe DR**, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, Bultman SJ. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab* 2011; **13**: 517-526 [PMID: 21531334 DOI: 10.1016/j.cmet.2011.02.018]

27 **Seo B**, Jeon K, Moon S, Lee K, Kim WK, Jeong H, Cha KH, Lim MY, Kang W, Kweon MN, Sung J, Kim W, Park JH, Ko G. Roseburia spp. Abundance Associates with Alcohol Consumption in Humans and Its Administration Ameliorates Alcoholic Fatty Liver in Mice. *Cell Host Microbe* 2020; **27**: 25-40. e6 [PMID: 31866426 DOI: 10.1016/j.chom.2019.11.001]

28 **Quan Y**, Song K, Zhang Y, Zhu C, Shen Z, Wu S, Luo W, Tan B, Yang Z, Wang X. Roseburia intestinalis-derived flagellin is a negative regulator of intestinal inflammation. *Biochem Biophys Res Commun* 2018; **501**: 791-799 [PMID: 29772233 DOI: 10.1016/j.bbrc.2018.05.075]

29 **Ananthakrishnan AN**, Luo C, Yajnik V, Khalili H, Garber JJ, Stevens BW, Cleland T, Xavier RJ. Gut Microbiome Function Predicts Response to Anti-integrin Biologic Therapy in Inflammatory Bowel Diseases. *Cell Host Microbe* 2017; **21**: 603-610. e3 [PMID: 28494241 DOI: 10.1016/j.chom.2017.04.010]

30 **Ruff WE**, Dehner C, Kim WJ, Pagovich O, Aguiar CL, Yu AT, Roth AS, Vieira SM, Kriegel C, Adeniyi O, Mulla MJ, Abrahams VM, Kwok WW, Nussinov R, Erkan D, Goodman AL, Kriegel MA. Pathogenic Autoreactive T and B Cells Cross-React with Mimotopes Expressed by a Common Human Gut Commensal to Trigger Autoimmunity. *Cell Host Microbe* 2019; **26**: 100-113. e8 [PMID: 31227334 DOI: 10.1016/j.chom.2019.05.003]

31 **Hayakawa M**, Asahara T, Henzan N, Murakami H, Yamamoto H, Mukai N, Minami Y, Sugano M, Kubota N, Uegaki S, Kamoshida H, Sawamura A, Nomoto K, Gando S. Dramatic changes of the gut flora immediately after severe and sudden insults. *Dig Dis Sci* 2011; **56**: 2361-2365 [PMID: 21384123 DOI: 10.1007/s10620-011-1649-3]

32 **Babrowski T**, Romanowski K, Fink D, Kim M, Gopalakrishnan V, Zaborina O, Alverdy JC. The intestinal environment of surgical injury transforms Pseudomonas aeruginosa into a discrete hypervirulent morphotype capable of causing lethal peritonitis. *Surgery* 2013; **153**: 36-43 [PMID: 22862900 DOI: 10.1016/j.surg.2012.06.022]

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ORIGINAL ARTICLE

Observational Study

Gut microbiota dysbiosis in Chinese children with type 1 diabetes mellitus: An observational study

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Abstract

BACKGROUND

Gut microbiota dysbiosis is reportedly actively involved in autoimmune diseases such as type 1 diabetes mellitus (T1DM). However, the alterations in the gut microbiota and their correlation with fasting blood glucose (FBG) in Chinese children with T1DM remain unclear.

AIM

To investigate alterations in the gut microbiota in Chinese children with T1DM

and their associations with clinical indicators.

METHODS
Samples from 51 children with T1DM and 47 age-matched and gender-matched healthy controls were obtained, to explore the structural and functional alterations in the fecal microbiota. The V3-V4 regions of the 16S rRNA gene were sequenced on a MiSeq instrument, and the association with FBG were analyzed.

RESULTS
We found that the bacterial diversity was significantly increased in the T1DM-associated fecal microbiota, and changes in the microbial composition were observed at different taxonomic levels. The T1DM-reduced differential taxa, such as *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, *Bacteroides xylanisolvens*, and *Flavonifractor plautii*, were negatively correlated with FBG, while the T1DM-enriched taxa, such as *Blautia*, *Eubacterium hallii* group, *Anaerostipes hadrus*, and *Dorea longicatena*, were positively correlated with FBG. *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, the *Eubacterium hallii* group, and *Anaerostipes hadrus*, either alone or in combination, could be used as noninvasive diagnostic biomarkers to discriminate children with T1DM from healthy controls. In addition, the functional changes in the T1DM-associated fecal microbiota also suggest that these fecal microbes were associated with altered functions and metabolic activities, such as glycan biosynthesis and metabolism and lipid metabolism, which might play vital roles in the pathogenesis and development of T1DM.

CONCLUSION
Our present comprehensive investigation of the T1DM-associated fecal microbiota provides novel insights into the pathogenesis of the disease and sheds light on the diagnosis and treatment of T1DM.

Key Words: Dysbiosis; Fasting blood glucose; Sequencing; Metabolism; Type 1 diabetes mellitus

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Core Tip: Alterations in the gut microbiota play vital roles in the development of autoimmune diseases such as type 1 diabetes mellitus (T1DM). Our present study explores the overall structure and composition of the fecal microbiota in Chinese children with T1DM and its association with fasting blood glucose (FBG). We found that the bacterial diversity increased significantly in children with T1DM and that several key functional taxa were correlated with FBG. These key functional bacteria could be used as noninvasive diagnostic biomarkers to discriminate T1DM patients from healthy controls. This comprehensive investigation of the T1DM-associated fecal microbiota provides novel insights into the pathogenesis of T1DM.

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INTRODUCTION

Type 1 diabetes mellitus (T1DM), a chronic autoimmune disease that usually begins in childhood, results from the destruction (or loss) of pancreatic β-cells, which leads to an inability to produce insulin and a need for the administration of exogenous insulin. T1DM ranks as the second most common autoimmune disease among children. The peak incidence of T1DM is clearly within the age group of 10–14 years and declines thereafter. One nationwide, population based study demonstrated that the incidence

guardian, provided informed written consent prior to study enrollment.

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Data sharing statement: No additional data are available.

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of T1DM in children is 1.93 (ranging from 0.83 to 3.03) per 100000 person-years in China, with an annual increase of approximately 6.5%[1]. T1DM can cause life-changing and life-threatening health complications in many organs and tissues rich in capillary vessels, such as the kidney, retina, and nerves, resulting in premature death. Children with T1DM often have to be treated for hypertension, dyslipidemia, microalbuminuria, and nephropathy, among other conditions[2]. Its early onset and chronicity make T1DM a disease of considerable importance. Currently, T1DM and its related comorbidities are considered major public health concerns.

Over the last few decades, considerable progress has been made in research on the pathogenesis and treatment of T1DM. However, its precise etiology and pathological mechanisms remain largely unclear. Both genetic susceptibility and environmental factors contribute to the development of T1DM[3]. The genetic susceptibility associated with T1DM is fairly well known, whereas the environmental factors remain poorly defined despite intensive research. Among the environmental risk factors associated with T1DM are industrial and economic advances (such as high levels of hygiene), changes in diet, and the emergence of more sedentary lifestyles[4], which can affect the environmental exposure of children. This altered environmental exposures can directly and indirectly influence the early-life gut microbiota.

Numerous clinical and experimental reports provide growing evidence of a close link between an altered gut microbiota (also defined as dysbiosis) and T1DM[5-12]. A previous study found that T1DM-related dysbiosis is associated with reduced integrity and increased permeability of the gut mucosa, which leads to bacterial penetration, and can stimulate the immune system to produce antibodies. Cross-reaction of these antibodies and surface antigens of pancreatic beta cells, as well as T cell cross-reactivity, results in the destruction of pancreatic β-cells and the development of T1DM[13]. Specifically, the Firmicutes/Bacteroidetes ratio is significantly reduced in Finnish children with T1DM[14]. Leiva-Gea *et al*[6] demonstrated that *Bacteroides* and *Veillonella* are markedly enriched in patients with TIDM, whereas *Faecalibacterium* and *Roseburia* are significantly reduced[6]. Livanos *et al*[11] showed that early-life antibiotic treatments alter the gut microbiota and its metabolic capacities, intestinal gene expression, and T-cell populations, thereby accelerating T1DM onset in non-obese diabetic mice[11]. Taken together, these findings show that early dysbiosis of the gut microbiota can be used as a promising biomarker for T1DM.

Most of the previous studies on the T1DM-associated microbiota were conducted in the United States and Europe. However, differences in lifestyle, dietary constitution, environmental exposures, and host genetic background between Chinese and Western populations may contribute to disparities in the baseline microbiota composition, which may influence the roles of specific bacteria in the etiopathology of T1DM. The present study aimed to explore the T1DM-associated gut microbiota in Chinese children by using the 16S rRNA gene high-throughput sequencing platform. We also evaluated the correlation between the T1DM-associated gut microbiota and fasting blood glucose (FBG). Our findings suggest novel targets for noninvasive early diagnosis and personalized treatment of T1DM.

MATERIALS AND METHODS

Participant selection

A total of 51 children with newly confirmed T1DM (aged 6-14 years) and 47 age-, gender-, and education-matched healthy controls were enrolled from Linyi People's Hospital (Linyi, China) and the First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China) from June 2019 to November 2019. The diagnosis of T1DM was based on the criteria of the American Diabetes Association: T1DM-associated autoimmunity (*i.e.*, formation of islet autoantibodies); the classic trio of symptoms associated with disease onset, *i.e.*, polydipsia, polyphagia, and polyuria along with hyperglycemia; an immediate need for exogenous insulin replacement and lifetime treatment. All the T1DM children were treated with only insulin. The FBG level of these participants was determined in the morning (Table 1). The following exclusion criteria were established: Body mass index (BMI) ≥ 30 kg/m^2; use of antibiotics, probiotics, prebiotics, or synbiotics in the previous month; known active infections such as bacterial, fungal, chlamydial, or viral infections; and other diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease or other autoimmune diseases. The protocols for the present study were approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. Informed written consent was obtained from the subjects' guardians before

Table 1 Fundamental information of subjects

Parameter	T1DM children (*n* = 51)	Healthy controls (*n* = 47)	*P* value
Age (yr)	10.38 ± 3.59	9.58 ± 4.35	0.284
Gender (male/female)	24/27	21/26	0.816
BMI (mean ± SD)	18.54 ± 4.21	18.98 ± 3.26	0.732
Age of onset (years, mean ± SD)	5.58 ± 3.45	-	-
FBG (mmol/L, mean ± SD)	11.87 ± 2.75	4.83 ± 0.41	0.000
Triglycerides (mmol/L, mean ± SD)	0.95 ± 0.38	0.88 ± 0.29	0.257
Antibiotics use, *n*	0	0	-
Insulin use, *n*	51	0	-
Other autoimmune diseases	0	0	-

T1DM: Type 1 diabetes mellitus; FBG: Fasting blood glucose; BMI: Body mass index; SD: Standard deviation.

enrollment.

Fecal sample collection and microbial DNA extraction

According to our previous studies, approximately 2 g of fresh fecal sample was collected in a sterile plastic cup, and stored at -80 °C within 15 min after preparation until use. Bacterial genomic DNA was extracted from 300 mg of homogenized feces using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with additional glass-bead beating steps on a Mini-Beadbeater (FastPrep; Thermo Electron Corporation, Boston, MA, United States). The amount of DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation); the integrity and size were checked by 1.0% agarose gel electrophoresis on a gel containing 0.5 mg/mL ethidium bromide. All DNA was stored at -20 °C before further analysis.

Amplicon library construction and sequencing

Amplicon libraries were constructed with the Illumina sequencing-compatible and barcode-indexed bacterial polymerase chain reaction primers 338F (5'-ACTCCTRCGG-GAGGCAGCAG-3') and 806R (5'-GGACTACCVGGGTATCTAAT-3'), which targeted the V3-V4 regions of 16S rRNA gene. All PCRs were performed with KAPA HiFi HotStart ReadyMix using the manufacturer's protocol (KAPA Biosystems, Wilmington, MA) and approximately 50 ng of extracted DNA was used per reaction. Thermocycling conditions were as follows: 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. All PCRs were performed in triplicate in a volume of 50 mL, and the samples were combined after PCR. The amplicon library was prepared using a TruSeq™ DNA Sample Preparation Kit (Illumina Inc, San Diego, CA, United States). Prior to sequencing, the PCR products were extracted with the MiniElute Gel Extraction Kit (Qiagen) and quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation) and Qubit 2.0 fluorometer (Invitrogen). The purified amplicons were then pooled in equimolar concentrations and the final concentration of the library was determined by Qubit (Invitrogen). Negative DNA extraction controls (lysis buffer and kit reagents only) were amplified and sequenced as contamination controls. Sequencing was performed on a MiSeq instrument (Illumina) using a 300 × 2 V3 kit together with PhiX Control V3 (Illumina)[15,16].

Bioinformatic analysis

The 16S rRNA gene sequence data set generated from the Illumina MiSeq platform was inputted to QIIME2 (version 2020.11), and all steps of sequence processing and quality control were performed in QIIME2 with default parameters[15,16]. Before the following data analysis, these reads of each sample were normalized to even sampling depths and annotated using the Greengenes reference database (version 13.8) with both the Ribosomal Database Project Classifier and UCLUST version 1.2.22 methods implemented in QIIME. Alpha diversity, including the observed species, abundance-based coverage estimator (ACE), Chao1 estimator, Shannon, Simpson, Evenness, and

PD whole tree indices, was calculated at a 97% similarity level. Beta diversity was measured by the unweighted UniFrac, weighted UniFrac, Jaccard, and Bray-Curtis distances calculated by QIIME2, which were visualized by principal coordinate analysis (PCoA). The differences in the composition of the fecal microbiota at different taxonomic levels were analyzed with Statistical Analysis of Metagenomic Profiles (STAMP) software package v2.1.3 and by the linear discriminant analysis effect size (LEfSe) method. PiCRUSt v1.0.0 was used to identify predicted gene families and associated pathways from inferred metagenomes of taxa of interest identified from the compositional analyses. The sparse compositional correlation (SparCC) algorithm was used for correlation analysis, and the results were visualized using Cytoscape v3.4.1.

Statistical analysis

For continuous variables, independent *t*-tests, White's nonparametric *t*-tests, and Mann-Whitney *U*-tests were applied. For categorical variables between groups, Pearson's chi-square test or Fisher's exact test was used, depending on assumption validity. For correlation analyses, Spearman's rank correlation test was used. Statistical analyses were performed using SPSS V19.0 (SPSS Inc., Chicago, IL, United States) and STAMP V2.1.3. GraphPad Prism version 6.0 (San Diego, CA, United States) was used to prepare graphs. All tests of significance were two sided, and $P < 0.05$ or corrected $P < 0.05$ was considered statistically significant.

Accession number

The sequence data from this study are deposited in the GenBank Sequence Read Archive with the accession number SRP287193.

RESULTS

Altered bacterial diversity in children with T1DM

No significant differences were noted in age, gender, race, BMI, or lipid levels between Chinese children with T1DM and healthy controls ($P > 0.05$). The FBG level was significantly higher in children with T1DM than in healthy controls (Figure 1; $P < 0.05$). A total of 3961145 high-quality reads (1857135 reads in healthy controls and 2104010 reads in children with T1DM) with an average of 40420 reads per sample were obtained for subsequent microbiota analysis. Deeper sequencing identified the majority of the bacterial phylotypes [640 operational taxonomic units (OTUs)] present in the fecal microbiota. The Good's estimator of coverage was 99.93%.

For bacterial diversity analyses, the Shannon and Simpson indices differed significantly between children with T1DM and healthy controls ($P < 0.05$; Figure 2A and B), with an increased diversity in the T1DM-associated fecal microbiota. However, richness indices, such as the ACE and Chao1 indices, showed no significant changes between the two groups ($P > 0.05$; Figure 2C and D). Owing to the significant interindividual variations, PCoA based on unweighted UniFrac, weighted UniFrac, and Bray–Curtis algorithms could not separate the two groups into different clusters (Figure 2E-G). Based on the Venn diagram in Figure 2H and Shannon rarefaction curves (Figure 2I), we observed a slightly higher number of OTUs in children with T1DM. Taken together, the results of our deeper sequencing analysis indicated increased fecal microbial diversity in children with T1DM.

Altered fecal microbiota composition in children with T1DM

The overall microbial compositions in children with T1DM and healthy controls were examined at different taxonomic levels from phylum to species. Using the RDP Classifier, sequences were annotated as follows: 11 phyla, 29 orders, 53 families, 184 genera, and 271 species. LEfSe identified many key bacterial phylotypes, mainly at the genus and species levels, that could potentially distinguish children with T1DM from healthy controls (Figure 3A and B). A representative cladogram demonstrated dysbiosis of the T1DM-associated fecal microbiota among children with T1DM.

Specifically, no one phylum was observed to differ significantly between children with T1DM and healthy controls. However, the dysbiotic indicator, the Firmicutes/Bacteroidetes (F/B) ratio, was markedly increased in children with T1DM (Supplementary Figure 1), which suggested that fecal microbial dysbiosis occurred in patients with T1DM. At the order level, we found that Erysipelotrichales, Enterobacteriales, and Coriobacteriales were enriched in children with T1DM, while Selenomonadales was markedly reduced ($P < 0.05$; Figure 3C). At the family level, Lachnospiraceae,

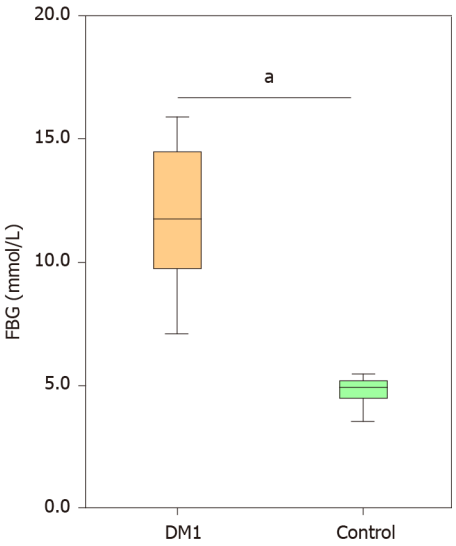


Figure 1 Comparison of the levels of fasting blood glucose between Chinese children with type 1 diabetes mellitus and healthy controls.
Data are presented as the mean ± SD. $^{a}P < 0.05$, compared with control group. FBG: Fasting blood glucose; DM1: Type 1 diabetes mellitus.

Erysipelotrichaceae, Enterobacteriaceae, and Coriobacteriaceae were significantly enriched in children with T1DM ($P < 0.05$; Figure 3C).

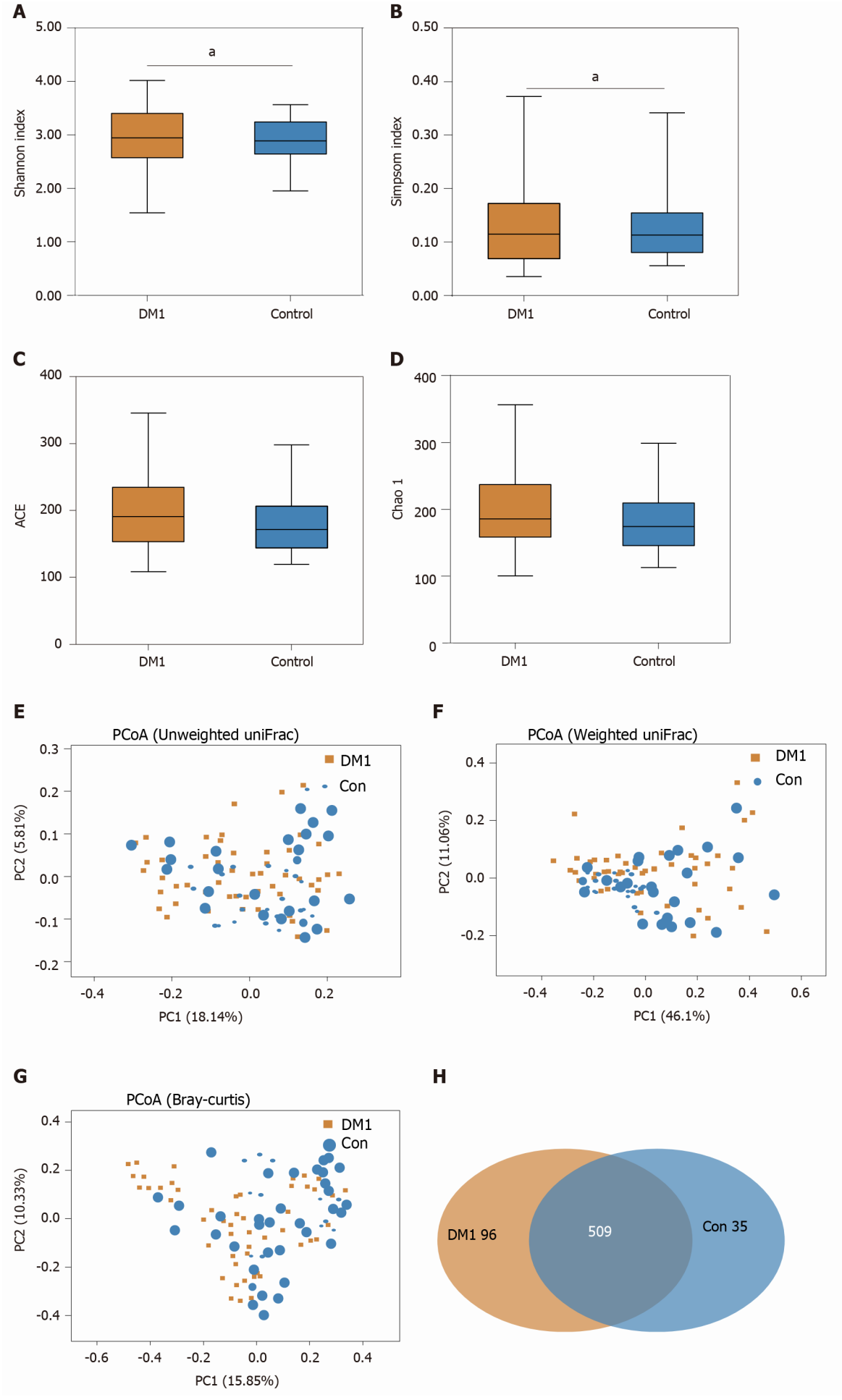
At the genus level, *Blautia*, *Anaerostipes*, unclassified Lachnospiraceae, the *Eubacterium hallii* group, unclassified Peptostreptococcaceae, *Dorea*, *Collinsella*, and *Klebsiella* were significantly enriched in children with T1DM, whereas *Parabacteroides*, *Flavonifractor*, and uncultured Ruminococcaceae were markedly reduced ($P < 0.05$; Figure 3C). At the species level, *Anaerostipes hadrus*, *Ruminococcus* sp._5_1_39BFAA, *Dorea longicatena*, and *Collinsella aerofaciens* were enriched in children with T1DM, while seven species, namely, *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, *Bacteroides xylanisolvens*, *Bacteroides dorei*, *Flavonifractor plautii*, *Parabacteroides merdae*, and *Parabacteroides distasonis* ATCC8503 were markedly reduced ($P < 0.05$; Figure 3C). Figure 4 shows a heatmap of bacterial genera in children with T1DM and healthy controls, presenting the relative percentages of most genera identified in each sample.

The overall structure of the T1DM-associated fecal microbiota was the result of dynamic interactions between community members. The SparCC algorithm with false discovery rate adjustment was employed to generate correlation-based microbial interaction networks based on the relative abundance of OTUs between the two groups (Figure 5). We found a more complex network of interactions in healthy controls than in children with T1DM. More positive and negative correlations among bacteria were found in the healthy controls. Based on our present findings, dysbiosis of the T1DM-associated fecal microbiota was observed in children with T1DM.

A fecal microbiota-based signature discriminates patients with T1DM from healthy controls

As mentioned above, several taxa were identified as key functional differentially abundant bacteria. These differentially abundant taxa, including *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, *Bacteroides xylanisolvens*, and *Flavonifractor plautii*, were negatively correlated with FBG, while *Blautia*, *Eubacterium hallii* group, *Anaerostipes hadrus*, and *Dorea longicatena* were positively correlated with FBG ($P < 0.05$; Figure 6). These correlation analyses indicated that key T1DM-associated functional bacteria actively participated in the regulation of glycemic levels in children.

We evaluated the potential value of the key functional differentially abundant taxa as biomarkers, including *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, the *Eubacterium hallii* group, and *Anaerostipes hadrus*. First, using only one of the differential bacteria as a predictor, we generated the receiver operating characteristic curves, with the area under the curve (AUC) ranging from 0.294 to 0.690 (Figure 7A). Multivariable stepwise logistic regression analysis was then performed to evaluate the list of T1DM-associated taxa, to distinguish T1DM patients from healthy controls (Figure 7B). We found that a combination of four taxa, including *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, the *Eubacterium hallii* group, and *Anaerostipes hadrus*, could significantly improve the predictive performance (AUC = 0.830). Based on our present findings, these key differentially abundant taxa could be used as potential


A
Shannon index
5.00
4.00
3.00
2.00
1.00
0.00
a
DM1
Control
B
Simpsom index
0.50
0.40
0.30
0.20
0.10
0.00
a
DM1
Control
C
ACE
400
300
200
100
0
DM1
Control
D
Chao 1
400
300
200
100
0
DM1
Control
E
PCoA (Unweighted uniFrac)
DM1
Con
PC2 (5.81%)
PC1 (18.14%)
F
PCoA (Weighted uniFrac)
DM1
Con
PC2 (11.06%)
PC1 (46.1%)
G
PCoA (Bray-curtis)
DM1
Con
PC2 (10.33%)
PC1 (15.85%)
H
DM1 96
509
Con 35

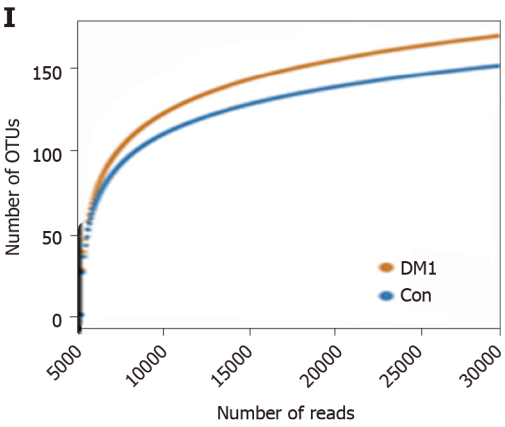


Figure 2 Altered bacterial diversity and richness of the fecal microbiota in Chinese children with type 1 diabetes mellitus. A-D: The diversity indices, such as Shannon (A), and Simpson (B), and the richness indices, such as the abundance-based coverage estimator (C), and Chao1 (D), were used to evaluate the overall structure of the fecal microbiota in patients with type 1 diabetes mellitus (T1DM) and healthy controls. Data are presented as the mean ± SD. Unpaired *t*-tests (two-tailed) were used to analyze variation between the two groups; E-G: Principal coordinate analysis plots of individual fecal microbiota based on unweighted (E) and weighted (F) UniFrac distance, and Bray–Curtis dissimilarity (G) in patients with T1DM and healthy controls. Each symbol represents a single sample; H: Venn diagram illustrating the overlap of operational taxonomic units (OTUs) in T1DM-associated fecal microbiota between the two groups; I: Rarefaction curves used to estimate the richness (at a 97% level of similarity) of T1DM-associated fecal microbiota between the two groups. The vertical axis shows the expected number of OTUs after sampling the number of tags or sequences shown on the horizontal axis. $^{a}P < 0.05$. OTUs: Operational taxonomic units; ACE: Abundance-based coverage estimator; PCoA: Principal coordinate analysis; Con: Control; DM1: Type 1 diabetes mellitus.

biomarkers for discriminating between T1DM patients and healthy controls.

T1DM-associated microbial functional alterations

To study the functional and metabolic changes in microbial communities between patients with T1DM and controls, we inferred the metagenomes from the 16S rRNA data and analyzed the functional potential of the fecal microbiota using PiCRUSt software, based on closed-reference OTU picking. We compared 64 Kyoto Encyclopedia of Genes and Genome (KEGG) pathways at level 2, and identified five KEGG categories with significantly differential abundances between children with T1DM and healthy controls. We found that glycan biosynthesis and metabolism and lipid metabolism were significantly reduced in children with T1DM ($P < 0.05$; Figure 8), which suggests that they might play crucial roles in the development of T1DM.

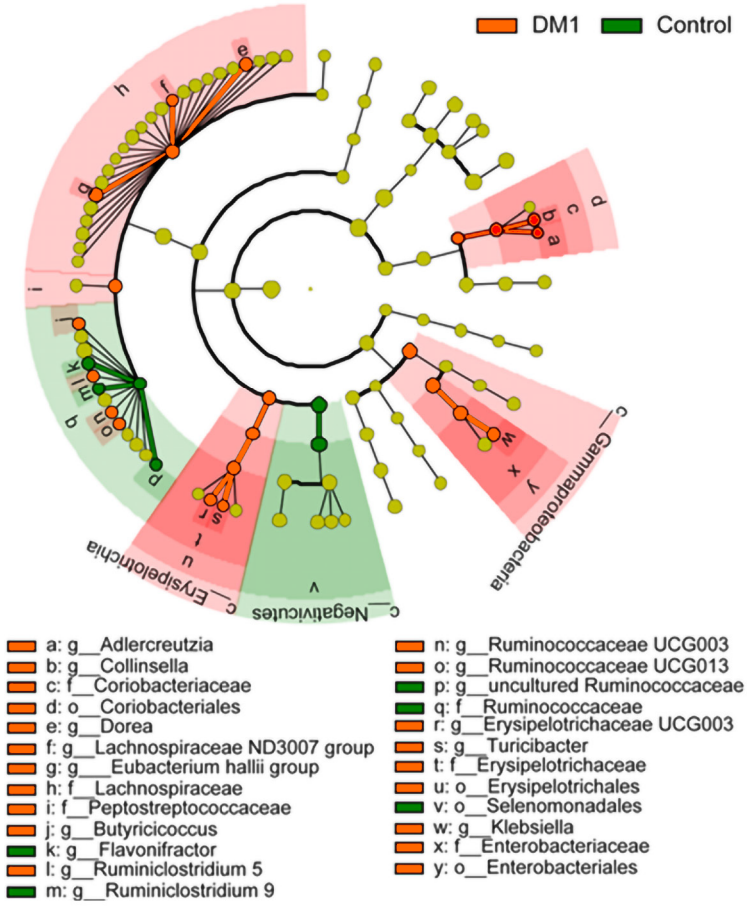
Specifically, nine pathways at level 3, including transcription factors, the phosphotransferase system, methane metabolism, and peptidoglycan biosynthesis, were significantly increased in the T1DM-associated fecal microbiota. Furthermore, 18 other pathways, including glycan degradation, glycosaminoglycan degradation, and insulin signaling pathway, were markedly reduced in the T1DM-associated microbiota. Together, these functional alterations in the fecal microbiota, especially that in glycan metabolism, were likely associated with the pathogenesis and development of T1DM.

DISCUSSION

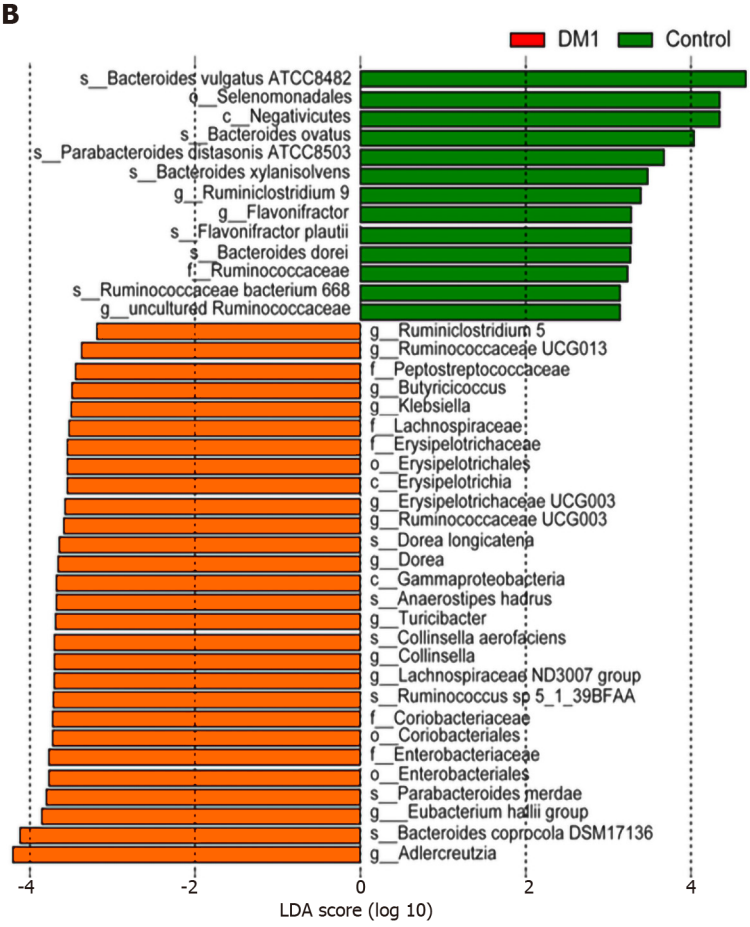
As a chronic autoimmune disease, T1DM is affected by genetic and non-genetic factors. With the advent of high-throughput sequencing technology, numerous studies have found that non-genetic factors, such as an optimal balance of the gut microbiota, play vital roles in regulating the host immune system and preventing the development of T1DM. A recent study demonstrated that a dysbiotic gut microbiota limits the effects of therapy in T1DM, while depletion of gut microbiota resistance enhances stem cell therapy in T1DM[17]. Our present T1DM-associated gut microbiota analysis excluded the influence of physiological factors such as the age, gender, and race of the enrolled participants. Most of the children with T1DM were newly diagnosed cases and were treated with only insulin. Without any additional treatment options, our present microbiota analysis could determine the actual correlations and roles of gut bacteria in the development of T1DM.

In the present study, the bacterial diversity of the T1DM-associated fecal microbiota was significantly increased[6,8,10,18], which is inconsistent with the findings of previous studies. Several case-control studies have reported that the bacterial diversity

A


DM1
Control
c__Negativicutes
c__Erysipelotrichia
c__Gammaproteobacteria
a: g__Adlercreutzia
b: g__Collinsella
c: f__Coriobacteriaceae
d: o__Coriobacteriales
e: g__Dorea
f: g__Lachnospiraceae ND3007 group
g: g__Eubacterium hallii group
h: f__Lachnospiraceae
i: f__Peptostreptococcaceae
j: g__Butyricicoccus
k: g__Flavonifractor
l: g__Ruminiclostridium 5
m: g__Ruminiclostridium 9
n: g__Ruminococcaceae UCG003
o: g__Ruminococcaceae UCG013
p: g__uncultured Ruminococcaceae
q: f__Ruminococcaceae
r: g__Erysipelotrichaceae UCG003
s: g__Turicibacter
t: f__Erysipelotrichaceae
u: o__Erysipelotrichales
v: o__Selenomonadales
w: g__Klebsiella
x: f__Enterobacteriaceae
y: o__Enterobacteriales

B


DM1
Control
s__Bacteroides vulgatus ATCC8482
o__Selenomonadales
c__Negativicutes
s__Bacteroides ovatus
s__Parabacteroides distasonis ATCC8503
s__Bacteroides xylanisolvens
g__Ruminiclostridium 9
g__Flavonifractor
s__Flavonifractor plautii
s__Bacteroides dorei
f__Ruminococcaceae
s__Ruminococcaceae bacterium 668
g__uncultured Ruminococcaceae
g__Ruminiclostridium 5
g__Ruminococcaceae UCG013
f__Peptostreptococcaceae
g__Butyricicoccus
g__Klebsiella
f__Lachnospiraceae
f__Erysipelotrichaceae
o__Erysipelotrichales
c__Erysipelotrichia
g__Erysipelotrichaceae UCG003
g__Ruminococcaceae UCG003
s__Dorea longicatena
g__Dorea
c__Gammaproteobacteria
s__Anaerostipes hadrus
g__Turicibacter
s__Collinsella aerofaciens
g__Collinsella
g__Lachnospiraceae ND3007 group
s__Ruminococcus sp 5_1_39BFAA
f__Coriobacteriaceae
o__Coriobacteriales
f__Enterobacteriaceae
o__Enterobacteriales
s__Parabacteroides merdae
g__Eubacterium hallii group
s__Bacteroides coprocola DSM17136
g__Adlercreutzia
-4
-2
0
2
4
LDA score (log 10)

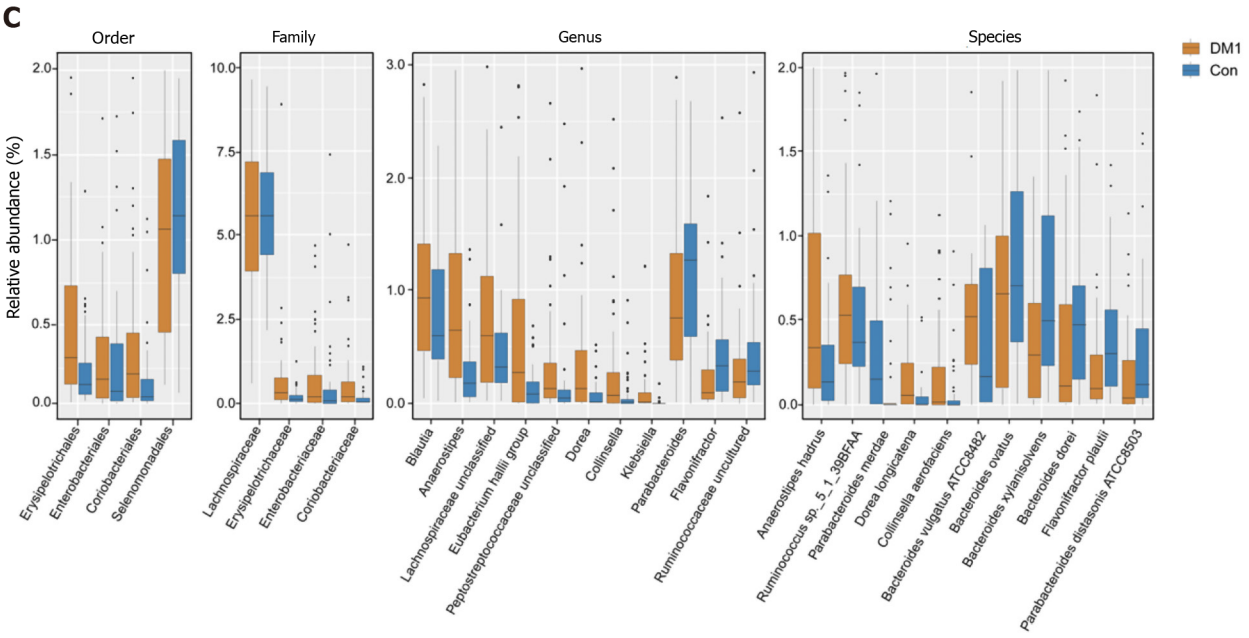


Figure 3 Differential bacterial taxa between Chinese children with type 1 diabetes mellitus and healthy controls. The linear discriminant analysis effect size identifies the taxa with the greatest differences in abundance between children with type 1 diabetes mellitus (T1DM) and healthy controls. A and B: Only the taxa meeting a significant linear discriminant analysis threshold value of > 2 are shown; C: Comparisons of the relative abundance of abundant bacterial taxa at the levels of the order, family, genus, and species. Data are presented as the mean ± SD. Mann–Whitney *U*-tests were used to analyze variation between children with T1DM and healthy controls. Con: Control; DM1: Type 1 diabetes mellitus.

of the gut microbiota is not significantly different in children with T1DM[8,10,18]. However, Leiva-Gea *et al*[6] found that T1DM is associated with a significantly lower microbiota diversity[6]. Furthermore, de Goffau *et al*[5] indicated that the age of children may influence bacterial diversity, and older children with T1DM tend to have a higher bacterial diversity[5]. In addition, differences in geographic location could influence the gut microbiota in early childhood, which might explain the disparity in bacterial diversity among different T1DM-associated microbiota studies. As significant inter-personal variations were observed, our PCoA could not separate the children with T1DM from healthy controls in these case-control studies. This indicates that β-diversity was similar between children with T1DM and healthy controls. In contrast to microbiota shifts in other childhood diseases, such as antibiotic-associated diarrhea, our present α- and β-diversity analyses showed increased bacterial diversity in children with T1DM, which could be used as a potential target dietary intervention in T1DM.

Inconsistent with previous findings regarding bacterial diversity, our LEfSe analysis showed that several taxa could be used as biomarkers to discriminate T1DM children from healthy controls. Consistent with previous human and animal studies, Bacteroidetes and Firmicutes were the two most predominant phyla, accounting for more than 93% of the gut microbiota in children. Most of the differentially abundant taxa at the genus and species levels belonged to these two phyla. However, the dominant phyla showed no significant differences between the two groups. The composition of the gut microbiota at the phylum level varied with age, and a greater relative abundance of Bacteroidetes was observed among older individuals. Despite the absence of differentially abundant phyla, the F/B ratio was significantly higher in children with T1DM. The F/B ratio is an indicator of gut dysbiosis, which is positively correlated with BMI[19]. Alterations in the F/B ratio may be important, as this ratio can influence efficiency in the processing of indigestible complex polysaccharides in the diet[20,21]. A previous study reported that the F/B ratio showed a significant decline in adults with type 2 diabetes mellitus (T2DM)[22]. Although the change patterns are not always consistent in patients with diabetes, an altered F/B ratio demonstrates a dysbiotic gut microbiota compared with healthy controls.

Specifically, we found that several genera and species of the phyla Firmicutes and Bacteroidetes were significantly altered in the T1DM-associated fecal microbiota. Interestingly, the co-network analysis indicated that interactions among altered bacterial species and abundant species play an important role in shaping the overall structure and composition of the T1DM-associated fecal microbiota. We found a more

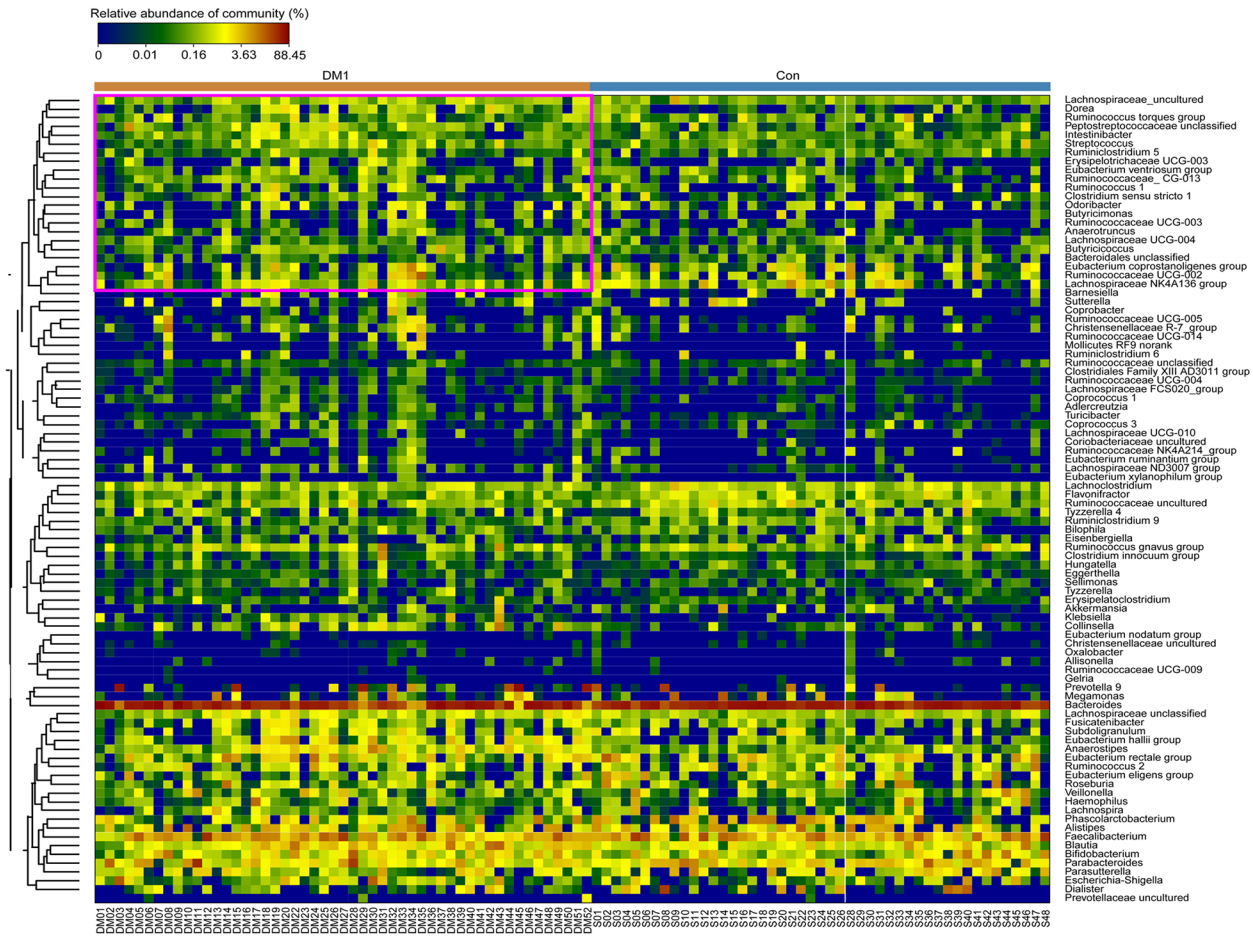


Figure 4 Heatmap of the type 1 diabetes mellitus-associated fecal microbiota at the genus level. The color of the spots in the panel represents the relative abundance (normalized and log10-transformed) of the genus in each sample. Relative abundance of the bacteria in each genus is indicated by a gradient of color from blue (low abundance) to red (high abundance). Genera were organized according to Spearman's correlation analysis, based on their relative abundances. Taxonomic classifications of the genus are shown on the right.

complex network of interactions in healthy controls than in children with T1DM, with more positive and negative correlations in healthy controls. These differentially abundant bacterial species played vital roles in regulating blood glucose in children. *Bacteroides vulgatus* ATCC8482, a highly abundant gram-negative obligate anaerobe, constitutes part of the core gut microbiota in healthy humans and is generally considered beneficial[23]. We found that the level of *Bacteroides vulgatus* ATCC8482 was significantly reduced in the T1DM-associated fecal microbiota and negatively correlated with FBG. Leiva-Gea *et al*[6] also found that the prevalence of *Bacteroides vulgatus* was significantly reduced in patients with T2DM, which can be considered a gut microbiota signature associated with the development of T2DM. Pedersen *et al*[24] identified *Bacteroides vulgatus* as the main species driving the association between the biosynthesis of branched-chain amino acids (BCAAs) and insulin resistance, suggesting that it may directly impact host metabolism[24]. Similar to our present findings, Yoshida *et al*[25] also revealed a significantly lower abundance of *Bacteroides vulgatus* in patients with coronary artery disease. Gavage with live *Bacteroides vulgatus* can attenuate atherosclerotic lesion formation in atherosclerosis-prone mice. Such action can thereby markedly ameliorate endotoxemia, directly reduce gut microbial lipopolysaccharide (LPS) production, and effectively suppress proinflammatory immune responses. These studies suggest that *Bacteroides vulgatus* plays a beneficial role in regulating blood glucose and preventing the development of T1DM.

Another dominant species of the genus *Bacteroides* in the human gut microbiota, *Bacteroides dorei*, shares similar 16S rRNA sequencing patterns with *Bacteroides vulgatus*. Leonard *et al*[26] found that cesarean section delivery is associated with a reduced abundance of the beneficial species, *Bacteroides vulgatus* and *Bacteroides dorei* [27]. Altered relative abundance of *Bacteroides dorei* can significantly influence the composition of the gut microbiota, and this species can be considered a keystone species[27]. A previous study showed that increased abundance of *Bacteroides dorei*

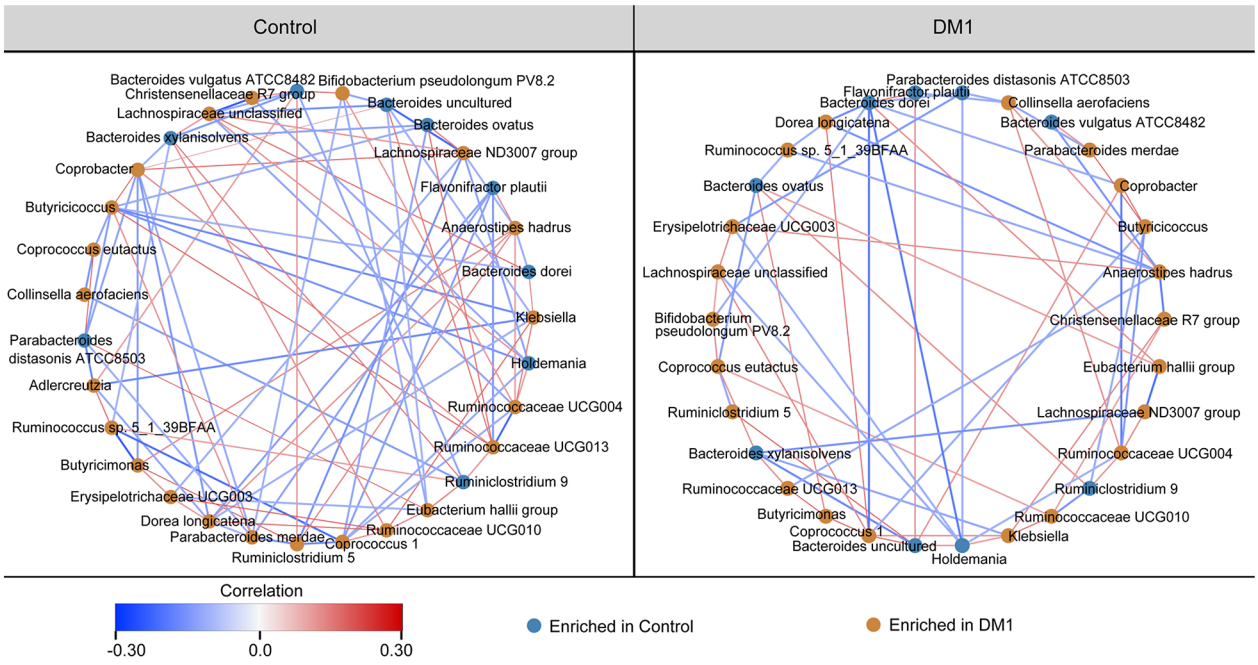


Figure 5 Correlation strengths of the abundant fecal microbiota in Chinese children with type 1 diabetes mellitus and healthy controls. Correlation network of the abundant fecal microbiota in healthy controls and children with type 1 diabetes mellitus (T1DM) is shown. Correlation coefficients were calculated with the sparse correlation for compositional data algorithm. The Cytoscape version 3.4.0 software was used for network construction. Red and blue lines represent positive and negative correlations, respectively. The correlation network became simpler in T1DM. DM1: Type 1 diabetes mellitus.

leads to reduced gut microbial production of LPS, which improves immune function through mechanisms such as major histocompatibility complex production and T cell activation[25]. Anonye *et al*[28] also found that *Clostridium difficile* growth is significantly reduced in the presence of *Bacteroides dorei*[28]. However, a recent study on gestational diabetes identified *Bacteroides dorei* as a putative biomarker of impaired carbohydrate tolerance, and suggested that it played a role in the regulation of glucose tolerance in pregnant women[29]. However, our present correlation analysis found no significant association between *Bacteroides dorei* and FBG. Inconsistent with our present alteration patterns, previous studies have found that higher levels of *Bacteroides dorei* may contribute to the onset of T1DM, and may be potential monitoring and therapeutic microbial markers for T1DM[30]. *Bacteroides ovatus* is a common member of the human gut microbiota, with a broad capability to degrade complex glycans[31]. It makes considerable contributions to the overall differences between T1DM cases and controls. Consistent with our results, Giongo *et al*[14] found that *Bacteroides ovatus* accounted for nearly 24% of the total increase in the phylum Bacteroidetes among children with T1DM[14] and for the first time established a causal relationship between *Bacteroides ovatus* and metabolic homeostasis. Their findings demonstrated that *Bacteroides ovatus* may be a potentially beneficial intestinal bacterial species. Similar to *Bacteroides vulgatus*, *Bacteroides ovatus* can also regulate BCAA metabolism, and alleviate metabolic syndrome. A recent study performed by Yang *et al*[32] demonstrated that specific strains of *Bacteroides ovatus* are capable of inducing high levels of mucosal immunoglobulin A (IgA) production in the large intestine, which can be used to modulate the host immune response[32]. In addition, as one of the active immunomodulators, oral gavage of *Bacteroides ovatus* could significantly increase the efficacy of erlotinib and induce the expression of CXCL9 and interferon-gamma in a murine lung cancer model, which was positively correlated with treatment outcomes[33]. Another *Bacteroides* species with reduced levels in T1DM, *Bacteroides xylanisolvens*, is a xylan-degrading bacterium isolated from human feces. Following a safety evaluation of a *Bacteroides xylanisolvens* strain (DSM 23964), a previous study reported its potential probiotic properties[34]. Consistent with a previous study on patients with atherosclerosis[35], *Bacteroides xylanisolvens* is reportedly an important contributor to folate transformations II and glycolysis III, and it is significantly more abundant in healthy controls than in patients with T1DM. Qiao *et al*[36] also found that *Bacteroides xylanisolvens* can alleviate nonalcoholic hepatic steatosis and provided evidence of the benefits of the gut *Bacteroides*-folate-liver pathway[36]. *Bacteroides xylanisolvens* is considered a probiotic bacterium that is positively correlated with anti-

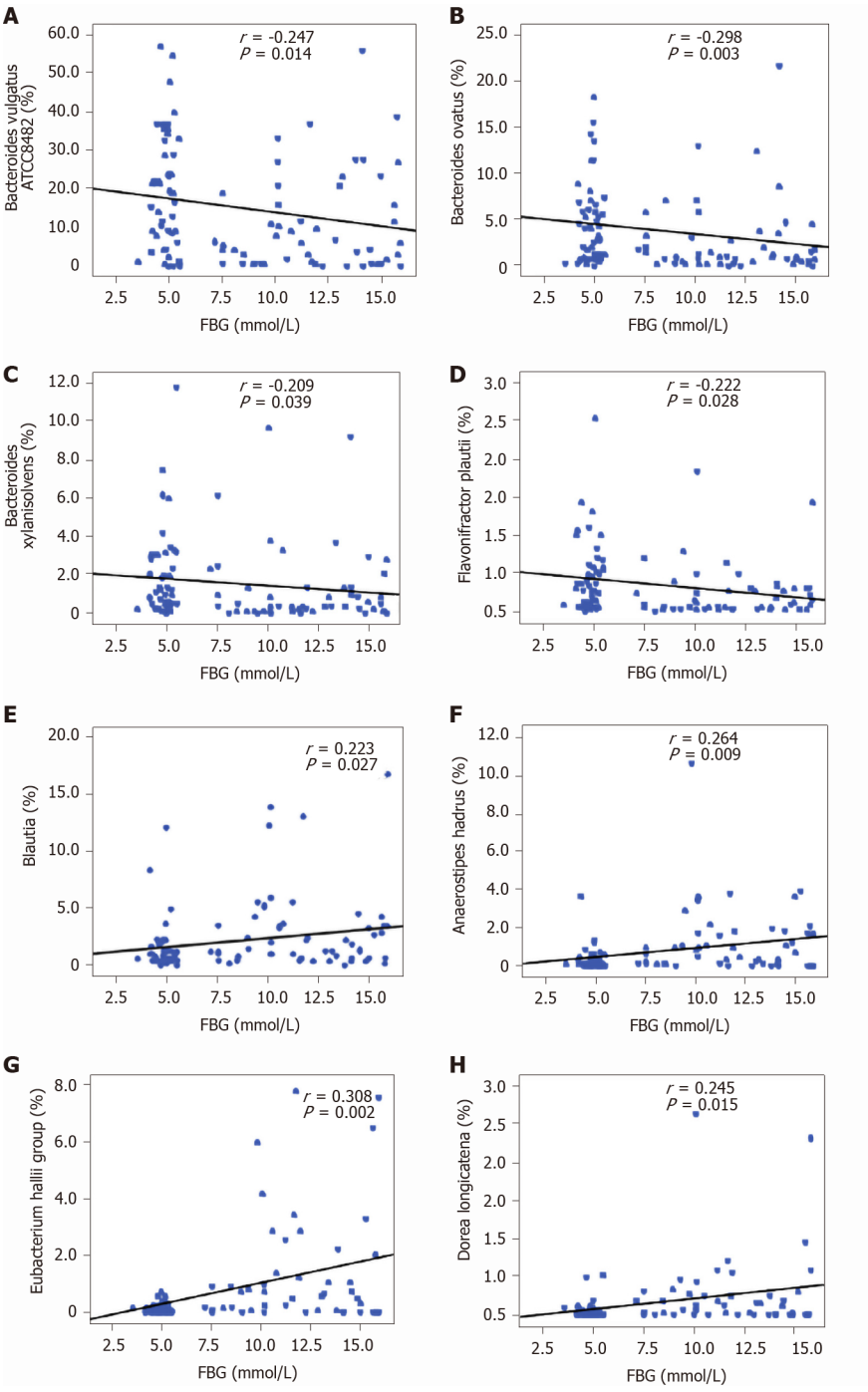


Figure 6 Correlation between key bacteria in type 1 diabetes mellitus-associated fecal microbiota and fasting blood glucose. The different taxa, including *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, *Bacteroides xylanisolvens*, and *Flavonifractor plautii*, were negatively correlated with fasting blood glucose (FBG), while *Blautia*, the *Eubacterium hallii* group, *Anaerostipes hadrus*, and *Dorea longicatena* were positively correlated with FBG. Spearman's rank correlation and probability were used to evaluate statistical significance. A: *Bacteroides vulgatus* ATCC8482; B: *Bacteroides ovatus*; C: *Bacteroides xylanisolvens*; D: *Flavonifractor plautii*; E: *Blautia*; F: *Anaerostipes hadrus*; G: *Eubacterium hallii*; H: *Dorea longicatena*. FBG: Fasting blood glucose.

inflammatory/tumor markers and negatively correlated with proinflammatory/tumor markers[37]. Sufficient evidence has supported and facilitated authorization of the use of heat-inactivated *Bacteroides xylanisolvens* in the European Union[38]. *Flavonifractor plautii*, a Gram-positive anaerobic bacterium, is a member of *Clostridium* cluster IV in the Ruminococcaceae family, and has been isolated worldwide from human feces. Our data showed a lower level of *Flavonifractor plautii* in children with T1DM than in healthy controls. This finding suggests that *Flavonifractor plautii* plays a beneficial role in regulating the metabolism of blood glucose. Similar to our present findings, Borgo *et al*[39] found that *Flavonifractor plautii* is negatively correlated with BMI[39]. Kasai *et al*[40] found that the fraction of *Flavonifractor plautii* is significantly lower in feces from obese subjects than in feces from non-obese[40]. Recently, Mikami *et al*[41] suggested

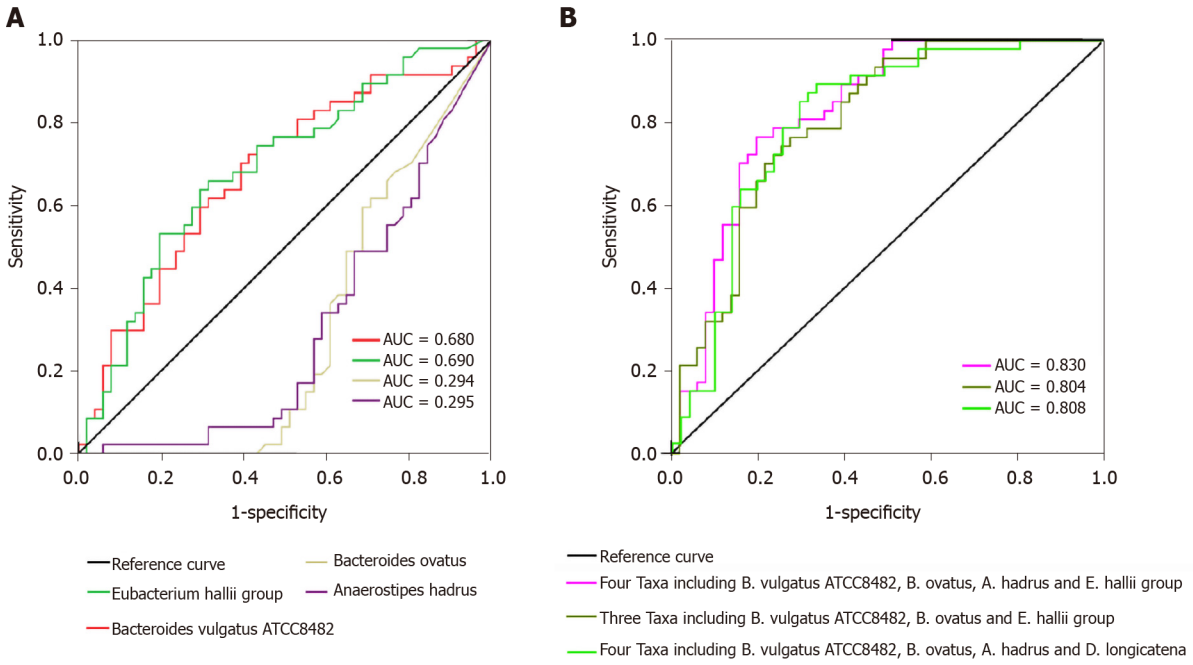


Figure 7 Receiver operating characteristic curves for different taxa, including *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, *Anaerostipes hadrus*, and the *Eubacterium hallii* group, which were used either alone or in combination to discriminate between patients with type 1 diabetes mellitus and healthy controls. A: Alone; B: Combination. AUC: Area under the curve.

that oral administration of *Flavonifractor plautii* prevents the accumulation of tumor necrosis factor-α-encoding transcripts in the adipose tissue of obese mice, thereby suppressing adipose tissue-associated chronic inflammation[41]. In addition, their group also found that *Flavonifractor plautii* alleviates antigen-induced Th2 immune responses, and can be used as a potential anti-allergic probiotic[42]. *Flavonifractor plautii* abundance in fecal samples has now been proposed as a biomarker of health status[39]. *Parabacteroides distasonis*, a core member of the gut microbiota in humans, is also reportedly a beneficial commensal gut microorganism in different pathophysiological models due to its anti-inflammatory and barrier restorative abilities. The abundance of *Parabacteroides distasonis* is relatively low in patients affected by obesity, nonalcoholic fatty liver disease (NAFLD), and multiple sclerosis[43-45]. A recent study indicated that *Parabacteroides distasonis* modulates host metabolism and alleviates obesity and metabolic dysfunctions *via* the production of succinate and secondary bile acids[46]. Colonization of antibiotic-treated or germ-free mice with a single *Parabacteroides distasonis* strain induced Treg differentiation[43]. The abundance of another *Parabacteroides* species, *Parabacteroides merdae*, was also reduced in children with T1DM, indicating its beneficial role during T1DM development. Wang *et al*[47] recently reported that enrichment of *Parabacteroides merdae* is positively correlated with longevity[47]. These bacteria exhibit promising potential beneficial effects on human health in a strain-dependent manner. Thus, several strains could contribute to the development of chronic diseases.

The proportional abundances of four species, namely, *Anaerostipes hadrus*, *Ruminococcus* sp. 5_1_39BFAA, *Dorea longicatena*, and *Collinsella aerofaciens*, were significantly increased in children with T1DM. *Anaerostipes hadrus*, one of the core species isolated from human feces, is able to produce large amounts of butyrate from both L-sorbose and xylitol and can consume acetate[48]. Zhang *et al*[49] found that *Anaerostipes hadrus* can significantly aggravate colitis in dextran sulfate sodium-treated mice, but exerts no detrimental effects in healthy mice[49]. Recently, Zeevi *et al*[50] observed that several genomic structural variants of *Anaerostipes hadrus* are negatively correlated with weight, waist circumference, median blood glucose levels, and BMI and positively correlated with HDL cholesterol levels[50]. The functions of *Anaerostipes hadrus* structural variants are not consistent with those of the wild type, which is strongly associated with lower metabolic risk. *Ruminococcus* sp. 5_1_39BFAA, a member of the genus *Blautia*, is positively associated with FBG. A previous study reported that *Ruminococcus* sp. 5_1_39BFAA is enriched among elderly patients with hypertension and reduced exercise capacity[51]. To date, no studies have extensively investigated the roles and mechanisms of *Ruminococcus* sp. 5_1_39BFAA. *Blautia* is associated with

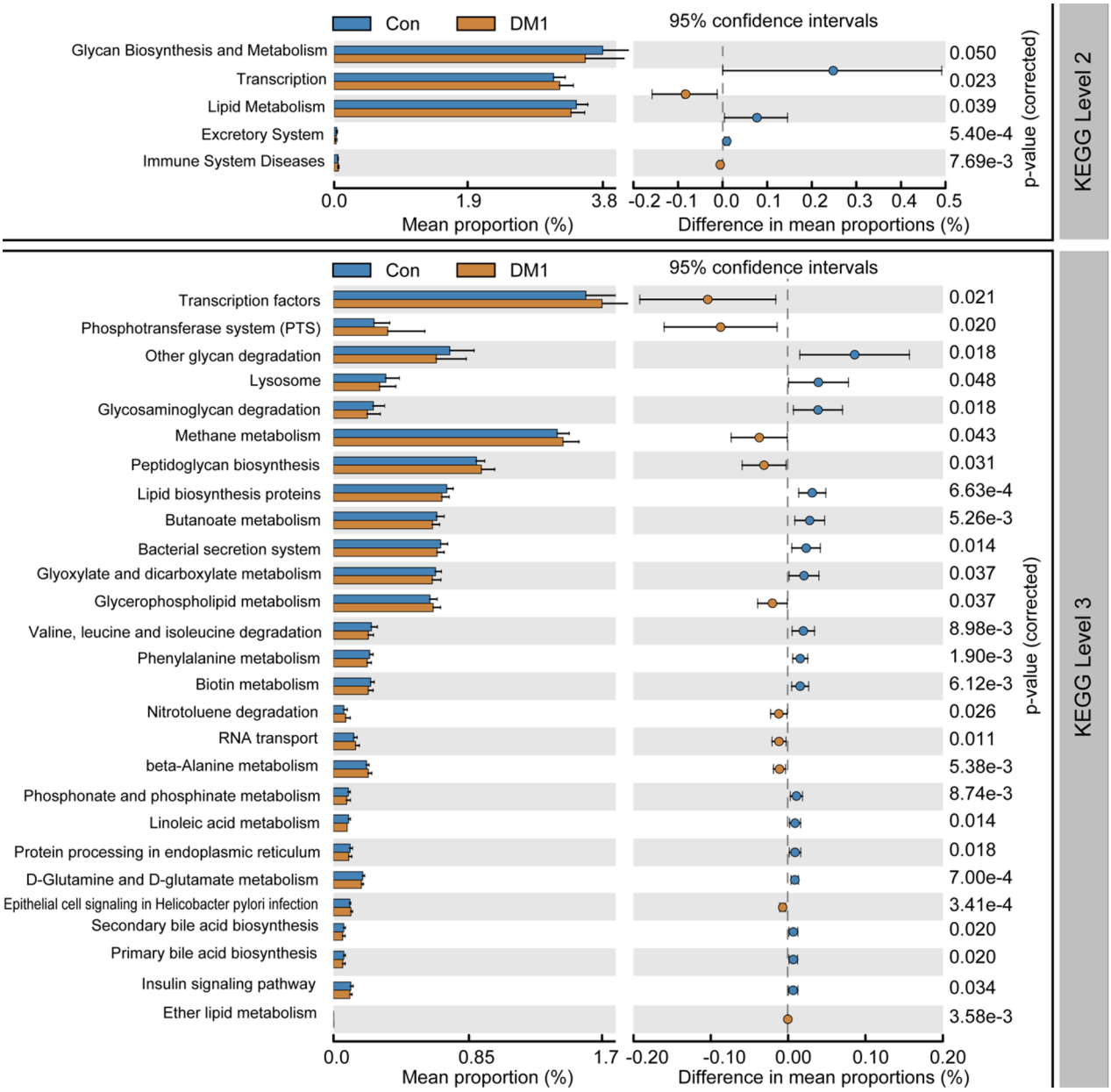


Figure 8 PiCRUSt-based fecal microbiome study among Chinese children with type 1 diabetes mellitus and healthy controls. Different bacterial functions were evaluated based on the two-sided Welch's *t*-test. Comparisons between the two groups for each Kyoto Encyclopedia of Genes and Genome functional category (level 2 and level 3) are shown by percentage. The Benjamini–Hochberg method was used for multiple testing correction, based on the false discovery rate using the Statistical Analysis of Metagenomic Profiles software. KEGG: Kyoto Encyclopedia of Genes and Genome; Con: Control; DM1: Type 1 diabetes mellitus.

blood glucose regulation, lipid metabolism, and regulation of T cell differentiation [6,52]. However, increased proportions of *Blautia* have been reported in various diseases, such as IBS, NAFLD, and Crohn's disease. A previous case-control study also found that the abundance of *Blautia* is increased in children with T1DM, and is positively correlated with HbA1c, the number of T1DM autoantibodies, and the titers of tyrosine phosphatase autoantibodies[52]. Consistent with the findings of Kostic *et al*[53], the present study demonstrated that *Blautia* is positively correlated with the levels of FBG in children with T1DM. *Dorea longicatena*, a new member of *Clostridium* cluster XIVa in the Lachnospiraceae family, can produce acetate as a fermentation product. Mortaş *et al*[54] found that *Dorea longicatena* is significantly more abundant in individuals working the night shift[54]. Yang *et al*[55] found that *Dorea* is a biomarker of the risk for colorectal cancer[55]. Higher abundance of the *Dorea* genus is associated with increased intestinal permeability. In contrast with our present findings, Brahe *et al*[56] reported that *Dorea longicatena* is negatively correlated with markers for insulin resistance, such as glucose and insulin, in obese female participants [56]. Nevertheless, the increased abundance of *Dorea longicatena* in T1DM could be actively

involved in regulating host metabolism. *Collinsella aerofaciens*, one of the most abundant Actinobacteria in the gastrointestinal tract of healthy humans, shows increased abundance in the feces of patients with T1DM; one of its subspecies is capable of butyrate production[57]. Increased *Collinsella* abundance has been associated with both positive and negative health conditions, but there is no consensus on its health effects. Cohort studies have identified increases in *Collinsella* abundance in the fecal microbiota of patients with T2DM, atherosclerosis, and IBS[58-60]. Turnbaugh *et al*[61] reported that the enrichment of *Collinsella aerofaciens* is linked to BMI, with an increased prominence of *Collinsella aerofaciens* in obese individuals than in lean twins and their mothers[61]. Another abundant bacterium in T1DM, the *Eubacterium hallii* group (an anaerobic, Gram-positive, catalase-negative bacterium of the Lachnospiraceae family), is a metabolically versatile species that can contribute to intestinal butyrate and propionate formation[62,63]. We observed a positive correlation between the *Eubacterium hallii* group and FBG. Consistent with the present data, Ye *et al*[64] found that the abundance of the *Eubacterium hallii* group was significantly higher in patients with gestational diabetes mellitus (GDM) who failed to make lifestyle modifications for glycemic control, which is also positively correlated with FBG[64]. They also showed that the *Eubacterium hallii* group can be used to distinguish GDM patients and patients who failed glycemic control from healthy controls. As mentioned by Schwab *et al*[63], the *Eubacterium hallii* group may actively contribute to metabolic interactions[63]. However, Udayappan *et al*[65] observed that oral treatment with the *Eubacterium hallii* group can improve insulin sensitivity in db/db mice[65], which is inconsistent with the present findings. Our ROC analysis found that the bacteria mentioned above, such as *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, the *Eubacterium hallii* group, and *Anaerostipes hadrus*, can be used as potential biomarkers to discriminate children with T1DM from healthy controls. These bacteria in patients with T1DM might contribute to alterations in microbial functions and actively participate in the development of T1DM. Therefore, they could be used as novel targets for non-invasive diagnostic biomarkers and personalized treatment of T1DM in the future.

There are several limitations of the present study. First, our case-control study only explored the characteristics of the T1DM-associated fecal microbiota, whereas alterations in the fecal microbiota after successful treatment were not investigated. Second, the fecal microbial signature and corresponding metabolites, as well as the diagnostic model associated with T1DM, still require further clinical studies with a larger sample size to validate the results. Third, the relatively weak correlations between key differential functional bacteria and FBG could not indicate an obvious primary or secondary relationship. More clinical indicators should be added into these correlation analyses in future studies. Fourth, culturomics should be used to identify T1DM-associated bacteria. Further animal experiments could help to determine the cause-effect relationship between these bacteria and the pathogenesis of T1DM.

CONCLUSION

In summary, the present study investigated the altered profiles of fecal microbiota in children with T1DM. High-throughput sequencing identified the detailed composition and diversity of the T1DM-associated fecal microbiota at a much deeper level. We found that bacterial diversity was significantly increased in the T1DM-associated fecal microbiota. We also observed microbial compositional changes at different taxonomic levels. The proportions of *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, *Bacteroides xylanisolvens*, *Flavonifractor plautii*, *Anaerostipes hadrus*, and *Dorea longicatena* at the species level and *Blautia* and the *Eubacterium hallii* group at the genus level showed significant differences between children with T1DM and healthy controls, and were markedly correlated with FBG. Furthermore, *Bacteroides vulgatus* ATCC8482 and *Bacteroides ovatus*, either alone or in combination, can be used as non-invasive diagnostic biomarkers to distinguish between patients with T1DM and healthy controls. In addition, functional changes in the T1DM-associated fecal microbiota suggest that the alterations are associated with the functions and metabolic activities of the microbiota, which might play vital roles in the pathogenesis and development of T1DM. Thus, our comprehensive investigation of the T1DM-associated fecal microbiota provides novel insights into the pathogenesis of T1DM.

ARTICLE HIGHLIGHTS

Research background

Gut microbiota dysbiosis is reportedly actively involved in autoimmune diseases such as type 1 diabetes mellitus (T1DM). However, the alterations in the gut microbiota and their correlation with fasting blood glucose (FBG) in Chinese children with T1DM remain unclear.

Research motivation

Most of the previous studies on the T1DM-associated microbiota were conducted in the United States and Europe. However, differences in lifestyle, dietary constitution, environmental exposure, and host genetic background between Chinese and Western populations may contribute to disparities in the baseline microbiota composition, which may influence the roles of specific bacteria in the etiopathology of T1DM.

Research objectives

Our present study aimed to investigate alterations in the gut microbiota in Chinese children with T1DM and their associations with clinical indicators.

Research methods

Samples from 51 children with T1DM and 47 age- and gender-matched healthy controls were obtained to explore the structural and functional alterations in the fecal microbiota. The V3-V4 regions of the 16S rRNA gene were sequenced on a MiSeq instrument and the association with FBG was analyzed.

Research results

We found that the bacterial diversity was significantly increased in the T1DM-associated fecal microbiota, and changes in the microbial composition were observed at different taxonomic levels. The T1DM-reduced differentially abundant taxa, such as *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, *Bacteroides xylanisolvens*, and *Flavonifractor plautii*, were negatively correlated with FBG, while the T1DM-enriched taxa, such as *Blautia*, *Eubacterium hallii* group, *Anaerostipes hadrus*, and *Dorea longicatena*, were positively correlated with FBG. *Bacteroides vulgatus* ATCC8482, *Bacteroides ovatus*, the *Eubacterium hallii* group, and *Anaerostipes hadrus*, either alone or in combination, could be used as non-invasive diagnostic biomarkers to discriminate children with T1DM from healthy controls. In addition, the functional changes in the T1DM-associated fecal microbiota also suggest that these fecal microbes were associated with altered functions and metabolic activities, such as glycan biosynthesis and metabolism and lipid metabolism, which might play vital roles in the pathogenesis and development of T1DM.

Research conclusions

Our present comprehensive investigation of the T1DM-associated fecal microbiota provides novel insights into the pathogenesis of the disease and sheds light on the diagnosis and treatment of T1DM.

Research perspectives

Further causal-development studies based on the present results will expand the knowledge on the pathogenesis of T1DM, which will help to provide microbiota-targeted T1DM diagnosis and treatment.

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REFERENCES

1 **Weng J**, Zhou Z, Guo L, Zhu D, Ji L, Luo X, Mu Y, Jia W; T1D China Study Group. Incidence of type 1 diabetes in China, 2010-13: population based study. *BMJ* 2018; **360**: j5295 [PMID: 29298776 DOI: 10.1136/bmj.j5295]

2 **Rak K**, Bronkowska M. Immunomodulatory Effect of Vitamin D and Its Potential Role in the

Prevention and Treatment of Type 1 Diabetes Mellitus-A Narrative Review. *Molecules* 2018; **24** [PMID: 30586887 DOI: 10.3390/molecules24010053]

3 **Ilonen J**, Lempainen J, Veijola R. The heterogeneous pathogenesis of type 1 diabetes mellitus. *Nat Rev Endocrinol* 2019; **15**: 635-650 [PMID: 31534209 DOI: 10.1038/s41574-019-0254-y]

4 **Gowd V**, Xie L, Zheng X, Chen W. Dietary fibers as emerging nutritional factors against diabetes: focus on the involvement of gut microbiota. *Crit Rev Biotechnol* 2019; **39**: 524-540 [PMID: 30810398 DOI: 10.1080/07388551.2019.1576025]

5 **de Goffau MC**, Fuentes S, van den Bogert B, Honkanen H, de Vos WM, Welling GW, Hyöty H, Harmsen HJ. Aberrant gut microbiota composition at the onset of type 1 diabetes in young children. *Diabetologia* 2014; **57**: 1569-1577 [PMID: 24930037 DOI: 10.1007/s00125-014-3274-0]

6 **Leiva-Gea I**, Sánchez-Alcoholado L, Martín-Tejedor B, Castellano-Castillo D, Moreno-Indias I, Urda-Cardona A, Tinahones FJ, Fernández-García JC, Queipo-Ortuño MI. Gut Microbiota Differs in Composition and Functionality Between Children With Type 1 Diabetes and MODY2 and Healthy Control Subjects: A Case-Control Study. *Diabetes Care* 2018; **41**: 2385-2395 [PMID: 30224347 DOI: 10.2337/dc18-0253]

7 **Demirci M**, Bahar Tokman H, Taner Z, Keskin FE, Çağatay P, Ozturk Bakar Y, Özyazar M, Kiraz N, Kocazeybek BS. Bacteroidetes and Firmicutes levels in gut microbiota and effects of hosts TLR2/TLR4 gene expression levels in adult type 1 diabetes patients in Istanbul, Turkey. *J Diabetes Complications* 2020; **34**: 107449 [PMID: 31677982 DOI: 10.1016/j.jdiacomp.2019.107449]

8 **Murri M**, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, Queipo-Ortuño MI. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. *BMC Med* 2013; **11**: 46 [PMID: 23433344 DOI: 10.1186/1741-7015-11-46]

9 **Wen L**, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, Hu C, Wong FS, Szot GL, Bluestone JA, Gordon JI, Chervonsky AV. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 2008; **455**: 1109-1113 [PMID: 18806780 DOI: 10.1038/nature07336]

10 **Huang Y**, Li SC, Hu J, Ruan HB, Guo HM, Zhang HH, Wang X, Pei YF, Pan Y, Fang C. Gut microbiota profiling in Han Chinese with type 1 diabetes. *Diabetes Res Clin Pract* 2018; **141**: 256-263 [PMID: 29733871 DOI: 10.1016/j.diabres.2018.04.032]

11 **Livanos AE**, Greiner TU, Vangay P, Pathmasiri W, Stewart D, McRitchie S, Li H, Chung J, Sohn J, Kim S, Gao Z, Barber C, Kim J, Ng S, Rogers AB, Sumner S, Zhang XS, Cadwell K, Knights D, Alekseyenko A, Bäckhed F, Blaser MJ. Antibiotic-mediated gut microbiome perturbation accelerates development of type 1 diabetes in mice. *Nat Microbiol* 2016; **1**: 16140 [PMID: 27782139 DOI: 10.1038/nmicrobiol.2016.140]

12 **Vatanen T**, Franzosa EA, Schwager R, Tripathi S, Arthur TD, Vehik K, Lernmark Å, Hagopian WA, Rewers MJ, She JX, Toppari J, Ziegler AG, Akolkar B, Krischer JP, Stewart CJ, Ajami NJ, Petrosino JF, Gevers D, Lähdesmäki H, Vlamakis H, Huttenhower C, Xavier RJ. The human gut microbiome in early-onset type 1 diabetes from the TEDDY study. *Nature* 2018; **562**: 589-594 [PMID: 30356183 DOI: 10.1038/s41586-018-0620-2]

13 **Cole DK**, Bulek AM, Dolton G, Schauenberg AJ, Szomolay B, Rittase W, Trimby A, Jothikumar P, Fuller A, Skowera A, Rossjohn J, Zhu C, Miles JJ, Peakman M, Wooldridge L, Rizkallah PJ, Sewell AK. Hotspot autoimmune T cell receptor binding underlies pathogen and insulin peptide cross-reactivity. *J Clin Invest* 2016; **126**: 2191-2204 [PMID: 27183389 DOI: 10.1172/JCI85679]

14 **Giongo A**, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Drew JC, Ilonen J, Knip M, Hyöty H, Veijola R, Simell T, Simell O, Neu J, Wasserfall CH, Schatz D, Atkinson MA, Triplett EW. Toward defining the autoimmune microbiome for type 1 diabetes. *ISME J* 2011; **5**: 82-91 [PMID: 20613793 DOI: 10.1038/ismej.2010.92]

15 **Ling Z**, Shao L, Liu X, Cheng Y, Yan C, Mei Y, Ji F. Regulatory T Cells and Plasmacytoid Dendritic Cells Within the Tumor Microenvironment in Gastric Cancer Are Correlated With Gastric Microbiota Dysbiosis: A Preliminary Study. *Front Immunol* 2019; **10**: 533 [PMID: 30936882 DOI: 10.3389/fimmu.2019.00533]

16 **Liu X**, Shao L, Liu X, Ji F, Mei Y, Cheng Y, Liu F, Yan C, Li L, Ling Z. Alterations of gastric mucosal microbiota across different stomach microhabitats in a cohort of 276 patients with gastric cancer. *EBioMedicine* 2019; **40**: 336-348 [PMID: 30584008 DOI: 10.1016/j.ebiom.2018.12.034]

17 **Lv W**, Graves DT, He L, Shi Y, Deng X, Zhao Y, Dong X, Ren Y, Liu X, Xiao E, Zhang Y. Depletion of the diabetic gut microbiota resistance enhances stem cells therapy in type 1 diabetes mellitus. *Theranostics* 2020; **10**: 6500-6516 [PMID: 32483466 DOI: 10.7150/thno.44113]

18 **Mejía-León ME**, Petrosino JF, Ajami NJ, Domínguez-Bello MG, de la Barca AM. Fecal microbiota imbalance in Mexican children with type 1 diabetes. *Sci Rep* 2014; **4**: 3814 [PMID: 24448554 DOI: 10.1038/srep03814]

19 **Koliada A**, Syzenko G, Moseiko V, Budovska L, Puchkov K, Perederiy V, Gavalko Y, Dorofeyev A, Romanenko M, Tkach S, Sineok L, Lushchak O, Vaiserman A. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *BMC Microbiol* 2017; **17**: 120 [PMID: 28532414 DOI: 10.1186/s12866-017-1027-1]

20 **Turnbaugh PJ**, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; **444**: 1027-1031 [PMID: 17183312 DOI: 10.1038/nature05414]

21 **Turnbaugh PJ**, Bäckhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* 2008; **3**: 213-223

[PMID: 18407065 DOI: 10.1016/j.chom.2008.02.015]

22 **Larsen N**, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, Al-Soud WA, Sørensen SJ, Hansen LH, Jakobsen M. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS One* 2010; **5**: e9085 [PMID: 20140211 DOI: 10.1371/journal.pone.0009085]

23 **Bittinger K**, Zhao C, Li Y, Ford E, Friedman ES, Ni J, Kulkarni CV, Cai J, Tian Y, Liu Q, Patterson AD, Sarkar D, Chan SHJ, Maranas C, Saha-Shah A, Lund P, Garcia BA, Mattei LM, Gerber JS, Elovitz MA, Kelly A, DeRusso P, Kim D, Hofstaedter CE, Goulian M, Li H, Bushman FD, Zemel BS, Wu GD. Bacterial colonization reprograms the neonatal gut metabolome. *Nat Microbiol* 2020; **5**: 838-847 [PMID: 32284564 DOI: 10.1038/s41564-020-0694-0]

24 **Pedersen HK**, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BA, Forslund K, Hildebrand F, Prifti E, Falony G, Le Chatelier E, Levenez F, Doré J, Mattila I, Plichta DR, Pöhö P, Hellgren LI, Arumugam M, Sunagawa S, Vieira-Silva S, Jørgensen T, Holm JB, Trošt K; MetaHIT Consortium, Kristiansen K, Brix S, Raes J, Wang J, Hansen T, Bork P, Brunak S, Oresic M, Ehrlich SD, Pedersen O. Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* 2016; **535**: 376-381 [PMID: 27409811 DOI: 10.1038/nature18646]

25 **Yoshida N**, Emoto T, Yamashita T, Watanabe H, Hayashi T, Tabata T, Hoshi N, Hatano N, Ozawa G, Sasaki N, Mizoguchi T, Amin HZ, Hirota Y, Ogawa W, Yamada T, Hirata KI. Bacteroides vulgatus and Bacteroides dorei Reduce Gut Microbial Lipopolysaccharide Production and Inhibit Atherosclerosis. *Circulation* 2018; **138**: 2486-2498 [PMID: 30571343 DOI: 10.1161/CIRCULATIONAHA.118.033714]

26 **Leonard MM**, Karathia H, Pujolassos M, Troisi J, Valitutti F, Subramanian P, Camhi S, Kenyon V, Colucci A, Serena G, Cucchiara S, Montuori M, Malamisura B, Francavilla R, Elli L, Fanelli B, Colwell R, Hasan N, Zomorrodi AR, Fasano A; CD-GEMM Team. Multi-omics analysis reveals the influence of genetic and environmental risk factors on developing gut microbiota in infants at risk of celiac disease. *Microbiome* 2020; **8**: 130 [PMID: 32917289 DOI: 10.1186/s40168-020-00906-w]

27 **Gutiérrez N**, Garrido D. Species Deletions from Microbiome Consortia Reveal Key Metabolic Interactions between Gut Microbes. *mSystems* 2019; **4** [PMID: 31311843 DOI: 10.1128/mSystems.00185-19]

28 **Anonye BO**, Hassall J, Patient J, Detamornrat U, Aladdad AM, Schüller S, Rose FRAJ, Unnikrishnan M. Probing *Clostridium difficile* Infection in Complex Human Gut Cellular Models. *Front Microbiol* 2019; **10**: 879 [PMID: 31114553 DOI: 10.3389/fmicb.2019.00879]

29 **Wu Y**, Bible PW, Long S, Ming WK, Ding W, Long Y, Wen X, Li X, Deng X, Deng Y, Guo S, Doçi CL, Wei L, Chen H, Wang Z. Metagenomic analysis reveals gestational diabetes mellitus-related microbial regulators of glucose tolerance. *Acta Diabetol* 2020; **57**: 569-581 [PMID: 31820107 DOI: 10.1007/s00592-019-01434-2]

30 T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, Kolde R, Vlamakis H, Arthur TD, Hämäläinen AM, Peet A, Tillmann V, Uibo R, Mokurov S, Dorshakova N, Ilonen J, Virtanen SM, Szabo SJ, Porter JA, Lähdesmäki H, Huttenhower C, Gevers D, Cullen TW, Knip M; DIABIMMUNE Study Group, Xavier RJ. Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell* 2016; **165**: 842-853 [PMID: 27133167 DOI: 10.1016/j.cell.2016.04.007]

31 **Qin J**, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Jian M, Zhou Y, Li Y, Zhang X, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J; MetaHIT Consortium, Bork P, Ehrlich SD. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010; **464**: 59-65 [PMID: 20203603 DOI: 10.1038/nature08821]

32 **Yang C**, Mogno I, Contijoch EJ, Borgerding JN, Aggarwala V, Li Z, Siu S, Grasset EK, Helmus DS, Dubinsky MC, Mehandru S, Cerutti A, Faith JJ. Fecal IgA Levels Are Determined by Strain-Level Differences in Bacteroides ovatus and Are Modifiable by Gut Microbiota Manipulation. *Cell Host Microbe* 2020; **27**: 467-475. e6 [PMID: 32075742 DOI: 10.1016/j.chom.2020.01.016]

33 **Heshiki Y**, Vazquez-Uribe R, Li J, Ni Y, Quainoo S, Imamovic L, Sørensen M, Chow BKC, Weiss GJ, Xu A, Sommer MOA, Panagiotou G. Predictable modulation of cancer treatment outcomes by the gut microbiota. *Microbiome* 2020; **8**: 28 [PMID: 32138779 DOI: 10.1186/s40168-020-00811-2]

34 **Ulsemer P**, Toutounian K, Schmidt J, Karsten U, Goletz S. Preliminary safety evaluation of a new Bacteroides xylanisolvens isolate. *Appl Environ Microbiol* 2012; **78**: 528-535 [PMID: 22101046 DOI: 10.1128/AEM.06641-11]

35 **Liu S**, Zhao W, Liu X, Cheng L. Metagenomic analysis of the gut microbiome in atherosclerosis patients identify cross-cohort microbial signatures and potential therapeutic target. *FASEB J* 2020; **34**: 14166-14181 [PMID: 32939880 DOI: 10.1096/fj.202000622R]

36 **Qiao S**, Bao L, Wang K, Sun S, Liao M, Liu C, Zhou N, Ma K, Zhang Y, Chen Y, Liu SJ, Liu H. Activation of a Specific Gut Bacteroides-Folate-Liver Axis Benefits for the Alleviation of Nonalcoholic Hepatic Steatosis. *Cell Rep* 2020; **32**: 108005 [PMID: 32783933 DOI: 10.1016/j.celrep.2020.108005]

37 **Huang G**, Khan I, Li X, Chen L, Leong W, Ho LT, Hsiao WLW. Ginsenosides Rb3 and Rd reduce polyps formation while reinstate the dysbiotic gut microbiota and the intestinal microenvironment in $Apc^{Min/+}$ mice. *Sci Rep* 2017; **7**: 12552 [PMID: 28970547 DOI: 10.1038/s41598-017-12644-5]

38 **Brodmann T**, Endo A, Gueimonde M, Vinderola G, Kneifel W, de Vos WM, Salminen S, Gómez-Gallego C. Safety of Novel Microbes for Human Consumption: Practical Examples of Assessment in the European Union. *Front Microbiol* 2017; **8**: 1725 [PMID: 28955311 DOI: 10.3389/fmicb.2017.01725]

39 **Borgo F**, Garbossa S, Riva A, Severgnini M, Luigiano C, Benetti A, Pontiroli AE, Morace G, Borghi E. Body Mass Index and Sex Affect Diverse Microbial Niches within the Gut. *Front Microbiol* 2018; **9**: 213 [PMID: 29491857 DOI: 10.3389/fmicb.2018.00213]

40 **Kasai C**, Sugimoto K, Moritani I, Tanaka J, Oya Y, Inoue H, Tameda M, Shiraki K, Ito M, Takei Y, Takase K. Comparison of the gut microbiota composition between obese and non-obese individuals in a Japanese population, as analyzed by terminal restriction fragment length polymorphism and next-generation sequencing. *BMC Gastroenterol* 2015; **15**: 100 [PMID: 26261039 DOI: 10.1186/s12876-015-0330-2]

41 **Mikami A**, Ogita T, Namai F, Shigemori S, Sato T, Shimosato T. Oral administration of Flavonifractor plautii attenuates inflammatory responses in obese adipose tissue. *Mol Biol Rep* 2020; **47**: 6717-6725 [PMID: 32808115 DOI: 10.1007/s11033-020-05727-6]

42 **Ogita T**, Yamamoto Y, Mikami A, Shigemori S, Sato T, Shimosato T. Oral Administration of *Flavonifractor plautii* Strongly Suppresses Th2 Immune Responses in Mice. *Front Immunol* 2020; **11**: 379 [PMID: 32184789 DOI: 10.3389/fimmu.2020.00379]

43 **Cekanaviciute E**, Yoo BB, Runia TF, Debelius JW, Singh S, Nelson CA, Kanner R, Bencosme Y, Lee YK, Hauser SL, Crabtree-Hartman E, Sand IK, Gacias M, Zhu Y, Casaccia P, Cree BAC, Knight R, Mazmanian SK, Baranzini SE. Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. *Proc Natl Acad Sci USA* 2017; **114**: 10713-10718 [PMID: 28893978 DOI: 10.1073/pnas.1711235114]

44 **Del Chierico F**, Nobili V, Vernocchi P, Russo A, De Stefanis C, Gnani D, Furlanello C, Zandonà A, Paci P, Capuani G, Dallapiccola B, Miccheli A, Alisi A, Putignani L. Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach. *Hepatology* 2017; **65**: 451-464 [PMID: 27028797 DOI: 10.1002/hep.28572]

45 **Verdam FJ**, Fuentes S, de Jonge C, Zoetendal EG, Erbil R, Greve JW, Buurman WA, de Vos WM, Rensen SS. Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. *Obesity (Silver Spring)* 2013; **21**: E607-E615 [PMID: 23526699 DOI: 10.1002/oby.20466]

46 **Wang K**, Liao M, Zhou N, Bao L, Ma K, Zheng Z, Wang Y, Liu C, Wang W, Wang J, Liu SJ, Liu H. Parabacteroides distasonis Alleviates Obesity and Metabolic Dysfunctions *via* Production of Succinate and Secondary Bile Acids. *Cell Rep* 2019; **26**: 222-235. e5 [PMID: 30605678 DOI: 10.1016/j.celrep.2018.12.028]

47 **Wang N**, Li R, Lin H, Fu C, Wang X, Zhang Y, Su M, Huang P, Qian J, Jiang F, Wang H, Jiang L, Yu X, Liu J, Chen Y, Jiang Q. Enriched taxa were found among the gut microbiota of centenarians in East China. *PLoS One* 2019; **14**: e0222763 [PMID: 31639130 DOI: 10.1371/journal.pone.0222763]

48 **Sato T**, Kusuhara S, Yokoi W, Ito M, Miyazaki K. Prebiotic potential of L-sorbose and xylitol in promoting the growth and metabolic activity of specific butyrate-producing bacteria in human fecal culture. *FEMS Microbiol Ecol* 2017; **93** [PMID: 27810878 DOI: 10.1093/femsec/fiw227]

49 **Zhang Q**, Wu Y, Wang J, Wu G, Long W, Xue Z, Wang L, Zhang X, Pang X, Zhao Y, Zhao L, Zhang C. Accelerated dysbiosis of gut microbiota during aggravation of DSS-induced colitis by a butyrate-producing bacterium. *Sci Rep* 2016; **6**: 27572 [PMID: 27264309 DOI: 10.1038/srep27572]

50 **Zeevi D**, Korem T, Godneva A, Bar N, Kurilshikov A, Lotan-Pompan M, Weinberger A, Fu J, Wijmenga C, Zhernakova A, Segal E. Structural variation in the gut microbiome associates with host health. *Nature* 2019; **568**: 43-48 [PMID: 30918406 DOI: 10.1038/s41586-019-1065-y]

51 **Yu Y**, Mao G, Wang J, Zhu L, Lv X, Tong Q, Fang Y, Lv Y, Wang G. Gut dysbiosis is associated with the reduced exercise capacity of elderly patients with hypertension. *Hypertens Res* 2018; **41**: 1036-1044 [PMID: 30291307 DOI: 10.1038/s41440-018-0110-9]

52 **Qi CJ**, Zhang Q, Yu M, Xu JP, Zheng J, Wang T, Xiao XH. Imbalance of Fecal Microbiota at Newly Diagnosed Type 1 Diabetes in Chinese Children. *Chin Med J (Engl)* 2016; **129**: 1298-1304 [PMID: 27231166 DOI: 10.4103/0366-6999.182841]

53 **Kostic AD**, Gevers D, Siljander H, Vatanen T, Hyötyläinen T, Hämäläinen AM, Peet A, Tillmann V, Pöhö P, Mattila I, Lähdesmäki H, Franzosa EA, Vaarala O, de Goffau M, Harmsen H, Ilonen J, Virtanen SM, Clish CB, Orešič M, Huttenhower C, Knip M; DIABIMMUNE Study Group, Xavier RJ. The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. *Cell Host Microbe* 2015; **17**: 260-273 [PMID: 25662751 DOI: 10.1016/j.chom.2015.01.001]

54 **Mortaş H**, Bilici S, Karakan T. The circadian disruption of night work alters gut microbiota consistent with elevated risk for future metabolic and gastrointestinal pathology. *Chronobiol Int* 2020; **37**: 1067-1081 [PMID: 32602753 DOI: 10.1080/07420528.2020.1778717]

55 **Yang J**, McDowell A, Kim EK, Seo H, Lee WH, Moon CM, Kym SM, Lee DH, Park YS, Jee YK, Kim YK. Development of a colorectal cancer diagnostic model and dietary risk assessment through gut microbiome analysis. *Exp Mol Med* 2019; **51**: 1-15 [PMID: 31582724 DOI: 10.1038/s12276-019-0313-4]

56 **Brahe LK**, Le Chatelier E, Prifti E, Pons N, Kennedy S, Hansen T, Pedersen O, Astrup A, Ehrlich SD, Larsen LH. Specific gut microbiota features and metabolic markers in postmenopausal women with obesity. *Nutr Diabetes* 2015; **5**: e159 [PMID: 26075636 DOI: 10.1038/nutd.2015.9]

57 **Qin P**, Zou Y, Dai Y, Luo G, Zhang X, Xiao L. Characterization a Novel Butyric Acid-Producing Bacterium *Collinsella aerofaciens* Subsp. *Shenzhenensis* Subsp. Nov. *Microorganisms* 2019; **7** [PMID: 30871249 DOI: 10.3390/microorganisms7030078]

58 **Candela M**, Biagi E, Soverini M, Consolandi C, Quercia S, Severgnini M, Peano C, Turroni S, Rampelli S, Pozzilli P, Pianesi M, Fallucca F, Brigidi P. Modulation of gut microbiota dysbioses in type 2 diabetic patients by macrobiotic Ma-Pi 2 diet. *Br J Nutr* 2016; **116**: 80-93 [PMID: 27151248 DOI: 10.1017/S0007114516001045]

59 **Karlsson FH**, Fåk F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, Bäckhed F, Nielsen J. Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nat Commun* 2012; **3**: 1245 [PMID: 23212374 DOI: 10.1038/ncomms2266]

60 **Masoodi I**, Alshanqeeti AS, Alyamani EJ, AlLehibi AA, Alqutub AN, Alsayari KN, Alomair AO. Microbial dysbiosis in irritable bowel syndrome: A single-center metagenomic study in Saudi Arabia. *JGH Open* 2020; **4**: 649-655 [PMID: 32782952 DOI: 10.1002/jgh3.12313]

61 **Turnbaugh PJ**, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. *Nature* 2009; **457**: 480-484 [PMID: 19043404 DOI: 10.1038/nature07540]

62 **Shetty SA**, Ritari J, Paulin L, Smidt H, De Vos WM. Complete Genome Sequence of *Eubacterium hallii* Strain L2-7. *Genome Announc* 2017; **5** [PMID: 29074659 DOI: 10.1128/genomeA.01167-17]

63 **Schwab C**, Ruscheweyh HJ, Bunesova V, Pham VT, Beerenwinkel N, Lacroix C. Trophic Interactions of Infant Bifidobacteria and *Eubacterium hallii* during L-Fucose and Fucosyllactose Degradation. *Front Microbiol* 2017; **8**: 95 [PMID: 28194144 DOI: 10.3389/fmicb.2017.00095]

64 **Ye G**, Zhang L, Wang M, Chen Y, Gu S, Wang K, Leng J, Gu Y, Xie X. The Gut Microbiota in Women Suffering from Gestational Diabetes Mellitus with the Failure of Glycemic Control by Lifestyle Modification. *J Diabetes Res* 2019; **2019**: 6081248 [PMID: 31772944 DOI: 10.1155/2019/6081248]

65 **Udayappan S**, Manneras-Holm L, Chaplin-Scott A, Belzer C, Herrema H, Dallinga-Thie GM, Duncan SH, Stroes ESG, Groen AK, Flint HJ, Backhed F, de Vos WM, Nieuwdorp M. Oral treatment with *Eubacterium hallii* improves insulin sensitivity in *db/db* mice. *NPJ Biofilms Microbiomes* 2016; **2**: 16009 [PMID: 28721246 DOI: 10.1038/npjbiofilms.2016.9]

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META-ANALYSIS

Selection of first-line systemic therapies for advanced hepatocellular carcinoma: A network meta-analysis of randomized controlled trials

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Abstract

BACKGROUND

The majority of clinical trials of first-line systemic treatments for hepatocellular carcinoma (HCC) used placebo or sorafenib as comparators, and there are limited data providing a cross comparison of treatments in this setting, especially for newly-approved immune checkpoint inhibitor and vascular endothelial growth factor inhibitor combination treatments.

AIM

To systematically review and compare response rates, survival outcomes, and safety of first-line systemic therapies for advanced hepatocellular carcinoma.

METHODS

We searched PubMed, Science Direct, the Cochrane Database, Excerpta Medica Database, and abstracts from the American Society of Clinical Oncology 2020 annual congress. Eligible studies were randomized controlled trials of systemic therapy enrolling adults with advanced/unresectable HCC. Risk of bias was assessed with the Cochrane risk of bias tool for randomized controlled trials. A network meta-analysis was used to synthesize data and perform direct and indirect comparisons between treatments. *P* value, a frequentist analog to the surface under the cumulative ranking curve, was used to rank treatments.

RESULTS

In total, 1398 articles were screened and 27 included. Treatments compared were atezolizumab plus bevacizumab, brivanib, donafenib, dovitinib, FOLFOX4, lenvatinib, linifanib, nintedanib, nivolumab, sorafenib, sunitinib, vandetanib, 11 sorafenib combination therapies, and three other combination therapies. For

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overall response rate, lenvatinib ranked 1/19, followed by atezolizumab plus bevacizumab and nivolumab. For progression-free survival (PFS), atezolizumab + bevacizumab was ranked 1/15, followed by lenvatinib. With the exception of atezolizumab + bevacizumab [hazard ratios $(HR)_{PFS}$ = 0.90; 95% confidence interval (CI): 0.64-1.25], the estimated HRs for PFS for all included treatments *vs* lenvatinib were > 1; however, the associated 95%CI passed through unity for bevacizumab plus erlotinib, linifanib, and FOLFOX4. For overall survival, atezolizumab plus bevacizumab was ranked 1/25, followed by vandetanib 100 mg/d and donafinib, with lenvatinib ranked 6/25. Atezolizumab + bevacizumab was associated with a lower risk of death *vs* lenvatinib (HR_{os} = 0.63; 95%CI: 0.44-0.89), while the HR for overall survival for most other treatments *vs* lenvatinib had associated 95%CIs that passed through unity. Vandetanib 300 mg/d and 100 mg/d were ranked 1/13 and 2/13, respectively, for the lowest incidence of treatment terminations due to adverse events, followed by sorafenib (5/13), lenvatinib (10/13), and atezolizumab + bevacizumab (13/13).

CONCLUSION

There is not one single first-line treatment for advanced HCC associated with superior outcomes across all outcome measurements. Therefore, first-line systemic treatment should be selected based on individualized treatment goals.

Key Words: Hepatocellular carcinoma; Systemic therapy; Meta-analysis; Lenvatinib; First-line; Immune therapy

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Core Tip: The present network meta-analysis is the first to compare data from randomized trials of all first-line systemic therapies for hepatocellular carcinoma including chemotherapy, targeted drugs, immunotherapy, and combination therapies. Furthermore, the analysis represents a comprehensive cross comparison of outcomes, including tumor response rates, survival, and safety and included a sub-analysis in patients with hepatitis B virus infection. Our results showed that atezolizumab plus bevacizumab was ranked first for progression-free survival and overall survival but also had the highest rate of discontinuations due to adverse events. Lenvatinib ranked first for overall response rate and second for progression-free survival.

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INTRODUCTION

Liver cancer is the sixth most common cancer globally, accounting for 4.7% of all new cancer cases in 2018, and represents the third most common cause of cancer-related death worldwide behind lung and colorectal cancer[1]. Of the primary liver cancers, hepatocellular carcinoma (HCC) is the most prevalent histological subtype and accounts for 80%-85% of cases[2]. Surgical resection and liver transplant are associated with the best survival outcomes for patients with HCC, and are potentially curative treatments[3]. Locoregional therapies including arterially directed therapies, ablation, and radiotherapy are also associated with good survival outcomes in patients with unresectable disease confined to the liver[4]. However, over 50% of patients with HCC are diagnosed at an advanced stage or with other characteristics that preclude surgical or locoregional treatment[5]. For these patients, systemic therapy is usually the recommended treatment option[4,6].

Over the past 3 years, the number of approved first-line systemic therapies for patients with HCC has expanded greatly, and numerous drugs and drug combinations have been evaluated in this setting[7]. Between 2007 and 2018, sorafenib was the only

approved systemic treatment for HCC based on the results of the Phase III SHARP trial, which showed a survival benefit for sorafenib *vs* placebo[8]. In the decade following the approval of sorafenib, numerous unsuccessful trials of systemic therapies in advanced HCC were conducted until the approval of lenvatinib in 2018[9]. Lenvatinib was approved for first-line use in advanced HCC following the successful outcome of the Phase III REFLECT trial. In this trial, lenvatinib showed a non-inferior overall survival (OS) *vs* sorafenib for the treatment of advanced HCC[10]. Since the approval of lenvatinib, the immunotherapy drugs nivolumab and pembrolizumab, as well as other tyrosine kinase inhibitors (TKI), have been approved for the second-line treatment of HCC. Most recently, combination therapy with the anti-PD-L1 agent atezolizumab plus bevacizumab demonstrated better OS and progression-free survival (PFS) than sorafenib in the Phase III IMbrave 150 trial[11].

The expansion of first-line treatment options for advanced HCC represents a significant advance in the treatment of this disease. However, further data would be useful to inform treatment selection. Most clinical trials of first-line therapies for HCC used placebo or sorafenib as comparators and there are limited data providing a cross comparison of the efficacy and safety of drugs in this setting. Furthermore, although lenvatinib is widely seen as a standard of care in real clinical practice and is a recommended first-line therapy in most international treatment guidelines[4,12,13], there are limited head-to-head data comparing lenvatinib with other systemic therapies. Finally, although historically systemic treatments for HCC were associated with low tumor response rates, recently approved therapies have been associated with response rates > 30%[14]. This has led to renewed interest in tumor response rates in HCC, and investigation of downstaging and conversion therapy strategies. A comparison of response rates for all currently available therapies would therefore be of clinical value.

This network meta-analysis was conducted to systematically review and compare the response rates, survival outcomes, and safety reported by randomized trials of first-line systemic therapies in patients with advanced unresectable HCC, and to provide a comparison between lenvatinib and other systemic therapies in this setting. Two recent meta-analyses have investigated a similar topic to the present study; however, one did not include data on atezolizumab plus bevacizumab and excluded non-targeted therapies[15], and a more recent analysis focused on treatment sequencing by investigating survival outcomes only[16]. Therefore, although there is some overlap with the present analysis, these studies are complementary to each other. In particular, the present analysis is the first to include data on donafenib, a Chinese drug that has shown a superior OS to sorafenib in a Phase III trial[17]. Furthermore, our analysis includes data on survival, response rate, and safety, which in combination are important for treatment decision-making, particularly for patients who may be candidates for downstaging. Finally, the present meta-analysis included a sub-group analysis of patients with HBV infection, which is an important population in the Asia-Pacific region and has not been covered by other current meta-analyses.

MATERIALS AND METHODS

The analysis methods and inclusion criteria for this study were specified in advance and the protocol was prospectively submitted for registration in the PROSPERO database on May 26, 2020. This report has been written in line with the PRISMA guidelines for network meta-analyses.

Eligibility criteria

This analysis included randomized controlled trials conducted in adult patients (age ≥ 18 years) with advanced or unresectable HCC not eligible for, or with disease progression after, surgical or locoregional therapies. Eligible studies included patients with Child-Pugh Class A or B liver function, ≥ 1 measurable lesion, and no evidence of untreated brain or meningeal metastases. Eligible studies were also required to report at least an assessment of tumor response, survival [OS, PFS, or time to progression (TTP)], and safety. The analysis excluded studies including patients with Child-Pugh Class C liver function, patients receiving anticoagulation therapy or antiretroviral therapy for HIV, and patients who had received previous systemic treatment. These broad eligibility criteria covered a number of trials reporting negative results *vs* sorafenib. Although the analysis therefore includes multiple therapies that failed clinical trials in HCC, this allowed the collection of data for sorafenib from studies conducted over a wide time range, which improved the precision of the analysis.

Information sources, search strategy, and study selection

Studies were identified by searching the following electronic databases: PubMed, Science Direct, and the Cochrane Database, and Excerpta Medica Database Abstracts from the American Society of Clinical Oncology (ASCO) 2020 annual congress were also searched. The search was completed on May 21, 2020 using the search terms shown in Figure 1.

The Cochrane risk of bias tool for randomized controlled trials was used to assess the quality and risk of bias of studies included in the analysis[18].

Data extraction

Data were independently extracted by two evaluators (Luo JF and Huang XQ) and cross-checked. In the case of disagreement, the original documents were checked and the correct data confirmed. General information extracted included journal name, document title, publication time, author, country, region where the lead author was located, and the country and region where the research was conducted. Demographics and baseline characteristics extracted were patient age, gender, Barcelona clinic liver cancer classification, Eastern Co-operative Oncology Group performance status, prevalence of HBV infection, and presence of extrahepatic vascular infiltration and extrahepatic metastasis. Details of interventions extracted included dosage and dose schedule. Efficacy and safety endpoints extracted (where available) were overall response rate [ORR; assessed by response evaluation criteria in solid tumours (RECIST) v1/1.1 for all included studies], OS, PFS, TTP, incidence of Grade ≥ 3 adverse events (AE), incidence of treatment interruption due to adverse events (AEs), and incidence of dose reductions due to AEs.

Statistical analysis

For OS, PFS, TTP, and other survival endpoints, hazard ratios (HR) were estimated to compare treatments. For discrete variables such as ORR, and incidence of AEs, estimated risk ratios were calculated to compare treatments. Selection of a fixed effect or random effect model was based on the level of heterogeneity in the data, assessed using the Higgins I^2 statistic and defined as $I^2 \leq 50\%$ and $P > 0.1$. If no obvious data heterogeneity was found, a fixed effect model was adopted, otherwise a random effect model was utilized. For endpoints reported in a relatively small number of studies (< 6), a fixed effects model was adopted.

A network meta-analysis was used to synthesize information from the included studies, and perform direct and indirect comparisons using a method based on the frequency school of Rücker *et al*[19,20]. The Q statistic was used to assess the consistency of direct and indirect evidence in the treatment network(s) studied. If no obvious inconsistency ($P > 0.1$) was found, a fixed effect model was adopted, otherwise a random effect model was utilized. *P* value, a frequentist analog to the surface under the cumulative ranking curve, was used to rank treatments[21]. A funnel chart was used to evaluate publication bias; a symmetrical graph indicates a low influence of publication bias and an asymmetric graph indicates possible publication bias. A post-hoc analysis of all studies reporting data from patients with HBV-related HCC was also included to assess OS, PFS, and safety in these patients.

All statistical analyses were performed using Rv3.6. The Robias toolkit was used for evaluation of literature quality and Netmeta was used for the network meta-analysis.

RESULTS

Studies included in the analysis

In total, 1398 articles were screened: PubMed/MEDLINE, *n* = 114; Science Direct, *n* = 312; Cochrane Database, *n* = 355; Excerpta Medica Database, *n* = 561; and the ASCO 2020 abstract book, *n* = 12 (Figure 1). After removing duplicates and top-line screening of abstracts for suitability, a total of 86 articles were reviewed in detail, of which 27 met the full inclusion criteria (Table 1). These 27 articles corresponded to 27 different studies (Supplementary Figure 1).

Study characteristics

Of the 27 studies included, 25 investigated targeted treatment regimens (nintedanib, mapatumumab + sorafenib, atezolizumab + bevacizumab, doxorubicin + sorafenib, dovitinib, tigatuzumab + sorafenib, vandetanib, brivanib, linifanib, lenvatinib, nivolumab, sunitinib, sorafenib + erlotinib, sorafenib (two studies), nintedanib,

Table 1 Details of included studies

Ref.	Year	Experimental arm(s)	Comparator arm	Primary endpoint	Analysis timing	Survival outcomes, mo
Yen *et al*[47]	2018	Nintedanib	Sorafenib	TTP	PFS: Final; OS: Final	PFS: 2.7 *vs* 3.7; OS: 10.2 *vs* 1.1
Ciuleanu *et al*[48]	2016	Mapatumumab + sorafenib	Placebo + sorafenib	TTP	PFS: Final; OS: Final	PFS: 3.2 *vs* 4.3; OS: 10.0 *vs* 10.1
Finn *et al*[11]	2020	Atezolizumab + bevacizumab	Sorafenib	OS and PFS	PFS: Final; OS: Final	PFS: 6.8 *vs* 4.3; OS: NE *vs* 13.2
Abou-Alfa *et al*[49]	2010	Doxorubicin + sorafenib	Doxorubicin + placebo	TTP	PFS: Final; OS: Final	PFS: 6.0 *vs* 2.7; OS: 13.7 *vs* 6.5
Cheng *et al*[50]	2016	Dovitinib	Sorafenib	OS and TTP	TTP: Final; OS: Final	TTP: 4.1 *vs* 4.1; OS: 8.0 *vs* 8.4
Cheng *et al*[28]	2015	Tigatuzumab (6 + 2) + sorafenib; Tigatuzumab (6 + 6) + sorafenib	Sorafenib	TTP	TTP: Final; OS: Final	TTP: 3.0 *vs* 3.9 *vs* 2.8; OS: 8.2 *vs* 12.2 *vs* 8.2
Hsu *et al*[51]	2012	Vandetanib 300 mg/d; Vandetanib 100 mg/d	Placebo	Tumor stabilization rate	PFS: Final; OS: Final	PFS: 1.1 *vs* 0.7 *vs* 1.0; OS: 6.0 *vs* 5.8 *vs* 4.3
Johnson *et al*[22]	2013	Sorafenib	Brivanib	OS	PFS: No; OS: Final	PFS: 4.1 *vs* 4.2; OS: 9.9 *vs* 9.5
Cainap *et al*[24]	2015	Linifanib	Sorafenib	OS	PFS: Final; OS: Final	PFS: 4.2 *vs* 2.9; OS: 9.1 *vs* 9.8
Kudo *et al*[10]	2018	Lenvatinib	Sorafenib	OS	PFS: No; OS: Final	PFS: 7.4 *vs* 3.7; OS: 13.6 *vs* 12.3
Yau *et al*[23]	2019	Nivolumab	Sorafenib	OS	PFS: Final; OS: Final	PFS: 3.7 *vs* 3.8; OS: 16.4 *vs* 14.7
Cheng *et al*[29]	2013	Sunitinib	Sorafenib	OS	PFS: Final;OS: Final	PFS: 3.6 *vs* 3.0; OS: 7.9 *vs* 10.2
Zhu *et al*[26]	2015	Sorafenib + erlotinib	Sorafenib + placebo	OS	TTP: Final; OS: Final	TTP: 3.2 *vs* 4.0; OS: 9.5 *vs* 8.5
Llovet *et al*[52]	2008	Sorafenib	Placebo	OS and TTP	TTP: Final; OS: Final	TSP: 5.5 *vs* 2.8; OS: 10.7 *vs* 7.9
Cheng *et al*[25]	2009	Sorafenib	Placebo	-	TTP: Final; OS: Final	TTP: 2.8 *vs* 1.4; OS: 6.5 *vs* 4.2
Palmer *et al*[53]	2018	Nintedanib	Sorafenib	TTP	PFS: Final; OS: Final	PFS: 5.3 *vs* 3.9; OS: 11.9 *vs* 11.4
Thomas *et al*[54]	2018	Bevacizumab + erlotinib	Sorafenib	OS	PFS: No; OS: Final	PFS: 4.4 *vs* 2.8; OS: 8.6 *vs* 8.6
Abou-Alfa *et al*[55]	2019	Sorafenib + doxorubicin	Sorafenib	OS	PFS: Final; OS: Final	PFS: 4.0 *vs* 3.7; OS: 9.3 vs. 9.4
Tak *et al*[27]	2018	Sorafenib	Sorafenib + resminostat	TTP	TTP: Final; OS: Final	TTP: 2.8 *vs* 2.8; OS: 14.1 *vs* 11.8
Jouve *et al*[56]	2019	Sorafenib + pravastatin	Sorafenib	OS	PFS: Final; OS: Final	PFS: 5.0 *vs* 5.4; OS: 10.7 *vs* 10.5
Lee *et al*[57]	2016	AEG35156 + sorafenib	Sorafenib	PFS	PFS: Final; OS: Final	PFS: 4.0 *vs* 2.6; OS: 6.5 *vs* 5.4
Assenat *et al*[58]	2019	Sorafenib + GEMOX	Sorafenib	PFS	PFS: Final; OS: Final	PFS: 6.2 *vs* 4.6; OS:13.5 *vs* 14.8
Azim *et al*[59]	2018	Sorafenib + tegafur–uracil	Sorafenib	TTP	PFS: Final; OS: Final	PFS: 6.0 *vs* 6.0; OS: 8.2 *vs* 10.5
Koeberle *et al*[60]	2016	Sorafenib	Sorafenib + everolimus	PFS	PFS: Final; OS: Final	PFS: 6.6 *vs* 5.7; OS: 10.0 *vs* 12
Bi *et al*[17]	2020	Donafinib	Sorafenib	OS	PFS: Final; OS: Final	PFS: 3.7 *vs* 3.6; OS: 21.1 *vs* 10.3
Qin *et al*[61]	2013	FOLFOX4	Doxorubicin	OS	PFS: Final; OS: Final	PFS: 2.9 *vs* 1.8; OS: 6.4 *vs* 5.0

Yeo *et al*[62]	2005	Doxorubicin	PIAF	OS	PFS: No; OS: Final	OS: 6.8 *vs* 8.7

FOLFOX4: Oxaliplatin/folinic acid/5-fluorouracil; GEMOX: Gemcitabine and oxaliplatin; NE, Not reported; OS, Overall survival; PFS, Progression-free survival; PIAF, Cisplatin/interferon α-2b/doxorubicin/5-fluorouracil; TTP, Time to progression.

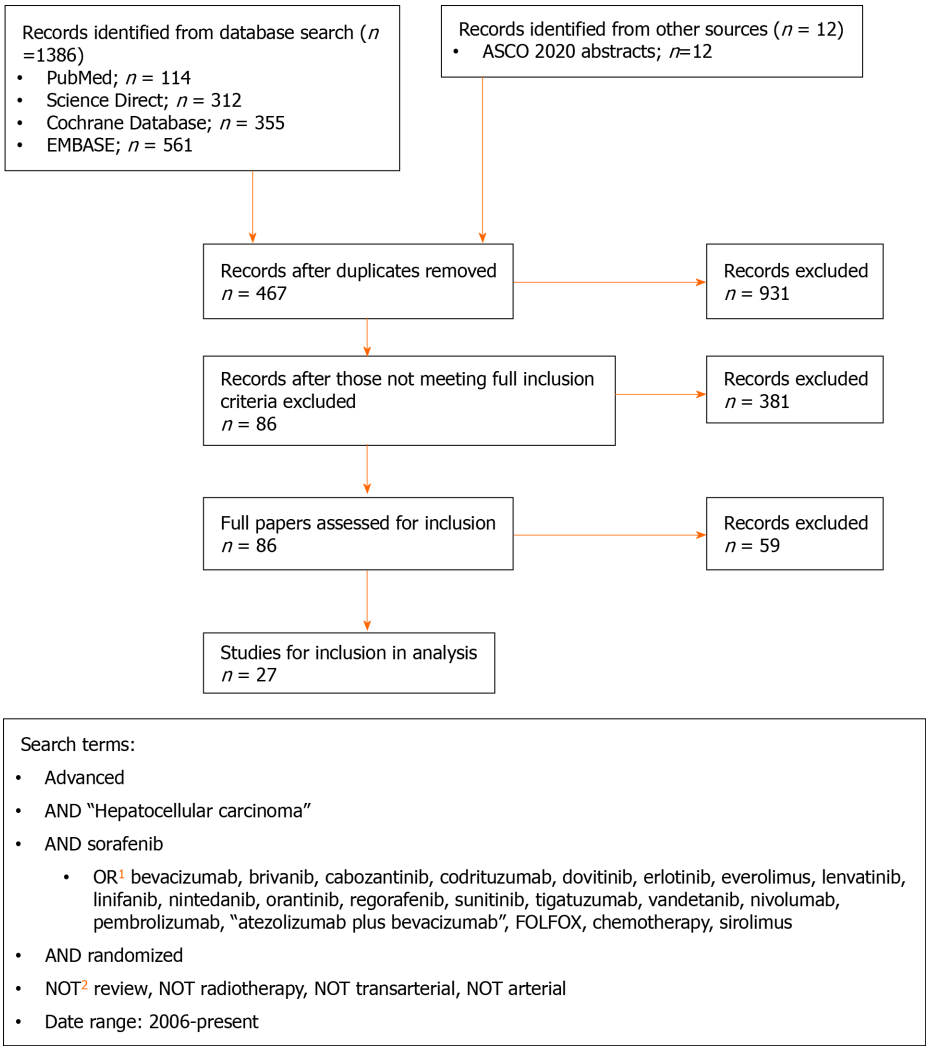


Figure 1 Flow chart of study selection and search terms. Different therapeutics were searched for using individual searches to allow easier processing of the results; NOT was used for databases allowing use of this operator. ASCO: American Society of Clinical Oncology.

bevacizumab + erlotinib, sorafenib + doxorubicin, sorafenib + resminostat, sorafenib + pravastatin, AEG35156 (a second-generation synthetic antisense oligonucleotide inhibitor of cellular expression of the X-linked inhibitor of apoptosis protein) + sorafenib, sorafenib + gemcitabine and cisplatin, sorafenib + tegafur–uracil, sorafenib + everolimus, and donafinib) and two investigated combination chemotherapy regimens [oxaliplatin/folinic acid/5-fluorouracil (FOLFOX4) and cisplatin/interferon α-2b/doxorubicin/5-fluorouracil] (Table 1). Twenty-one of the included studies used sorafenib as the comparator treatment, three used doxorubicin, and three studies were placebo controlled (including the two Phase III studies of sorafenib). The majority of the studies had OS (n = 12) or PFS/TTP (n = 10) as the primary endpoint, and almost all had reported final data for these endpoints.

Quality assessment

Study design characteristics are summarized in Supplementary Table 1. In brief, all 27 studies selected for inclusion were randomized controlled studies (20 provided details of the randomization scheme used and seven articles did not specify), seven of the studies used double blinding and 20 were open label, and 24 included a data flow

chart. Overall, the quality of the included studies was considered relatively high (Figure 1).

Patient description

All of the studies included patients with advanced HCC who had not received previous treatment. Overall, the total of 10256 patients included in the analysis were predominantly male and had median ages ranging from 49 to 68 years, and most of the studies included > 50% of patients with extrahepatic metastasis (Table 2).

Evaluation of efficacy

Overall response rate: A total of 18 studies reported ORR, including 19 interventions and allowing 20 comparisons (Figure 2A). No significant heterogeneity was detected between the studies (tau-squared = 0; I^2 = 0%; P = 0.9502) and a fixed effect model was selected. P value for ORR showed that lenvatinib was associated with the best ORR among all treatments included in the analysis (P = 0.9042) (Figure 2B). Atezolizumab + bevacizumab ranked second (P = 0.8045) and nivolumab ranked third (P = 0.7834). Using lenvatinib as the comparator, all treatments included in the analysis had an estimated risk ratio for ORR (RR_{ORR}) of < 1, except for AEG35156 + sorafenib, which had an estimated RR_{ORR} of 1.3451 [95% confidence interval (CI): 0.07-25.21] (Figure 2B).

Progression-free survival: A total of 15 studies reported PFS, including 15 interventions and allowing 15 comparisons (Figure 3A). No significant heterogeneity was detected (tau-squared = 0; I^2 = 0%; P = 0.7361) and a fixed effect model was selected. Atezolizumab + bevacizumab was ranked first for PFS (P = 0.9501), followed by lenvatinib (P = 0.9041). Nivolumab ranked sixth (P = 0.558) (Figure 3B). With the exception of atezolizumab + bevacizumab (HR_{PFS} = 0.90; 95%CI: 0.64-1.25), the estimated HRs for PFS for all included treatments *vs* lenvatinib were > 1; however, the associated 95%CI passed through unity for bevacizumab plus erlotinib, linifanib, and FOLFOX4.

Time to progression: A total of 17 studies reported TTP, including 17 interventions and allowing 19 comparisons (Figure 3C). No significant heterogeneity was detected between studies (tau-squared = 0; I^2 = 0%; P = 0.9028) and a fixed effect model was selected. Lenvatinib was ranked first for TTP (P = 0.9888) followed by linifanib (P = 0.9067) and sorafenib + doxorubicin (P = 0.7344) (Figure 3D). When compared with lenvatinib, all other treatments in the analysis had an estimated HR_{TTP} > 1, although the associated 95%CI passed through unity for linifanib and sorafenib plus tegafur–uracil.

Overall survival: A total of 24 studies reported OS, including 25 interventions and allowing 28 comparisons (Figure 3E). No significant heterogeneity was detected between studies (tau-squared = 0; I^2 = 0%; P = 0.9802) and a fixed effect model was selected. Atezolizumab + bevacizumab was ranked highest for OS (P = 0.9651) followed by vandetanib 100 mg/d (P = 0.8653), donafinib (P = 0.7958), and nivolumab (P = 0.7701) (Figure 3F). Lenvatinib ranked sixth (P = 0.6675). Atezolizumab + bevacizumab was associated with a lower risk of death *vs* lenvatinib (HR_{os} = 0.63; 95%CI: 0.44-0.89), and the HR_{os} for most other treatments *vs* lenvatinib had associated 95%CIs that passed through unity.

Outcomes in patients with HBV infection: Ten studies included sub-analyses of patients with HBV infection, including data on the following treatments: atezolizumab + bevacizumab[11], brivanib[22], nivolumab[23], lenvatinib[10], linifanib[24], sorafenib[25], sorafenib + erlotinib[26], sorafenib + resminostat[27], tigatuzumab + sorafenib[28], and sunitinib[29]. A total of three studies reported PFS in patients with HBV infection, including four interventions and three comparisons (Supplementary Figure 2A). A fixed effect model was selected for the analysis. Lenvatinib ranked first for PFS (P = 0.8786) followed by atezolizumab + bevacizumab (P = 0.7746) and donafinib (P = 0.2972) (Figure 2B). A comparison of HRs for PFS *vs* lenvatinib is shown in Supplementary Figure 2B. A total of nine studies reported OS, including ten interventions and allowing nine comparisons (Supplementary Figure 2C). A random effect model was selected for the analysis. Atezolizumab + bevacizumab ranked first (P = 0.9751), followed by lenvatinib (P = 0.8308) and nivolumab (P = 0.7732) (Supplementary Figure 2D). Comparison with lenvatinib revealed that atezolizumab + bevacizumab had an estimated HR_{OS} < 1 and all other interventions had an HR_{OS} > 1 (Supplementary Figure 2D).

Table 2 Patient characteristics in the included studies

Ref.	Year	Treatments	*n*	Age, median	Males, %	ECOG 0/1–2, %	Extrahepatic disease, %
Yen *et al*[47]	2018	Nintedanib	63	58	91	55.6/44.5	68.3
		Sorafenib	32	62	81	56.3/43.8	68.3
Ciuleanu *et al*[48]	2016	Mapatumumab + sorafenib	50	60	52	36.0/64.0	66.0
		Placebo + sorafenib	51	61	77	33.3/66.6	49.0
Finn *et al*[11]	2020	Atezolizumab + bevacizumab	336	64	82	62.0/38.0	63.0
		Sorafenib	165	66	83	62.0/38.0	56.0
Abou-Alfa *et al*[49]	2010	Doxorubicin + sorafenib	47	66	66	-	51.1
		Doxorubicin + placebo	49	65	86	-	79.6
Cheng *et al*[50]	2016	Dovitinib	82	56	89	63.0/37.0	-
		Sorafenib	83	56	81	64.0/35.0	-
Cheng *et al*[28]	2015	Tigatuzumab (6 + 2) + sorafenib	53	63	85	60.4/39.6	-
		Tigatuzumab (6 + 6) + sorafenib	54	63	83	57.4/42.6	-
		Sorafenib	55	66	80	54.5/45.5	-
Hsu *et al*[51]	2012	Vandetanib 300 mg/d	19	55	95	-	-
		Vandetanib 100 mg/d	25	61	68	-	-
		Placebo	23	56	87	-	-
Johnson *et al*[22]	2013	Sorafenib	578	60	84	61.0/39.0	62.0
		Brivanib	577	61	84	64.0/36.0	63.0
Cainap *et al*[24]	2015	Linifanib	514	59	86	62.8/37.2	59.7
		Sorafenib	521	60	84	66.2/33.8	56.8
Kudo *et al*[10]	2018	Lenvatinib	478	63	85	-	-
		Sorafenib	476	62	84	-	-
Yau *et al*[23]	2019	Nivolumab	371	65	85	-	-
		Sorafenib	372	65	85	-	-
Cheng *et al*[29]	2013	Sunitinib	530	59	82	52.5/46.8	78.9
		Sorafenib	544	59	84	52.9/46.7	76.3
Zhu *et al*[26]	2015	Sorafenib + erlotinib	362	60	82	61.3/38.7	56.6
		Sorafenib + placebo	358	61	80	60.3/39.7	61.2
Llovet *et al*[52]	2008	Sorafenib	299	65	87	54.0/46.0	53.0
		Placebo	303	66	87	54.0/46.0	50.0
Cheng *et al*[25]	2009	Sorafenib	150	51	85	25.3/74.6	68.7
		Placebo	76	52	87	27.6/72.4	68.4
Palmer *et al*[53]	2018	Nintedanib	62	66	77	51.6/48.4	64.5
		Sorafenib	31	64	84	58.1/33.0	67.7
Thomas *et al*[54]	2018	Bevacizumab + erlotinib	47	61	NR	32.0/68.0	40.0
		Sorafenib	43	61	NR	40.0/60.0	25.0
Abou-Alfa *et al*[55]	2019	Sorafenib + doxorubicin	180	62	85	36.1/63.9	-
		Sorafenib	176	62	87	39.8/60.2	-

Tak *et al*[27]	2018	Sorafenib	84	62	87	-	56.0
		Sorafenib + resminostat	86	65	80	-	51.8
Jouve *et al*[56]	2019	Sorafenib + pravastatin	162	68	96	-	29.0
		Sorafenib	161	68	88	-	30.4
Lee *et al*[57]	2016	AEG35156 + sorafenib	31	61	87	3.2/96.8	-
		Sorafenib	17	54	88	11.8/88.3	-
Assenat *et al*[58]	2019	Sorafenib + GEMOX	39	62	86	-	77.0
		Sorafenib	44	65	92	-	61.0
Azim *et al*[59]	2018	Sorafenib + tegafur–uracil	36	59	86	69.4/30.6	52.8
		Sorafenib	38	59	90	65.8/34.2	47.4
Koeberle *et al*[60]	2016	Sorafenib	46	65	87	72.0/28.0	57.0
		Sorafenib + everolimus	59	66	81	59.0/41.0	54.0
Bi *et al*[17]	2020	Donafinib	328	53	86	61.3/38.7	-
		Sorafenib	331	53	88	66.8/33.2	-
Qin *et al*[61]	2013	FOLFOX4	184	50	90	-	-
		Doxorubicin	187	49	87	-	-
Yeo *et al*[62]	2005	Doxorubicin	94	54	90	87.2/12.8	-
		PIAF	94	49	93	92.6/7.4	-

ECOG: Eastern Co-operative Oncology Group; FOLFOX4: Oxaliplatin/folinic acid/5-fluorouracil; GEMOX: Gemcitabine and oxaliplatin; NR: Not reported; PIAF: Cisplatin/interferon α-2b/doxorubicin/5-fluorouracil.

Safety

Grade ≥ 3 adverse events: In total, 17 studies reported data on the incidence of Grade ≥ 3 AEs, including 19 interventions and allowing 21 comparisons (Supplementary Figure 3A). No significant heterogeneity was detected between studies (tau-squared = 0; I^2 = 0%; P = 0.4493) and a fixed effect model was selected. Nivolumab ranked 2/19 (P = 0.9351), sorafenib ranked 8/19 (P = 0.5040), atezolizumab + bevacizumab ranked 11/19 (P = 0.4167), and lenvatinib ranked 16/19 (P = 0.2468) for incidence of Grade ≥ 3 AEs (higher ranking indicated a lower incidence of AEs) (Supplementary Figure 3B).

Treatment termination due to adverse events: A total of 13 studies reported the incidence of treatment termination due to AEs, including 13 interventions and allowing 15 comparisons (Supplementary Figure 3C). A degree of heterogeneity was detected between studies (tau-squared = 0.1536; I^2 = 65%; P = 0.0573) and a random effect model was selected. After ranking all interventions from the lowest to highest incidence of terminations due to AEs, vandetanib 300 mg/d and 100 mg/d were ranked first and second (P = 0.8036 and 0.7252, respectively), sorafenib ranked 5/13 (P = 0.5372), nintedanib ranked 8/13 (P = 0.4251), lenvatinib ranked 10/13 (P = 0.3907), and atezolizumab + bevacizumab ranked 13/13 (P = 0.2584) (Supplementary Figure 3D).

DISCUSSION

Following an expansion of first-line systemic treatment options for HCC over the past decade, international treatment guidelines now recommend sorafenib, lenvatinib, and atezolizumab plus bevacizumab in this setting, as well as nivolumab and FOLFOX (off-label use in many countries, but approved by the China National Medical Products Administration) for selected patients[4,12,13]. Numerous other therapies and combinations of therapies have also been unsuccessfully investigated in first-line advanced HCC management. However, most trials of systemic therapy for HCC used sorafenib as the comparator, as it was the only approved systemic therapy available at the time, and this limits the clinicians' ability to compare currently available treatment options. The present study represents one of the most comprehensive systematic

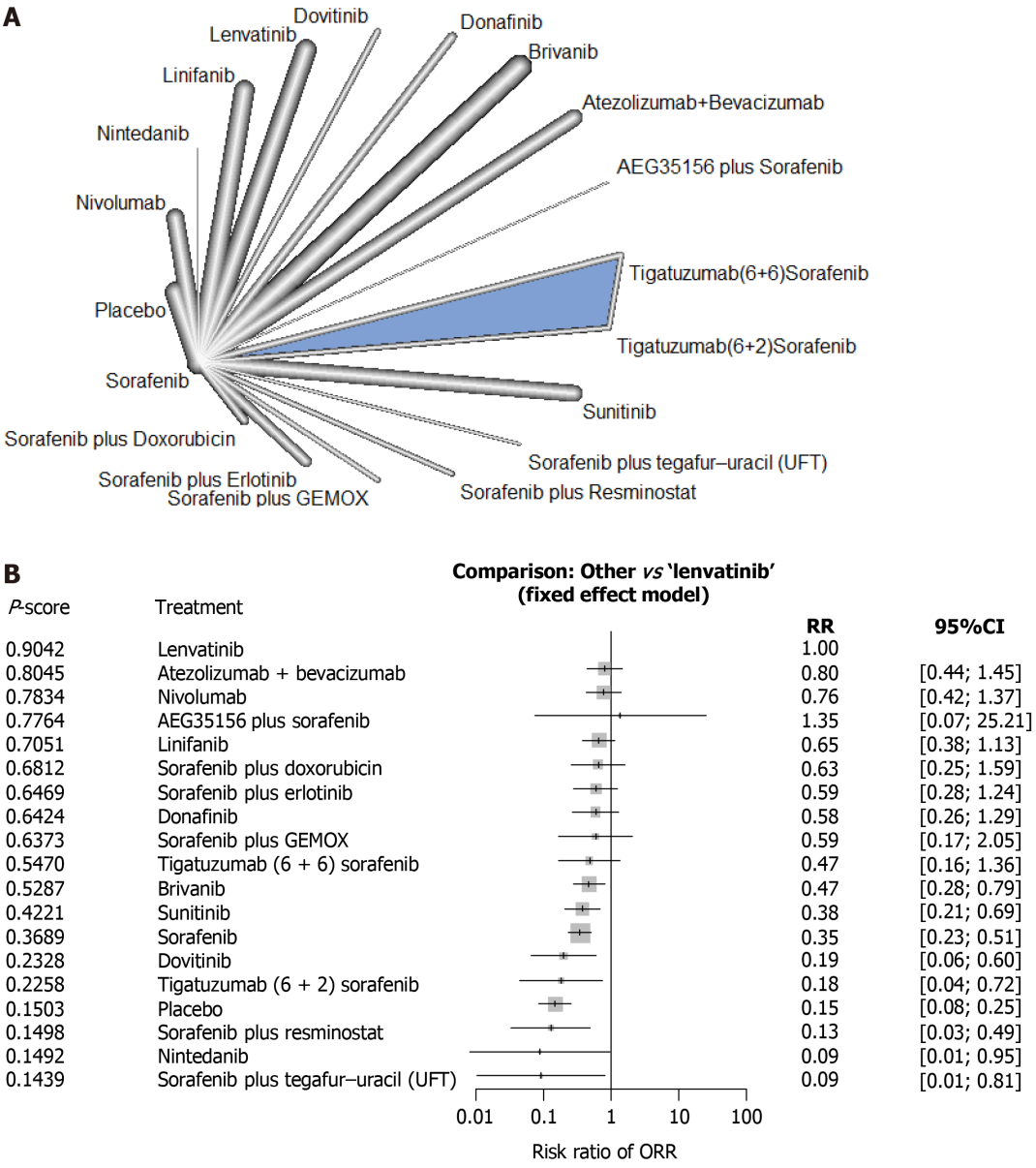
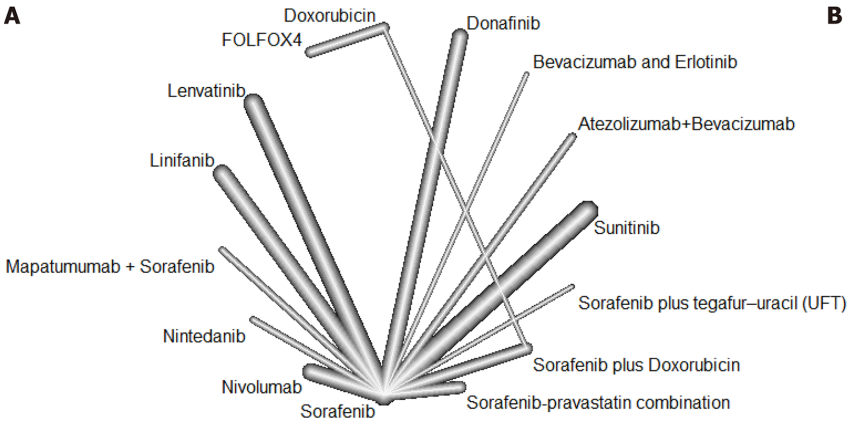


Figure 2 Response rates of first-line systemic therapy in patients with advanced hepatocellular carcinoma. A: Network diagram; B: Interventions ranked by *P* value with risk ratios and 95% confidence interval for overall response rate for each treatment *vs* lenvatinib. CI: Confidence interval; GEMOX: Gemcitabine and oxaliplatin; ORR: Overall response rate; RR: Risk ratio.

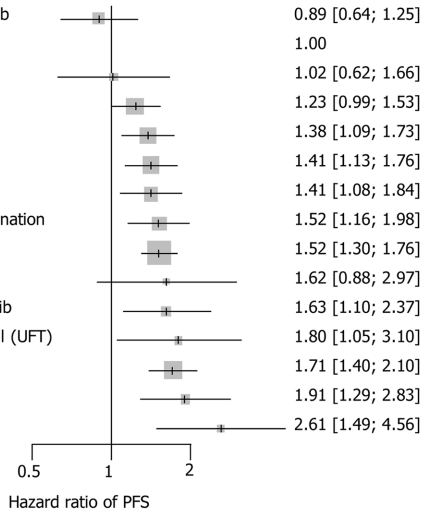
reviews and meta-analyses of first-line systemic treatments for advanced unresectable HCC conducted to date, and compares the treatment outcomes and safety of lenvatinib with multiple other systemic therapies, including immunotherapy (nivolumab) and combined therapy with immunotherapy and a TKI (atezolizumab + bevacizumab).

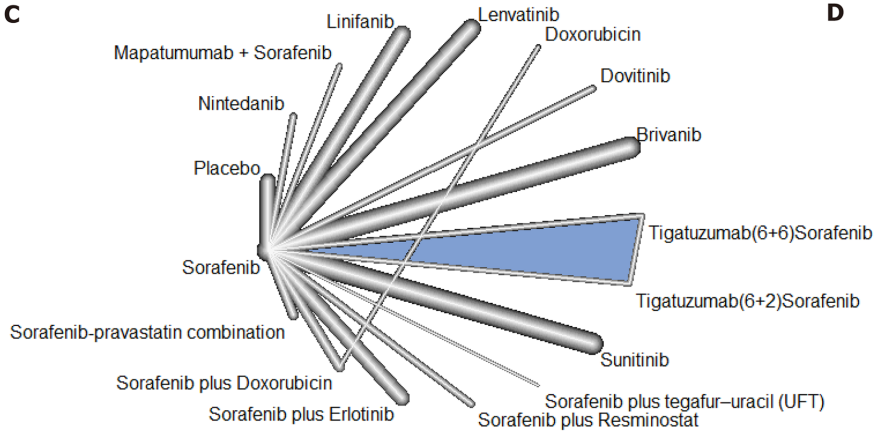
Our results show that atezolizumab plus bevacizumab is associated with the best OS outcomes of all therapies included in the analysis. This result is supported by findings from a recent meta-analysis that investigated optimal treatment sequencing for HCC and also reported that atezolizumab plus bevacizumab had a higher OS benefit *vs* lenvatinib (HR_{OS} = 0.63; 95%CI: 0.44-0.89), nivolumab (HR_{OS} = 0.68; 95%CI: 0.48-0.98), and sorafenib (HR_{OS} = 0.58; 95%CI: 0.42-0.80)[16]. Atezolizumab plus bevacizumab is the first combined immunotherapy and vascular-targeted regimen to be recommended as a first-line treatment option in the National Comprehensive Cancer Network HCC guidelines[4]. The long OS associated with atezolizumab plus bevacizumab may be related to the 'long tail' effect characteristic of immune checkpoint inhibitors, which was also observed in the Phase III Checkmate 459 study of nivolumab. A number of studies have identified several mechanisms by which angiogenesis-related processes can enhance immune checkpoint inhibitors therapy, including vascular normalization, reduction of hypoxia, and increasing tumor infilt-



B

P-score	Treatment	HR [95%CI]
0.9501	Atezolizumab + bevacizumab	0.89 [0.64; 1.25]
0.9041	Lenvatinib	1.00
0.8490	Bevacizumab and erlotinib	1.02 [0.62; 1.66]
0.7411	Linifanib	1.23 [0.99; 1.53]
0.5899	Donafinib	1.38 [1.09; 1.73]
0.5548	Nivolumab	1.41 [1.13; 1.76]
0.5511	Sorafenib plus doxorubicin	1.41 [1.08; 1.84]
0.4347	Sorafenib-pravastatin combination	1.52 [1.16; 1.98]
0.4246	Sorafenib	1.52 [1.30; 1.76]
0.3951	FOLFOX4	1.62 [0.88; 2.97]
0.3599	Mapatumumab plus sorafenib	1.63 [1.10; 2.37]
0.2738	Sorafenib plus tegafur–uracil (UFT)	1.80 [1.05; 3.10]
0.2422	Sunitinib	1.71 [1.40; 2.10]
0.1883	Nintedanib	1.91 [1.29; 2.83]
0.0414	Doxorubicin	2.61 [1.49; 4.56]

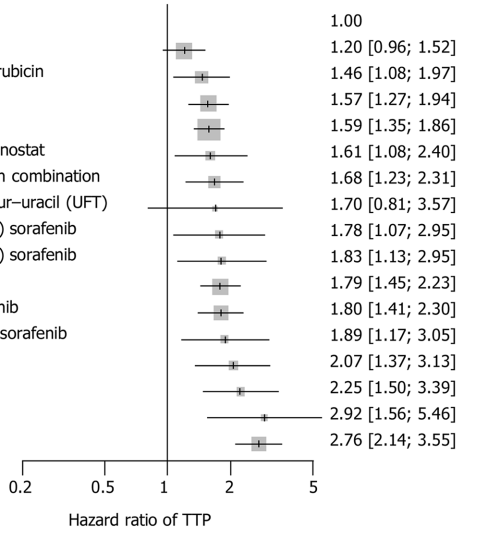




D

Comparison: Other *vs* 'lenvatinib' (fixed effect model)

P-score	Treatment	HR [95%CI]
0.9888	Lenvatinib	1.00
0.9067	Linifanib	1.20 [0.96; 1.52]
0.7344	Sorafenib plus doxorubicin	1.46 [1.08; 1.97]
0.6562	Brivanib	1.57 [1.27; 1.94]
0.6484	Sorafenib	1.59 [1.35; 1.86]
0.5944	Sorafenib plus resminostat	1.61 [1.08; 2.40]
0.5426	Sorafenib-pravastatin combination	1.68 [1.23; 2.31]
0.5265	Sorafenib plus tegafur–uracil (UFT)	1.70 [0.81; 3.57]
0.4797	Tigatuzumab (6 + 2) sorafenib	1.78 [1.07; 2.95]
0.4473	Tigatuzumab (6 + 6) sorafenib	1.83 [1.13; 2.95]
0.4370	Sunitinib	1.79 [1.45; 2.23]
0.4360	Sorafenib plus erlotinib	1.80 [1.41; 2.30]
0.4081	Mapatumumab plus sorafenib	1.89 [1.17; 3.05]
0.3013	Nintedanib	2.07 [1.37; 3.13]
0.2222	Dovitinib	2.25 [1.50; 3.39]
0.1003	Doxorubicin	2.92 [1.56; 5.46]
0.0702	Placebo	2.76 [2.14; 3.55]



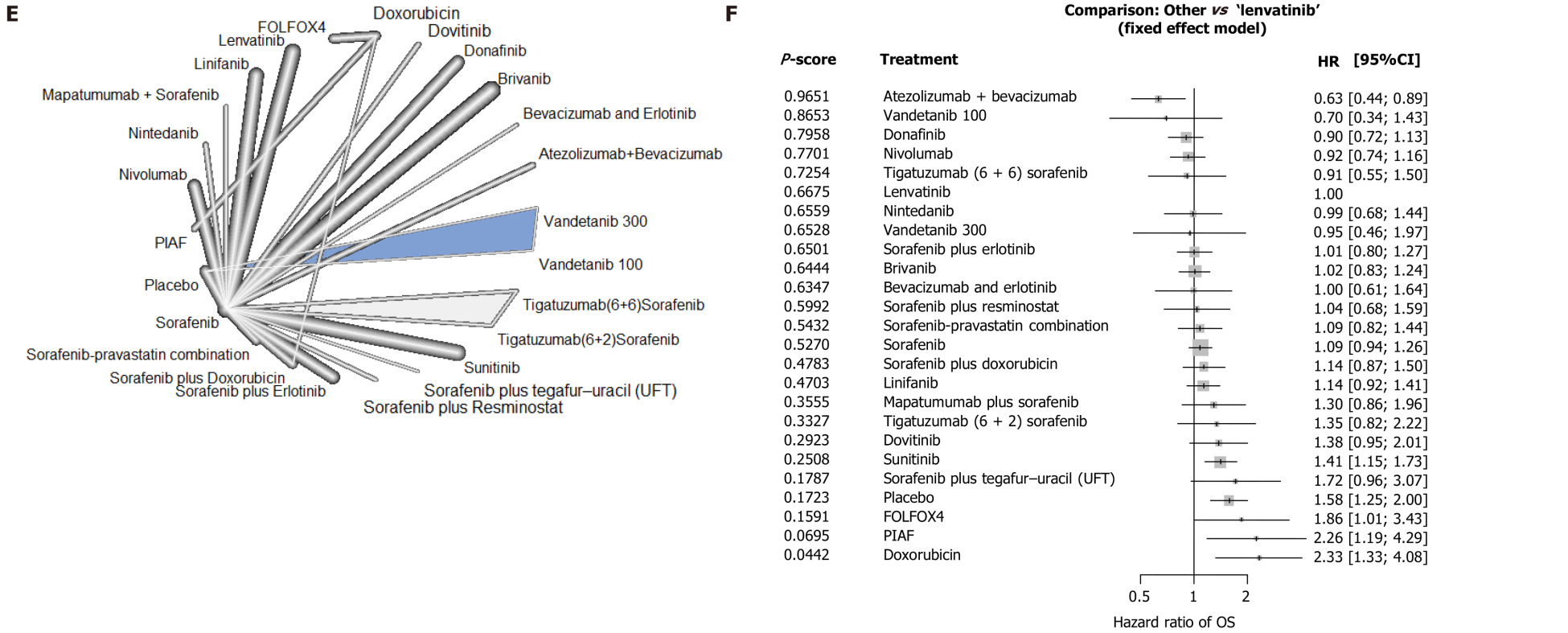


Figure 3 Survival outcomes in patients with advanced hepatocellular carcinoma following first-line systemic therapy. A, C, and E: Network diagrams; B: Interventions ranked by *P* value with hazard ratios for progression-free survival, D: Time to progression and F: overall survival for each treatment *vs* lenvatinib. CI: Confidence interval; FOLFOX4: Oxaliplatin/folinic acid/5-fluorouracil; HCC: Hepatocellular carcinoma; HR: Hazard ratio; OS: Overall survival; PIAF: Cisplatin/interferon α-2b/doxorubicin/5-fluorouracil; TTP: Time to progression.

rating lymphocytes[30]. Although bevacizumab monotherapy failed Phase II trials in unresectable HCC, in combination with atezolizumab it led to superior efficacy compared with bevacizumb monotherapy[31]. However, consideration of treatment safety and tolerability is also an important factor in clinical decision-making. Our analysis revealed that atezolizumab plus bevacizumab was associated with the highest incidence of discontinuation due to AEs. This may be associated with the relatively long time to progression and duration of treatment reported for atezolizumab plus bevacizumab, but as treatment discontinuations due to AEs usually involve uncontrolled Grade ≥ 3 AEs, this would likely be a weak association. In addition, the prescribing information for bevacizumab highlights a possible risk of bleeding, and requires termination of bevacizumab at least 4 wk before surgery[32]. Therefore, in

patients with high risk of gastric esophageal varices and patients with the potential to undergo any surgical procedures, atezolizumab plus bevacizumab should be used carefully, to manage the risk of bleeding events. Atezolizumab plus bevacizumab may be more suitable for patients who are unsuitable for surgery but with good liver function and limited cirrhosis, who have the potential to achieve a long-term survival benefit with systemic therapy.

The results of this meta-analysis show that there is currently not one single systemic treatment for advanced HCC associated with superior outcomes across all outcome measurements (ORR, OS, PFS, and safety). This highlights the importance of individualized treatment selection based on specific treatment goals. For example, a number of studies have shown that lenvatinib or lenvatinib combination therapy[33] can allow patients to achieve downstaging and become eligible for surgery[34-36]. For patients with HCC ineligible for surgical intervention at diagnosis, we are of the opinion that treatment selection should be objective based. In patients without serious underlying liver disease and for whom surgery may be possible, systemic treatments with the highest ORR are the optimal choice. Conversely, for patients with poor liver function, underlying liver disease, or local advanced HCC, selection of therapies based on longer OS may provide the most benefit.

In our analysis, lenvatinib had superior short-term efficacy compared with all other systemic therapies investigated. Lenvatinib ranked first for ORR and TTP, and second for PFS after atezolizumab plus bevacizumab. This finding is supported by the results of another recent network meta-analysis presented at the ASCO Gastrointestinal Symposium 2021 that also ranked atezolizumab plus bevacizumab first for OS but lenvatinib first for ORR[37]. In addition, although direct comparison of the ORRs (RECIST v1.1) reported for atezolizumab plus bevacizumab and lenvatinib in the IMbrave 100 and REFLECT studies appears to show a moderately higher ORR for atezolizumab plus bevacizumab (27% *vs* 18%)[10,11], our network analysis provides a more robust comparison of the two therapies by comparing both to sorafenib. There are several possible mechanistic explanations for this finding. First, preclinical studies show that lenvatinib has multiple targets including VEGFR1-3, FGFR1-4, PDGFRα, RET, and KIT, and this broad spectrum of activity may be one factor explaining the high response rates associated with this therapy[38]. Furthermore, lenvatinib is a type V TKI with fast binding and relatively slow dissociation compared with other TKIs[39]. In addition to anti-vascular effects, lenvatinib also has a regulatory effect on the immune microenvironment of liver cancer[40]. Preclinical research has shown that, compared with sorafenib, lenvatinib has a significant anti-tumor effect in immunodeficient mice, suggesting that lenvatinib may activate immune function by decreasing the number of tumor-associated macrophages, increasing the proportion of activated CD8+ cells[40], and increasing activation and infiltration of natural killer cells[41].

HBV-related liver cancer is particularly prevalent in Asian populations, especially in China where 69%-80% of liver cancers have an HBV etiology[42,43]. Our meta-analysis of data from patients with HCC and HBV infection suggested that, in terms of OS, atezolizumab plus bevacizumab, lenvatinib, and nivolumab are the three most effective treatments in this patient population. For PFS, lenvatinib ranked first over atezolizumab plus bevacizumab. This finding supports previous meta-analyses that have shown lenvatinib to have a particularly strong anti-tumor effect *vs* sorafenib in patients with HBV-related HCC[44,45]. It is unclear why lenvatinib may have a particularly good anti-tumor effect in HBV-related HCC, but it may be due to the impact of lenvatinib on the immune microenvironment, as described above. In addition, the China National Health and Health Commission guidelines for diagnosis and treatment of primary liver cancer in China (2019 edition) recommend lenvatinib as a systemic therapy with good efficacy in patients with HBV-related liver cancer[46].

This network meta-analysis had several possible limitations. First, the quality of studies included in the analysis had some heterogeneity; for example, the analysis included both large Phase III clinical trials, such as REFLECT and Checkmate 459, and smaller Phase II clinical studies. Second, there was also heterogeneity in the patient populations included in the analysis, including patients from different geographic regions, of different races, and different proportions of patients with HBV infection. Additionally, it should be noted that second-line therapeutic options for HCC have greatly improved over the past decade. As a result, estimates of first-line OS from older studies are generally shorter than those from more recent studies. However, among the therapies included in this analysis that are currently approved for first-line HCC, only sorafenib has OS data old enough to potentially be biased by this phenomenon. Fortunately, our analysis was based on pooled data from the pivotal study of sorafenib in 2008 and comparator arms of trials conducted between 2008 and 2020, which limits the potential effect of bias from improvements in second-line

therapies[47-62]. Finally, because multiple interventions were included in the analysis, several had data from only one study and therefore a relatively small sample size, which may have led to bias.

CONCLUSION

This network meta-analysis of first-line systemic therapies for advanced HCC revealed that atezolizumab plus bevacizumab is associated with the best OS and PFS, but also with a high incidence of discontinuation due to AEs. The results also showed that lenvatinib is associated with the best ORR of all systemic therapies included in the analysis, as well as a relatively high PFS, particularly in patients with HBV-related liver cancer in whom lenvatinib ranked first for PFS, over atezolizumab plus bevacizumab. Therefore, in patients with unresectable advanced HCC, systemic treatment should be selected based on the individualized treatment goals of each patient.

ARTICLE HIGHLIGHTS

Research background

The recent expansion of first-line systemic therapy options for patients with advanced hepatocellular carcinoma represents a significant advance in the treatment of this disease. However, the majority of clinical trials in first-line hepatocellular carcinoma management used placebo or sorafenib as comparators, and there are limited data providing a cross comparison of the efficacy and safety of treatments in this setting, especially for newly-approved immune checkpoint inhibitor and vascular endothelial growth factor inhibitor combination treatments.

Research motivation

Clinical trials of recently-approved therapies for hepatocellular carcinoma have revealed differing profiles of efficacy and safety, and comparative data to inform selection of first-line treatments for individual patients are limited. Furthermore, although lenvatinib is widely seen as a standard of care in real clinical practice, and is a recommended first-line therapy in most international treatment guidelines, there are limited head-to-head data comparing lenvatinib with other systemic therapies.

Research objectives

The objectives of this network meta-analysis were to systematically review and compare the response rates, survival outcomes, and safety of first-line systemic therapies for advanced hepatocellular carcinoma, and to provide a comparison between lenvatinib and other systemic therapies in this setting. The study also included a sub-group analysis of patients with hepatitis B virus infection, which is an important population in the Asia-Pacific region and has not been covered by other current meta-analyses.

Research methods

We searched PubMed, Science Direct, the Cochrane Database, Excerpta Medica Database, and abstracts from the American Society of Clinical Oncology 2020 annual congress. Eligible studies were randomized controlled trials of systemic therapy enrolling adults with advanced/unresectable hepatocellular carcinoma. A network meta-analysis was used to synthesize data and perform direct and indirect comparisons between treatments for endpoints including (where available) overall response rate, overall survival, progression-free survival, time-to-progression, incidence of Grade ≥ 3 adverse events, incidence of treatment interruptions due to adverse events, and incidence of dose reductions due to adverse events. *P* value, a frequentist analog to the surface under the cumulative ranking curve, was used to rank treatments.

Research results

Treatments included in the analysis were atezolizumab plus bevacizumab, brivanib, donafenib, dovitinib, FOLFOX4, lenvatinib, linifanib, nintedanib, nivolumab, sorafenib, sunitinib, vandetanib, 11 sorafenib combination therapies, and three other

combination therapies. Atezolizumab plus bevacizumab was ranked first for progression-free survival and overall survival but also had the highest rate of discontinuations due to adverse events. Lenvatinib ranked first for overall response rate and second for progression-free survival. Our findings show that first-line systemic treatment should be selected based on individualized treatment goals and provide valuable comparative data that can help to inform treatment decisions.

Research conclusions

Our findings suggest that there is no one single first-line treatment for advanced hepatocellular carcinoma associated with superior outcomes across all outcome measurements. Therefore, first-line systemic treatment should be selected based on individualized treatment goals.

Research perspectives

Future research should continue to evaluate new therapeutic strategies for hepatocellular carcinoma in the context of existing treatments, and provide further information to support treatment selection for individual patients.

REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Zhou M**, Wang H, Zhu J, Chen W, Wang L, Liu S, Li Y, Liu Y, Yin P, Liu J, Yu S, Tan F, Barber RM, Coates MM, Dicker D, Fraser M, González-Medina D, Hamavid H, Hao Y, Hu G, Jiang G, Kan H, Lopez AD, Phillips MR, She J, Vos T, Wan X, Xu G, Yan LL, Yu C, Zhao Y, Zheng Y, Zou X, Naghavi M, Wang Y, Murray CJ, Yang G, Liang X. Cause-specific mortality for 240 causes in China during 1990-2013: a systematic subnational analysis for the Global Burden of Disease Study 2013. *Lancet* 2016; **387**: 251-272 [PMID: 26510778 DOI: 10.1016/S0140-6736(15)00551-6]

3 **Allemann P**, Demartines N, Bouzourene H, Tempia A, Halkic N. Long-term outcome after liver resection for hepatocellular carcinoma larger than 10 cm. *World J Surg* 2013; **37**: 452-458 [PMID: 23188527 DOI: 10.1007/s00268-012-1840-5]

4 **National Comprehensive Cancer Network**. Hepatobiliary cancers 2020 [cited 20 March 2021]. Available from: https://www.abc-directory.com/site/1034756

5 **Park JW**, Chen M, Colombo M, Roberts LR, Schwartz M, Chen PJ, Kudo M, Johnson P, Wagner S, Orsini LS, Sherman M. Global patterns of hepatocellular carcinoma management from diagnosis to death: the BRIDGE Study. *Liver Int* 2015; **35**: 2155-2166 [PMID: 25752327 DOI: 10.1111/Liv.12818]

6 **Forner A**, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018; **391**: 1301-1314 [PMID: 29307467 DOI: 10.1016/S0140-6736(18)30010-2]

7 **Li D**, Sedano S, Allen R, Gong J, Cho M, Sharma S. Current Treatment Landscape for Advanced Hepatocellular Carcinoma: Patient Outcomes and the Impact on Quality of Life. *Cancers (Basel)* 2019; **11** [PMID: 31216701 DOI: 10.3390/cancers11060841]

8 **Bruix J**, Raoul JL, Sherman M, Mazzaferro V, Bolondi L, Craxi A, Galle PR, Santoro A, Beaugrand M, Sangiovanni A, Porta C, Gerken G, Marrero JA, Nadel A, Shan M, Moscovici M, Voliotis D, Llovet JM. Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. *J Hepatol* 2012; **57**: 821-829 [PMID: 22727733 DOI: 10.1016/j.jhep.2012.06.014]

9 **Bruix J**, da Fonseca LG, Reig M. Insights into the success and failure of systemic therapy for hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 617-630 [PMID: 31371809 DOI: 10.1038/s41575-019-0179-x]

10 **Kudo M**, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, Baron A, Park JW, Han G, Jassem J, Blanc JF, Vogel A, Komov D, Evans TRJ, Lopez C, Dutcus C, Guo M, Saito K, Kraljevic S, Tamai T, Ren M, Cheng AL. Lenvatinib *vs* sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 2018; **391**: 1163-1173 [PMID: 29433850 DOI: 10.1016/S0140-6736(18)30207-1]

11 **Finn RS**, Qin S, Ikeda M, Galle PR, Ducreux M, Kim TY, Kudo M, Breder V, Merle P, Kaseb AO, Li D, Verret W, Xu DZ, Hernandez S, Liu J, Huang C, Mulla S, Wang Y, Lim HY, Zhu AX, Cheng AL; IMbrave150 Investigators. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *N Engl J Med* 2020; **382**: 1894-1905 [PMID: 32402160 DOI: 10.1056/NEJMoa1915745]

12 **Heimbach JK**. Overview of the Updated AASLD Guidelines for the Management of HCC. *Gastroenterol Hepatol (N Y)* 2017; **13**: 751-753 [PMID: 29339953]

13 **Vogel A**, Cervantes A, Chau I, Daniele B, Llovet JM, Meyer T, Nault JC, Neumann U, Ricke J, Sangro B, Schirmacher P, Verslype C, Zech CJ, Arnold D, Martinelli E; ESMO Guidelines Committee. Hepatocellular carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2018; **29**: iv238-iv255 [PMID: 30285213 DOI: 10.1093/annonc/mdy308]

14 **Kudo M**. Systemic Therapy for Hepatocellular Carcinoma: Latest Advances. *Cancers (Basel)* 2018; **10** [PMID: 30380773 DOI: 10.3390/cancers10110412]

15 **Ding W**, Tan Y, Qian Y, Xue W, Wang Y, Jiang P, Xu X. First-line targ veted therapies of advanced hepatocellular carcinoma: A Bayesian network analysis of randomized controlled trials. *PLoS One* 2020; **15**: e0229492 [PMID: 32134981 DOI: 10.1371/journal.pone.0229492]

16 **Sonbol MB**, Riaz IB, Naqvi SAA, Almquist DR, Mina S, Almasri J, Shah S, Almader-Douglas D, Uson Junior PLS, Mahipal A, Ma WW, Jin Z, Mody K, Starr J, Borad MJ, Ahn DH, Murad MH, Bekaii-Saab T. Systemic Therapy and Sequencing Options in Advanced Hepatocellular Carcinoma: A Systematic Review and Network Meta-analysis. *JAMA Oncol* 2020; **6**: e204930 [PMID: 33090186 DOI: 10.1001/jamaoncol.2020.4930]

17 **Bi F**, Qin SK, Gu SZ, Bai YX, Chen ZD, Wang ZS, Ying J, Lu YY, Meng ZHQ, Pan HM, Yang P, Zhang HL, Chen X, Xu AB, Liu XF, Meng Q, Wu LQ, Chen F. Donafenib *vs* Sorafenib as first-line therapy in advanced hepatocellular carcinoma: an open-label, randomized, multicentre phase II/III trial. *J Clin Oncol* 2020; **38**: 4506-4506 [DOI: 10.1200/JCO.2020.38.15_suppl.4506]

18 **Higgins JP**, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, Savovic J, Schulz KF, Weeks L, Sterne JA; Cochrane Bias Methods Group; Cochrane Statistical Methods Group. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ* 2011; **343**: d5928 [PMID: 22008217 DOI: 10.1136/bmj.d5928]

19 **Rücker G**. Network meta-analysis, electrical networks and graph theory. *Res Synth Methods* 2012; **3**: 312-324 [PMID: 26053424 DOI: 10.1002/jrsm.1058]

20 **Rücker G**, Schwarzer G. Reduce dimension or reduce weights? *Stat Med* 2014; **33**: 4353-4369 [PMID: 24942211 DOI: 10.1002/sim.6236]

21 **Rücker G**, Schwarzer G. Ranking treatments in frequentist network meta-analysis works without resampling methods. *BMC Med Res Methodol* 2015; **15**: 58 [PMID: 26227148 DOI: 10.1186/s12874-015-0060-8]

22 **Johnson PJ**, Qin S, Park JW, Poon RT, Raoul JL, Philip PA, Hsu CH, Hu TH, Heo J, Xu J, Lu L, Chao Y, Boucher E, Han KH, Paik SW, Robles-Aviña J, Kudo M, Yan L, Sobhonslidsuk A, Komov D, Decaens T, Tak WY, Jeng LB, Liu D, Ezzeddine R, Walters I, Cheng AL. Brivanib *vs* sorafenib as first-line therapy in patients with unresectable, advanced hepatocellular carcinoma: results from the randomized phase III BRISK-FL study. *J Clin Oncol* 2013; **31**: 3517-3524 [PMID: 23980084 DOI: 10.1200/JCO.2012.48.4410]

23 **Yau T**, Park JW, Finn RS, Cheng AL, Mathurin P, Edeline J, Kudo M, Han KH, Harding JJ, Merle P, Rosmorduc O, Wyrwicz L, Schott E, Choo SP, Kelley RK, Begic D, Chen G, Neely J, Anderson J, Sangro B. CheckMate 459: A randomized, multi-center phase III study of nivolumab (NIVO) *vs* sorafenib (SOR) as first-line (1L) treatment in patients (pts) with advanced hepatocellular carcinoma (aHCC). *Ann Oncol* 2019; **30**: 874 [DOI: 10.1093/annonc/mdz394.029]

24 **Cainap C**, Qin S, Huang WT, Chung IJ, Pan H, Cheng Y, Kudo M, Kang YK, Chen PJ, Toh HC, Gorbunova V, Eskens FA, Qian J, McKee MD, Ricker JL, Carlson DM, El-Nowiem S. Linifanib *vs* Sorafenib in patients with advanced hepatocellular carcinoma: results of a randomized phase III trial. *J Clin Oncol* 2015; **33**: 172-179 [PMID: 25488963 DOI: 10.1200/JCO.2013.54.3298]

25 **Cheng AL**, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25-34 [PMID: 19095497 DOI: 10.1016/S1470-2045(08)70285-7]

26 **Zhu AX**, Rosmorduc O, Evans TR, Ross PJ, Santoro A, Carrilho FJ, Bruix J, Qin S, Thuluvath PJ, Llovet JM, Leberre MA, Jensen M, Meinhardt G, Kang YK. SEARCH: a phase III, randomized, double-blind, placebo-controlled trial of sorafenib plus erlotinib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 2015; **33**: 559-566 [PMID: 25547503 DOI: 10.1200/JCO.2013.53.7746]

27 **Tak WY**, Ryoo BY, Lim HY, Kim DY, Okusaka T, Ikeda M, Hidaka H, Yeon JE, Mizukoshi E, Morimoto M, Lee MA, Yasui K, Kawaguchi Y, Heo J, Morita S, Kim TY, Furuse J, Katayama K, Aramaki T, Hara R, Kimura T, Nakamura O, Kudo M. Phase I/II study of first-line combination therapy with sorafenib plus resminostat, an oral HDAC inhibitor, *vs* sorafenib monotherapy for advanced hepatocellular carcinoma in east Asian patients. *Invest New Drugs* 2018; **36**: 1072-1084 [PMID: 30198057 DOI: 10.1007/s10637-018-0658-x]

28 **Cheng AL**, Kang YK, He AR, Lim HY, Ryoo BY, Hung CH, Sheen IS, Izumi N, Austin T, Wang Q, Greenberg J, Shiratori S, Beckman RA, Kudo M; Investigators' Study Group. Safety and efficacy of tigatuzumab plus sorafenib as first-line therapy in subjects with advanced hepatocellular carcinoma: A phase 2 randomized study. *J Hepatol* 2015; **63**: 896-904 [PMID: 26071796 DOI: 10.1016/j.jhep.2015.06.001]

29 **Cheng AL**, Kang YK, Lin DY, Park JW, Kudo M, Qin S, Chung HC, Song X, Xu J, Poggi G, Omata M, Pitman Lowenthal S, Lanzalone S, Yang L, Lechuga MJ, Raymond E. Sunitinib *vs* sorafenib in advanced hepatocellular cancer: results of a randomized phase III trial. *J Clin Oncol* 2013; **31**: 4067-4075 [PMID: 24081937 DOI: 10.1200/JCO.2012.45.8372]

30 **Yi M**, Jiao D, Qin S, Chu Q, Wu K, Li A. Synergistic effect of immune checkpoint blockade and anti-angiogenesis in cancer treatment. *Mol Cancer* 2019; **18**: 60 [PMID: 30925919 DOI: 10.1186/s12943-019-0974-6]

31 **Lee MS**, Ryoo BY, Hsu CH, Numata K, Stein S, Verret W, Hack SP, Spahn J, Liu B, Abdullah H,

Wang Y, He AR, Lee KH; GO30140 investigators. Atezolizumab with or without bevacizumab in unresectable hepatocellular carcinoma (GO30140): an open-label, multicentre, phase 1b study. *Lancet Oncol* 2020; **21**: 808-820 [PMID: 32502443 DOI: 10.1016/S1470-2045(20)30156-X]

32 **ten Doesschate T**, van Haren E, Wijma RA, Koch BCP. The effectiveness of nitrofurantoin, fosfomycin and trimethoprim for the treatment of cystitis in relation to renal function. *Clin Microbiol Infec* 2020; **26** [DOI: 10.1016/j.cmi.2020.03.001]

33 **Kudo M**, Ueshima K, Chan S, Minami T, Chishina H, Aoki T, Takita M, Hagiwara S, Minami Y, Ida H, Takenaka M, Sakurai T, Watanabe T, Morita M, Ogawa C, Wada Y, Ikeda M, Ishii H, Izumi N, Nishida N. Lenvatinib as an Initial Treatment in Patients with Intermediate-Stage Hepatocellular Carcinoma Beyond Up-To-Seven Criteria and Child-Pugh A Liver Function: A Proof-Of-Concept Study. *Cancers (Basel)* 2019; **11** [PMID: 31370183 DOI: 10.3390/cancers11081084]

34 **Sun HC**, Zhu XD, Huang C, Shen YH, Ge NL, Chen Y, Tan CJ, Zhou J, Fan J. Combination therapy with lenvatinib and anti-PD-1 antibodies for unresectable or advanced hepatocellular carcinoma: A real-world study. *J Clin Oncol* 2020; **38**: e16610-e16610 [DOI: 10.1200/JCO.2020.38.15_suppl.e16610]

35 **Chen X**, Zhang Y, Zhang N, Ge Y, Jia W. Lenvatinib combined nivolumab injection followed by extended right hepatectomy is a feasible treatment for patients with massive hepatocellular carcinoma: a case report. *Onco Targets Ther* 2019; **12**: 7355-7359 [PMID: 31686845 DOI: 10.2147/OTT.S217123]

36 **He M**, Li Q, Zou R, Shen J, Fang W, Tan G, Zhou Y, Wu X, Xu L, Wei W, Le Y, Zhou Z, Zhao M, Guo Y, Guo R, Chen M, Shi M. Sorafenib Plus Hepatic Arterial Infusion of Oxaliplatin, Fluorouracil, and Leucovorin *vs* Sorafenib Alone for Hepatocellular Carcinoma With Portal Vein Invasion: A Randomized Clinical Trial. *JAMA Oncol* 2019; **5**: 953-960 [PMID: 31070690 DOI: 10.1001/jamaoncol.2019.0250]

37 **Park R**, Silva L, Nissaisorakarn V, Riano I, Saeed A. Comparison of systemic therapy efficacy in advanced hepatocellular carcinoma: Systematic review and frequentist network meta-analysis of randomized controlled trials. *J Clin Oncol* 2021; **39**: 293-293 [DOI: 10.1200/JCO.2021.39.3_suppl.293]

38 **Yamamoto Y**, Matsui J, Matsushima T, Obaishi H, Miyazaki K, Nakamura K, Tohyama O, Semba T, Yamaguchi A, Hoshi SS, Mimura F, Haneda T, Fukuda Y, Kamata JI, Takahashi K, Matsukura M, Wakabayashi T, Asada M, Nomoto KI, Watanabe T, Dezso Z, Yoshimatsu K, Funahashi Y, Tsuruoka A. Lenvatinib, an angiogenesis inhibitor targeting VEGFR/FGFR, shows broad antitumor activity in human tumor xenograft models associated with microvessel density and pericyte coverage. *Vasc Cell* 2014; **6**: 18 [PMID: 25197551 DOI: 10.1186/2045-824X-6-18]

39 **Okamoto K**, Ikemori-Kawada M, Jestel A, von König K, Funahashi Y, Matsushima T, Tsuruoka A, Inoue A, Matsui J. Distinct binding mode of multikinase inhibitor lenvatinib revealed by biochemical characterization. *ACS Med Chem Lett* 2015; **6**: 89-94 [PMID: 25589937 DOI: 10.1021/mL500394m]

40 **Kato Y**, Tabata K, Kimura T, Yachie-Kinoshita A, Ozawa Y, Yamada K, Ito J, Tachino S, Hori Y, Matsuki M, Matsuoka Y, Ghosh S, Kitano H, Nomoto K, Matsui J, Funahashi Y. Lenvatinib plus anti-PD-1 antibody combination treatment activates CD8+ T cells through reduction of tumor-associated macrophage and activation of the interferon pathway. *PLoS One* 2019; **14**: e0212513 [PMID: 30811474 DOI: 10.1371/journal.pone.0212513]

41 **Zhang Q**, Liu H, Wang H, Lu M, Miao Y, Ding J, Li H, Gao X, Sun S, Zheng J. Lenvatinib promotes antitumor immunity by enhancing the tumor infiltration and activation of NK cells. *Am J Cancer Res* 2019; **9**: 1382-1395 [PMID: 31392076]

42 **Zhu RX**, Seto WK, Lai CL, Yuen MF. Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region. *Gut Liver* 2016; **10**: 332-339 [PMID: 27114433 DOI: 10.5009/gnl15257]

43 **Fan JH**, Wang JB, Jiang Y, Xiang W, Liang H, Wei WQ, Qiao YL, Boffetta P. Attributable causes of liver cancer mortality and incidence in china. *Asian Pac J Cancer Prev* 2013; **14**: 7251-7256 [PMID: 24460283 DOI: 10.7314/apjcp.2013.14.12.7251]

44 **Casadei Gardini A**, Puzzoni M, Montagnani F, Marisi G, Tamburini E, Cucchetti A, Solaini L, Foschi FG, Conti F, Ercolani G, Cascinu S, Scartozzi M. Profile of lenvatinib in the treatment of hepatocellular carcinoma: design, development, potential place in therapy and network meta-analysis of hepatitis B and hepatitis C in all Phase III trials. *Onco Targets Ther* 2019; **12**: 2981-2988 [PMID: 31118665 DOI: 10.2147/OTT.S192572]

45 **Park J**, Cho J, Lim JH, Lee MH, Kim J. Relative Efficacy of Systemic Treatments for Patients with Advanced Hepatocellular Carcinoma According to Viral Status: A Systematic Review and Network Meta-Analysis. *Target Oncol* 2019; **14**: 395-403 [PMID: 31290003 DOI: 10.1007/s11523-019-00651-7]

46 **Department of Medical Administration**, National Health and Health Commission of the People's Republic of China. [Guidelines for diagnosis and treatment of primary liver cancer in China (2019 edition)]. *Zhonghua Gan Zang Bing Za Zhi* 2020; **28**: 112-128 [PMID: 32164061 DOI: 10.3760/cma.j.issn.1007-3418.2020.02.004]

47 **Yen CJ**, Kim TY, Feng YH, Chao Y, Lin DY, Ryoo BY, Huang DC, Schnell D, Hocke J, Loembé AB, Cheng AL. A Phase I/Randomized Phase II Study to Evaluate the Safety, Pharmacokinetics, and Efficacy of Nintedanib *vs* Sorafenib in Asian Patients with Advanced Hepatocellular Carcinoma. *Liver Cancer* 2018; **7**: 165-178 [PMID: 29888206 DOI: 10.1159/000486460]

48 **Ciuleanu T**, Bazin I, Lungulescu D, Miron L, Bondarenko I, Deptala A, Rodriguez-Torres M, Giantonio B, Fox NL, Wissel P, Egger J, Ding M, Kalyani RN, Humphreys R, Gribbin M, Sun W. A

randomized, double-blind, placebo-controlled phase II study to assess the efficacy and safety of mapatumumab with sorafenib in patients with advanced hepatocellular carcinoma. *Ann Oncol* 2016; **27**: 680-687 [PMID: 26802147 DOI: 10.1093/annonc/mdw004]

49 **Abou-Alfa GK**, Johnson P, Knox JJ, Capanu M, Davidenko I, Lacava J, Leung T, Gansukh B, Saltz LB. Doxorubicin plus sorafenib *vs* doxorubicin alone in patients with advanced hepatocellular carcinoma: a randomized trial. *JAMA* 2010; **304**: 2154-2160 [PMID: 21081728 DOI: 10.1001/jama.2010.1672]

50 **Cheng AL**, Thongprasert S, Lim HY, Sukeepaisarnjaroen W, Yang TS, Wu CC, Chao Y, Chan SL, Kudo M, Ikeda M, Kang YK, Pan H, Numata K, Han G, Balsara B, Zhang Y, Rodriguez AM, Wang Y, Poon RT. Randomized, open-label phase 2 study comparing frontline dovitinib *vs* sorafenib in patients with advanced hepatocellular carcinoma. *Hepatology* 2016; **64**: 774-784 [PMID: 27082062 DOI: 10.1002/hep.28600]

51 **Hsu C**, Yang TS, Huo TI, Hsieh RK, Yu CW, Hwang WS, Hsieh TY, Huang WT, Chao Y, Meng R, Cheng AL. Vandetanib in patients with inoperable hepatocellular carcinoma: a phase II, randomized, double-blind, placebo-controlled study. *J Hepatol* 2012; **56**: 1097-1103 [PMID: 22245891 DOI: 10.1016/j.jhep.2011.12.013]

52 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

53 **Palmer DH**, Ma YT, Peck-Radosavljevic M, Ross P, Graham J, Fartoux L, Deptala A, Studeny M, Schnell D, Hocke J, Loembé AB, Meyer T. A multicentre, open-label, phase-I/randomised phase-II study to evaluate safety, pharmacokinetics, and efficacy of nintedanib vs. sorafenib in European patients with advanced hepatocellular carcinoma. *Br J Cancer* 2018; **118**: 1162-1168 [PMID: 29563636 DOI: 10.1038/s41416-018-0051-8]

54 **Thomas MB**, Garrett-Mayer E, Anis M, Anderton K, Bentz T, Edwards A, Brisendine A, Weiss G, Siegel AB, Bendell J, Baron A, Duddalwar V, El-Khoueiry A. A Randomized Phase II Open-Label Multi-Institution Study of the Combination of Bevacizumab and Erlotinib Compared to Sorafenib in the First-Line Treatment of Patients with Advanced Hepatocellular Carcinoma. *Oncology* 2018; **94**: 329-339 [PMID: 29719302 DOI: 10.1159/000485384]

55 **Abou-Alfa GK**, Shi Q, Knox JJ, Kaubisch A, Niedzwiecki D, Posey J, Tan BR Jr, Kavan P, Goel R, Lammers PE, Bekaii-Saab TS, Tam VC, Rajdev L, Kelley RK, El Dika I, Zemla T, Potaracke RI, Balletti J, El-Khoueiry AB, Harding JH, Suga JM, Schwartz LH, Goldberg RM, Bertagnolli MM, Meyerhardt J, O'Reilly EM, Venook AP. Assessment of Treatment With Sorafenib Plus Doxorubicin *vs* Sorafenib Alone in Patients With Advanced Hepatocellular Carcinoma: Phase 3 CALGB 80802 Randomized Clinical Trial. *JAMA Oncol* 2019 [PMID: 31486832 DOI: 10.1001/jamaoncol.2019.2792]

56 **Jouve JL**, Lecomte T, Bouché O, Barbier E, Khemissa Akouz F, Riachi G, Nguyen Khac E, Ollivier-Hourmand I, Debette-Gratien M, Faroux R, Villing AL, Vergniol J, Ramee JF, Bronowicki JP, Seitz JF, Legoux JL, Denis J, Manfredi S, Phelip JM; PRODIGE-11 investigators/collaborators. Pravastatin combination with sorafenib does not improve survival in advanced hepatocellular carcinoma. *J Hepatol* 2019; **71**: 516-522 [PMID: 31125576 DOI: 10.1016/j.jhep.2019.04.021]

57 **Lee FA**, Zee BC, Cheung FY, Kwong P, Chiang CL, Leung KC, Siu SW, Lee C, Lai M, Kwok C, Chong M, Jolivet J, Tung S. Randomized Phase II Study of the X-linked Inhibitor of Apoptosis (XIAP) Antisense AEG35156 in Combination With Sorafenib in Patients With Advanced Hepatocellular Carcinoma (HCC). *Am J Clin Oncol* 2016; **39**: 609-613 [PMID: 24977690 DOI: 10.1097/coc.0000000000000099]

58 **Assenat E**, Pageaux GP, Thézenas S, Peron JM, Bécouarn Y, Seitz JF, Merle P, Blanc JF, Bouché O, Ramdani M, Poujol S, de Forges H, Ychou M, Boige V. Sorafenib alone vs. sorafenib plus GEMOX as 1st-line treatment for advanced HCC: the phase II randomised PRODIGE 10 trial. *Br J Cancer* 2019; **120**: 896-902 [PMID: 30944458 DOI: 10.1038/s41416-019-0443-4]

59 **Azim HA**, Omar A, Atef H, Zawahry H, Shaker MK, Abdelmaksoud AK, EzzElarab M, Abdel-Rahman O, Ismail M, Kassem L, Waked I. Sorafenib plus tegafur-uracil (UFT) *vs* sorafenib as first line systemic treatment for patients with advanced stage HCC: a Phase II trial (ESLC01 study). *J Hepatocell Carcinoma* 2018; **5**: 109-119 [PMID: 30510922 DOI: 10.2147/JHC.S169285]

60 **Koeberle D**, Dufour JF, Demeter G, Li Q, Ribi K, Samaras P, Saletti P, Roth AD, Horber D, Buehlmann M, Wagner AD, Montemurro M, Lakatos G, Feilchenfeldt J, Peck-Radosavljevic M, Rauch D, Tschanz B, Bodoky G; Swiss Group for Clinical Cancer Research (SAKK). Sorafenib with or without everolimus in patients with advanced hepatocellular carcinoma (HCC): a randomized multicenter, multinational phase II trial (SAKK 77/08 and SASL 29). *Ann Oncol* 2016; **27**: 856-861 [PMID: 26884590 DOI: 10.1093/annonc/mdw054]

61 **Qin S**, Bai Y, Lim HY, Thongprasert S, Chao Y, Fan J, Yang TS, Bhudhisawasdi V, Kang WK, Zhou Y, Lee JH, Sun Y. Randomized, multicenter, open-label study of oxaliplatin plus fluorouracil/Leucovorin *vs* doxorubicin as palliative chemotherapy in patients with advanced hepatocellular carcinoma from Asia. *J Clin Oncol* 2013; **31**: 3501-3508 [PMID: 23980077 DOI: 10.1200/JCO.2012.44.5643]

62 **Yeo W**, Mok TS, Zee B, Leung TW, Lai PB, Lau WY, Koh J, Mo FK, Yu SC, Chan AT, Hui P, Ma B, Lam KC, Ho WM, Wong HT, Tang A, Johnson PJ. A randomized phase III study of doxorubicin

vs cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. *J Natl Cancer Inst* 2005; **97**: 1532-1538 [PMID: 16234567 DOI: 10.1093/jnci/dji315]

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REVIEW

Role of modern radiotherapy in managing patients with hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. Several treatment options are available for managing HCC patients, classified roughly as local, local-regional, and systemic therapies. The high post-monotherapy recurrence rate of HCC urges the need for the use of combined modalities to increase tumor control and patient survival. Different international guidelines offer treatment recommendations based on different points of view and classification systems. Radiotherapy (RT) is a well-known local-regional treatment modality for managing many types of cancers, including HCC. However, only some of these treatment guidelines include RT, and the role of combined modalities is rarely mentioned. Hence, the present study reviewed clinical evidence for the use of different combined modalities in managing HCC, focusing on modern RT's role. Modern RT has an increased utility in managing HCC patients, mainly due to two driving forces. First, technological advancement (*e.g.*, stereotactic body radiotherapy and advanced proton-beam therapy) enables precise delivery of radiation to increase tumor control and reduce side effects in the surrounding normal tissue. Second, the boom in developing target therapies and checkpoint-blockade immunotherapy prolongs overall survival in HCC patients, re-emphasizing the importance of local tumor control. Remarkably, RT combines with systemic therapies to generate the systemic therapy augmented by radiotherapy effect, a benefit now being actively investigated.

Key Words: Hepatocellular carcinoma; Stereotactic body radiotherapies; Radiotherapy;

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Core Tip: Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and treatments outcome are often unsatisfactory. The high post-monotherapy recurrence rate points to the need to combine modalities, including radiotherapy (RT). The international guidelines from North America, Europe and Asia offer treatment recommendations based on different classification systems. However, not all of these treatment guidelines include RT and the role of combined modalities. Hence, the present study reviewed clinical evidence of different combined modalities in managing HCC, focusing on RT's role and especially modern advanced RT techniques and the interaction with game-changing immunotherapy.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, ranking as the sixth leading cause of cancer incidence and the fourth most common etiology of cancer death worldwide; overall, around 953000 cases were diagnosed in 2017 globally[1]. The majority of HCC patients are found in the Asia–Pacific region, and around 75%-90% of cases are associated with chronic viral hepatitis (mainly hepatitis B and C) infection[2-4]. Therefore, the management of HCC requires considering detailed information from three main domains together: Cancer characteristics, liver function reserve, and overall patient condition.

Several treatment modalities are available for managing HCC patients; these modalities are generally classified as local, local-regional, and systemic therapies. Local therapy includes surgical resection, ablative therapies, and radiotherapy (RT). Ablative therapies include radiofrequency ablation (RFA)[5-7], percutaneous ethanol injection therapy[8], cryoablation[9], and microwave[10]; these modalities can be performed through percutaneous, laparoscopic, or open approaches. Local-regional or so-called arterially directed therapies include trans-arterial embolization (TAE), trans-arterial chemoembolization (TACE)[5], drug-eluting beads (DEB)-TACE[11-13], trans-arterial radioembolization (TARE) with yttrium 90[14,15], and hepatic arterial infusion chemotherapy (HAIC)[16-18]. Systemic therapy includes target therapy (*e.g.*, sorafenib[19], lenvatinib[20], and regorafenib[21]) and immunotherapy (*e.g.*, nivolumab[22]); more notably, the use of several such types of agents has been investigated aggressively.

The preferred curative surgical modalities include liver resection and liver transplantation, generating a 5-year overall survival rate of up to 70%-75%[23,24]. RFA is another frequently used curative modality, with a 5-year overall survival rate of around 40%-70%[24]. However, local control of RFA is decreased in patients with large tumors, especially those larger than 3 cm[25,26]. It is recognized that only 10%-30% of HCC patients are candidates for curative surgical options because most HCCs are diagnosed at an intermediate or advanced stage[27,28]. Even though many treatment options can be chosen for HCC patients and each treatment modality has seen advancement in past decades, 5-year overall survival rates are still unsatisfactory, being less than 20%[29].

A high treatment failure (*i.e.*, recurrence) rate is reported in the heterogeneous group of patients with intermediate- to advanced-stage HCC. Five-year tumor recurrence rates are more than 50% after liver resection and may be up to 80% after RFA[30]. This high recurrence rate includes patients who underwent TACE or some other singular local treatment modality. Of note, intrahepatic recurrence is the most common recurrence pattern. This unsatisfactory tumor control rate suggests that

combining different locoregional therapies may improve treatment outcomes[31-33]. This review focuses mainly on RT's current role in managing HCC, not only as a monotherapy but also as an essential part of combined modalities, especially stereotactic body radiotherapy (SBRT)/stereotactic ablative radiotherapy (SABR).

CURRENT TREATMENT GUIDELINES FOR HCC

Currently, useful treatment guidelines for managing HCC include recommendations from the National Comprehensive Cancer Network (NCCN)[34], the American Association for the Study of Liver Diseases (AASLD)[35], the European Association for the Study of the Liver (EASL)[36], the Japan Society of Hepatology (JSH)-HCC[37], the Korean Liver Cancer Association (KLCA) and National Cancer Center (NCC)[38], the National Health and Family Planning Commission (NHFPC) of the People's Republic of China HCC guideline[39], and the Taiwan Liver Cancer Association (TLCA)[40].

In practice, these guidelines implemented different classification systems to stratify HCC patients for appropriate management. For example, the NCCN grouped patients as potentially resectable/transplantable *vs* unresectable/inoperable to guide treatment options. The AASLD and EASL guidelines used the Barcelona clinic liver cancer (BCLC) stage to lead management recommendations. The KLCA and NCC guidelines used a modified Union for International Cancer staging system adapted from the Liver Cancer Study Group of Japan[41,42]. Both the JSH-HCC and TLCA guidelines adopted hepatic functional reserve, extra-hepatic metastasis, vascular invasion, tumor number, and tumor size to guide treatment choice using a step-by-step manner and the Chinese guideline added general health status to the previously-mentioned risk factors[37,39,40]. Due to the variety of classification systems used in these different guidelines, we summarized treatment recommendations according to the BCLC stage in Figure 1.

COMBINED MODALITY FOR HCC

None of the guidelines mentioned above well declare the role of the combined treatment strategies used frequently in daily practice. Clinically, the choice of different combined modalities is based not only on guidelines or evidence, but also on the individual patient's condition, the liver function preservation, the tumor characteristics, and the treatment perspective including the availability of resources within the facility and the therapeutic ratio. Overview of therapeutic options and the consideration behind the combination of different modalities for liver cancer are illustrated in Figure 2.

For very early and early stage HCC

Some anatomic locations of HCC, such as tumors adjacent to the gallbladder, liver hilum, bowel, stomach, and other critical organs, may limit the use of RFA as an intervention[43]. On the other hand, RT can be delivered to tumors that arise from any location, so that it can compensate for or combine with RFA.

For intermediate stage HCC

Many patients receive TACE as their first local-regional therapy. However, TACE alone seldom achieves satisfactory tumor control[44]. Therefore, several combined modalities have been reported to increase treatment outcomes, and they are subdivided into three main categories, as follows.

Local-regional plus local therapy: TACE combined with RFA achieves a complete response (CR) rate of 55-65% at the time of the first or second post-treatment check[31,32]. TACE combined with conventionally fractionated radiotherapy (CFRT) demonstrates a better 1-year survival rate than TACE alone[45]. Remarkably, TACE combined with SBRT shows promising results[46]. In contrast, the role of HAIC in conjunction with RT is still under investigation[47].

Local-regional plus another local-regional therapy: TACE combined with TARE has been reported as a safe and effective treatment modality for bi-lobar HCC[48].

Local-regional plus systemic therapy: This type of combination includes such options as TACE combined with target therapy[44,49,50], TARE combined with target

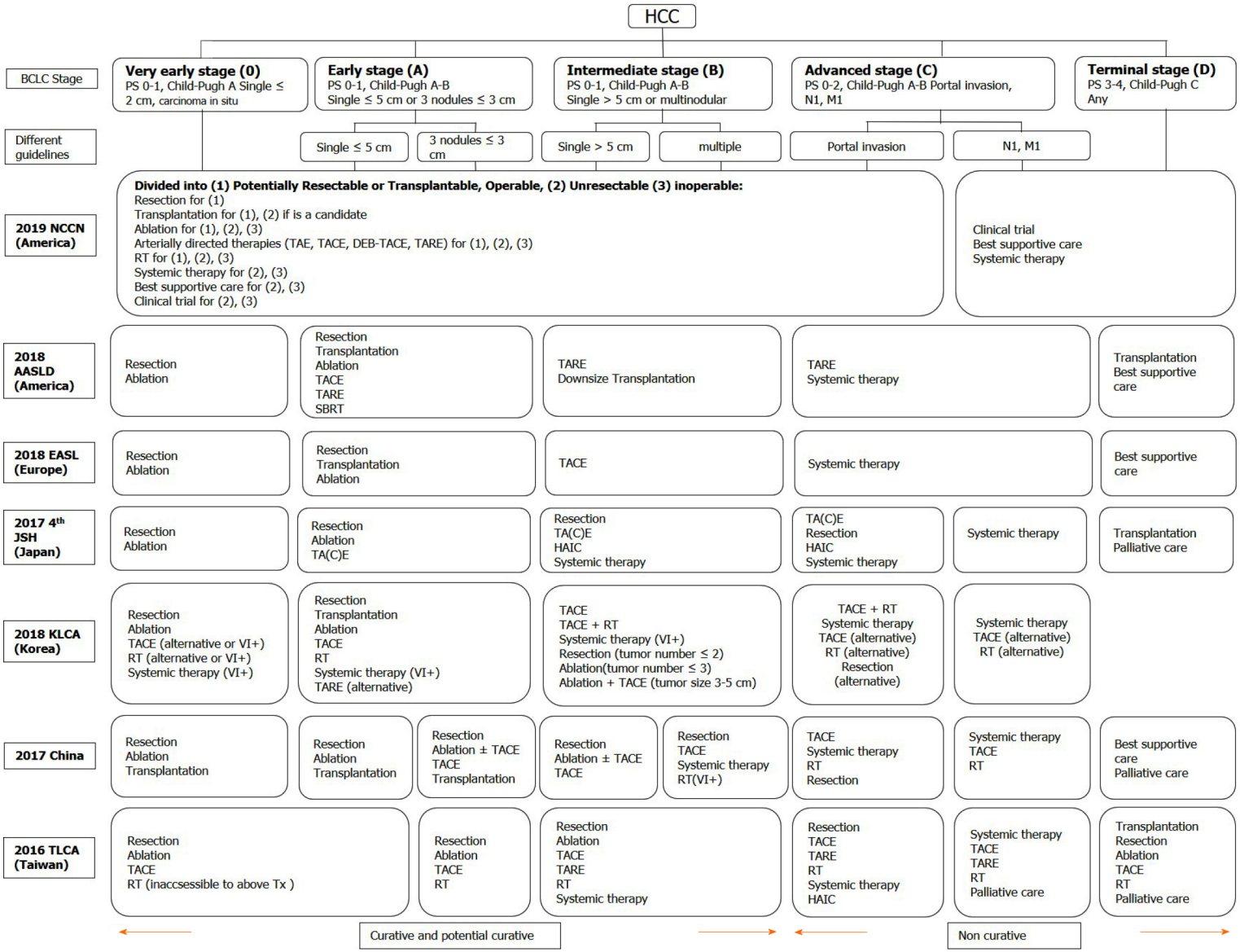


Figure 1 Treatment recommendations modified in different guidelines according to the Barcelona clinic liver cancer stage. HCC: Hepatocellular carcinoma; BCLC: Barcelona clinic liver cancer; PS: Performance status; TA(C)E:

Trans-arterial (chemo)embolization; TARE: Trans-arterial radioembolization; DEB-TACE: Trans-arterial chemoembolisation with drug-eluting beads; RT: Radiotherapy; SBRT: Stereotactic body radiation therapy; HAIC: Hepatic arterial infusion chemotherapy; NCCN: National Comprehensive Cancer Network; AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver; JSH: Japan Society of Hepatology; KLCA: Korean Liver Cancer Association; TLCA: Taiwan Liver Cancer Association; Tx: Treatments; VI+: Positive vascular or bile duct invasion.

therapy[51-53], TACE combined with immunotherapy[54-56], and TARE combined with immunotherapy[57].

For advanced HCC

Only limited HCC patients are responsive to immune checkpoint inhibitors, and a combination of these with RT may enhance the immune response; this phenomenon is named the systemic therapy augmented by radiotherapy (STAR) effect[58]. Overall, RT or SBRT combined with other treatment modalities has been applied increasingly in HCC patients.

THE ROLE OF RT IN HCC

RT was used as a salvage or palliative treatment in the past, and only a few guidelines mention the role of RT. However, in the modern era, RT is indicated across all stages (*i.e.*, from very early to end-stage HCC)[34,35,37,40]. Notably, RT can be used as a single therapy or as an essential component of a combined modality. Current treatment recommendations based on the BCLC stage and RT's potential roles are summarized in Figure 3.

Different RT techniques

Photon therapy: The most commonly available treatment beam of RT is photons. In managing patients with HCC, several photon-beam delivery systems of external-beam radiation therapy (EBRT) are clinical available, such as conventional fractionated RT (CFRT), hypo-fractionated RT (HFRT), and SBRT. CFRT is usually delivered with daily fractions of 1.8-2 Gy, and HFRT is characterized by a large daily dose (*i.e.*, > 2 Gy) in the context of precise RT. Clinically, HFRT is a useful strategy for improving dose intensity and then tumor control. Both CFRT and HFRT can be delivered by using three-dimensional conformal radiotherapy (3DCRT), intensity-modulated radiotherapy (IMRT), and Volumetric-modulated Arc Therapy (VMAT).

Remarkably, SBRT, or so-called SABR, is an advanced technique of EBRT that delivers a very high dose of irradiation in a very precise way in a limited number of treatment fractions (*i.e.*, usually 3-6 fractions and > 5 Gy per fraction) over a treatment course of 1-2 wk. For more focused and accurate delivery of SBRT, advancements across the whole RT department should be provided, including imaging, immobilization, target delineation, treatment planning, on-board image guidance, and

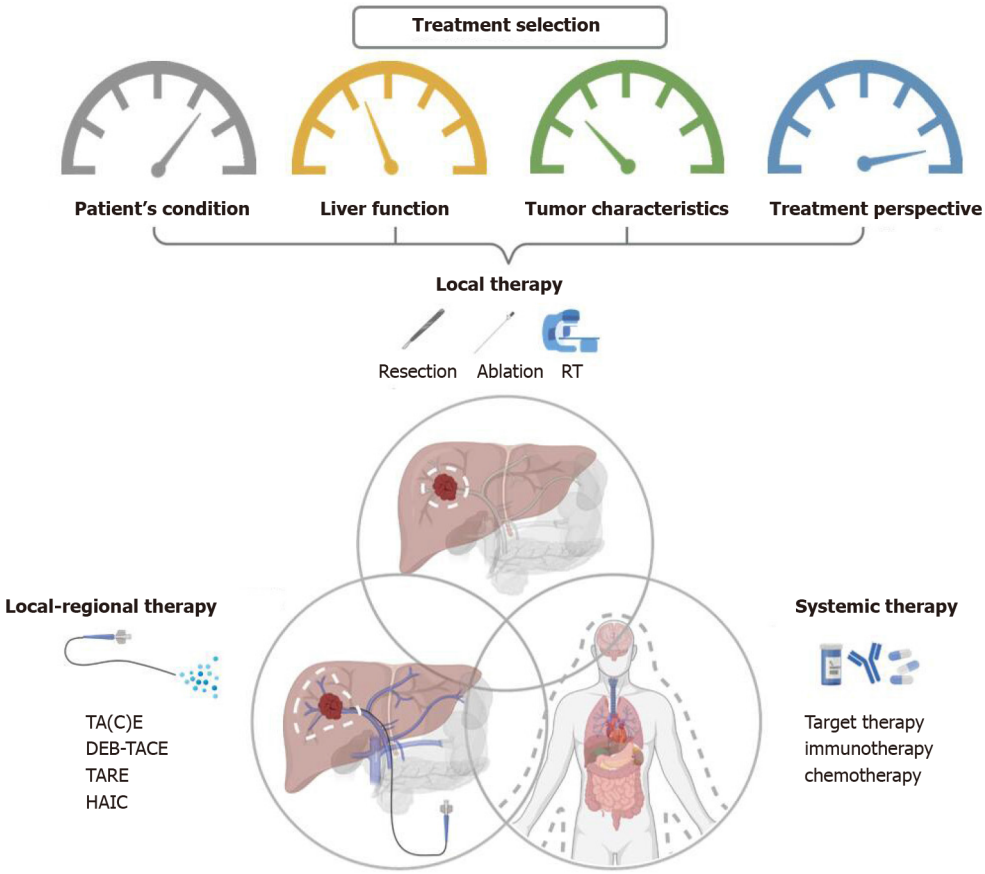


Figure 2 Overview of therapeutic options and the consideration behind the combination of different combined modalities for liver cancer. RT: Radiotherapy; TA(C)E: Trans-arterial (chemo)embolization; TARE: Trans-arterial radioembolization; DEB-TACE: Trans-arterial chemoembolisation with drug-eluting beads; HAIC: Hepatic arterial infusion chemotherapy.

respiratory motion management (RMM). Only advanced IMRT or VMAT with or without non-coplanar beam designs can be used for delivering SBRT. These advancements result in better dose distribution, deliver a higher dose within the tumor, and generate a rapid dose fall-off outside the target. Thus, SBRT can improve tumor control and reduce the irradiation dose to the surrounding normal tissue, to decrease RT toxicity. Owing to this double benefit of enhancing therapeutic gain, SBRT is highly recommended in managing HCC patients treated with curative intent.

For patients who cannot be treated successfully with SBRT, CFRT combined with two or more advanced irradiation techniques, such as combined VMAT and Simultaneously Integrated inner-Escalated Boost (SIEB), may be helpful to achieve a better therapeutic gain (*i.e.*, better tumor control with minimal RT toxicity) compared to conventional CFRT, including for elderly HCC patients who have inoperable disease[59].

Proton therapy: Charged particle irradiations, including proton-beam therapy (PBT) and carbon-ion RT, have unique dosimetric characteristics. That is, they eliminate the low-dose bath volume distal to the target area that is associated with photons. This elimination is because the characteristic Bragg peak of charged particles deposits irradiation energy mainly within the targeted tumor area and results in a near-zero dose beyond the end of its path[60]. Therefore, charge particle irradiation represents an excellent option for improving normal liver sparing and minimizing side effects such as radiation-induced liver disease (RILD). It also makes possible dose escalation for curing unresectable huge HCC.

Park *et al*[61] reported that dose escalation could enhance HCC tumor control. Kim *et al*[62] further confirmed that proton dose escalation is safe and effective; they suggested an EQD2 ≥ 78 Gy-equivalents (GyE) could be delivered to achieve reasonable tumor control. According to the tumor location, the University of Tsukuba proton team developed different PBT dose protocols[63-65]. Extrapolating on the concept of a lung cancer SBRT "No Fly Zone"[66], peripheral liver tumors located at > 2 cm from the hepatic portal region or gastrointestinal (GI) tract can be treated with

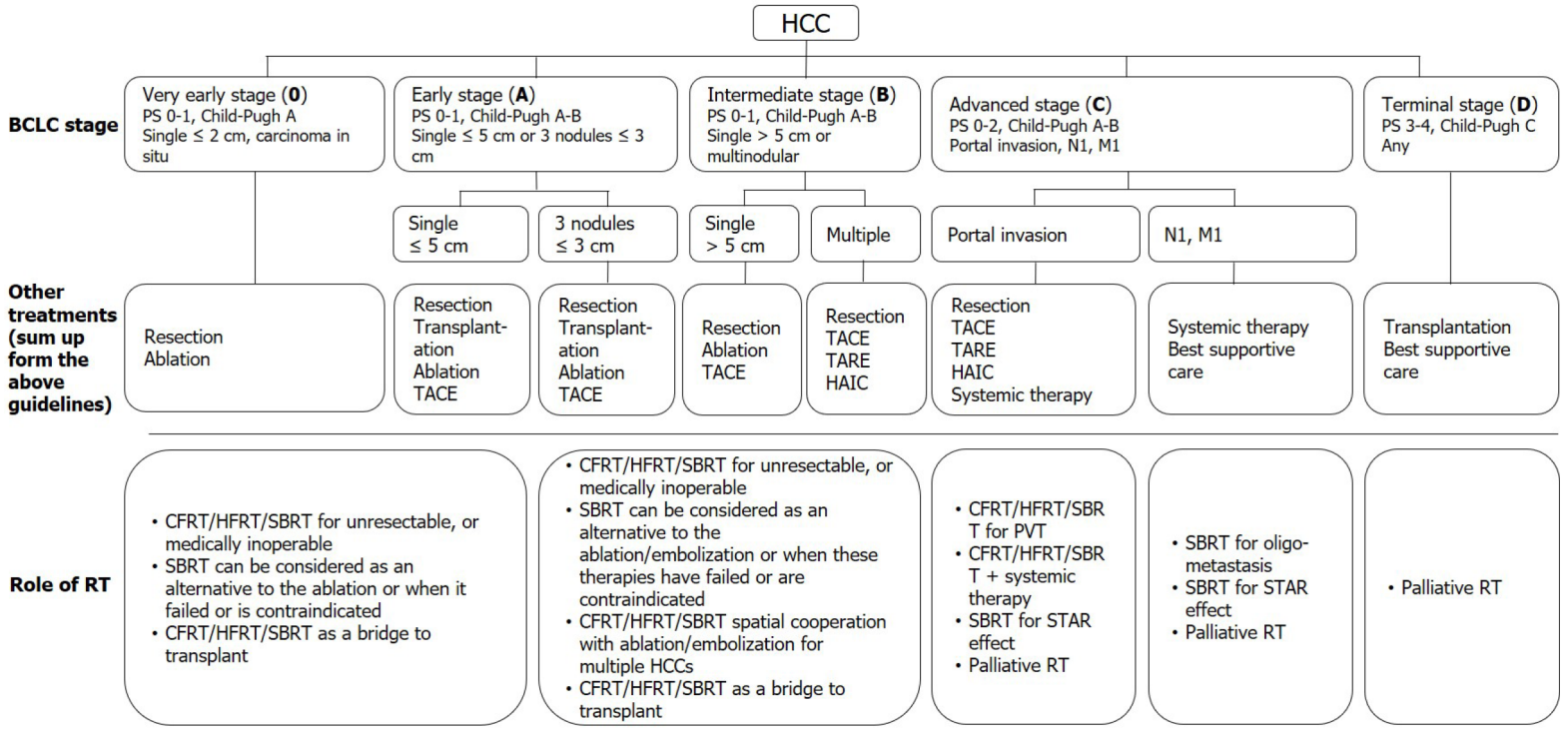


Figure 3 Current treatment recommendations based on the Barcelona clinic liver cancer stage and the potential roles of radiotherapy. HCC: Hepatocellular carcinoma; BCLC: Barcelona clinic liver cancer; PS: Performance status; TACE: trans-arterial chemoembolization; TARE: Trans-arterial radioembolization; HAIC: Hepatic arterial infusion chemotherapy; RT: Radiotherapy; CFRT: Conventionally fractionated radiotherapy; HFRT: Hypo-fractionated radiotherapy; SBRT: Stereotactic body radiation therapy; PVT: Portal vein thrombosis; STAR effect: Systemic therapy augmented by radiotherapy effect.

hypofractionated proton 66 GyE in 10 fractions. On the other hand, for tumors located within 2 cm adjacent to the hepatic portal region, small doses per fraction with 72.6 GyE in 22 fractions should be considered. For tumors located within 2 cm of the GI tract, 77.0 GyE in 35 fractions may be given[63-65].

Several studies report using PBT for localized HCC with excellent local control ranging from 80% to 100%, even for huge unresectable HCCs, due to dose escalation and sparing of more liver function[67-69]. Furthermore, Sanford *et al*[70] reported that the overall survival (OS) benefit of proton-RT over photon-RT might be due to decreasing the incidence of RILD. Hsieh *et al*[71] further identified the predictors of RILD in HCC patients treated with PBT beyond the conventional concept of minimizing the mean liver dose. A "volume-response" relationship between unirradiated liver volume (ULV)/standard liver volume (SLV) and RILD was found: For Child-Pugh A patients, it was < 50%; for Child-Pugh B patients, it was < 30%[71].

Both photon and PBT can achieve high rates of local control with acceptable toxicity. However, PBT has better potential to deliver a higher dose while maximizing the volume of unirradiated liver. Clinically, the reduced normal liver dose achieved by PBT is not critically required for all patients, since some may benefit from a smaller irradiated target volume when normal liver constraints can be met. The 2018 Miami Liver Proton Therapy Conference reached the consensus that the patients who should be strongly prioritized for PBT include those with: At least Child-Pugh B cirrhosis, a high tumor-to-liver ratio (*i.e.*, larger tumor size or smaller uninvolved liver volume), a greater number of tumors, or prior RT to the liver[72]. The dose comparison of proton therapy *vs* SBRT *vs* conventional RT for liver tumors are illustrated in Figure 4.

The role of RT in very early and early stage HCC

Very early and early stage HCCs include those with BCLC classification 0-A, as follows: Carcinoma in situ; a single tumor of ≤ 2 cm, a single tumor of ≤ 5 cm, or three tumors of < 3 cm; and tumor burden laying within the Milan criteria, with Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 as well as Child-Pugh classification A-B[73]. For these HCC patients, although the standard of care is still surgery and RFA, definitive SBRT is a potential third curative treatment modality for medically inoperable, technically unresectable, and difficult to RFA conditions; more notably, it can serve as a bridge to liver transplantation[26,74].

The role of RT in intermediate stage HCCs

Intermediate stage HCCs include those with BCLC classification B, as follows: Multi-tumors, a single tumor of > 5 cm, good patient condition (*i.e.*, PS 0-1), as well as good liver reserve (*i.e.*, Child-Pugh A-B). Tumor burden can be further subdivided, as follows: (1) Beyond Milan criteria but within the Up-to-7 criteria; and (2) Tumors beyond the Up-to-7 criteria[75]. For these patients, only limited cases can be treated by surgery or RFA. Several combined-modality approaches, including local therapy, local-regional therapy, and systemic therapy, have been reported in conjunction with CFRT and SBRT[76,77].

The role of RT in advanced stage HCCs

Advanced stage HCCs include those with BCLC classification C, with the criteria of portal vein invasion, inferior vena cava/heart invasion or thrombosis, lymph node metastasis, distant metastasis, and Child-Pugh A-B. SBRT or conventional RT may be applied in conjunction with other local, local-regional, and systemic therapies to serve as a potentially curative or palliative treatment[76-78].

The role of RT in terminal stage HCCs

Terminal stage HCCs include those with BCLC classification D, with the criteria of Child-Pugh C or ECOG PS 3-4. For these patients, SBRT with careful planning is safe as a bridge to liver transplantation in selected patients with a Child-Pugh score of ≥ 8. Additionally, SBRT or conventional RT can be used to treat symptoms[79-81].

THE ROLE OF SBRT IN MANAGING PATIENTS WITH HCC

SABR/SBRT

Recently, clinical evidence has rapidly grown for the use of SBRT in managing all stage HCC patients, with curative, potentially curative, or palliative intent[33,77,78,82]. Prospective clinical trials have demonstrated that SBRT effectively treats HCC, resulting in satisfactory local control, ranging from 75% to 100% at 1 year and 65% to 100% at 2 years[33,77,78]. Local control of HCC using SBRT is typically defined as no progression or no recurrent disease within the irradiated field according to the Response Evaluation Criteria in Solid Tumors (RECIST) or its modification (mRECIST)[83-85]. Moreover, SBRT showed a benefit of limited toxicity, with a severe late toxicity rate of < 15%; thus, SBRT is considered a safe modality for treating elderly patients[86]. In the literature, most patients treated with SBRT have Child-Pugh A disease and limited lesions (often < 3 tumors), and delivering SBRT in Child-Pugh B patients increases toxicity rates[80]. However, if dose modification is done to meet the more conservative (*i.e.*, strict) normal tissue constraints, SBRT may be allowed for patients with small HCCs with Child-Pugh B or those with relatively larger tumors with a Child-Pugh score of 7[80].

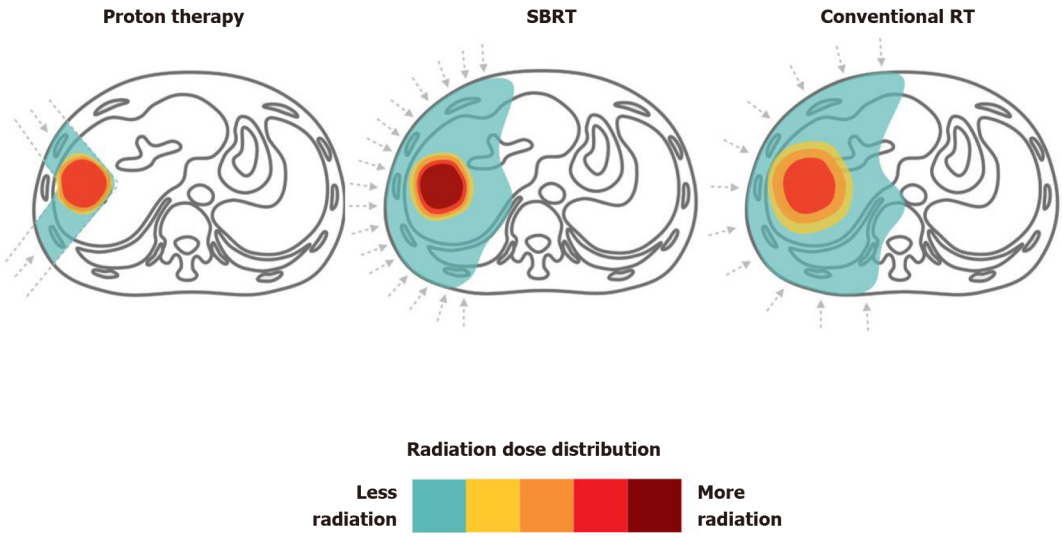


Figure 4 Proton therapy *vs* stereotactic body radiation therapy *vs* conventional radiotherapy for liver tumors. Dose distributions for a proton (left), stereotactic body radiation therapy (middle) and conventional radiotherapy (right) hepatocellular carcinoma radiotherapy plan are illustrated for comparison. RT: Radiotherapy; SBRT: Stereotactic body radiation therapy.

SBRT as an alternative treatment modality to ablation therapy (i.e., RFA)

Ablative therapy is curative in treating small tumors (*i.e.*, ≤ 3 cm) that locate in a feasible location, achieving excellent local control rates of around 70-90%; these results are similar to that of surgical resection[87]. Under such conditions, ablative therapy is considered an alternative treatment to surgical resection or liver transplantation.

Recently, SBRT showed comparable outcomes with RFA. Wahl *et al*[26] compared treatment outcomes between SBRT and RFA; they declared that both are effective for patients with inoperable HCC, with comparable freedom from local progression (FFLP) and comparable OS rates. The 1- and 2-year FFLP rates of SBRT were 97.4% and 83.8% *vs* 83.6% and 80.2% for RFA, respectively. The 1- and 2-year OS rates were also similar between the two treatment modalities. Remarkably, for larger tumors of ≥ 2 cm, SBRT demonstrated a better FFLP than that of RFA [hazard ratio (HR), 3.35; 95% confidence Interval (CI): 1.17-9.62; $P = 0.025$][26].

Hara *et al*[88] used propensity score matching (PSM) to assess the pre-treatment characteristics of BCLC stage, computed tomography (CT) status, and tumor size; they reported comparable 3-year OS rates between RFA and SBRT. Kim *et al*[89] also applied PSM to compare treatment results of SBRT and RFA. Two-year FFLP rates were 74.9% for the SBRT group and 64.9% for the RFA group. Taking these data together, SBRT demonstrates an emerging role as a curative treatment modality that is an alternative to ablative therapy for managing HCC patients.

SBRT as an alternative or adjuvant therapy to arterially directed therapies

Among arterially directed therapies, TACE is the most widely used treatment modality for managing HCC patients with an intermediate stage, applied in 50%-60% of patients[90]. However, TACE alone demonstrates unsatisfactory local control. This unacceptably low response rate suggests the use of local therapy, such as SBRT, as an alternative or adjuvant therapy to improve local control.

SBRT as an alternative therapy to TACE: Sapir *et al*[91] used PSM with inverse treatment weighting probability to compare SBRT and TACE in HCC patients with 1-2 tumors. They found that SBRT demonstrated better 1- and 2-year local control rates when compared with TACE: 97% and 91% *vs* 47% and 23% (HR, 66.5; 95%CI: 18.99-233.0; $P < 0.001$), respectively. However, the difference in OS did not significantly differ between the two treatment modalities.

SBRT combined with TACE: Several retrospective studies and reviews have demonstrated increased local control rates by adding conventional RT to TACE[45,92,93]. However, studies using SBRT to replace conventional RT are scarce because SBRT is a new technique.

A pilot study and preliminary results of prospective studies also confirmed the safety and efficacy of SBRT in patients with HCC that failed to respond to TACE[94-96]. Kang *et al*[46] published a phase-II trial that enrolled 47 patients who

received TACE 1 to 5 times before SBRT. The result showed a good response, with a 2-year local control rate of 94.6%. Moreover, 38.3% of patients achieved a complete response within 6 mo. However, there is still no published data from head-to-head trials that compare TACE plus SBRT with TACE alone. Several clinical trials are ongoing (ClinicalTrials.gov Identifier: NCT02762266, NCT02323360, NCT02323360, NCT02794337, NCT02921139).

SBRT combined with TARE: Previously, there has been much concern over increasing radiation-related toxicity when using SBRT after TARE. Hardy-Abeloos *et al*[97] recently reported that TARE followed by SBRT has comparable safety and efficacy to TACE followed by SBRT.

SBRT as a bridge to liver transplantation

One of the preferred gold-standard treatments for managing HCC patients is liver transplantation, but only a few patients have a chance to receive a transplant due to an insufficient supply of donor livers[23]. Therefore, several local and local-regional therapies for HCC have been used to bridge care in patients seeking a transplant, to delay tumor progression[24]. Several studies have shown that SBRT may be an excellent alternative to conventional therapies as a bridge to transplantation[81,98]. Sapisochin *et al*[98] reported 1-, 3-, and 5-year actuarial patient survival rates from the time of listing of 83%, 61%, and 61% in the SBRT group, respectively, rates which were comparable with those of the TACE or RFA groups.

SBRT for macroscopic vascular invasion or portal vein thrombosis

In the past decades, two landmark randomized trials revealed that sorafenib yielded modest survival prolongation in patients with portal vein thrombosis (PVT)[99,100]. However, the response rate was unsatisfactory (only 2%). CFRT has been widely used in advanced HCC with macroscopic vascular invasion or PVT because it can be delivered regardless of tumor location, and major vessels demonstrate high radiation tolerance[24,78,79].

Rim *et al*[101] published a meta-analysis and systematic review which compared 3DCRT, TARE, and SBRT in patients with PVT. No significant differences in OS were observed among the three treatment modalities, but SBRT demonstrated the highest response rate of around 70%. More notably, toxicities of more than grade 3 were rare in the SBRT group[101]. These data revealed that SBRT could be safely applied in patients with PVT, with a better response rate than CFRT.

SBRT and sorafenib

SBRT compared with sorafenib: Bettinger *et al*[102] used PSM to compare SBRT and sorafenib. SBRT showed a superior survival benefit to that of sorafenib: median overall survival was 17.0 mo (range, 10.8-23.2) *vs* 9.6 mo (range, 8.6-10.7), respectively, with or without adjusting for different baseline characteristics. A cost-effectiveness study also reported that SBRT had better cost-effectiveness than sorafenib for patients with advanced HCC[103].

SBRT combined with sorafenib: Brade *et al*[104] initiated a phase-I trial to evaluate the combination of sorafenib and SBRT. Sorafenib was delivered before, during, and after SBRT. The researchers found that concurrent use of SBRT with sorafenib significantly increased side effects, *e.g.*, grade 3+ bowel toxicity and tumor rupture. Thus, they did not recommend using this combination outside of a clinical trial. A clinical trial (RTOG 1112) is ongoing to compare SBRT followed by sorafenib with sorafenib alone in patients with advanced HCC (ClinicalTrials.gov Identifier: NCT01730937).

SBRT combined with immunotherapy

SBRT as an immunostimulator: Preclinical data demonstrated that RT could augment the intra-tumor cell surface expression of immunogenicity markers (*e.g.*, dendritic cells) and enhance therapeutic efficacy[105-107]. Recently, immunotherapy combined with RT is an active field in managing HCC and has shown promising results[106,108,109]. SBRT may stimulate the release of tumor antigens and increase antigen-presenting cells to enhance the immune response to cancer cells provided by the immunotherapy[110]. This effect is also termed STAR[58] or "immunotherapy and stereotactic ablative radiotherapy (ISABR)"[111]. Investigations exploring in detail the underlying mechanisms of these effects are ongoing aggressively. Potential mechanism of SBRT combined with systemic therapy to induce the STAR effect (ISABR) for liver tumors is illustrated in Figure 5.

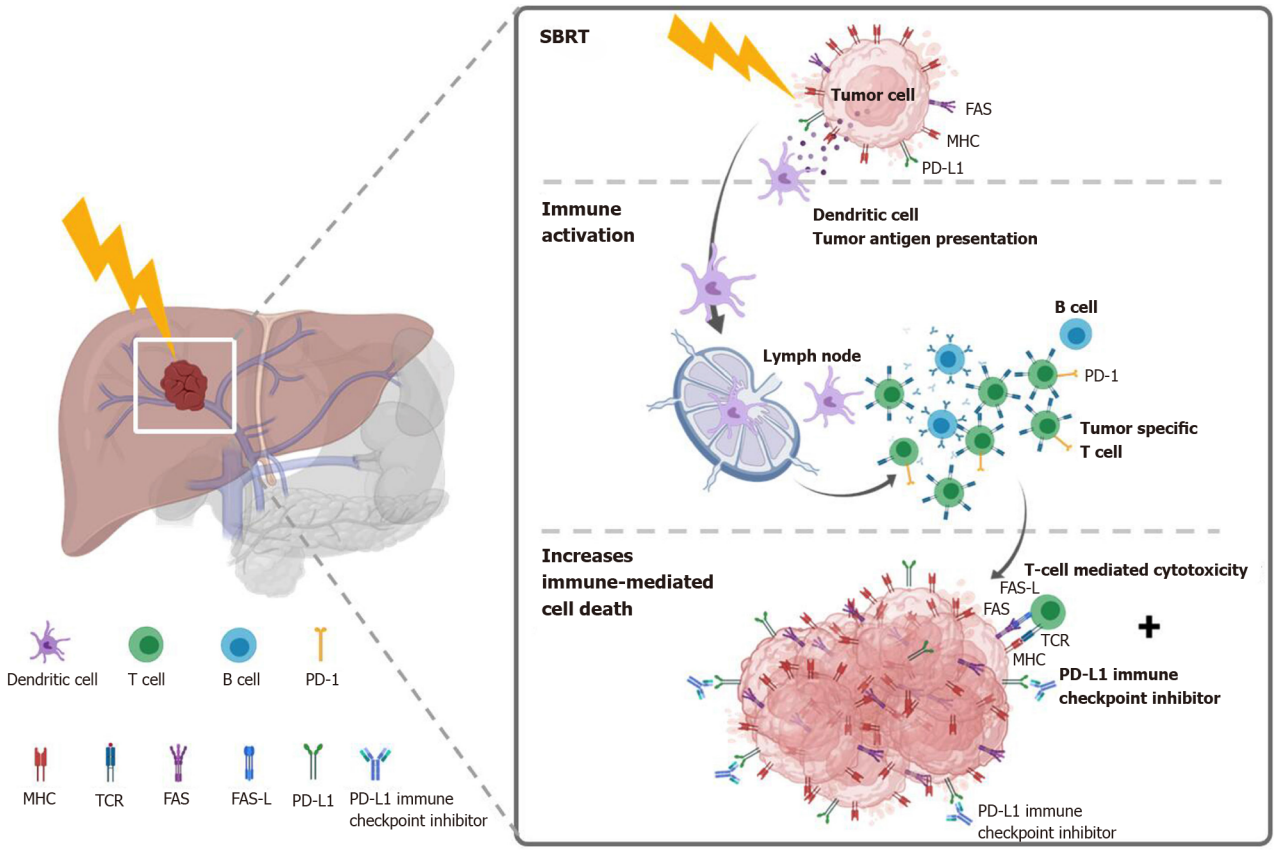


Figure 5 Potential mechanism of stereotactic body radiation therapy combined with systemic therapy to induce the systemic therapy augmented by radiotherapy effect (also known as immunotherapy and stereotactic ablative radiotherapy) for liver tumors. Stereotactic body radiation therapy (SBRT) induces antigen release and immunogenic cell death, activation of several transcription factors and signal pathways, as well as dendritic cell antigen presentation and maturation, resulting in proliferation of tumor-specific T cells and immune-mediated cytotoxicity. SBRT combined with Immune-checkpoint inhibitors augmented the tumoricidal effect by upregulates major histocompatibility complex and FAS on tumor cells, increasing susceptibility to T-cell-mediated cell death. MHC: Major histocompatibility complex; TCR: T cell receptor; FAS-L: FAS ligand.

SBRT is more immunogenic than conventional RT: SBRT is more immunogenic and has a beneficial outcome than the conventionally fractionated RT[112,113]. By using ablative SBRT in a mouse model, Lee *et al*[113] observed that a 20-Gy single fraction dose leads to a reduction in the tumor burden in both primary and distant metastases in a CD8+ T-cell–dependent fashion. However, conventionally fractionated irradiation showed a CD8+-depleted condition. In another preclinical study that compared soluble PD-L1 (sPD-L1) between the SBRT and conventional RT groups[114], Kim and colleagues found that the sPD-L1 Level increases persistently for 1 mo in the SBRT group. In comparison, it increased initially after irradiation but decreased after 1 mo in the conventional RT group.

Potential optimal SBRT dose and treatment sequences for induced immunogenicity: The optimal window of radiation immunogenicity is determined by the levels of double-strand DNA (dsDNA) *vs* Trex1[115,116]. SBRT doses up to 10-12 Gy (*e.g.*, 8 Gy x 3 fractions) can up-regulate dsDNA accumulation in cancer cells *via* the cGAS/STING pathway, turning on RT-driven immune responses. However, higher doses above 12–18 Gy, such as 20-30 Gy in a single fraction, may induce the exonuclease TREX1, which down-regulates the immunogenicity by degrading cytosolic DNA, turning off RT-driven immune responses[115]. Otherwise, increasing the dose to higher than 10 Gy per fraction can rapidly reduce the tumor blood perfusion, leading to a tumoricidal effect *via* severe vascular damage[117].

The optimal sequence of SBRT and immunotherapy is still unclear. Both concurrent and sequential combination have been applied[118-120]. In other cancers, Wegner *et al*[121] found that OS was improved when immunotherapy was given more than 3 wk after initiating SBRT/SRS in patients with stage IV non-small-cell lung cancer. In a study designed by Tang *et al*[120], SBRT was given either concurrently or sequentially with ipilimumab in patients with advanced solid tumors. However, there are limited

studies specifically focused on HCC. Chiang *et al*[122] added nivolumab at 2 wk after completed SBRT and continuously applied every 2 wk until disease progression. Currently, the optimal timing of SBRT and immunotherapy remains under active investigation.

Clinical data and ongoing trials for SBRT combined with immunotherapy: Recently, two phase-II studies, CheckMate 040[123] and KEYNOTE-224[124], have shown that the anti-PD-1 immune checkpoint inhibitors of nivolumab and pembrolizumab demonstrate favorable tumor responses (15%-20%) in managing HCC patients. The two agents have been approved by the Food and Drug Administration (FDA) for patients previously treated with sorafenib. A phase-III randomized trial, KEYNOTE-240[125], then compared pembrolizumab and placebo in 413 patients previously treated with sorafenib. A median OS was 13.9 mo for pembrolizumab *vs* 10.6 mo for placebo (HR, 0.781; 95%CI: 0.611-0.998; P = 0.0238) and the median PFS was 3.0 *vs* 2.8 mo (HR, 0.718; 95%CI: 0.570-0.904; P = 0.0022). However, neither study endpoint reached statistical significance according to the trial-specified criteria (one-sided significance threshold, P = 0.0174, for the final analysis). Another phase-III trial, CheckMate 459, comparing nivolumab *vs* sorafenib as first-line treatment in advanced HCC, is ongoing (ClinicalTrials.gov Identifier: NCT02576509). Abstracts of CheckMate 459 in the 44th European Society for Medical Oncology Congress revealed that the primary endpoint of OS was statistically insignificant, but the objective response rate was double in the nivolumab group (15% *vs* 7%, respectively)[126].

Although the immune checkpoint inhibitor's objective response rate was higher than that of sorafenib, 15%-20% is still relatively insufficient[123-125]. Hence, the combination of immunotherapy with SBRT has been proposed to improve the efficacy of immunotherapy, to combine the direct tumoricidal effects with the immunogenic STAR effect[110,111,127,128]. As a novel combined treatment modality, only one published HCC study is currently available, revealing encouraging results. Chiang *et al*[122] reported 5 retrospective cases with unresectable HCC treated with SBRT 27.5-35 Gy in 5 fractions, followed by nivolumab. The median follow-up time was 14.9 mo. An objective response rate of 100% was reported, with 2 complete response and 3 partial responses. The 1-year OS and LC rates were both 100%. In addition, several phase-I or -II trials that combine SBRT and immunotherapy are ongoing (ClinicalTrials.gov Identifier: NCT03817736, NCT03316872, NCT03482102, and NCT03203304).

SBRT for metastatic disease

SBRT becomes much more important in managing cancer patients with oligometastatic disease[129-134], including those with HCC[76]. Recently, a landmark trial applied SBRT to manage oligometastatic cancers, defined as primary controlled tumors with only 1-5 metastatic lesions, PS ≤ 1, and life expectancy > 6 mo[135]. Median OS of the SBRT group was better than that of the control group (41 *vs* 28 mo; HR, 0.57; 95%CI: 0.30-1.10; P = 0.090)[135]. Therefore, it is reasonable to treat more aggressively those HCC patients with oligometastasis, oligo-progression, and oligo-recurrence, *i.e.*, treat with potentially curative intent by using SBRT if individual conditions allow.

How to combine SBRT?

Clinically, managing HCC patients with a combination of different treatment modalities, including SBRT, remains a challenge. Information from several domains should be judged together carefully, such as the individual patient's condition, the tumor location/characteristics, liver function reservation, facility resources, and the irradiation techniques available. A multidisciplinary evaluation before initiating treatment is crucial[34]. Moreover, to individualize the treatment combination into the best available option, clinicians must discuss options with patients and their families, using shared decision making (SDM)[136].

COMBINED MODERN RT TECHNIQUES FOR PATIENTS IN WHOM SBRT CANNOT BE DONE SAFELY

As mentioned above, for vulnerable patients with unresectable bulky HCCs (*i.e.*, > 5 cm), clinicians may hesitate to use SBRT because of the potentially greater risk of severe non-classic RILD or GI tract toxicity[61,137]. Under such conditions, a combination of modern irradiation techniques, such as VMAT, RMM[138], and modified simultaneously integrated boost (SIB)[139-142], has been reported to be useful.

In the literature, SIB dose prescription has been recommended for dose escalation[143]. The original SIB prescription delivers different homogenous doses to separate target regions in the same fraction numbers[144]. That is, the high-, intermediate-, and low-risk target volumes are simultaneously irradiated with high, intermediate, and low doses, respectively, per fraction (Figure 6A).

Recently, a modified SIB technique simultaneously applied a heterogeneous dose per fraction on peripheral (lower dose per fraction, *e.g.*, traditional 200 centigray [cGy]) and intra-tumor zones (escalated higher dose per fraction, *e.g.*, 240 cGy) (Figure 6B) [139-143]. Modified SIB simultaneously delivers an intra-tumor boost dose to the irradiated tumor's inner region to maximally enhance the possibility of tumor control in treating bulky tumors. This type of planned intra-tumor heterogenous dose distribution of modified SIB differs from the original SIB. This modified SIB has shown a potential role in managing bulky tumors, such as bulky pelvic tumor[139], retroperitoneal mass[140], breast cancer[141], and liver tumor[142,143,145]. Recently, higher doses per fraction, *e.g.*, 2.4 Gy on peripheral and 3 Gy on intra-tumor boost zones, have been prescribed for managing very bulky tumors[145].

To gain better tumor control without the cost of increasing irradiation-associated critical organ toxicity, a type of combined SIB and simultaneously integrated protection (SIP) was developed (Figure 6C)[146,147]. In conjunction with an SIB to the intra-tumor volume, SIP includes an attenuated dose per fraction on the overlapping sites of PTV and the extended critical organ volume, to gain a double benefit from clinical irradiation. Several unresectable bulky cancers have benefitted from this modern RT dose prescription technique, including HCC[142,146,148].

Following this line of dose prescription, a secondary-modified SIB technique (*i.e.*, SIEB; Figure 6D) was developed[59]. SIEB secondly remodeled the modified SIB to further expand the double benefits of SIB in managing unresectable bulky tumors. That is, SIEB simultaneously maintained an intra-tumor escalated boost of the modified SIB to gain maximal tumor control (*e.g.*, 220-240 cGy per fraction). Moreover, SIEB includes prescribing a planned attenuated peri-gross-tumor dose to all adjacent normal tissues (*e.g.*, 120-150 cGy per fraction). Note that this intended protection is not limited only to critical organs/structures[59]. As a result, in theory, SIEB's therapeutic gain is further enhanced even over that of modified SIB (Figure 6B) or combined SIB and SIP (Figure 6C). However, prospective randomized clinical trials should be conducted to confirm the effectiveness of SIEB in managing patients with unresectable HCC.

One topic for further investigation in modified SIB and SIEB is whether the intra-tumor boost volume should be guided by using metabolic images, such as positron emission tomography (PET)[149,150]. For example, F-18-labeled fluoromisonidazole PET/CT images have been used to guide an SIB-escalated boost to the hypoxia tumor region[150]. However, using metabolic images to guide the intra-tumor boost volume has some limitations in daily clinical use, such as resource availability and the dynamic change in the PET-guided increased-uptake region during the treatment course of irradiation. As a result, multiple sessions of PET images and adaptive RT treatment planning may be required. Hence, in practice, it is feasible to deliver an intra-tumor boost to the geometric-central zone to maintain treatment efficacy, enhance tumor control, and minimize irradiation toxicity[59,143,145].

CONCLUSION

Currently, two driving forces have come together to improve the treatment efficacy of RT in HCC. One is technological advancement, which enables the precise delivery of SBRT, increasing tumor control and reducing the side effects to the surrounding normal tissue. The other is the boom in the development of target therapies and checkpoint-blockade immunotherapy, which prolongs HCC patients' survival and re-emphasizes the importance of local tumor control. Currently, the role of RT in HCC treatment is actively being investigating in combination with systemic therapies to generate the STAR effect.

Remarkably, the development and use of combined modalities to increase liver reserve and patient tolerance may have the best chance to improve treatment outcomes. For HCC patients with any stage disease, RT plays a crucial role, whether delivered alone or in combination with other treatment modalities, because it is not limited by tumor location. Currently, a lack of level-III evidence is the main barrier to recommending SBRT as a standard of care in most international treatment guidelines. In this regard, several clinical trials of SBRT for HCC are ongoing. In the modern era,

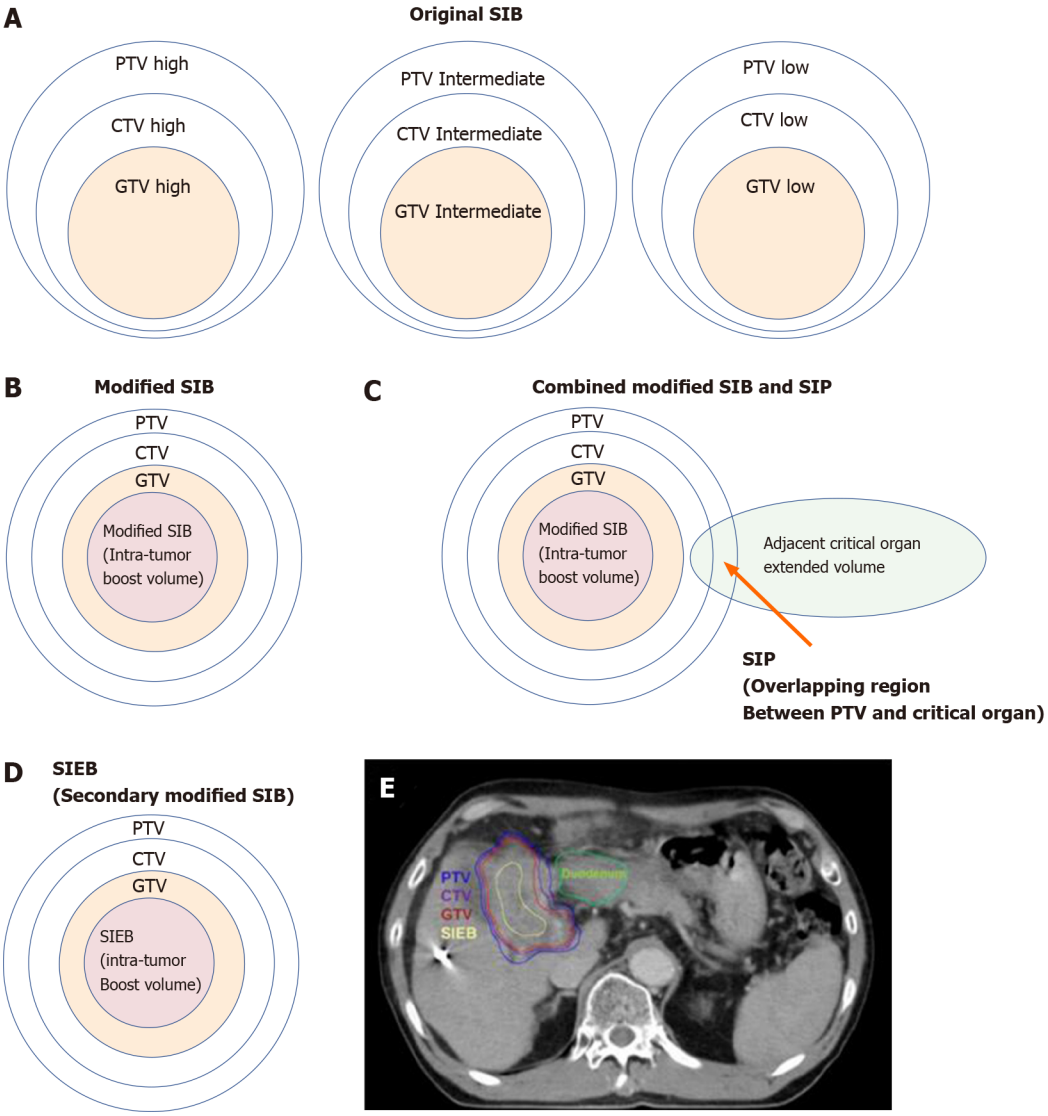


Figure 6 Cartoon and case illustrations of simultaneously integrated boost, modified simultaneously integrated boost, combined simultaneously integrated boost and simultaneously integrated protection, and simultaneously integrated inner-escalated boost dose-prescription techniques. A: A simple cartoon figure representing original simultaneously integrated boost (SIB) dose prescription. Original SIB is prescribed in different doses per fraction to different target regions according to the risk levels of recurrence. For example, uniform doses per fraction may be given at planning target volume (PTV) high, intermediate, and low with 240 centigray (cGy) (simultaneously integrated boost), 180-200 cGy (traditional dose per fraction), and 160 cGy (inferior to the traditional dose) in the same fractions, respectively; B: A simple cartoon figure representing a modified SIB dose prescription. Traditionally, radiotherapy is prescribed as a uniform dose per fraction (*e.g.*, 200 cGy) on PTV, which provides a homogeneous dose to cover clinical target volume (CTV) and gross target volume (GTV). Recently, to maximally enhance the possibility of tumor control, a modified SIB technique is applied using a planned non-homologous dose distribution, *i.e.*, escalating a simultaneous intra-tumor boost (*e.g.*, 220-240 cGy per fraction) in addition to a traditional covering dose to the PTV (*e.g.*, 200 cGy per fraction) in the same treatment fractions; C: A simple cartoon figure representing combined modified SIB and simultaneously integrated protection (SIP) dose prescription. To reduce treatment toxicities to adjacent critical organs/tissue, SIP was developed in conjunction with modified SIB. SIP prescribes an inferior-to-traditional dose per fraction, *e.g.*, 150 cGy, to the overlapping region between the PTV and the extended critical organ volume (as shown by the long arrow); D: A simple cartoon figure representing simultaneously integrated inner-escalated boost (SIEB) dose prescription. For further enhanced therapeutic gain (*i.e.*, increased tumor control and decreased treatment toxicity) in managing patients with unresectable liver tumors, we applied a secondary modified SIB (also termed SIEB). SIEB further escalates the intra-tumor boost (*e.g.*, 240-260 cGy per fraction) based on a planned generally attenuated peri-tumor dose (*e.g.*, 120-150 cGy per fraction delivered to the PTV), administered simultaneously. The intra-tumor SIEB boost volume is delineated as a uniform-inner-shrinkage area from the GTV with a margin of 1-10 mm (*i.e.*, a geometrically central zone), depending on the tumor size, the intensity of the dose escalation, the level of liver preservation, the closeness of the gastrointestinal organs to the irradiation targets, and the patient's condition. Note that an additional most-inner SIEB boost volume with the highest dose per fraction (*e.g.*, 260-300 cGy or higher) might be considered for highly selected patients with very bulky tumors. E: A clinical case with SIEB dose prescription. The blue, purple, and red lines show the PTV, CTV, and GTV of the irradiating target, respectively. In this case, based on the physician's choice and the patient's condition, a dose per fraction of 120 cGy was prescribed to the PTV, 150 cGy to the CTV, and 200 cGy to the GTV. Finally, in the yellow-outlined region, 280 cGy per fraction was simultaneously delivered to the SIEB boost volume. A total of 30 fractions were given, generating total doses levels of 3600 cGy, 4500 cGy, 6000 cGy, and 7400 cGy to the PTC, CTV, GTV, and SIEB boost volume, respectively. Note that the most peripheral dose per fraction of 120 cGy was chosen mainly due to a very close distance between the PTV and an adjacent critical organ, *i.e.*, the duodenum. This short-distance closeness could easily lead irradiation to harm the duodenum under the context of daily organ motion. SIB: Simultaneously integrated boost; SIP: Simultaneously integrated protection; SIEB: Simultaneously integrated inner-escalated boost; PTV: Planning target volume; CTV: Clinical target volume; GTV: Gross target volume.

the role of photon- and proton-based precise RT in managing patients with HCC is shifting continuously from palliative to curative intent.

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REFERENCES

1 **Global Burden of Disease Cancer Collaboration**, Fitzmaurice C, Abate D, Abbasi N, Abbastabar H, Abd-Allah F, Abdel-Rahman O, Abdelalim A, Abdoli A, Abdollahpour I, Abdulle ASM, Abebe ND, Abraha HN, Abu-Raddad LJ, Abualhasan A, Adedeji IA, Advani SM, Afarideh M, Afshari M, Aghaali M, Agius D, Agrawal S, Ahmadi A, Ahmadian E, Ahmadpour E, Ahmed MB, Akbari ME, Akinyemiju T, Al-Aly Z, AlAbdulKader AM, Alahdab F, Alam T, Alamene GM, Alemnew BTT, Alene KA, Alinia C, Alipour V, Aljunid SM, Bakeshei FA, Almadi MAH, Almasi-Hashiani A, Alsharif U, Alsowaidi S, Alvis-Guzman N, Amini E, Amini S, Amoako YA, Anbari Z, Anber NH, Andrei CL, Anjomshoa M, Ansari F, Ansariadi A, Appiah SCY, Arab-Zozani M, Arabloo J, Arefi Z, Aremu O, Areri HA, Artaman A, Asayesh H, Asfaw ET, Ashagre AF, Assadi R, Ataeinia B, Atalay HT, Ataro Z, Atique S, Ausloos M, Avila-Burgos L, Avokpaho EFGA, Awasthi A, Awoke N, Ayala Quintanilla BP, Ayanore MA, Ayele HT, Babaee E, Bacha U, Badawi A, Bagherzadeh M, Bagli E, Balakrishnan S, Balouchi A, Bärnighausen TW, Battista RJ, Behzadifar M, Bekele BB, Belay YB, Belayneh YM, Berfield KKS, Berhane A, Bernabe E, Beuran M, Bhakta N, Bhattacharyya K, Biadgo B, Bijani A, Bin Sayeed MS, Birungi C, Bisignano C, Bitew H, Bjørge T, Bleyer A, Bogale KA, Bojia HA, Borzì AM, Bosetti C, Bou-Orm IR, Brenner H, Brewer JD, Briko AN, Briko NI, Bustamante-Teixeira MT, Butt ZA, Carreras G, Carrero JJ, Carvalho F, Castro C, Castro F, Catalá-López F, Cerin E, Chaiah Y, Chanie WF, Chattu VK, Chaturvedi P, Chauhan NS, Chehrazi M, Chiang PP, Chichiabellu TY, Chido-Amajuoyi OG, Chimed-Ochir O, Choi JJ, Christopher DJ, Chu DT, Constantin MM, Costa VM, Crocetti E, Crowe CS, Curado MP, Dahlawi SMA, Damiani G, Darwish AH, Daryani A, das Neves J, Demeke FM, Demis AB, Demissie BW, Demoz GT, Denova-Gutiérrez E, Derakhshani A, Deribe KS, Desai R, Desalegn BB, Desta M, Dey S, Dharmaratne SD, Dhimal M, Diaz D, Dinberu MTT, Djalalinia S, Doku DT, Drake TM, Dubey M, Dubljanin E, Duken EE, Ebrahimi H, Effiong A, Eftekhari A, El Sayed I, Zaki MES, El-Jaafary SI, El-Khatib Z, Elemineh DA, Elkout H, Ellenbogen RG, Elsharkawy A, Emamian MH, Endalew DA, Endries AY, Eshrati B, Fadhil I, Fallah Omrani V, Faramarzi M, Farhangi MA, Farioli A, Farzadfar F, Fentahun N, Fernandes E, Feyissa GT, Filip I, Fischer F, Fisher JL, Force LM, Foroutan M, Freitas M, Fukumoto T, Futran ND, Gallus S, Gankpe FG, Gayesa RT, Gebrehiwot TT, Gebremeskel GG, Gedefaw GA, Gelaw BK, Geta B, Getachew S, Gezae KE, Ghafourifard M, Ghajar A, Ghashghaee A, Gholamian A, Gill PS, Ginindza TTG, Girmay A, Gizaw M, Gomez RS, Gopalani SV, Gorini G, Goulart BNG, Grada A, Ribeiro Guerra M, Guimaraes ALS, Gupta PC, Gupta R, Hadkhale K, Haj-Mirzaian A, Hamadeh RR, Hamidi S, Hanfore LK, Haro JM, Hasankhani M, Hasanzadeh A, Hassen HY, Hay RJ, Hay SI, Henok A, Henry NJ, Herteliu C, Hidru HD, Hoang CL, Hole MK, Hoogar P, Horita N, Hosgood HD, Hosseini M, Hosseinzadeh M, Hostiuc M, Hostiuc S, Househ M, Hussen MM, Ileanu B, Ilic MD, Innos K, Irvani SSN, Iseh KR, Islam SMS, Islami F, Jafari Balalami N, Jafarinia M, Jahangiry L, Jahani MA, Jahanmehr N, Jakovljevic M, James SL, Javanbakht M, Jayaraman S, Jee SH, Jenabi E, Jha RP, Jonas JB, Jonnagaddala J, Joo T, Jungari SB, Jürisson M, Kabir A, Kamangar F, Karch A, Karimi N, Karimian A, Kasaeian A, Kasahun GG, Kassa B, Kassa TD, Kassaw MW, Kaul A, Keiyoro PN, Kelbore AG, Kerbo AA, Khader YS, Khalilarjmandi M, Khan EA, Khan G, Khang YH, Khatab K, Khater A, Khayamzadeh M, Khazaee-Pool M, Khazaei S, Khoja AT, Khosravi MH, Khubchandani J, Kianipour N, Kim D, Kim YJ, Kisa A, Kisa S, Kissimova-Skarbek K, Komaki H, Koyanagi A, Krohn KJ, Bicer BK, Kugbey N, Kumar V, Kuupiel D, La Vecchia C, Lad DP, Lake EA, Lakew AM, Lal DK, Lami FH, Lan Q, Lasrado S, Lauriola P, Lazarus JV, Leigh J, Leshargie CT, Liao Y, Limenih MA, Listl S, Lopez AD, Lopukhov PD, Lunevicius R, Madadin M, Magdeldin S, El Razek HMA, Majeed A, Maleki A, Malekzadeh R, Manafi A, Manafi N, Manamo WA, Mansourian M, Mansournia MA, Mantovani LG, Maroufizadeh S, Martini SMS, Mashamba-Thompson TP, Massenburg BB, Maswabi MT, Mathur MR, McAlinden C, McKee M, Meheretu HAA, Mehrotra R, Mehta V, Meier T, Melaku YA, Meles GG, Meles HG, Melese A, Melku M, Memiah PTN, Mendoza W, Menezes RG, Merat S, Meretoja TJ, Mestrovic T, Miazgowski B, Miazgowski T, Mihretie KMM, Miller TR, Mills EJ, Mir SM, Mirzaei H, Mirzaei HR, Mishra R, Moazen B, Mohammad DK, Mohammad KA, Mohammad Y, Darwesh AM, Mohammadbeigi A, Mohammadi H, Mohammadi M, Mohammadian M, Mohammadian-Hafshejani

A, Mohammadoo-Khorasani M, Mohammadpourhodki R, Mohammed AS, Mohammed JA, Mohammed S, Mohebi F, Mokdad AH, Monasta L, Moodley Y, Moosazadeh M, Moossavi M, Moradi G, Moradi-Joo M, Moradi-Lakeh M, Moradpour F, Morawska L, Morgado-da-Costa J, Morisaki N, Morrison SD, Mosapour A, Mousavi SM, Muche AA, Muhammed OSS, Musa J, Nabhan AF, Naderi M, Nagarajan AJ, Nagel G, Nahvijou A, Naik G, Najafi F, Naldi L, Nam HS, Nasiri N, Nazari J, Negoi I, Neupane S, Newcomb PA, Nggada HA, Ngunjiri JW, Nguyen CT, Nikniaz L, Ningrum DNA, Nirayo YL, Nixon MR, Nnaji CA, Nojomi M, Nosratnejad S, Shiadeh MN, Obsa MS, Ofori-Asenso R, Ogbo FA, Oh IH, Olagunju AT, Olagunju TO, Oluwasanu MM, Omonisi AE, Onwujekwe OE, Oommen AM, Oren E, Ortega-Altamirano DDV, Ota E, Otstavnov SS, Owolabi MO, P A M, Padubidri JR, Pakhale S, Pakpour AH, Pana A, Park EK, Parsian H, Pashaei T, Patel S, Patil ST, Pennini A, Pereira DM, Piccinelli C, Pillay JD, Pirestani M, Pishgar F, Postma MJ, Pourjafar H, Pourmalek F, Pourshams A, Prakash S, Prasad N, Qorbani M, Rabiee M, Rabiee N, Radfar A, Rafiei A, Rahim F, Rahimi M, Rahman MA, Rajati F, Rana SM, Raoofi S, Rath GK, Rawaf DL, Rawaf S, Reiner RC, Renzaho AMN, Rezaei N, Rezapour A, Ribeiro AI, Ribeiro D, Ronfani L, Roro EM, Roshandel G, Rostami A, Saad RS, Sabbagh P, Sabour S, Saddik B, Safiri S, Sahebkar A, Salahshoor MR, Salehi F, Salem H, Salem MR, Salimzadeh H, Salomon JA, Samy AM, Sanabria J, Santric Milicevic MM, Sartorius B, Sarveazad A, Sathian B, Satpathy M, Savic M, Sawhney M, Sayyah M, Schneider IJC, Schöttker B, Sekerija M, Sepanlou SG, Sepehrimanesh M, Seyedmousavi S, Shaahmadi F, Shabaninejad H, Shahbaz M, Shaikh MA, Shamshirian A, Shamsizadeh M, Sharafi H, Sharafi Z, Sharif M, Sharifi A, Sharifi H, Sharma R, Sheikh A, Shirkoohi R, Shukla SR, Si S, Siabani S, Silva DAS, Silveira DGA, Singh A, Singh JA, Sisay S, Sitas F, Sobngwi E, Soofi M, Soriano JB, Stathopoulou V, Sufiyan MB, Tabarés-Seisdedos R, Tabuchi T, Takahashi K, Tamtaji OR, Tarawneh MR, Tassew SG, Taymoori P, Tehrani-Banihashemi A, Temsah MH, Temsah O, Tesfay BE, Tesfay FH, Teshale MY, Tessema GA, Thapa S, Tlaye KG, Topor-Madry R, Tovani-Palone MR, Traini E, Tran BX, Tran KB, Tsadik AG, Ullah I, Uthman OA, Vacante M, Vaezi M, Varona Pérez P, Veisani Y, Vidale S, Violante FS, Vlassov V, Vollset SE, Vos T, Vosoughi K, Vu GT, Vujcic IS, Wabinga H, Wachamo TM, Wagnew FS, Waheed Y, Weldegebreal F, Weldesamuel GT, Wijeratne T, Wondafrash DZ, Wonde TE, Wondmieneh AB, Workie HM, Yadav R, Yadegar A, Yadollahpour A, Yaseri M, Yazdi-Feyzabadi V, Yeshaneh A, Yimam MA, Yimer EM, Yisma E, Yonemoto N, Younis MZ, Yousefi B, Yousefifard M, Yu C, Zabeh E, Zadnik V, Moghadam TZ, Zaidi Z, Zamani M, Zandian H, Zangeneh A, Zaki L, Zendehdel K, Zenebe ZM, Zewale TA, Ziapour A, Zodpey S, Murray CJL. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2017: A Systematic Analysis for the Global Burden of Disease Study. *JAMA Oncol* 2019; **5**: 1749-1768 [PMID: 31560378 DOI: 10.1001/jamaoncol.2019.2996]

2 **Chiang CJ**, Yang YW, Chen JD, You SL, Yang HI, Lee MH, Lai MS, Chen CJ. Significant reduction in end-stage liver diseases burden through the national viral hepatitis therapy program in Taiwan. *Hepatology* 2015; **61**: 1154-1162 [PMID: 25476749 DOI: 10.1002/hep.27630]

3 **Omata M**, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK, Sarin SK. Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. *Hepatol Int* 2017; **11**: 317-370 [PMID: 28620797 DOI: 10.1007/s12072-017-9799-9]

4 **Yuen MF**, Hou JL, Chutaputti A; Asia Pacific Working Party on Prevention of Hepatocellular Carcinoma. Hepatocellular carcinoma in the Asia pacific region. *J Gastroenterol Hepatol* 2009; **24**: 346-353 [PMID: 19220670 DOI: 10.1111/j.1440-1746.2009.05784.x]

5 **Yun BY**, Lee HW, Min IK, Kim SU, Park JY, Kim DY, Ahn SH, Kim BK. Prognosis of Early-Stage Hepatocellular Carcinoma: Comparison between Trans-Arterial Chemoembolization and Radiofrequency Ablation. *Cancers (Basel)* 2020; **12** [PMID: 32899584 DOI: 10.3390/cancers12092527]

6 **Lu LH**, Mei J, Kan A, Ling YH, Li SH, Wei W, Chen MS, Zhang YF, Guo RP. Treatment optimization for recurrent hepatocellular carcinoma: Repeat hepatic resection versus radiofrequency ablation. *Cancer Med* 2020; **9**: 2997-3005 [PMID: 32108433 DOI: 10.1002/cam4.2951]

7 **Huang Y**, Song J, Zheng J, Jiang L, Yan L, Yang J, Zeng Y, Wu H. Comparison of Hepatic Resection Combined with Intraoperative Radiofrequency Ablation, or Hepatic Resection Alone, for Hepatocellular Carcinoma Patients with Multifocal Tumors Meeting the University of California San Francisco (UCSF) Criteria: A Propensity Score-Matched Analysis. *Ann Surg Oncol* 2020; **27**: 2334-2345 [PMID: 32016632 DOI: 10.1245/s10434-020-08231-0]

8 **Yu SJ**, Yoon JH, Lee JM, Lee JY, Kim SH, Cho YY, Yoo JJ, Lee M, Lee DH, Cho Y, Cho EJ, Lee JH, Kim YJ, Kim CY. Percutaneous ethanol injection therapy is comparable to radiofrequency ablation in hepatocellular carcinoma smaller than 1.5 cm: A matched case-control comparative analysis. *Medicine (Baltimore)* 2016; **95**: e4551 [PMID: 27583865 DOI: 10.1097/MD.0000000000004551]

9 **Qi C**, Gao H, Zhao Q, Zhang L. Computed Tomography-Guided Percutaneous Cryoablation for Subcardiac Hepatocellular Carcinoma: Safety, Efficacy, Therapeutic Results and Risk Factors for Survival Outcomes. *Cancer Manag Res* 2020; **12**: 3333-3342 [PMID: 32494197 DOI: 10.2147/CMAR.S250652]

10 **Qi C**, Li S, Zhang L. Development and Validation of a Clinicopathological-Based Nomogram to

Predict the Survival Outcome of Patients with Recurrent Hepatocellular Carcinoma After Hepatectomy Who Underwent Microwave Ablation. *Cancer Manag Res* 2020; **12**: 7589-7600 [PMID: 32904581 DOI: 10.2147/CMAR.S266052]

11 **Peisen F**, Maurer M, Grosse U, Nikolaou K, Syha R, Ketelsen D, Artzner C, Bitzer M, Horger M, Grözinger G. Predictive performance of the mHAP-II score in a real-life western cohort with hepatocellular carcinoma following trans-arterial chemoembolisation with drug-eluting beads (DEB-TACE). *Eur Radiol* 2020; **30**: 3782-3792 [PMID: 32125515 DOI: 10.1007/s00330-020-06734-8]

12 **Nakano MM**, Yamamoto A, Nishida N, Hamuro M, Hamamoto S, Jogo A, Sohgawa E, Kageyama K, Minami T, Miki Y. Risk factors for local recurrence of hepatocellular carcinoma after transcatheter arterial chemoembolization with drug-eluting beads (DEB-TACE). *Jpn J Radiol* 2019; **37**: 543-548 [PMID: 31055710 DOI: 10.1007/s11604-019-00840-4]

13 **Vesselle G**, Quirier-Leleu C, Velasco S, Charier F, Silvain C, Boucebci S, Ingrand P, Tasu JP. Predictive factors for complete response of chemoembolization with drug-eluting beads (DEB-TACE) for hepatocellular carcinoma. *Eur Radiol* 2016; **26**: 1640-1648 [PMID: 26455721 DOI: 10.1007/s00330-015-3982-y]

14 **Adcock CS**, Puneky LV, Campbell GS. Favorable Response of Metastatic Hepatocellular Carcinoma to Treatment with Trans-arterial Radioembolization Followed by Sorafenib and Nivolumab. *Cureus* 2019; **11**: e4083 [PMID: 31019861 DOI: 10.7759/cureus.4083]

15 **Rognoni C**, Ciani O, Sommariva S, Bargellini I, Bhoori S, Cioni R, Facciorusso A, Golfieri R, Gramenzi A, Mazzaferro V, Mosconi C, Ponziani F, Sacco R, Trevisani F, Tarricone R. Trans-arterial radioembolization for intermediate-advanced hepatocellular carcinoma: a budget impact analysis. *BMC Cancer* 2018; **18**: 715 [PMID: 29976149 DOI: 10.1186/s12885-018-4636-7]

16 **Fornaro L**, Vivaldi C, Lorenzoni G, Masi G, Bargellini I. Moving beyond sorafenib alone in advanced hepatocellular carcinoma: is hepatic arterial infusion chemotherapy the best option? *Ann Oncol* 2017; **28**: 667 [PMID: 27993799 DOI: 10.1093/annonc/mdw664]

17 **Ikeda M**, Shimizu S, Sato T, Morimoto M, Kojima Y, Inaba Y, Hagihara A, Kudo M, Nakamori S, Kaneko S, Sugimoto R, Tahara T, Ohmura T, Yasui K, Sato K, Ishii H, Furuse J, Okusaka T. Sorafenib plus hepatic arterial infusion chemotherapy with cisplatin *vs* sorafenib for advanced hepatocellular carcinoma: randomized phase II trial. *Ann Oncol* 2016; **27**: 2090-2096 [PMID: 27573564 DOI: 10.1093/annonc/mdw323]

18 **Ishikawa T**, Kubota T, Abe S, Watanabe Y, Sugano T, Inoue R, Iwanaga A, Seki K, Honma T, Yoshida T. Hepatic arterial infusion chemotherapy with cisplatin before radical local treatment of early hepatocellular carcinoma (JIS score 0/1) improves survival. *Ann Oncol* 2014; **25**: 1379-1384 [PMID: 24737779 DOI: 10.1093/annonc/mdu155]

19 **Labeur TA**, Hofsink Q, Takkenberg RB, van Delden OM, Mathôt RAA, Schinner R, Malfertheiner P, Amthauer H, Schütte K, Basu B, Kuhl C, Mayerle J, Ricke J, Klümpen HJ. The value of sorafenib trough levels in patients with advanced hepatocellular carcinoma - a substudy of the SORAMIC trial. *Acta Oncol* 2020; **59**: 1028-1035 [PMID: 32366155 DOI: 10.1080/0284186X.2020.1759826]

20 **Briggs A**, Daniele B, Dick K, Evans TRJ, Galle PR, Hubner RA, Lopez C, Siebert U, Tremblay G. Covariate-adjusted analysis of the Phase 3 REFLECT study of lenvatinib versus sorafenib in the treatment of unresectable hepatocellular carcinoma. *Br J Cancer* 2020; **122**: 1754-1759 [PMID: 32265508 DOI: 10.1038/s41416-020-0817-7]

21 **Fung AS**, Tam VC, Meyers DE, Sim HW, Knox JJ, Zaborska V, Davies J, Ko YJ, Batuyong E, Samawi H, Cheung WY, Lee-Ying R. Second-line treatment of hepatocellular carcinoma after sorafenib: Characterizing treatments used over the past 10 years and real-world eligibility for cabozantinib, regorafenib, and ramucirumab. *Cancer Med* 2020; **9**: 4640-4647 [PMID: 32378799 DOI: 10.1002/cam4.3116]

22 **Choi WM**, Choi J, Lee D, Shim JH, Lim YS, Lee HC, Chung YH, Lee YS, Park SR, Ryu MH, Ryoo BY, Lee SJ, Kim KM. Regorafenib Versus Nivolumab After Sorafenib Failure: Real-World Data in Patients With Hepatocellular Carcinoma. *Hepatol Commun* 2020; **4**: 1073-1086 [PMID: 32626838 DOI: 10.1002/hep4.1523]

23 **Duffy JP**, Vardanian A, Benjamin E, Watson M, Farmer DG, Ghobrial RM, Lipshutz G, Yersiz H, Lu DS, Lassman C, Tong MJ, Hiatt JR, Busuttil RW. Liver transplantation criteria for hepatocellular carcinoma should be expanded: a 22-year experience with 467 patients at UCLA. *Ann Surg* 2007; **246**: 502-509; discussion 509 [PMID: 17717454 DOI: 10.1097/SLA.0b013e318148c704]

24 **Omata M**, Lesmana LA, Tateishi R, Chen PJ, Lin SM, Yoshida H, Kudo M, Lee JM, Choi BI, Poon RT, Shiina S, Cheng AL, Jia JD, Obi S, Han KH, Jafri W, Chow P, Lim SG, Chawla YK, Budihusodo U, Gani RA, Lesmana CR, Putranto TA, Liaw YF, Sarin SK. Asian Pacific Association for the Study of the Liver consensus recommendations on hepatocellular carcinoma. *Hepatol Int* 2010; **4**: 439-474 [PMID: 20827404 DOI: 10.1007/s12072-010-9165-7]

25 **Shiina S**, Tateishi R, Arano T, Uchino K, Enooku K, Nakagawa H, Asaoka Y, Sato T, Masuzaki R, Kondo Y, Goto T, Yoshida H, Omata M, Koike K. Radiofrequency ablation for hepatocellular carcinoma: 10-year outcome and prognostic factors. *Am J Gastroenterol* 2012; **107**: 569-77; quiz 578 [PMID: 22158026 DOI: 10.1038/ajg.2011.425]

26 **Wahl DR**, Stenmark MH, Tao Y, Pollom EL, Caoili EM, Lawrence TS, Schipper MJ, Feng M. Outcomes After Stereotactic Body Radiotherapy or Radiofrequency Ablation for Hepatocellular Carcinoma. *J Clin Oncol* 2016; **34**: 452-459 [PMID: 26628466 DOI: 10.1200/JCO.2015.61.4925]

27 **Delis SG**, Dervenis C. Selection criteria for liver resection in patients with hepatocellular carcinoma and chronic liver disease. *World J Gastroenterol* 2008; **14**: 3452-3460 [PMID: 18567070 DOI:

10.3748/wjg.14.3452]
28 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J; EASL Panel of Experts on HCC. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430 [PMID: 11592607 DOI: 10.1016/s0168-8278(01)00130-1]
29 **McMasters KM**, Vauthey JN. Hepatocellular Carcinoma: Targeted Therapy and Multidisciplinary Care. New York: Springer, 2011
30 **Chan AC**, Chan SC, Chok KS, Cheung TT, Chiu DW, Poon RT, Fan ST, Lo CM. Treatment strategy for recurrent hepatocellular carcinoma: salvage transplantation, repeated resection, or radiofrequency ablation? *Liver Transpl* 2013; **19**: 411-419 [PMID: 23447460 DOI: 10.1002/lt.23605]
31 **Veltri A**, Moretto P, Doriguzzi A, Pagano E, Carrara G, Gandini G. Radiofrequency thermal ablation (RFA) after transarterial chemoembolization (TACE) as a combined therapy for unresectable non-early hepatocellular carcinoma (HCC). *Eur Radiol* 2006; **16**: 661-669 [PMID: 16228211 DOI: 10.1007/s00330-005-0029-9]
32 **Guo W**, He X, Li Z, Li Y. Combination of Transarterial Chemoembolization (TACE) and Radiofrequency Ablation (RFA) *vs* Surgical Resection (SR) on Survival Outcome of Early Hepatocellular Carcinoma: A Meta-Analysis. *Hepatogastroenterology* 2015; **62**: 710-714 [PMID: 26897959]
33 **Doi H**, Beppu N, Kitajima K, Kuribayashi K. Stereotactic Body Radiation Therapy for Liver Tumors: Current Status and Perspectives. *Anticancer Res* 2018; **38**: 591-599 [PMID: 29374681 DOI: 10.21873/anticanres.12263]
34 **Benson AB**, D'Angelica MI, Abbott DE, Abrams TA, Alberts SR, Anaya DA, Anders R, Are C, Brown D, Chang DT, Cloyd J, Covey AM, Hawkins W, Iyer R, Jacob R, Karachristos A, Kelley RK, Kim R, Palta M, Park JO, Sahai V, Schefter T, Sicklick JK, Singh G, Sohal D, Stein S, Tian GG, Vauthey JN, Venook AP, Hammond LJ, Darlow SD. Guidelines Insights: Hepatobiliary Cancers, Version 2.2019. *J Natl Compr Canc Netw* 2019; **17**: 302-310 [PMID: 30959462 DOI: 10.6004/jnccn.2019.0019]
35 **Marrero JA**, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; **68**: 723-750 [PMID: 29624699 DOI: 10.1002/hep.29913]
36 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]
37 **Kokudo N**, Takemura N, Hasegawa K, Takayama T, Kubo S, Shimada M, Nagano H, Hatano E, Izumi N, Kaneko S, Kudo M, Iijima H, Genda T, Tateishi R, Torimura T, Igaki H, Kobayashi S, Sakurai H, Murakami T, Watadani T, Matsuyama Y. Clinical practice guidelines for hepatocellular carcinoma: The Japan Society of Hepatology 2017 (4th JSH-HCC guidelines) 2019 update. *Hepatol Res* 2019; **49**: 1109-1113 [PMID: 31336394 DOI: 10.1111/hepr.13411]
38 **Korean Liver Cancer Association**; National Cancer Center. 2018 Korean Liver Cancer Association-National Cancer Center Korea Practice Guidelines for the Management of Hepatocellular Carcinoma. *Gut Liver* 2019; **13**: 227-299 [PMID: 31060120 DOI: 10.5009/gnl19024]
39 **Zhou J**, Sun HC, Wang Z, Cong WM, Wang JH, Zeng MS, Yang JM, Bie P, Liu LX, Wen TF, Han GH, Wang MQ, Liu RB, Lu LG, Ren ZG, Chen MS, Zeng ZC, Liang P, Liang CH, Chen M, Yan FH, Wang WP, Ji Y, Cheng WW, Dai CL, Jia WD, Li YM, Li YX, Liang J, Liu TS, Lv GY, Mao YL, Ren WX, Shi HC, Wang WT, Wang XY, Xing BC, Xu JM, Yang JY, Yang YF, Ye SL, Yin ZY, Zhang BH, Zhang SJ, Zhou WP, Zhu JY, Liu R, Shi YH, Xiao YS, Dai Z, Teng GJ, Cai JQ, Wang WL, Dong JH, Li Q, Shen F, Qin SK, Fan J. Guidelines for Diagnosis and Treatment of Primary Liver Cancer in China (2017 Edition). *Liver Cancer* 2018; **7**: 235-260 [PMID: 30319983 DOI: 10.1159/000488035]
40 **Surveillance group**. ; Diagnosis group; Staging group; Surgery group; Local ablation group; TACE/TARE/HAI group; Target therapy/systemic therapy group; Radiotherapy group; Prevention group; Drafting group. Management consensus guideline for hepatocellular carcinoma: 2016 updated by the Taiwan Liver Cancer Association and the Gastroenterological Society of Taiwan. *J Formos Med Assoc* 2018; **117**: 381-403 [PMID: 29074347 DOI: 10.1016/j.jfma.2017.09.007]
41 **Ueno S**, Tanabe G, Nuruki K, Hamanoue M, Komorizono Y, Oketani M, Hokotate H, Inoue H, Baba Y, Imamura Y, Aikou T. Prognostic performance of the new classification of primary liver cancer of Japan (4th edition) for patients with hepatocellular carcinoma: a validation analysis. *Hepatol Res* 2002; **24**: 395-403 [PMID: 12479938 DOI: 10.1016/s1386-6346(02)00144-4]
42 **Kudo M**, Kitano M, Sakurai T, Nishida N. General Rules for the Clinical and Pathological Study of Primary Liver Cancer, Nationwide Follow-Up Survey and Clinical Practice Guidelines: The Outstanding Achievements of the Liver Cancer Study Group of Japan. *Dig Dis* 2015; **33**: 765-770 [PMID: 26488173 DOI: 10.1159/000439101]
43 **Künzli BM**, Abitabile P, Maurer CA. Radiofrequency ablation of liver tumors: Actual limitations and potential solutions in the future. *World J Hepatol* 2011; **3**: 8-14 [PMID: 21307982 DOI: 10.4254/wjh.v3.i1.8]

44 **Zhang X**, Wang K, Wang M, Yang G, Ye X, Wu M, Cheng S. Transarterial chemoembolization (TACE) combined with sorafenib versus TACE for hepatocellular carcinoma with portal vein tumor thrombus: a systematic review and meta-analysis. *Oncotarget* 2017; **8**: 29416-29427 [PMID: 28177886 DOI: 10.18632/oncotarget.15075]

45 **Huo YR**, Eslick GD. Transcatheter Arterial Chemoembolization Plus Radiotherapy Compared With Chemoembolization Alone for Hepatocellular Carcinoma: A Systematic Review and Meta-analysis. *JAMA Oncol* 2015; **1**: 756-765 [PMID: 26182200 DOI: 10.1001/jamaoncol.2015.2189]

46 **Kang JK**, Kim MS, Cho CK, Yang KM, Yoo HJ, Kim JH, Bae SH, Jung DH, Kim KB, Lee DH, Han CJ, Kim J, Park SC, Kim YH. Stereotactic body radiation therapy for inoperable hepatocellular carcinoma as a local salvage treatment after incomplete transarterial chemoembolization. *Cancer* 2012; **118**: 5424-5431 [PMID: 22570179 DOI: 10.1002/cncr.27533]

47 **Li MF**, Leung HW, Chan AL, Wang SY. Network meta-analysis of treatment regimens for inoperable advanced hepatocellular carcinoma with portal vein invasion. *Ther Clin Risk Manag* 2018; **14**: 1157-1168 [PMID: 30013351 DOI: 10.2147/TCRM.S162898]

48 **Kwon JH**, Kim GM, Han K, Won JY, Kim MD, Lee DY, Lee J, Choi W, Kim YS, Kim DY, Han KH. Safety and Efficacy of Transarterial Radioembolization Combined with Chemoembolization for Bilobar Hepatocellular Carcinoma: A Single-Center Retrospective Study. *Cardiovasc Intervent Radiol* 2018; **41**: 459-465 [PMID: 29067511 DOI: 10.1007/s00270-017-1826-7]

49 **Qu XD**, Chen CS, Wang JH, Yan ZP, Chen JM, Gong GQ, Liu QX, Luo JJ, Liu LX, Liu R, Qian S. The efficacy of TACE combined sorafenib in advanced stages hepatocellullar carcinoma. *BMC Cancer* 2012; **12**: 263 [PMID: 22721173 DOI: 10.1186/1471-2407-12-263]

50 **Pan T**, Li XS, Xie QK, Wang JP, Li W, Wu PH, Zhao M. Safety and efficacy of transarterial chemoembolization plus sorafenib for hepatocellular carcinoma with portal venous tumour thrombus. *Clin Radiol* 2014; **69**: e553-e561 [PMID: 25304928 DOI: 10.1016/j.crad.2014.09.007]

51 **Salman A**, Simoneau E, Hassanain M, Chaudhury P, Boucher LM, Valenti D, Cabrera T, Nudo C, Metrakos P. Combined sorafenib and yttrium-90 radioembolization for the treatment of advanced hepatocellular carcinoma. *Curr Oncol* 2016; **23**: e472-e480 [PMID: 27803608 DOI: 10.3747/co.23.2827]

52 **Ricke J**, Bulla K, Kolligs F, Peck-Radosavljevic M, Reimer P, Sangro B, Schott E, Schütte K, Verslype C, Walecki J, Malfertheiner P; SORAMIC study group. Safety and toxicity of radioembolization plus Sorafenib in advanced hepatocellular carcinoma: analysis of the European multicentre trial SORAMIC. *Liver Int* 2015; **35**: 620-626 [PMID: 24930619 DOI: 10.1111/liv.12622]

53 **Lorenzin D**, Pravisani R, Leo CA, Bugiantella W, Soardo G, Carnelutti A, Umberto B, Risaliti A. Complete Remission of Unresectable Hepatocellular Carcinoma After Combined Sorafenib and Adjuvant Yttrium-90 Radioembolization. *Cancer Biother Radiopharm* 2016; **31**: 65-69 [PMID: 26986817 DOI: 10.1089/cbr.2015.1905]

54 **Floudas CS**, Brar G, Greten TF. Immunotherapy: Current Status and Future Perspectives. *Dig Dis Sci* 2019; **64**: 1030-1040 [PMID: 30830521 DOI: 10.1007/s10620-019-05516-7]

55 **Okusaka T**, Ikeda M. Immunotherapy for hepatocellular carcinoma: current status and future perspectives. *ESMO Open* 2018; **3**: e000455 [PMID: 30622744 DOI: 10.1136/esmoopen-2018-000455]

56 **Ikeda M**, Morizane C, Ueno M, Okusaka T, Ishii H, Furuse J. Chemotherapy for hepatocellular carcinoma: current status and future perspectives. *Jpn J Clin Oncol* 2018; **48**: 103-114 [PMID: 29253194 DOI: 10.1093/jjco/hyx180]

57 **Wehrenberg-Klee E**, Goyal L, Dugan M, Zhu AX, Ganguli S. Y-90 Radioembolization Combined with a PD-1 Inhibitor for Advanced Hepatocellular Carcinoma. *Cardiovasc Intervent Radiol* 2018; **41**: 1799-1802 [PMID: 29845347 DOI: 10.1007/s00270-018-1993-1]

58 **Torok JA**, Salama JK. Combining immunotherapy and radiotherapy for the STAR treatment. *Nat Rev Clin Oncol* 2019; **16**: 666-667 [PMID: 31541199 DOI: 10.1038/s41571-019-0277-2]

59 **Lin YH**, Hung SK, Chiou WY, Lee MS, Shen BJ, Chen LC, Liu DW, Tsai WT, Lin PH, Shih YT, Hsu FC, Tsai SJ, Chan MWY, Lin HY. Significant symptoms alleviation and tumor volume reduction after combined simultaneously integrated inner-escalated boost and volumetric-modulated arc radiotherapy in a patient with unresectable bulky hepatocellular carcinoma: A care-compliant case report. *Medicine (Baltimore)* 2016; **95**: e4717 [PMID: 27559982 DOI: 10.1097/MD.0000000000004717]

60 **Frank SJ**, Zhu XR. Proton Therapy: Indications, Techniques, and Outcomes. Elsevier Health Sciences, 2020

61 **Park HC**, Seong J, Han KH, Chon CY, Moon YM, Suh CO. Dose-response relationship in local radiotherapy for hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2002; **54**: 150-155 [PMID: 12182985 DOI: 10.1016/s0360-3016(02)02864-x]

62 **Kim TH**, Park JW, Kim YJ, Kim BH, Woo SM, Moon SH, Kim SS, Koh YH, Lee WJ, Park SJ, Kim JY, Kim DY, Kim CM. Phase I dose-escalation study of proton beam therapy for inoperable hepatocellular carcinoma. *Cancer Res Treat* 2015; **47**: 34-45 [PMID: 25381830 DOI: 10.4143/crt.2013.218]

63 **Mizumoto M**, Tokuuye K, Sugahara S, Nakayama H, Fukumitsu N, Ohara K, Abei M, Shoda J, Tohno E, Minami M. Proton beam therapy for hepatocellular carcinoma adjacent to the porta hepatis. *Int J Radiat Oncol Biol Phys* 2008; **71**: 462-467 [PMID: 18243571 DOI: 10.1016/j.ijrobp.2007.09.056]

64 **Fukumitsu N**, Sugahara S, Nakayama H, Fukuda K, Mizumoto M, Abei M, Shoda J, Thono E, Tsuboi K, Tokuuye K. A prospective study of hypofractionated proton beam therapy for patients with hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 2009; **74**: 831-836 [PMID: 19304408 DOI: 10.1016/j.ijrobp.2008.10.073]

65 **Nakayama H**, Sugahara S, Fukuda K, Abei M, Shoda J, Sakurai H, Tsuboi K, Matsuzaki Y, Tokuuye K. Proton beam therapy for hepatocellular carcinoma located adjacent to the alimentary tract. *Int J Radiat Oncol Biol Phys* 2011; **80**: 992-995 [PMID: 21543162 DOI: 10.1016/j.ijrobp.2010.03.015]

66 **Chang JY**, Li QQ, Xu QY, Allen PK, Rebueno N, Gomez DR, Balter P, Komaki R, Mehran R, Swisher SG, Roth JA. Stereotactic ablative radiation therapy for centrally located early stage or isolated parenchymal recurrences of non-small cell lung cancer: how to fly in a "no fly zone". *Int J Radiat Oncol Biol Phys* 2014; **88**: 1120-1128 [PMID: 24661665 DOI: 10.1016/j.ijrobp.2014.01.022]

67 **Hong TS**, Wo JY, Yeap BY, Ben-Josef E, McDonnell EI, Blaszkowsky LS, Kwak EL, Allen JN, Clark JW, Goyal L, Murphy JE, Javle MM, Wolfgang JA, Drapek LC, Arellano RS, Mamon HJ, Mullen JT, Yoon SS, Tanabe KK, Ferrone CR, Ryan DP, DeLaney TF, Crane CH, Zhu AX. Multi-Institutional Phase II Study of High-Dose Hypofractionated Proton Beam Therapy in Patients With Localized, Unresectable Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. *J Clin Oncol* 2016; **34**: 460-468 [PMID: 26668346 DOI: 10.1200/JCO.2015.64.2710]

68 **Nakayama H**, Sugahara S, Tokita M, Fukuda K, Mizumoto M, Abei M, Shoda J, Sakurai H, Tsuboi K, Tokuuye K. Proton beam therapy for hepatocellular carcinoma: the University of Tsukuba experience. *Cancer* 2009; **115**: 5499-5506 [PMID: 19645024 DOI: 10.1002/cncr.24619]

69 **Kawashima M**, Furuse J, Nishio T, Konishi M, Ishii H, Kinoshita T, Nagase M, Nihei K, Ogino T. Phase II study of radiotherapy employing proton beam for hepatocellular carcinoma. *J Clin Oncol* 2005; **23**: 1839-1846 [PMID: 15774777 DOI: 10.1200/JCO.2005.00.620]

70 **Sanford NN**, Pursley J, Noe B, Yeap BY, Goyal L, Clark JW, Allen JN, Blaszkowsky LS, Ryan DP, Ferrone CR, Tanabe KK, Qadan M, Crane CH, Koay EJ, Eyler C, DeLaney TF, Zhu AX, Wo JY, Grassberger C, Hong TS. Protons *vs* Photons for Unresectable Hepatocellular Carcinoma: Liver Decompensation and Overall Survival. *Int J Radiat Oncol Biol Phys* 2019; **105**: 64-72 [PMID: 30684667 DOI: 10.1016/j.ijrobp.2019.01.076]

71 **Hsieh CE**, Venkatesulu BP, Lee CH, Hung SP, Wong PF, Aithala SP, Kim BK, Rao A, Tung-Chieh Chang J, Tsang NM, Wang CC, Lee CC, Lin CC, Tseng JH, Chou WC, Wang YC, Krishnan S, Hong JH. Predictors of Radiation-Induced Liver Disease in Eastern and Western Patients With Hepatocellular Carcinoma Undergoing Proton Beam Therapy. *Int J Radiat Oncol Biol Phys* 2019; **105**: 73-86 [PMID: 30797890 DOI: 10.1016/j.ijrobp.2019.02.032]

72 **Chuong MD**, Kaiser A, Khan F, Parikh P, Ben-Josef E, Crane C, Brunner T, Okumura T, Schreuder N, Bentzen SM, Gutierrez A, Mendez Romero A, Yoon SM, Sharma N, Kim TH, Kishi K, Moeslein F, Hoffe S, Schefter T, Hanish S, Scorsetti M, Apisarnthanarax S. Consensus Report From the Miami Liver Proton Therapy Conference. *Front Oncol* 2019; **9**: 457 [PMID: 31214502 DOI: 10.3389/fonc.2019.00457]

73 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699 [PMID: 8594428 DOI: 10.1056/NEJM199603143341104]

74 **Rajyaguru DJ**, Borgert AJ, Smith AL, Thomes RM, Conway PD, Halfdanarson TR, Truty MJ, Kurup AN, Go RS. Radiofrequency Ablation Versus Stereotactic Body Radiotherapy for Localized Hepatocellular Carcinoma in Nonsurgically Managed Patients: Analysis of the National Cancer Database. *J Clin Oncol* 2018; **36**: 600-608 [PMID: 29328861 DOI: 10.1200/JCO.2017.75.3228]

75 **Mazzaferro V**, Llovet JM, Miceli R, Bhoori S, Schiavo M, Mariani L, Camerini T, Roayaie S, Schwartz ME, Grazi GL, Adam R, Neuhaus P, Salizzoni M, Bruix J, Forner A, De Carlis L, Cillo U, Burroughs AK, Troisi R, Rossi M, Gerunda GE, Lerut J, Belghiti J, Boin I, Gugenheim J, Rochling F, Van Hoek B, Majno P; Metroticket Investigator Study Group. Predicting survival after liver transplantation in patients with hepatocellular carcinoma beyond the Milan criteria: a retrospective, exploratory analysis. *Lancet Oncol* 2009; **10**: 35-43 [PMID: 19058754 DOI: 10.1016/S1470-2045(08)70284-5]

76 **Burkoň P**, Slavik M, Kazda T, Pospíšil P, Prochazka T, Vrzal M, Šlampa P. Stereotactic Body Radiotherapy - Current Indications. *Klin Onkol* 2019; **32**: 10-24 [PMID: 30894002 DOI: 10.14735/amko201910]

77 **Murray LJ**, Dawson LA. Advances in Stereotactic Body Radiation Therapy for Hepatocellular Carcinoma. *Semin Radiat Oncol* 2017; **27**: 247-255 [PMID: 28577832 DOI: 10.1016/j.semradonc.2017.02.002]

78 **Ohri N**, Dawson LA, Krishnan S, Seong J, Cheng JC, Sarin SK, Kinkhabwala M, Ahmed MM, Vikram B, Coleman CN, Guha C. Radiotherapy for Hepatocellular Carcinoma: New Indications and Directions for Future Study. *J Natl Cancer Inst* 2016; **108** [PMID: 27377923 DOI: 10.1093/jnci/djw133]

79 **Chen CP**. Role of Radiotherapy in the Treatment of Hepatocellular Carcinoma. *J Clin Transl Hepatol* 2019; **7**: 183-190 [PMID: 31293919 DOI: 10.14218/JCTH.2018.00060]

80 **Culleton S**, Jiang H, Haddad CR, Kim J, Brierley J, Brade A, Ringash J, Dawson LA. Outcomes following definitive stereotactic body radiotherapy for patients with Child-Pugh B or C

hepatocellular carcinoma. *Radiother Oncol* 2014; **111**: 412-417 [PMID: 24906626 DOI: 10.1016/j.radonc.2014.05.002]

81 **Gresswell S**, Tobillo R, Hasan S, Uemura T, Machado L, Thai N, Kirichenko A. Stereotactic body radiotherapy used as a bridge to liver transplant in patients with hepatocellular carcinoma and Child-Pugh score ≥8 cirrhosis. *J Radiosurg SBRT* 2018; **5**: 261-267 [PMID: 30538886]

82 **National Comprehensive Cancer Network**. NCCN Clinical Practice Guidelines in Oncology: Hepatobiliary Cancers. [cited 7 July 2020]. Available from: http://www.nccn.org/professionals/physician_gls

83 **Ronot M**, Bouattour M, Wassermann J, Bruno O, Dreyer C, Larroque B, Castera L, Vilgrain V, Belghiti J, Raymond E, Faivre S. Alternative Response Criteria (Choi, European association for the study of the liver, and modified Response Evaluation Criteria in Solid Tumors [RECIST]) Versus RECIST 1.1 in patients with advanced hepatocellular carcinoma treated with sorafenib. *Oncologist* 2014; **19**: 394-402 [PMID: 24652387 DOI: 10.1634/theoncologist.2013-0114]

84 **Liu M**, Lin MX, Lu MD, Xu ZF, Zheng KG, Wang W, Kuang M, Zhuang WQ, Xie XY. Comparison of contrast-enhanced ultrasound and contrast-enhanced computed tomography in evaluating the treatment response to transcatheter arterial chemoembolization of hepatocellular carcinoma using modified RECIST. *Eur Radiol* 2015; **25**: 2502-2511 [PMID: 25702094 DOI: 10.1007/s00330-015-3611-9]

85 **Edeline J**, Boucher E, Rolland Y, Vauléon E, Pracht M, Perrin C, Le Roux C, Raoul JL. Comparison of tumor response by Response Evaluation Criteria in Solid Tumors (RECIST) and modified RECIST in patients treated with sorafenib for hepatocellular carcinoma. *Cancer* 2012; **118**: 147-156 [PMID: 21713764 DOI: 10.1002/cncr.26255]

86 **Teraoka Y**, Kimura T, Aikata H, Daijo K, Osawa M, Honda F, Nakamura Y, Morio K, Morio R, Hatooka M, Kobayashi T, Nakahara T, Murakami E, Nagaoki Y, Kawaoka T, Tsuge M, Hiramatsu A, Imamura M, Kawakami Y, Nagata Y, Chayama K. Clinical outcomes of stereotactic body radiotherapy for elderly patients with hepatocellular carcinoma. *Hepatol Res* 2018; **48**: 193-204 [PMID: 28544062 DOI: 10.1111/hepr.12916]

87 **Chen MS**, Li JQ, Zheng Y, Guo RP, Liang HH, Zhang YQ, Lin XJ, Lau WY. A prospective randomized trial comparing percutaneous local ablative therapy and partial hepatectomy for small hepatocellular carcinoma. *Ann Surg* 2006; **243**: 321-328 [PMID: 16495695 DOI: 10.1097/01.sla.0000201480.65519.b8]

88 **Hara K**, Takeda A, Tsurugai Y, Saigusa Y, Sanuki N, Eriguchi T, Maeda S, Tanaka K, Numata K. Radiotherapy for Hepatocellular Carcinoma Results in Comparable Survival to Radiofrequency Ablation: A Propensity Score Analysis. *Hepatology* 2019; **69**: 2533-2545 [PMID: 30805950 DOI: 10.1002/hep.30591]

89 **Kim N**, Kim HJ, Won JY, Kim DY, Han KH, Jung I, Seong J. Retrospective analysis of stereotactic body radiation therapy efficacy over radiofrequency ablation for hepatocellular carcinoma. *Radiother Oncol* 2019; **131**: 81-87 [PMID: 30773192 DOI: 10.1016/j.radonc.2018.12.013]

90 **Elshaarawy O**, Gomaa A, Omar H, Rewisha E, Waked I. Intermediate stage hepatocellular carcinoma: a summary review. *J Hepatocell Carcinoma* 2019; **6**: 105-117 [PMID: 31372364 DOI: 10.2147/JHC.S168682]

91 **Sapir E**, Tao Y, Schipper MJ, Bazzi L, Novelli PM, Devlin P, Owen D, Cuneo KC, Lawrence TS, Parikh ND, Feng M. Stereotactic Body Radiation Therapy as an Alternative to Transarterial Chemoembolization for Hepatocellular Carcinoma. *Int J Radiat Oncol Biol Phys* 2018; **100**: 122-130 [PMID: 29066120 DOI: 10.1016/j.ijrobp.2017.09.001]

92 **Lu L**, Zeng J, Wen Z, Tang C, Xu N. Transcatheter arterial chemoembolisation followed by three-dimensional conformal radiotherapy versus transcatheter arterial chemoembolisation alone for primary hepatocellular carcinoma in adults. *Cochrane Database Syst Rev* 2019; **2**: CD012244 [PMID: 30776082 DOI: 10.1002/14651858.CD012244.pub2]

93 **Xie H**, Yu H, Tian S, Yang X, Wang X, Yang Z, Wang H, Guo Z. What is the best combination treatment with transarterial chemoembolization of unresectable hepatocellular carcinoma? *Oncotarget* 2017; **8**: 100508-100523 [PMID: 29245997 DOI: 10.18632/oncotarget.20119]

94 **Paik EK**, Kim MS, Jang WI, Seo YS, Cho CK, Yoo HJ, Han CJ, Park SC, Kim SB, Kim YH. Benefits of stereotactic ablative radiotherapy combined with incomplete transcatheter arterial chemoembolization in hepatocellular carcinoma. *Radiat Oncol* 2016; **11**: 22 [PMID: 26896371 DOI: 10.1186/s13014-016-0597-7]

95 **Jacob R**, Turley F, Redden DT, Saddekni S, Aal AK, Keene K, Yang E, Zarzour J, Bolus D, Smith JK, Gray S, White J, Eckhoff DE, DuBay DA. Adjuvant stereotactic body radiotherapy following transarterial chemoembolization in patients with non-resectable hepatocellular carcinoma tumours of ≥ 3 cm. *HPB (Oxford)* 2015; **17**: 140-149 [PMID: 25186290 DOI: 10.1111/hpb.12331]

96 **Lo CH**, Huang WY, Lee MS, Lin KT, Lin TP, Chang PY, Fan CY, Jen YM. Stereotactic ablative radiotherapy for unresectable hepatocellular carcinoma patients who failed or were unsuitable for transarterial chemoembolization. *Eur J Gastroenterol Hepatol* 2014; **26**: 345-352 [PMID: 24384685 DOI: 10.1097/MEG.0000000000000032]

97 **Hardy-Abeloos C**, Lazarev S, Ru M, Kim E, Fischman A, Moshier E, Rosenzweig K, Buckstein M. Safety and Efficacy of Liver Stereotactic Body Radiation Therapy for Hepatocellular Carcinoma After Segmental Transarterial Radioembolization. *Int J Radiat Oncol Biol Phys* 2019; **105**: 968-976 [PMID: 31536781 DOI: 10.1016/j.ijrobp.2019.09.006]

98 **Sapisochin G**, Barry A, Doherty M, Fischer S, Goldaracena N, Rosales R, Russo M, Beecroft R,

Ghanekar A, Bhat M, Brierley J, Greig PD, Knox JJ, Dawson LA, Grant DR. Stereotactic body radiotherapy *vs* TACE or RFA as a bridge to transplant in patients with hepatocellular carcinoma. An intention-to-treat analysis. *J Hepatol* 2017; **67**: 92-99 [PMID: 28257902 DOI: 10.1016/j.jhep.2017.02.022]

99 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

100 **Cheng AL**, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25-34 [PMID: 19095497 DOI: 10.1016/S1470-2045(08)70285-7]

101 **Rim CH**, Kim CY, Yang DS, Yoon WS. Comparison of radiation therapy modalities for hepatocellular carcinoma with portal vein thrombosis: A meta-analysis and systematic review. *Radiother Oncol* 2018; **129**: 112-122 [PMID: 29233562 DOI: 10.1016/j.radonc.2017.11.013]

102 **Bettinger D**, Pinato DJ, Schultheiss M, Sharma R, Rimassa L, Pressiani T, Burlone ME, Pirisi M, Kudo M, Park JW, Buettner N, Neumann-Haefelin C, Boettler T, Abbasi-Senger N, Alheit H, Baus W, Blanck O, Gerum S, Guckenberger M, Habermehl D, Ostheimer C, Riesterer O, Tamihardja J, Grosu AL, Thimme R, Brunner TB, Gkika E. Stereotactic Body Radiation Therapy as an Alternative Treatment for Patients with Hepatocellular Carcinoma Compared to Sorafenib: A Propensity Score Analysis. *Liver Cancer* 2019; **8**: 281-294 [PMID: 31602371 DOI: 10.1159/000490260]

103 **Leung HW**, Liu CF, Chan AL. Cost-effectiveness of sorafenib versus SBRT for unresectable advanced hepatocellular carcinoma. *Radiat Oncol* 2016; **11**: 69 [PMID: 27193904 DOI: 10.1186/s13014-016-0644-4]

104 **Brade AM**, Ng S, Brierley J, Kim J, Dinniwell R, Ringash J, Wong RR, Cho C, Knox J, Dawson LA. Phase 1 Trial of Sorafenib and Stereotactic Body Radiation Therapy for Hepatocellular Carcinoma. *Int J Radiat Oncol Biol Phys* 2016; **94**: 580-587 [PMID: 26867886 DOI: 10.1016/j.ijrobp.2015.11.048]

105 **Teitz-Tennenbaum S**, Li Q, Rynkiewicz S, Ito F, Davis MA, McGinn CJ, Chang AE. Radiotherapy potentiates the therapeutic efficacy of intratumoral dendritic cell administration. *Cancer Res* 2003; **63**: 8466-8475 [PMID: 14679011]

106 **Chi KH**, Liu SJ, Li CP, Kuo HP, Wang YS, Chao Y, Hsieh SL. Combination of conformal radiotherapy and intratumoral injection of adoptive dendritic cell immunotherapy in refractory hepatoma. *J Immunother* 2005; **28**: 129-135 [PMID: 15725956 DOI: 10.1097/01.cji.0000154248.74383.5e]

107 **Teitz-Tennenbaum S**, Li Q, Okuyama R, Davis MA, Sun R, Whitfield J, Knibbs RN, Stoolman LM, Chang AE. Mechanisms involved in radiation enhancement of intratumoral dendritic cell therapy. *J Immunother* 2008; **31**: 345-358 [PMID: 18391761 DOI: 10.1097/CJI.0b013e318163628c]

108 **Rai V**, Abdo J, Alsuwaidan AN, Agrawal S, Sharma P, Agrawal DK. Cellular and molecular targets for the immunotherapy of hepatocellular carcinoma. *Mol Cell Biochem* 2018; **437**: 13-36 [PMID: 28593566 DOI: 10.1007/s11010-017-3092-z]

109 **Choi C**, Yoo GS, Cho WK, Park HC. Optimizing radiotherapy with immune checkpoint blockade in hepatocellular carcinoma. *World J Gastroenterol* 2019; **25**: 2416-2429 [PMID: 31171886 DOI: 10.3748/wjg.v25.i20.2416]

110 **Kreidieh M**, Zeidan YH, Shamseddine A. The Combination of Stereotactic Body Radiation Therapy and Immunotherapy in Primary Liver Tumors. *J Oncol* 2019; **2019**: 4304817 [PMID: 31182960 DOI: 10.1155/2019/4304817]

111 **Bernstein MB**, Krishnan S, Hodge JW, Chang JY. Immunotherapy and stereotactic ablative radiotherapy (ISABR): a curative approach? *Nat Rev Clin Oncol* 2016; **13**: 516-524 [PMID: 26951040 DOI: 10.1038/nrclinonc.2016.30]

112 **Sharabi AB**, Nirschl CJ, Kochel CM, Nirschl TR, Francica BJ, Velarde E, Deweese TL, Drake CG. Stereotactic Radiation Therapy Augments Antigen-Specific PD-1-Mediated Antitumor Immune Responses *via* Cross-Presentation of Tumor Antigen. *Cancer Immunol Res* 2015; **3**: 345-355 [PMID: 25527358 DOI: 10.1158/2326-6066.CIR-14-0196]

113 **Lee Y**, Auh SL, Wang Y, Burnette B, Meng Y, Beckett M, Sharma R, Chin R, Tu T, Weichselbaum RR, Fu YX. Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: changing strategies for cancer treatment. *Blood* 2009; **114**: 589-595 [PMID: 19349616 DOI: 10.1182/blood-2009-02-206870]

114 **Kim HJ**, Park S, Kim KJ, Seong J. Clinical significance of soluble programmed cell death ligand-1 (sPD-L1) in hepatocellular carcinoma patients treated with radiotherapy. *Radiother Oncol* 2018; **129**: 130-135 [PMID: 29366520 DOI: 10.1016/j.radonc.2017.11.027]

115 **Vanpouille-Box C**, Formenti SC, Demaria S. TREX1 dictates the immune fate of irradiated cancer cells. *Oncoimmunology* 2017; **6**: e1339857 [PMID: 28932642 DOI: 10.1080/2162402X.2017.1339857]

116 **Vanpouille-Box C**, Alard A, Aryankalayil MJ, Sarfraz Y, Diamond JM, Schneider RJ, Inghirami G, Coleman CN, Formenti SC, Demaria S. DNA exonuclease Trex1 regulates radiotherapy-induced

tumour immunogenicity. *Nat Commun* 2017; **8**: 15618 [PMID: 28598415 DOI: 10.1038/ncomms15618]

117 **Park HJ**, Griffin RJ, Hui S, Levitt SH, Song CW. Radiation-induced vascular damage in tumors: implications of vascular damage in ablative hypofractionated radiotherapy (SBRT and SRS). *Radiat Res* 2012; **177**: 311-327 [PMID: 22229487 DOI: 10.1667/rr2773.1]

118 **Dovedi SJ**, Adlard AL, Lipowska-Bhalla G, McKenna C, Jones S, Cheadle EJ, Stratford IJ, Poon E, Morrow M, Stewart R, Jones H, Wilkinson RW, Honeychurch J, Illidge TM. Acquired resistance to fractionated radiotherapy can be overcome by concurrent PD-L1 blockade. *Cancer Res* 2014; **74**: 5458-5468 [PMID: 25274032 DOI: 10.1158/0008-5472.CAN-14-1258]

119 **Tian S**, Switchenko JM, Buchwald ZS, Patel PR, Shelton JW, Kahn SE, Pillai RN, Steuer CE, Owonikoko TK, Behera M, Curran WJ, Higgins KA. Lung Stereotactic Body Radiation Therapy and Concurrent Immunotherapy: A Multicenter Safety and Toxicity Analysis. *Int J Radiat Oncol Biol Phys* 2020; **108**: 304-313 [PMID: 31982496 DOI: 10.1016/j.ijrobp.2019.12.030]

120 **Tang C**, Welsh JW, de Groot P, Massarelli E, Chang JY, Hess KR, Basu S, Curran MA, Cabanillas ME, Subbiah V, Fu S, Tsimberidou AM, Karp D, Gomez DR, Diab A, Komaki R, Heymach JV, Sharma P, Naing A, Hong DS. Ipilimumab with Stereotactic Ablative Radiation Therapy: Phase I Results and Immunologic Correlates from Peripheral T Cells. *Clin Cancer Res* 2017; **23**: 1388-1396 [PMID: 27649551 DOI: 10.1158/1078-0432.CCR-16-1432]

121 **Wegner RE**, Abel S, Hasan S, White RJ, Finley G, Monga D, Colonias A, Verma V. Time from stereotactic radiotherapy to immunotherapy is a predictor for outcome in stage IV non-small cell lung cancer. *J Immunol Sci* 2019; **3**: 6-13 [DOI: 10.29245/2578-3009/2019/2.1171]

122 **Chiang CL**, Chan ACY, Chiu KWH, Kong FS. Combined Stereotactic Body Radiotherapy and Checkpoint Inhibition in Unresectable Hepatocellular Carcinoma: A Potential Synergistic Treatment Strategy. *Front Oncol* 2019; **9**: 1157 [PMID: 31799176 DOI: 10.3389/fonc.2019.01157]

123 **El-Khoueiry AB**, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]

124 **Zhu AX**, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, Verslype C, Zagonel V, Fartoux L, Vogel A, Sarker D, Verset G, Chan SL, Knox J, Daniele B, Webber AL, Ebbinghaus SW, Ma J, Siegel AB, Cheng AL, Kudo M; KEYNOTE-224 investigators. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. *Lancet Oncol* 2018; **19**: 940-952 [PMID: 29875066 DOI: 10.1016/S1470-2045(18)30351-6]

125 **Finn RS**, Ryoo BY, Merle P, Kudo M, Bouattour M, Lim HY, Breder V, Edeline J, Chao Y, Ogasawara S, Yau T, Garrido M, Chan SL, Knox J, Daniele B, Ebbinghaus SW, Chen E, Siegel AB, Zhu AX, Cheng AL; KEYNOTE-240 investigators. Pembrolizumab As Second-Line Therapy in Patients With Advanced Hepatocellular Carcinoma in KEYNOTE-240: A Randomized, Double-Blind, Phase III Trial. *J Clin Oncol* 2020; **38**: 193-202 [PMID: 31790344 DOI: 10.1200/JCO.19.01307]

126 **Yau T**, Park JW, Finn RS, Cheng A-L, Mathurin P, Edeline J, Kudo M, Han K-H, Harding JJ, Merle P, Rosmorduc O, Wyrwicz L, Schott E, Choo SP, Kelley RK, Begic D, Chen G, Neely J, Anderson J, Sangro B. LBA38_PR - CheckMate 459: A randomized, multi-center phase III study of nivolumab (NIVO) *vs* sorafenib (SOR) as first-line (1L) treatment in patients (pts) with advanced hepatocellular carcinoma (aHCC). *Ann Oncol* 2019; **30**: v874-v875 [DOI: 10.1093/annonc/mdz394.029]

127 **Waidmann O**. Recent developments with immunotherapy for hepatocellular carcinoma. *Expert Opin Biol Ther* 2018; **18**: 905-910 [PMID: 29995439 DOI: 10.1080/14712598.2018.1499722]

128 **Choi SH**, Seong J. Stereotactic Body Radiotherapy: Does It Have a Role in Management of Hepatocellular Carcinoma? *Yonsei Med J* 2018; **59**: 912-922 [PMID: 30187697 DOI: 10.3349/ymj.2018.59.8.912]

129 **Salama JK**, Milano MT. Radical irradiation of extracranial oligometastases. *J Clin Oncol* 2014; **32**: 2902-2912 [PMID: 25113765 DOI: 10.1200/JCO.2014.55.9567]

130 **Kissel M**, Martel-Lafay I, Lequesne J, Faivre JC, Le Péchoux C, Stefan D, Barraux V, Loiseau C, Grellard JM, Danhier S, Lerouge D, Chouaid C, Gervais R, Thariat J. Stereotactic ablative radiotherapy and systemic treatments for extracerebral oligometastases, oligorecurrence, oligopersistence and oligoprogression from lung cancer. *BMC Cancer* 2019; **19**: 1237 [PMID: 31856742 DOI: 10.1186/s12885-019-6449-8]

131 **Kam TY**, Chan OSH, Hung AWM, Yeung RMW. Utilization of stereotactic ablative radiotherapy in oligometastatic & oligoprogressive skeletal metastases: Results and pattern of failure. *Asia Pac J Clin Oncol* 2019; **15** Suppl 2: 14-19 [PMID: 30859749 DOI: 10.1111/ajco.13115]

132 **Cheung P**. Stereotactic body radiotherapy for oligoprogressive cancer. *Br J Radiol* 2016; **89**: 20160251 [PMID: 27556349 DOI: 10.1259/bjr.20160251]

133 **Berghen C**, Joniau S, Ost P, Poels K, Everaerts W, Decaestecker K, Haustermans K, Devos G, De Meerleer G. Progression-directed Therapy for Oligoprogression in Castration-refractory Prostate Cancer. *Eur Urol Oncol* 2021; **4**: 305-309 [PMID: 31558422 DOI: 10.1016/j.euo.2019.08.012]

134 **Wray J**, Hawamdeh RF, Hasija N, Dagan R, Yeung AR, Lightsey JL, Okunieff P, Daily KC, George TJ, Zlotecki RA, Trevino J, Dang LH. Stereotactic body radiation therapy for oligoprogression of metastatic disease from gastrointestinal cancers: A novel approach to extend

chemotherapy efficacy. *Oncol Lett* 2017; **13**: 1087-1094 [PMID: 28454218 DOI: 10.3892/ol.2016.5540]

135 **Palma DA**, Olson R, Harrow S, Gaede S, Louie AV, Haasbeek C, Mulroy L, Lock M, Rodrigues GB, Yaremko BP, Schellenberg D, Ahmad B, Griffioen G, Senthi S, Swaminath A, Kopek N, Liu M, Moore K, Currie S, Bauman GS, Warner A, Senan S. Stereotactic ablative radiotherapy versus standard of care palliative treatment in patients with oligometastatic cancers (SABR-COMET): a randomised, phase 2, open-label trial. *Lancet* 2019; **393**: 2051-2058 [PMID: 30982687 DOI: 10.1016/S0140-6736(18)32487-5]

136 **Molinari M**, De Coutere S, Krahn M, Helton S, Urbach DR. Patients' preferences and trade-offs for the treatment of early stage hepatocellular carcinoma. *J Surg Res* 2014; **189**: 57-67 [PMID: 24650457 DOI: 10.1016/j.jss.2014.02.015]

137 **Liang SX**, Zhu XD, Xu ZY, Zhu J, Zhao JD, Lu HJ, Yang YL, Chen L, Wang AY, Fu XL, Jiang GL. Radiation-induced liver disease in three-dimensional conformal radiation therapy for primary liver carcinoma: the risk factors and hepatic radiation tolerance. *Int J Radiat Oncol Biol Phys* 2006; **65**: 426-434 [PMID: 16690430 DOI: 10.1016/j.ijrobp.2005.12.031]

138 **Gargett M**, Haddad C, Kneebone A, Booth JT, Hardcastle N. Clinical impact of removing respiratory motion during liver SABR. *Radiat Oncol* 2019; **14**: 93 [PMID: 31159840 DOI: 10.1186/s13014-019-1300-6]

139 **Nomiya T**, Akamatsu H, Harada M, Ota I, Hagiwara Y, Ichikawa M, Miwa M, Kawashiro S, Hagiwara M, Chin M, Hashizume E, Nemoto K. Modified simultaneous integrated boost radiotherapy for an unresectable huge refractory pelvic tumor diagnosed as a rectal adenocarcinoma. *World J Gastroenterol* 2014; **20**: 18480-18486 [PMID: 25561820 DOI: 10.3748/wjg.v20.i48.18480]

140 **Nomiya T**, Akamatsu H, Harada M, Ota I, Hagiwara Y, Ichikawa M, Miwa M, Mizutani M, Kato T, Nagaoka A, Tomita Y, Nemoto K. Modified simultaneous integrated boost radiotherapy for large retroperitoneal malignant tumor: A case report. *Oncol Lett* 2015; **9**: 2520-2524 [PMID: 26137100 DOI: 10.3892/ol.2015.3095]

141 **Nomiya T**, Akamatsu H, Harada M, Ota I, Hagiwara Y, Ichikawa M, Miwa M, Suzuki A, Nemoto K. Modified simultaneous integrated boost radiotherapy for unresectable locally advanced breast cancer: preliminary results of a prospective clinical trial. *Clin Breast Cancer* 2015; **15**: 161-167 [PMID: 25459068 DOI: 10.1016/j.clbc.2014.09.011]

142 **Crane CH**, Koay EJ. Solutions that enable ablative radiotherapy for large liver tumors: Fractionated dose painting, simultaneous integrated protection, motion management, and computed tomography image guidance. *Cancer* 2016; **122**: 1974-1986 [PMID: 26950735 DOI: 10.1002/cncr.29878]

143 **Byun HK**, Kim HJ, Im YR, Kim DY, Han KH, Seong J. Dose escalation in radiotherapy for incomplete transarterial chemoembolization of hepatocellular carcinoma. *Strahlenther Onkol* 2020; **196**: 132-141 [PMID: 31286149 DOI: 10.1007/s00066-019-01488-9]

144 **Mohan R**, Wu Q, Manning M, Schmidt-Ullrich R. Radiobiological considerations in the design of fractionation strategies for intensity-modulated radiation therapy of head and neck cancers. *Int J Radiat Oncol Biol Phys* 2000; **46**: 619-630 [PMID: 10701741 DOI: 10.1016/s0360-3016(99)00438-1]

145 **Byun HK**, Kim HJ, Im YR, Kim DY, Han KH, Seong J. Dose escalation by intensity modulated radiotherapy in liver-directed concurrent chemoradiotherapy for locally advanced BCLC stage C hepatocellular carcinoma. *Radiother Oncol* 2019; **133**: 1-8 [PMID: 30935563 DOI: 10.1016/j.radonc.2018.12.025]

146 **Simoni N**, Micera R, Paiella S, Guariglia S, Zivelonghi E, Malleo G, Rossi G, Addari L, Giuliani T, Pollini T, Cavedon C, Salvia R, Milella M, Bassi C, Mazzarotto R. Hypofractionated Stereotactic Body Radiation Therapy With Simultaneous Integrated Boost and Simultaneous Integrated Protection in Pancreatic Ductal Adenocarcinoma. *Clin Oncol (R Coll Radiol)* 2021; **33**: e31-e38 [PMID: 32682686 DOI: 10.1016/j.clon.2020.06.019]

147 **Oehlke O**, Wucherpfennig D, Fels F, Frings L, Egger K, Weyerbrock A, Prokic V, Nieder C, Grosu AL. Whole brain irradiation with hippocampal sparing and dose escalation on multiple brain metastases: Local tumour control and survival. *Strahlenther Onkol* 2015; **191**: 461-469 [PMID: 25592907 DOI: 10.1007/s00066-014-0808-9]

148 **Mazzola R**, Ruggieri R, Figlia V, Rigo M, Giaj Levra N, Ricchetti F, Nicosia L, Corradini S, Alongi F. Stereotactic body radiotherapy of central lung malignancies using a simultaneous integrated protection approach : A prospective observational study. *Strahlenther Onkol* 2019; **195**: 719-724 [PMID: 30689026 DOI: 10.1007/s00066-018-01419-0]

149 **Li QS**, Liang N, Ouyang WW, Su SF, Ma Z, Geng YC, Yang WG, Hu YX, Li HQ, Lu B. Simultaneous integrated boost of intensity-modulated radiation therapy to Stage II-III non-small cell lung cancer with metastatic lymph nodes. *Cancer Med* 2020; **9**: 8364-8372 [PMID: 32902184 DOI: 10.1002/cam4.3446]

150 **Qiu J**, Lv B, Fu M, Wang X, Zheng X, Zhuo W. ^{18}F-Fluoromisonidazole positron emission tomography/CT-guided volumetric-modulated arc therapy-based dose escalation for hypoxic subvolume in nasopharyngeal carcinomas: A feasibility study. *Head Neck* 2017; **39**: 2519-2527 [PMID: 28963789 DOI: 10.1002/hed.24925]

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REVIEW

Open reading frame 3 protein of hepatitis E virus: Multi-function protein with endless potential

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Abstract

Hepatitis E virus (HEV), a fecal-orally transmitted foodborne viral pathogen, causes acute hepatitis in humans and is responsible for hepatitis E outbreaks worldwide. Since the identification of HEV as a zoonotic agent, this virus has been isolated from a variety of hosts with an ever-expanding host range. HEV-open reading frame (ORF) 3, the smallest ORF in HEV genomes, initially had been perceived as an unremarkable HEV accessory protein. However, as novel HEV-ORF3 function has been discovered that is related to the existence of a putative third virion structural form, referred to as "quasi-enveloped" HEV particles, HEV is challenging the conventional virion structure-based classification scheme, which assigns all viruses to two groups, "enveloped" or "non-enveloped". In this review, we systematically describe recent progress that has identified multiple pathogenic roles of HEV-ORF3, including roles in HEV virion release, biogenesis of quasi-enveloped virus, regulation of the host innate immune response, and interference with host signaling pathways. In addition, implications of HEV-ORF3-associated quasi-enveloped virions are discussed to guide future development of improved vaccines against zoonotic HEV infection.

Key Words: Hepatitis E virus; Zoonosis; Quasi-enveloped virion; Hepatitis E virus-open reading frame 3; Innate immunity

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Core Tip: Hepatitis E virus (HEV)-open reading frame (ORF) 3 was originally though as an accessory protein with limited function which is not essential for HEV replication. This view has been challenged by recent discoveries, such as HEV-ORF3-associated "quasi-enveloped" HEV particles, regulation of the host innate immune response, and interference with host signaling pathways. More novel function of HEV-ORF3 will be revealed.

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INTRODUCTION

Hepatitis E virus (HEV), a quasi-enveloped, single-stranded positive-sense RNA virus, is classified as a member of the family Hepeviridae[1]. Hepeviridae is a highly diverse family that contains several HEV and HEV-like virus species with zoonotic, anthropotropic, and animal-restricted tropisms[2]. Currently, nearly 3 million symptomatic cases of HEV infection are reported annually, resulting in approximately 70000 deaths and 3000 stillbirths in each year[3]. Generally, mortality of HEV ranges from 0.5% to 3% overall, but HEV mortality rates have approached 30% in pregnant women[4,5].

The viral genome of HEV is 7.2 kb in length and is an mRNA-like molecule (capped and poly-adenylated at 5' and 3' ends, respectively)[6]. To date, three well-defined open reading frames (ORFs) have been detected in all HEV genotypes studied (Figure 1)[7,8]. HEV-ORF1 protein is translated directly from HEV genome with HEV-ORF2 and-ORF3 proteins translated from subgenomic RNAs[9]. Moreover, ORF4, whose expression is promoted by an atypical internal ribosome entry site (IRES)-like element, completely overlaps with ORF1 and was identified recently only in HEV-1 isolates[10]. In addition to ORFs, HEV genome contains at least four cis-reactive elements (CREs) that are required for viral replication *in vivo*[11-13]. Two of these CREs, which are located within intergenic-junctional regions between HEV-ORFs, form "stem-loop" structures that act as promoter-like elements for initiation of subgenomic RNA synthesis[11,12]. Conversely, another two CREs within ORF1 and ORF2, function as a scaffold that generates specific signals that trigger recruitment of viral and host factors for a replication complex[13].

The latest classification system of HEVs includes two genera within the family Hepeviridae, *Orthohepevirus* (covering all HEV isolates with mammalian and avian origin) and *Piscihepevirus* (only HEV-like virus with cutthroat trout origin). All four previously characterized HEV genotypes (1-4) that cause human infection are categorized within the species *Orthohepevirus* A[1], with *Orthohepevirus* B, C, and D species encompassing HEV isolates found in other non-human hosts[1]. Within *Orthohepevirus* A, HEV-1 and HEV-2 isolates are anthropotropic viruses without any animal reservoirs, while HEV-3 and HEV-4 isolates are zoonotic[6,14]. The HEV isolates originally identified from Japanese wild boar, containing unique RNA sequences, are categorized into HEV-5 and 6, whereas camel HEV isolates belong to HEV-7 and HEV-8 genotypes[1,15]. Notably, HEV isolated from a human liver transplant patient has been reported to most closely match camel HEV, suggesting that camel HEVs may be zoonotic as well, although this concept requires further confirmation[16]. In addition to mammalian HEVs, two unique groups of HEV-like viruses that have been isolated from avian species and cutthroat trout (*Oncorhynchus clarkia*) have been assigned to species *Orthohepevirus* B and to genus *Piscihepevirus* (as the only member), respectively.

Initially, HEV was assumed to be solely restricted to humans, in whom it induced self-limiting hepatitis symptoms. However, the emergence of HEV and HEV-like isolates in swine and other animal species supports a much wider HEV host tropism, with some HEV genotypes identified as zoonotic pathogens[17]. Currently, hepatitis E cases have been frequently reported in both developing and developed countries and have occurred in step with increasingly more frequent observations of expanding host ranges[18-22]. Generally, inter-species transmission and infection of zoonotic type of virus from animal to humans is considered to be primary routing of HEV transmission

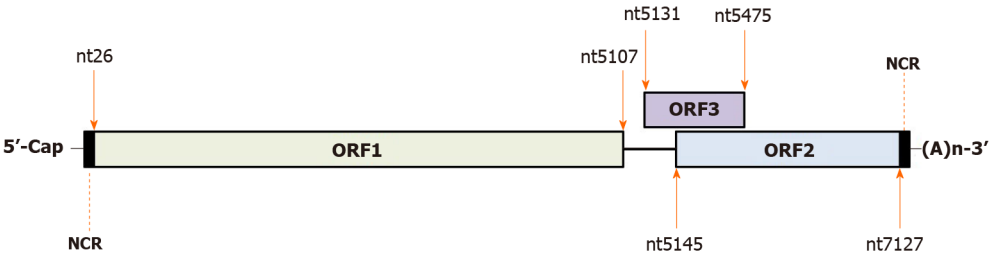


Figure 1 Schematic illustration of hepatitis E virus genome and three well-defined open reading frames. The numbers above or below the RNA boxes indicate nucleotide numbers based on hepatitis E virus-1 prototype Sar55 strain (GenBank accession number AF444002). NCR: Non-coding region; ORF: Open reading frame.

within Western worlds, while fecal-oral transmission appears to be the predominant route of HEV transmission within developing countries[23,24]. Notably, immune serological surveillance data support a high prevalence rate of previous HEV infection in the general population but demonstrate a declining trend in recent years that may be due to undetected endemic HEV circulation[25,26]. Meanwhile, frequent detection of recent cases of chronic HEV, HEV-related acute hepatic failure, and extrahepatic HEV manifestations supports this speculation as well[25,27-30]. Moreover, these observations imply that zoonotic HEV infection is a complicated pathogenic process underlying various forms of HEV-related disease. However, our understanding of HEV remains restricted due to the lack of a robust *in vitro* HEV cell system. Nevertheless, the HEV genome is known to contain three well-defined ORFs that have been found in all HEV genotypes[7,8]. HEV-ORF3, the smallest ORF found in HEV genomes, encodes a unique protein with multiple indispensable functional roles associated with viral replication and pathogenesis. In this review, we discuss the recent progress toward understanding HEV-ORF3 pathological roles in detail and provide new insights.

PROTEINS ENCODED BY HEV

HEV-ORF1 polyprotein as viral replicase

The HEV-ORF1 protein is the largest protein encoded by the HEV genome and can be directly translated from the mRNA-like genome of HEV[8]. Bioinformatics-based protein homology analysis indicated that at least eight function domains are present within HEV-ORF1 according to similarities to counterparts from other RNA viruses (Figure 2)[31]. These protein domains include methyltransferase domain, the Y domain, papain-like cysteine protease (PCP) domain, a hypervariable region containing previously assigned hypervariable domain and proline-rich domain, the X domain (also named macro-domain), RNA helicase domain, and a RNA polymerase domain (RdRp)[2,32]. The methyltransferase domain and Y domain together are thought to constitute the functional unit of RNA capping enzyme[2,32]. It remains unknown whether HEV-ORF1 protein acts alone to perform all putative viral replicase functions or is cleaved by host protease or viral protease (*via* PCP domains) to generate independent units resembling viral replicases similar to other positive-sense RNA viruses[2,33-35]. To date, available data suggests that proteolytic cleavage of the HEV-ORF1 product involves PCP domain encoded within HEV-ORF1[2,33-35]. Besides viral replication, another notable characteristic of HEV-ORF1 protein is its flexibility for insertions or deletions within the hypervariable region. The proline-rich domain within the hypervariable region was proposed to act as a linking hinge for the upstream PCP domain and downstream macro-domain, leading to formation of an unstable tertiary structure[31,36-38]. Conversely, the hypervariable region is considered an intrinsically disordered region containing extensive gene segment insertions or deletions and may participate in viral replication[39-42]. As a unique example, one HEV-3 isolate, Kernow-C1 p6 strain, which was originally isolated from an HIV patient chronically infected by HEV as well, contains a 174-nt insertion of a human ribosomal protein S17 sequence originating from the host[43], which confers ORF1 protein with novel nuclear/nucleolar trafficking capability and may promote viral replication *in vitro*[44,45].

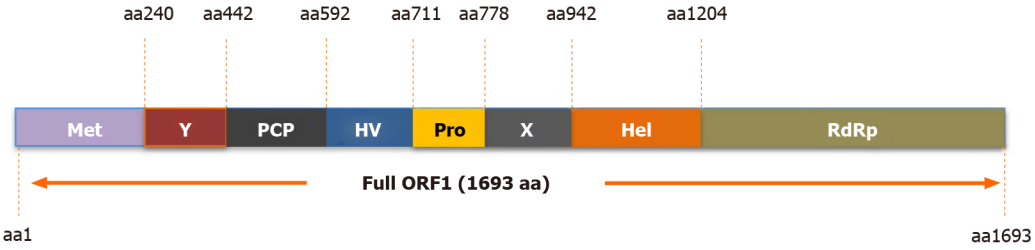


Figure 2 Function domains of hepatitis E virus-open reading frame 1 polyprotein. Putative functional domains of hepatitis E virus (HEV)-open reading frame 1 polyprotein based on HEV-1 prototype Sar55 strain are listed as follows: Methyltransferase domain; Y domain; papain-like cysteine protease; hypervariable region; proline-rich domain; X-domain; Hhelicase; RNA-dependent RNA polymerase. Met: Methyltransferase domain; Y: Y domain; PCP: Papain-like cysteine protease; HV: Hypervariable region; Pro: Proline-rich domain; X: X-domain; Hel: Helicase; RdRp: RNA polymerase; ORF: Open reading frame.

HEV-ORF2 encodes viral capsids

HEV-ORF2 encodes the putative capsid protein of HEV virions with a full-size of 660 aa residues, and has a predicted molecular mass of 72 kDa[46]. Notable, the full length ORF2 protein carries N-linked glycans at three putative glycosylation Asn residues at positions 137, 310, and 562, as well as a 15 aa signal peptide at the N-terminus directing full length ORF2 protein to the endoplasmic reticulum (ER)[47]. One notable characteristic of HEV-ORF2 is the existence of various forms of ORF2-derived proteins with multiple functions. Researchers observed very early that multiple processed ORF2-derived products were detected when recombinant ORF2 was expressed in different systems. The mature HEV capsid protein is generated from the full-length ORF2 precursor *via* proteolytic processing to remove the first N-terminal 111 aa and the last C-terminal 52 aa. However, at least two other forms of HEV-ORF2 have been detected in HEV infected cells and patients[48,49]. The first was a secreted form of ORF2 protein (ORF2s), which utilized an upstream start codon and contained a signal peptide that earmarked ORF2 for subsequent glycosylation and secretion[50]; the second was a capsid-associated truncated form of ORF2 (ORF2c), which was translated beginning at an internal methionine-encoding AUG start codon (aa16 of ORF2)[50]. Meanwhile, a truncated form of ORF2 (ORF2c) detected in HEV-infected cells may be secreted into the extracellular milieu, as is ORF2s[49].

The mature HEV capsid that lacks N-terminal 111 aa and the last C-terminal 52 aa of full ORF2 can form virus-like particles when expressed in insect cells[51,52]. Genetic analysis of ORF2 sequences of HEV genotypes 1-4 suggests that these proteins share greater than 85% similarity overall, with divergence mainly observed within the first N-terminal 111 aa, which are not incorporated into final virions[53]. A recombinant subunit vaccine using truncated HEV-1 ORF2 protein (HEV239) as major immunogen is licensed in China (Hecolin®)[2].

A multi-functional protein encoded by HEV-ORF3

ORF3, the smallest ORF among all HEV ORFs, partially overlaps with the N-terminus of ORF2 for about 300 nt and is translated from a different reading frame[9]. It was initially proposed that ORF3 protein contains 123 aa that are encoded by a subgenomic RNA distinct from the RNA encoding ORF2[7]. However, it was later confirmed that ORF3 protein is translated from a bicistronic subgenomic RNA to generate a 114-aa protein with a predicted molecular weight of 13 kDa (vp13), which is actually 9 aa shorter than the initially predicted length[9,54]. Basic sequence analysis of HEV-ORF3 protein indicated that there are two hydrophobic domains and two proline-rich domains present within the N-terminal half and C-terminal portion of HEV-ORF3 proteins, respectively[55,56], of which the first proline-rich domain contains a mitogen-activated protein kinase (MAPK) phosphorylation site (Ser71)[57]. Furthermore, two presenilin-associated protein (PSAP) motifs within the ORF3 protein were identified in HEV-1 prototype strain Sar55, with the first PSAP motif comprised of aa 86-89 and the second comprised of aa 95-98[58]. Although ORF3 protein is not required for viral RNA replication *in vitro*[59], it is irreplaceable for *in vivo* HEV infection and required for viral particle releasing[54,60,61]. In fact, most studies demonstrated that HEV-ORF3 is indispensable for viral particle egress and biogenesis of lipid membrane-wrapped HEV particles, which is now recognized as quasi-enveloped particles. Moreover, the second PSAP motif within the HEV-ORF3 protein has been shown to be required for the formation of membrane-associated HEV particles, a process that relies on an association of ORF3 with lipids[58,62]. Currently, HEV-ORF3 is thought to form an ion channel that shares key structural features with

class I viroporins that are required for virion particle release from cells during infection[63]. This observation aligns with the reported putative role of pORF3[61] and with other evidence indicating that HEV-ORF3 protein interacts with tumor susceptibility gene 101 (TSG101), the key component of host endosomal sorting complex required for transport (ESCRT) pathway, which is mainly employed by enveloped virus for budding and acquiring of viral envelope. Formation of ESCRT complex has been shown to lead to biogenesis of quasi-enveloped HEV particles[64-67].

HEV-1 specific ORF4

In recent years, a hidden ORF4 from HEV-1 was characterized[10]. Translation of HEV-ORF4 protein is promoted by a novel element located in HEV-ORF1 appearing to be an atypical IRES sequence and works in combination with a suboptimal Kozak sequence[10]. The exact function of HEV-1 specific pORF4 is still unclear. It was demonstrated that pORF4 stimulates ER stress upon HEV replication[10]. It also interacts with multiple ORF1 domains *in vitro* which are presumably to form a complex further enhancing RdRp activity [10]. Furthermore, HEV-pORF4 specific antibodies are detectable in HEV-infected patients[10]. Nonetheless, additional investigations are needed to understand functions of the ORF4 product that are unique to genotype 1 HEVs.

REGULATION OF HOST INNATE IMMUNITY AND SIGNALING BY HEV-ORF3 PROTEIN

Initially, HEV-ORF3 protein did not receive much attention, due to its presumed role as an accessory protein involved in regulation of host signaling to promote HEV replication and invasion. This hypothesis was partially evidenced by the fact that ORF3 protein was dispensable for *in vitro* replication of HEV-RNA[59]. However, subsequent research studies demonstrated that HEV-ORF3-associated putative interference mechanisms acted on multiple host cell signaling pathways, such as those involved in host innate immunity[2,68,69], indicating that HEV-ORF3 activities ultimately promote viral replication and pathogenesis.

As a multifunctional protein, HEV-ORF3 has been demonstrated to play both positive and negative roles in interferon (IFN) induction. In our previous research, we found that HEV-ORF3 protein could enhance retinoic acid-inducible gene I (RIG-I) activation to subsequently enhance IFN induction[68]. More specifically, HEV-1 ORF3 extended protein half-life of RIG-I and interacted with the RIG-I N-terminal portion to enhance ubiquitination-mediated RIG-I activation triggered by addition of the dsRNA analog poly (I:C)[68] (Figure 3A). Interestingly, it is notable that genotypic differences in HEV-ORF3-associated enhancement of RIG-I-mediated IFN induction were observed. For example, ORF3 proteins from the HEV-1 Sar55 strain and HEV-3 kernowC1 p6 strain could enhance RIG-I activation, while HEV-2 and HEV-4 ORF3 proteins could not[68], suggesting that HEV-ORF3 participated in genotype-specific HEV virulence and pathogenic effects. Moreover, these results also aligned with results of a more recent report demonstrating HEV-ORF3-associated increases of IFN-α/β and interferon-stimulated gene 15 levels in hepatoma cell line HepG2/C3A[70]. Conversely, other reports have demonstrated that overexpression of HEV-ORF3 downregulated Toll-like receptor (TLR) 3 and TLR7 and their downstream signaling pathways[71,72] (Figure 3B). Meanwhile, another study has demonstrated that ORF3 protein from an HEV-1 strain interacted with signal transducer and activator of transcription (STAT) 1 to block type I IFN-activated pathway[73] (Figure 3C). Furthermore, another study found that ORF3 proteins blocked nuclear translocation of STAT3 to down-regulate STAT3-dependent gene expression, including expression of acute-phase response proteins[74].

Besides the innate immune response, yeast two-hybrid-based screening detected that binding of ORF3 to Pyst1, a MAPK phosphatase, led to activation of MAPK pathways[75]. Thus, ORF3 appears to regulate host gene expression, as MAPK is related to host gene expression and signaling. Meanwhile, HEV-ORF3 may promote expression of glycolytic pathway enzymes by enhancing phosphorylation and transactivation function of p300/CREB-binding protein as well[76]. Additionally, microarray analysis of Huh7 cells has suggested that liver-specific genes may also be modulated by HEV-ORF3, since it modulated phosphorylation of hepatocyte nuclear factor 4[77]. Moreover, recent research has demonstrated that HEV-ORF3 plays a functional role in virus-cell interactions by influencing expression of integral membrane protein and basement membrane proteins to alter host cell processes

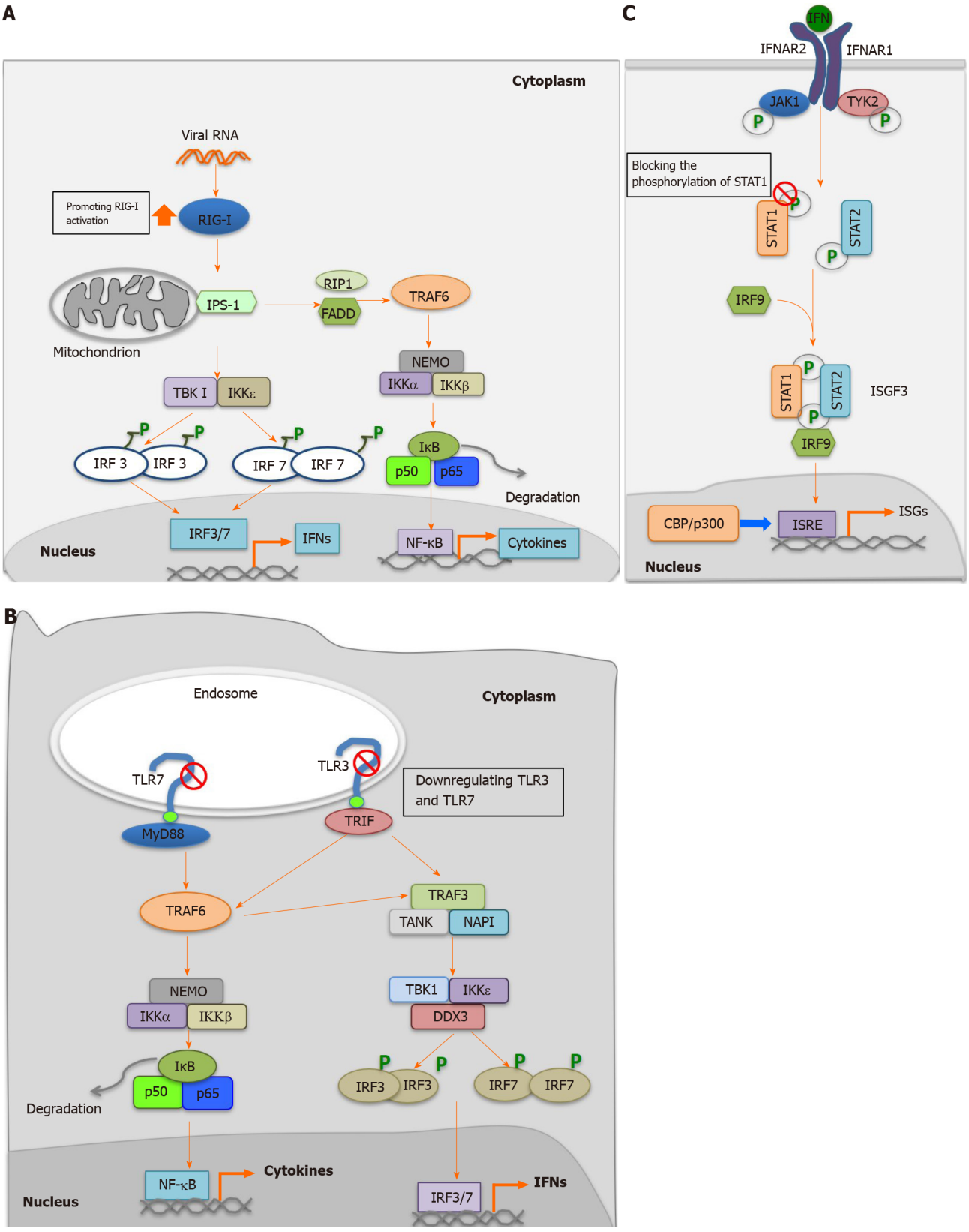


Figure 3 Regulation of host innate immune response by hepatitis E virus-open reading frame 3 proteins. A: Promotion of retinoic acid-inducible gene I mediated activation by hepatitis E virus (HEV)-open reading frame (ORF) 3; B: Inhibition of Toll-like receptor (TLR) 3 and TLR7 by HEV-ORF3; C: Blocking of the phosphorylation of signal transducer and activator of transcription (STAT) 1 to inhibit Janus kinase/STAT signaling. RIG-I: Retinoic acid-inducible gene I; RIP-I: Ribosome-inactivating proteins type I; FADD: Fas-associated protein with death domain; IKK: IkappaB kinase; TBK: Tank-Binding-Kinase; IRF: Interferon regulatory factor; NF-kB: Nuclear factor kB; IFN: Interferon; TLR: Toll-like receptor; TRIF: Toll-interleukin 1 receptor domain-containing adapter inducing interferon-beta; NAPI: Net anthropogenic phosphorus input; IFNAR: Inflammation-the type I interferon receptor; JAK: Janus kinase; TYK: Targeting tyrosine kinase; STAT: Signal transducer and activator of transcription; ISGF: Interferon-stimulated gene factor; ISRE: Interferon-stimulated response element; ISGs: Interferon-stimulated genes; CBP: CREB-binding protein.

associated with apoptosis and lipid metabolism[78]. Taken together, these data suggest that HEV-ORF3 modulates multiple signaling pathways, including those involved in host innate immunity, to ultimately promote HEV pathogenesis.

INVOVEMENT OF HEV-ORF3 PROTEIN IN BIOGENESIS OF QUASI-ENVELOPED HEV PARTICLES

The presence of a lipid layer-based envelope has long been used as the basic criterion for virus classification[66]. The presence of a lipid layer can be assessed through treatment of virus preparations with bile salts, a process that abrogates infectivity of enveloped virions, but not non-enveloped virions, by removing their surface lipid layers[66]. Generally, it is believed that during the virion budding process a viral envelope is formed from membranes of infected cells that contain molecules of at least one membrane-embedded virus-encoded glycoprotein (presented as peplomers). Viral envelopes interact with corresponding virus receptors located on target cells to promote membrane fusion of cellular membrane and viral envelop after initial interactions of virions and corresponding receptors. Meanwhile, surface glycoproteins located in viral envelope serve as antibody-neutralization targets in most cases[66], while the lipid layer of the virus envelope prevents internal virus antigens, such as nucleocapsid proteins, from serving as viral neutralization targets[79]. Thus, as compared to a non-enveloped virus, a quasi-enveloped virion would be perceived by the host immune system as antigenically distinct from a naked virion. For example, hepatitis A virus (HAV) was the first non-enveloped virus which is confirmed to hijack host cell membrane similar to enveloped virus as an enveloped form[80]. Biogenesis of enveloped HAV particles has been shown to depend on the ESCRT system[80,81], which is involved in budding of enveloped viruses. Membrane-wrapped or enveloped HAV particles mainly exist in circulation system during acute infection phase of HEV and envelopment confers protection of virus from recognition by neutralizing antibodies, which prevents impairment of virion infectivity[80]. Similar to HAV, HEV was originally classified solely as a non-enveloped virus before membrane-wrapped HEV particles were discovered, with HEV-ORF3 involvement in biogenesis of quasi-enveloped HEV virions confirmed only very recently.

It was observed very early that HEV-ORF3 protein is not required for viral RNA replication *in vitro*[59]; however, this protein is irreplaceable for HEV replication *in vivo* and is required for viral particle release from HEV infected cells *in vitro*[54,60,61]. Meanwhile, antibody-capture assays of HEV virions with or without detergent treatment demonstrated that HEV virion from either serum samples of patients or supernatants of HEV-infected cells were associated with lipid layer and ORF3 protein[82]. Subsequent screening to detect proteins interacting with HEV-ORF3 protein pinpointed the TSG101, a component of the ESCRT complex, as the potential interacting partner of HEV-ORF3[62]. Importantly, the ESCRT complex recognizes and earmarks ubiquitinated proteins for subsequent incorporation into multivesicular bodies (MVBs), an essential step for lysosomal degradation[83]. The ubiquitin E2 variant domain in TSG101 recognizes and interacts with the P(T/S)AP motif present in target proteins to recruit targets to the endosomal membrane[84].

Many enveloped viruses are equipped with a P(T/S)AP motif within their structural proteins that interact with TSG101 to redirect assembled viral components to cell membrane for virion release from infected cells[85]. Originally, two PSAP motifs comprised of aa 86-89 and aa 95-98 were identified within HEV-ORF3 proteins, the second of which was found to be conserved among HEV genotypes[58]. Replacement of the PSAP motif in HEV-ORF3 proteins with heterologous domain motifs (PPPY, YPDL, and PSAA) or mutated PSAP motifs has been shown to affect HEV virion release from infected cells[58,86], including avian HEV release[87]. Additionally, HEV-ORF3 expressed in cells was found to associate with the cellular cytoskeleton fraction, with deletion of the N-terminal hydrophobic domain of vp13 abolishing this association[57]. Moreover, a more recent study demonstrated that green fluorescent protein-tagged ORF3 protein interacted with cellular microtubules and modulated microtubule dynamics[55]. This microtubule-like filament of HEV-ORF3 protein indicated that it was potentially involved in a process that promotes virus egress; this process is reminiscent of the process by which the pUL37 protein of herpes virus interacts with dystonin, an important cytoskeleton cross-linker involved in microtubule-based transport of capsids during virion egress[88]. Although HEV was originally defined as a non-enveloped virus, such HEV-ORF3 functions that were formerly attributed only to enveloped viruses may now be related to HEV in its

recently discovered "quasi-enveloped" form[64-67].

Consistent with the aforementioned roles of HEV-ORF3 in virus egress[61], a recent study found that HEV-ORF3 shares key features with class I viroporins, including its function as an ion channel participating in viral particle egress or release[63], a function that had been previously demonstrated for the well-characterized viroporin of influenza A virus matrix-2 (M2) protein[63]. Meanwhile, a putative transmembrane region identified in pORF3 may be involved in ER localization of this protein[63]. Since viroporins of other viruses, such as M2 of influenza A virus, are components of virions, these observations imply that HEV-ORF3 is a structural HEV virion protein that exists in a membrane-associated state during the formation of envelope structures of quasi-enveloped HEV virions[89]. Interestingly, palmitoylation of cysteine residues within the N-terminal region of HEV-ORF3 has been shown to participate in its association with the membrane and is also required for infectious particle secretion[90].

The unique role that HEV-ORF3 plays during biogenesis of quasi-enveloped virus particles makes it a novel target candidate for antiviral drug development. In fact, one study has shown that the addition of a cyclic peptide inhibitor (CPI) to HEV-infected cells interrupted the interaction between the HEV-ORF3 PSAP motif and TSG101[85] and reduced virion release by over 90% when a 50% inhibitory concentration of CPI of 2 μM was used. Thus, HEV-OR3 has potential as a novel candidate for further development as an anti-HEV drug.

POTENTIAL ROLES OF HEV-ORF3 PROTEIN IN HEV HOST TROPSIM

Since the isolation of zoonotic HEV strains from swine HEV and discovery of other HEV-like viral isolates, HEVs have been continually identified from various mammalian hosts. Based on their ability to cause inter-species infection, HEV isolates can be divided three distinct groups: HEV-1 and 2 are only restricted in human; HEV-3, 4, and 7/8 are zoonotic types; while *Orthohepevirus* C is animal-restricted type. Based on reports in the literature, it implies that either factors or viral determinants contribute to HEV host tropisms and cross-species transmission events.

Among all ORFs, HEV-ORF1 encodes the largest HEV protein and appears to be indispensable for determining HEV host range. An *in vitro* study demonstrated that swapping of genetic fragment among HEV-1 and HEV-4 infectious clones indicated that chimeric virus formed from an HEV-1 infectious clone bearing surface HEV-4 ORF1 could replicate in porcine kidney cells, while the original HEV-1 virus could not[91]. Meanwhile, chimeric virus containing the junction region between ORF1/2 and the 3' non-coding region (NCR) of HEV-3 or the 3' end of the HEV-1 backbone failed to infect piglets, suggesting that the 5′ NCR and ORF1 are involved in HEV cross-species infection[92]. By contrast, a recent report demonstrated, *via* genetic fragment swapping of ORF1 regions between HEV-1 and HEV-3 infectious clones, that recombinant chimeric viruses could be generated *in vitro*. In any case, these chimeric viruses could not infect piglets *in vivo*[93], suggesting that ORF1 is not the only determinant that can confer cross-species infectivity of HEV *in vivo*.

As the viral capsid protein, HEV-ORF2 was initially thought to be an unlikely determinant of host tropism, since it is conserved among all major genotypes infecting humans[94]. However, results of *in vivo* reverse genetics-based studies that swapped segments between different HEV genotypes indicated that HEV-3 or HEV-4 based chimeric viruses inserting ORF2 from HEV-1 was incapable to cause effective infection in swine[95]. Thus, it appears that HEV-ORF2 is also involved in HEV-interspecies infectivity, in agreement with results of another report demonstrating that replacement of the HEV-3 capsid region spanning aa 456 to 605 (the putative virus receptor-binding region) with corresponding region from HEV-1 prevented chimeric virus from entering and infecting swine cells[96]. Therefore, these data imply that in addition to ORF1, viral capsid proteins also determine host preference. Nevertheless, until cellular receptors for HEV are identified, the link between viral capsid residues and cellular receptor determinants underlying HEV host tropism still requires further investigation.

In addition to ORF1/2, other reports have demonstrated that ORF3 proteins may be involved in determining host range of HEV. Up to date, the literature suggests that HEV-ORF3 protein acting as an ion channel essentially resembles viroporins involved in viral particle release during HEV infection[63]. This viroporin-like function depends on the highly conserved PSAP motif spanning aa 95-98 within *Orthohepevirus* A, which has been proposed to interact with host TSG101. However, truncation analysis has

indicated that the N-terminal 25 aa of HEV-1 ORF3 are required for its association with microtubules as well as virus release[55]. Meanwhile, alignment of ORF3 aa sequences of all eight HEV genotypes indicates that the region containing the M-terminal 25 aa of different HEV-ORF3 proteins are more conserved than the rest of HEV-ORF3. Therefore, the conservation of this region may reflect the conserved role of HEV-ORF3-dependent virion release across all genotypes. Nonetheless, a recent study demonstrated that rat HEV-ORF3 protein possessed the PxYPMP motif in place of the original PSAP motif found in human HEV-ORF3 proteins[97]. Intriguingly, unlike human HEV-ORF3 proteins, rat HEV-ORF3 proteins did not bind to TSG101, but instead utilized MVB-based sorting to achieve virion release; this mechanism differed from the aforementioned TSG101-dependent mechanism for effecting release of human HEV from infected cells[97]. Thus, these results taken together imply that HEV-ORF3 may have an important species-specific function.

Meanwhile, except for the abovementioned conserved motifs, less homology is observed elsewhere in the HEV-ORF3 protein, especially within its C-terminal half (aa 62 to aa 114) (Figure 4), a region that appears to be important for adaptation to various hosts. It is also notable that genomic locations of ORF3-encoding genes vary among species of *Orthohepevirus* (either of partially or fully overlapping with ORF2, Figure 5) [98,99], which implies a genotype-specific evolution pattern influencing functions of HEV-ORF3 that affect HEV host tropism. This speculation is in line with a genotype-specific enhancement of IFN induction by HEV-ORF3 proteins observed in our previous report[68]. Therefore, the mechanism by which a genotype-specific function of ORF3 product influences HEV host tropism requires further confirmation, although the accumulating literature indicates that a host-specific function exists that may influence host tropism by HEV-ORF3 proteins.

HEV-ORF PROTEIN AS VACCINE TARGET FOR HEV

Since the discovery of quasi-enveloped virions, researchers have tried to determine if these particles differ from classically enveloped virions, since the outer lipid bilayers of quasi-enveloped particles, such as those of HAV, are devoid of any viral proteins[66,80], while both HAV virion forms are equally infectious[80]. This apparent paradox raises the question of how membrane-wrapped particles can infect cells in the absence of viral peplomers that are generally thought to be required for enveloped virus infectivity[66]. Nevertheless, HEV appears to differ from HAV, since researchers observed very early before (prior to the identification of quasi-enveloped HEV particles) that HEV-ORF3 protein specific monoclonal antibody (mAb) could capture viral particles from serum samples of HEV patients or cell culture supernatants of HEV infected cells[89]. It is now clear that HEV-ORF3 protein associates with the lipid layer in quasi-enveloped virions produced both *in vitro* and *in vivo*, while HEV virions from feces fail to be captured by this mAb due to the lack of the HEV-ORF3-containing envelope[89]. This observation was recently confirmed by electron microscopy showing that immunogold-labeled mAb recognizing HEV-ORF3 proteins bound to quasi-enveloped HEV particles as well[100]. Moreover, although *in vitro* infectivity appears to be equivalent between quasi-enveloped HAV particles and naked counterparts, quasi-enveloped HEV particles infect fresh cells in a less efficient manner *in vitro*, as reflected by their need for a longer inoculation time to achieve maximal infectivity[67]. Meanwhile, it appears that cell entry by quasi-enveloped HEV virions depends on endosomal trafficking, which can be abrogated by blocking endosomal acidification[67]. Furthermore, additional investigations have demonstrated that HEV-ORF3 protein acts on ion channel protein and participates in the release of infectious virions from infected cells[63]; this role is similar to that of other well-characterized viroporins such as M2 protein of influenza A virus[63]. It is also notable that two hydrophobic domains located in N-terminal half of HEV-ORF3 demonstrated unique functions, whereby the first one is required associating with microtubules[55], while the second one contains a putative transmembrane region involved in ER localization[63]. Since viroporins of other viruses, such as M2 protein of influenza A virus, are components of virions, these observations imply that HEV-ORF3 is also a structural component of HEV virions, although it is not known if antibody-based neutralization mechanisms differ between the two types of HEV particles. Nevertheless, since HEV-ORF3 is present within quasi-enveloped HEV virions[89], it would be an interesting question to be determined if HEV-ORF3-specific antibodies are capable to neutralize quasi-enveloped HEV viral particles since capsid specific antibodies fail to do so.

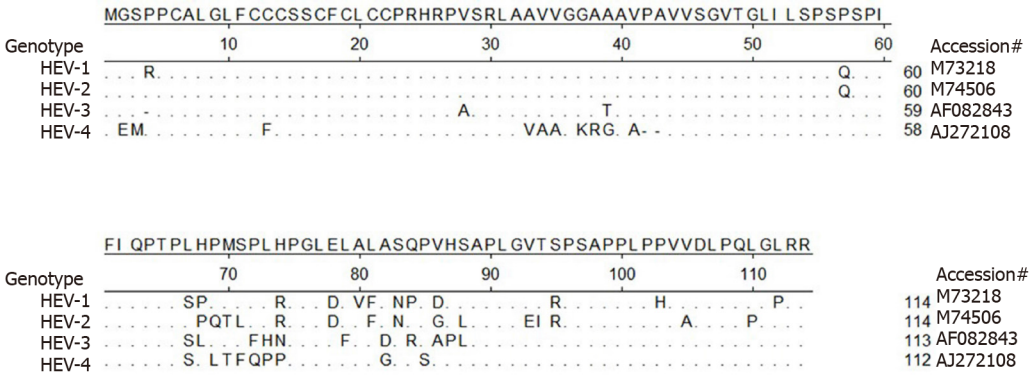


Figure 4 Alignment of amino acid sequence of hepatitis E virus-open reading frame 3 from 4 genotypes in *Orthohepevirus* A virus. Alignment of amino acid sequence of open reading frame 3 from all seven genotypes classified as *Orthohepevirus* A virus. Hepatitis E virus (HEV)-1 (reference sequence GenBank accession #M73218), HEV-2 (reference sequence GenBank accession #M74506), HEV-3 (reference sequence GenBank accession #AF082843), and HEV-4 (reference sequence GenBank accession #AJ272108) are shown. Those residues that are the same as consensus sequence are shown as ".". HEV: Hepatitis E virus.

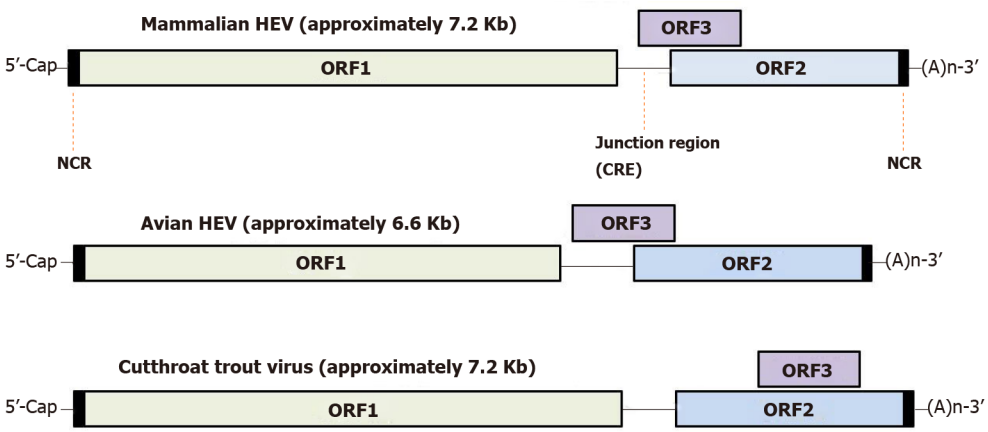


Figure 5 Hepatitis E virus genome organization. Genome location of open reading frame 3 among different hepeviruses. HEV: Hepatitis E virus; NCR: Non-coding region; CRE: Cis-reactive element; ORF: Open reading frame.

It remains unclear whether HEV-ORF3 acts as a potential neutralizing target for HEV. A previous report suggested that a recombinant vaccine candidate using HEV-4 ORF3 protein fused with interleukin-1β might confer partial protection against virus challenge[101]. Moreover, similar to vaccines based on HEV-4 ORF3, our research on avian HEV demonstrated that chickens immunized with recombinant avian HEV-ORF3 protein showed partial protection upon challenge and had milder disease symptoms than did controls[102]. However, it is notable that challenge experiments for these vaccines only employed virus stocks obtained from fecal samples which contained only naked viral particles (without envelopes containing HEV-ORF3 protein)[102]. Therefore, it is possible that antibodies induced by current ORF3-based vaccines cannot prevent first-round infection during initial challenge with naked virus, since naked HEV virions cannot be neutralized by antibodies specific for ORF3-HEV. Nevertheless, partial protection observed in both experiments may be due to ORF3-specific antibody-based neutralization of newly synthesized quasi-enveloped HEV virion entering into circulation after initial infection caused by naked HEV virion used for challenging. In any case, these observations raise the interesting question of whether an ORF2-based vaccine could protect hosts from challenge with quasi-enveloped HEV particles, a concept that warrants further investigation.

Up to date, available data indicated that ORF3 proteins (including avian HEV-ORF3) is highly immunogenic to evoke host humoral response, with most B-cell epitopes located at the C-terminal of HEV-ORF3[103-106]. Thus, the C-terminal half (about 60 aa) of HEV-ORF3 proteins appears to be a promising candidate as a recombinant subunit vaccine. However, an obstacle to employing this region for a vaccine candidate is the potential antigen variation issue as predicted by aa sequence

alignment of HEV-ORF3s from the four reference strains of HEV-1 to -4 (Figure 4)[68]. Therefore, systematic mapping of antigenic epitopes within HEV-ORF3 proteins of different HEV genotypes may be required before using immunogenic ORF3 epitopes as additional components of HEV subunit vaccines.

CONCLUSION

Although nearly three decades have elapsed since the identification and characterization of the complete genome sequence from the first HEV isolate, the full spectrum of this virus remains unclear and HEV infection now is a public health concern in developed countries as well. Currently, cross-species infection and host tropisms of different HEV genotypes remain elusive, due to the lack of easy-to-handle animal model and a robust *in vitro* system for studying HEV. Although many details about this virus and its pathology have been revealed in recent years, it is notable that HEV-ORF3 protein, the smallest ORF encoded by HEV, appears to have diverse functions and key roles in HEV virion release, biogenesis of quasi-enveloped virus, regulation of the host innate immune response, and neutralization of quasi-enveloped virus. These advances will guide further studies to reveal the basic biology of HEV, functions of HEV proteins, and HEV pathogenic factors toward the development of effective therapeutics and an improved vaccine.

REFERENCES

1 **Smith DB**, Simmonds P; Members Of The International Committee On The Taxonomy Of Viruses Study Group, Jameel S, Emerson SU, Harrison TJ, Meng XJ, Okamoto H, Van der Poel WHM, Purdy MA. Consensus proposals for classification of the family Hepeviridae. *J Gen Virol* 2014; **95**: 2223-2232 [PMID: 24989172 DOI: 10.1099/vir.0.068429-0]

2 **Nan Y**, Zhang YJ. Molecular Biology and Infection of Hepatitis E Virus. *Front Microbiol* 2016; **7**: 1419 [PMID: 27656178 DOI: 10.3389/fmicb.2016.01419]

3 **Dalton HR**, Saunders M, Woolson KL. Hepatitis E virus in developed countries: one of the most successful zoonotic viral diseases in human history? *J Virus Erad* 2015; **1**: 23-29 [PMID: 27482393]

4 **Jameel S**. Molecular biology and pathogenesis of hepatitis E virus. *Expert Rev Mol Med* 1999; **1999**: 1-16 [PMID: 14987355 DOI: 10.1017/S1462399499001271]

5 **Pillot J**, Türkoglu S, Dubreuil P, Cosson A, Lemaigre G, Meng J, Lazizi Y. Cross-reactive immunity against different strains of the hepatitis E virus transferable by simian and human sera. *C R Acad Sci III* 1995; **318**: 1059-1064 [PMID: 8556452]

6 **Ahmad I**, Holla RP, Jameel S. Molecular virology of hepatitis E virus. *Virus Res* 2011; **161**: 47-58 [PMID: 21345356 DOI: 10.1016/j.virusres.2011.02.011]

7 **Tam AW**, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* 1991; **185**: 120-131 [PMID: 1926770]

8 **Tsarev SA**, Emerson SU, Reyes GR, Tsareva TS, Legters LJ, Malik IA, Iqbal M, Purcell RH. Characterization of a prototype strain of hepatitis E virus. *Proc Natl Acad Sci USA* 1992; **89**: 559-563 [PMID: 1731327]

9 **Graff J**, Torian U, Nguyen H, Emerson SU. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. *J Virol* 2006; **80**: 5919-5926 [PMID: 16731930 DOI: 10.1128/JVI.00046-06]

10 **Nair VP**, Anang S, Subramani C, Madhvi A, Bakshi K, Srivastava A, Shalimar, Nayak B, Ranjith Kumar CT, Surjit M. Endoplasmic Reticulum Stress Induced Synthesis of a Novel Viral Factor Mediates Efficient Replication of Genotype-1 Hepatitis E Virus. *PLoS Pathog* 2016; **12**: e1005521 [PMID: 27035822 DOI: 10.1371/journal.ppat.1005521]

11 **Cao D**, Huang YW, Meng XJ. The nucleotides on the stem-loop RNA structure in the junction region of the hepatitis E virus genome are critical for virus replication. *J Virol* 2010; **84**: 13040-13044 [PMID: 20943962 DOI: 10.1128/JVI.01475-10]

12 **Parvez MK**. The intergenic-junction variant (genotype 2 isolate) of hepatitis E virus restores the CREX 'stem-loop' structural integrity, essential for viral life cycle. *Gene* 2015; **559**: 149-154 [PMID: 25597766 DOI: 10.1016/j.gene.2015.01.033]

13 **Ju X**, Xiang G, Gong M, Yang R, Qin J, Li Y, Nan Y, Yang Y, Zhang QC, Ding Q. Identification of functional cis-acting RNA elements in the hepatitis E virus genome required for viral replication. *PLoS Pathog* 2020; **16**: e1008488 [PMID: 32433693 DOI: 10.1371/journal.ppat.1008488]

14 **Meng XJ**. Recent advances in Hepatitis E virus. *J Viral Hepat* 2010; **17**: 153-161 [PMID: 20040046 DOI: 10.1111/j.1365-2893.2009.01257.x]

15 **Woo PC**, Lau SK, Teng JL, Cao KY, Wernery U, Schountz T, Chiu TH, Tsang AK, Wong PC, Wong EY, Yuen KY. New Hepatitis E Virus Genotype in Bactrian Camels, Xinjiang, China, 2013.

Emerg Infect Dis 2016; **22**: 2219-2221 [PMID: 27869607 DOI: 10.3201/eid2212.160979]
16 **Lee GH**, Tan BH, Teo EC, Lim SG, Dan YY, Wee A, Aw PP, Zhu Y, Hibberd ML, Tan CK, Purdy MA, Teo CG. Chronic Infection With Camelid Hepatitis E Virus in a Liver Transplant Recipient Who Regularly Consumes Camel Meat and Milk. *Gastroenterology* 2016; **150**: 355-7. e3 [PMID: 26551551 DOI: 10.1053/j.gastro.2015.10.048]
17 **Meng XJ**. Zoonotic and foodborne transmission of hepatitis E virus. *Semin Liver Dis* 2013; **33**: 41-49 [PMID: 23564388 DOI: 10.1055/s-0033-1338113]
18 **Park WJ**, Park BJ, Ahn HS, Lee JB, Park SY, Song CS, Lee SW, Yoo HS, Choi IS. Hepatitis E virus as an emerging zoonotic pathogen. *J Vet Sci* 2016; **17**: 1-11 [PMID: 27051334 DOI: 10.4142/jvs.2016.17.1.1]
19 **Montesano C**, Giovanetti M, Ciotti M, Cella E, Lo Presti A, Grifoni A, Zehender G, Angeletti S, Ciccozzi M. Hepatitis E Virus Circulation in Italy: Phylogenetic and Evolutionary Analysis. *Hepat Mon* 2016; **16**: e31951 [PMID: 27226798 DOI: 10.5812/hepatmon.31951]
20 **Anheyer-Behmenburg HE**, Szabo K, Schotte U, Binder A, Klein G, Johne R. Hepatitis E Virus in Wild Boars and Spillover Infection in Red and Roe Deer, Germany, 2013-2015. *Emerg Infect Dis* 2017; **23**: 130-133 [PMID: 27983488 DOI: 10.3201/eid2301.161169]
21 **Abravanel F**, Lhomme S, El Costa H, Schvartz B, Peron JM, Kamar N, Izopet J. Rabbit Hepatitis E Virus Infections in Humans, France. *Emerg Infect Dis* 2017; **23**: 1191-1193 [PMID: 28628452 DOI: 10.3201/eid2307.170318]
22 **Doceul V**, Bagdassarian E, Demange A, Pavio N. Zoonotic Hepatitis E Virus: Classification, Animal Reservoirs and Transmission Routes. *Viruses* 2016; **8** [PMID: 27706110 DOI: 10.3390/v8100270]
23 **Pavio N**, Meng XJ, Doceul V. Zoonotic origin of hepatitis E. *Curr Opin Virol* 2015; **10**: 34-41 [PMID: 25588602 DOI: 10.1016/j.coviro.2014.12.006]
24 **Salines M**, Andraud M, Rose N. From the epidemiology of hepatitis E virus (HEV) within the swine reservoir to public health risk mitigation strategies: a comprehensive review. *Vet Res* 2017; **48**: 31 [PMID: 28545558 DOI: 10.1186/s13567-017-0436-3]
25 **Sadik S**, van Rijckevorsel GG, van Rooijen MS, Sonder GJ, Bruisten SM. Seroprevalence of hepatitis E virus differs in Dutch and first generation migrant populations in Amsterdam, the Netherlands: a cross-sectional study. *BMC Infect Dis* 2016; **16**: 659 [PMID: 27825308 DOI: 10.1186/s12879-016-2007-z]
26 **Teshale EH**, Denniston MM, Drobeniuc J, Kamili S, Teo CG, Holmberg SD. Decline in hepatitis E virus antibody prevalence in the United States from 1988-1994 to 2009-2010. *J Infect Dis* 2015; **211**: 366-373 [PMID: 25147277 DOI: 10.1093/infdis/jiu466]
27 **Dalton HR**, Kamar N, van Eijk JJ, Mclean BN, Cintas P, Bendall RP, Jacobs BC. Hepatitis E virus and neurological injury. *Nat Rev Neurol* 2016; **12**: 77-85 [PMID: 26711839 DOI: 10.1038/nrneurol.2015.234]
28 **Geng Y**, Zhao C, Huang W, Harrison TJ, Zhang H, Geng K, Wang Y. Detection and assessment of infectivity of hepatitis E virus in urine. *J Hepatol* 2016; **64**: 37-43 [PMID: 26362822 DOI: 10.1016/j.jhep.2015.08.034]
29 **Feng Z**. Causation by HEV of extrahepatic manifestations remains unproven. *Liver Int* 2016; **36**: 477-479 [PMID: 27005694 DOI: 10.1111/liv.13085]
30 **Ripellino P**, Pasi E, Melli G, Staedler C, Fraga M, Moradpour D, Sahli R, Aubert V, Martinetti G, Bihl F, Bernasconi E, Terziroli Beretta-Piccoli B, Cerny A, Dalton HR, Zehnder C, Mathis B, Zecca C, Disanto G, Kaelin-Lang A, Gobbi C. Neurologic complications of acute hepatitis E virus infection. *Neurol Neuroimmunol Neuroinflamm* 2020; **7** [PMID: 31806684 DOI: 10.1212/NXI.0000000000000643]
31 **Koonin EV**, Gorbalenya AE, Purdy MA, Rozanov MN, Reyes GR, Bradley DW. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proc Natl Acad Sci USA* 1992; **89**: 8259-8263 [PMID: 1518855]
32 **Ahola T**, Karlin DG. Sequence analysis reveals a conserved extension in the capping enzyme of the alphavirus supergroup, and a homologous domain in nodaviruses. *Biol Direct* 2015; **10**: 16 [PMID: 25886938 DOI: 10.1186/s13062-015-0050-0]
33 **Parvez MK**. Molecular characterization of hepatitis E virus ORF1 gene supports a papain-like cysteine protease (PCP)-domain activity. *Virus Res* 2013; **178**: 553-556 [PMID: 23978667 DOI: 10.1016/j.virusres.2013.07.020]
34 **Paliwal D**, Panda SK, Kapur N, Varma SPK, Durgapal H. Hepatitis E virus (HEV) protease: a chymotrypsin-like enzyme that processes both non-structural (pORF1) and capsid (pORF2) protein. *J Gen Virol* 2014; **95**: 1689-1700 [PMID: 24795447 DOI: 10.1099/vir.0.066142-0]
35 **Parvez MK**. The hepatitis E virus nonstructural polyprotein. *Future Microbiol* 2017; **12**: 915-924 [PMID: 28686042 DOI: 10.2217/fmb-2017-0016]
36 **Dosztányi Z**, Chen J, Dunker AK, Simon I, Tompa P. Disorder and sequence repeats in hub proteins and their implications for network evolution. *J Proteome Res* 2006; **5**: 2985-2995 [PMID: 17081050 DOI: 10.1021/pr060171o]
37 **Dunker AK**, Oldfield CJ, Meng J, Romero P, Yang JY, Chen JW, Vacic V, Obradovic Z, Uversky VN. The unfoldomics decade: an update on intrinsically disordered proteins. *BMC Genomics* 2008; **9** Suppl 2: S1 [PMID: 18831774 DOI: 10.1186/1471-2164-9-S2-S1]
38 **Tsai CJ**, Ma B, Sham YY, Kumar S, Nussinov R. Structured disorder and conformational selection. *Proteins* 2001; **44**: 418-427 [PMID: 11484219]

39 **Purdy MA**. Evolution of the hepatitis E virus polyproline region: order from disorder. *J Virol* 2012; **86**: 10186-10193 [PMID: 22811526 DOI: 10.1128/JVI.01374-12]

40 **Purdy MA**, Lara J, Khudyakov YE. The hepatitis E virus polyproline region is involved in viral adaptation. *PLoS One* 2012; **7**: e35974 [PMID: 22545153 DOI: 10.1371/journal.pone.0035974]

41 **Pudupakam RS**, Huang YW, Opriessnig T, Halbur PG, Pierson FW, Meng XJ. Deletions of the hypervariable region (HVR) in open reading frame 1 of hepatitis E virus do not abolish virus infectivity: evidence for attenuation of HVR deletion mutants in vivo. *J Virol* 2009; **83**: 384-395 [PMID: 18945785 DOI: 10.1128/JVI.01854-08]

42 **Pudupakam RS**, Kenney SP, Córdoba L, Huang YW, Dryman BA, Leroith T, Pierson FW, Meng XJ. Mutational analysis of the hypervariable region of hepatitis e virus reveals its involvement in the efficiency of viral RNA replication. *J Virol* 2011; **85**: 10031-10040 [PMID: 21775444 DOI: 10.1128/JVI.00763-11]

43 **Shukla P**, Nguyen HT, Torian U, Engle RE, Faulk K, Dalton HR, Bendall RP, Keane FE, Purcell RH, Emerson SU. Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci USA* 2011; **108**: 2438-2443 [PMID: 21262830 DOI: 10.1073/pnas.1018878108]

44 **Kenney SP**, Meng XJ. The lysine residues within the human ribosomal protein S17 sequence naturally inserted into the viral nonstructural protein of a unique strain of hepatitis E virus are important for enhanced virus replication. *J Virol* 2015; **89**: 3793-3803 [PMID: 25609799 DOI: 10.1128/JVI.03582-14]

45 **Kenney SP**, Meng XJ. Identification and fine mapping of nuclear and nucleolar localization signals within the human ribosomal protein S17. *PLoS One* 2015; **10**: e0124396 [PMID: 25853866 DOI: 10.1371/journal.pone.0124396]

46 **Robinson RA**, Burgess WH, Emerson SU, Leibowitz RS, Sosnovtseva SA, Tsarev S, Purcell RH. Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. *Protein Expr Purif* 1998; **12**: 75-84 [PMID: 9473460 DOI: 10.1006/prep.1997.0817]

47 **Jameel S**, Zafrullah M, Ozdener MH, Panda SK. Expression in animal cells and characterization of the hepatitis E virus structural proteins. *J Virol* 1996; **70**: 207-216 [PMID: 8523527]

48 **Ankavay M**, Montpellier C, Sayed IM, Saliou JM, Wychowski C, Saas L, Duvet S, Aliouat-Denis CM, Farhat R, de Masson d'Autume V, Meuleman P, Dubuisson J, Cocquerel L. New insights into the ORF2 capsid protein, a key player of the hepatitis E virus lifecycle. *Sci Rep* 2019; **9**: 6243 [PMID: 31000788 DOI: 10.1038/s41598-019-42737-2]

49 **Montpellier C**, Wychowski C, Sayed IM, Meunier JC, Saliou JM, Ankavay M, Bull A, Pillez A, Abravanel F, Helle F, Brochot E, Drobecq H, Farhat R, Aliouat-Denis CM, Haddad JG, Izopet J, Meuleman P, Goffard A, Dubuisson J, Cocquerel L. Hepatitis E Virus Lifecycle and Identification of 3 Forms of the ORF2 Capsid Protein. *Gastroenterology* 2018; **154**: 211-223. e8 [PMID: 28958858 DOI: 10.1053/j.gastro.2017.09.020]

50 **Yin X**, Ying D, Lhomme S, Tang Z, Walker CM, Xia N, Zheng Z, Feng Z. Origin, antigenicity, and function of a secreted form of ORF2 in hepatitis E virus infection. *Proc Natl Acad Sci USA* 2018; **115**: 4773-4778 [PMID: 29669922 DOI: 10.1073/pnas.1721345115]

51 **Li TC**, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, Takeda N, Miyamura T. Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J Virol* 1997; **71**: 7207-7213 [PMID: 9311793]

52 **Li TC**, Takeda N, Miyamura T, Matsuura Y, Wang JC, Engvall H, Hammar L, Xing L, Cheng RH. Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. *J Virol* 2005; **79**: 12999-13006 [PMID: 16189002 DOI: 10.1128/JVI.79.20.12999-13006.2005]

53 **Mori Y**, Matsuura Y. Structure of hepatitis E viral particle. *Virus Res* 2011; **161**: 59-64 [PMID: 21440590 DOI: 10.1016/j.virusres.2011.03.015]

54 **Huang YW**, Opriessnig T, Halbur PG, Meng XJ. Initiation at the third in-frame AUG codon of open reading frame 3 of the hepatitis E virus is essential for viral infectivity in vivo. *J Virol* 2007; **81**: 3018-3026 [PMID: 17202216 DOI: 10.1128/JVI.02259-06]

55 **Kannan H**, Fan S, Patel D, Bossis I, Zhang YJ. The hepatitis E virus open reading frame 3 product interacts with microtubules and interferes with their dynamics. *J Virol* 2009; **83**: 6375-6382 [PMID: 19369329 DOI: 10.1128/JVI.02571-08]

56 **Holla RP**, Ahmad I, Ahmad Z, Jameel S. Molecular Virology of Hepatitis E Virus. *Semin Liver Dis* 2013; **33**: 3-14 [PMID: 23564385 DOI: 10.1055/s-0033-1338110]

57 **Zafrullah M**, Ozdener MH, Panda SK, Jameel S. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. *J Virol* 1997; **71**: 9045-9053 [PMID: 9371561]

58 **Nagashima S**, Takahashi M, Jirintai, Tanaka T, Yamada K, Nishizawa T, Okamoto H. A PSAP motif in the ORF3 protein of hepatitis E virus is necessary for virion release from infected cells. *J Gen Virol* 2011; **92**: 269-278 [PMID: 21068219 DOI: 10.1099/vir.0.025791-0]

59 **Emerson SU**, Nguyen H, Torian U, Purcell RH. ORF3 protein of hepatitis E virus is not required for replication, virion assembly, or infection of hepatoma cells in vitro. *J Virol* 2006; **80**: 10457-10464 [PMID: 16928762 DOI: 10.1128/JVI.00892-06]

60 **Graff J**, Nguyen H, Yu C, Elkins WR, St Claire M, Purcell RH, Emerson SU. The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. *J Virol* 2005; **79**: 6680-6689 [PMID: 15890906 DOI: 10.1128/JVI.79.11.6680-6689.2005]

61 **Yamada K**, Takahashi M, Hoshino Y, Takahashi H, Ichiyama K, Nagashima S, Tanaka T, Okamoto H. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. *J Gen Virol* 2009; **90**: 1880-1891 [PMID: 19339479 DOI: 10.1099/vir.0.010561-0]

62 **Nagashima S**, Takahashi M, Jirintai S, Tanaka T, Nishizawa T, Yasuda J, Okamoto H. Tumour susceptibility gene 101 and the vacuolar protein sorting pathway are required for the release of hepatitis E virions. *J Gen Virol* 2011; **92**: 2838-2848 [PMID: 21880841 DOI: 10.1099/vir.0.035378-0]

63 **Ding Q**, Heller B, Capuccino JM, Song B, Nimgaonkar I, Hrebikova G, Contreras JE, Ploss A. Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles. *Proc Natl Acad Sci USA* 2017; **114**: 1147-1152 [PMID: 28096411 DOI: 10.1073/pnas.1614955114]

64 **Nagashima S**, Takahashi M, Jirintai S, Tanggis, Kobayashi T, Nishizawa T, Okamoto H. The membrane on the surface of hepatitis E virus particles is derived from the intracellular membrane and contains trans-Golgi network protein 2. *Arch Virol* 2014; **159**: 979-991 [PMID: 24221250 DOI: 10.1007/s00705-013-1912-3]

65 **Hurley JH**. The ESCRT complexes. *Crit Rev Biochem Mol Biol* 2010; **45**: 463-487 [PMID: 20653365 DOI: 10.3109/10409238.2010.502516]

66 **Feng Z**, Hirai-Yuki A, McKnight KL, Lemon SM. Naked Viruses That Aren't Always Naked: Quasi-Enveloped Agents of Acute Hepatitis. *Annu Rev Virol* 2014; **1**: 539-560 [PMID: 26958733 DOI: 10.1146/annurev-virology-031413-085359]

67 **Yin X**, Ambardekar C, Lu Y, Feng Z. Distinct Entry Mechanisms for Nonenveloped and Quasi-Enveloped Hepatitis E Viruses. *J Virol* 2016; **90**: 4232-4242 [PMID: 26865708 DOI: 10.1128/JVI.02804-15]

68 **Nan Y**, Ma Z, Wang R, Yu Y, Kannan H, Fredericksen B, Zhang YJ. Enhancement of interferon induction by ORF3 product of hepatitis E virus. *J Virol* 2014; **88**: 8696-8705 [PMID: 24850742 DOI: 10.1128/JVI.01228-14]

69 **Lhomme S**, Migueres M, Abravanel F, Marion O, Kamar N, Izopet J. Hepatitis E Virus: How It Escapes Host Innate Immunity. *Vaccines (Basel)* 2020; **8** [PMID: 32731452 DOI: 10.3390/vaccines8030422]

70 **Wang M**, Huang Y, He M, Peng WJ, Tian DY. Effects of hepatitis E virus infection on interferon production *via* ISG15. *World J Gastroenterol* 2018; **24**: 2173-2180 [PMID: 29853735 DOI: 10.3748/wjg.v24.i20.2173]

71 **Lei Q**, Li L, Zhang S, Li T, Zhang X, Ding X, Qin B. HEV ORF3 downregulates TLR7 to inhibit the generation of type I interferon *via* impairment of multiple signaling pathways. *Sci Rep* 2018; **8**: 8585 [PMID: 29872132 DOI: 10.1038/s41598-018-26975-4]

72 **He M**, Wang M, Huang Y, Peng W, Zheng Z, Xia N, Xu J, Tian D. The ORF3 Protein of Genotype 1 Hepatitis E Virus Suppresses TLR3-induced NF-κB Signaling *via* TRADD and RIP1. *Sci Rep* 2016; **6**: 27597 [PMID: 27270888 DOI: 10.1038/srep27597]

73 **Dong C**, Zafrullah M, Mixson-Hayden T, Dai X, Liang J, Meng J, Kamili S. Suppression of interferon-α signaling by hepatitis E virus. *Hepatology* 2012; **55**: 1324-1332 [PMID: 22183878 DOI: 10.1002/hep.25530]

74 **Chandra V**, Kar-Roy A, Kumari S, Mayor S, Jameel S. The hepatitis E virus ORF3 protein modulates epidermal growth factor receptor trafficking, STAT3 translocation, and the acute-phase response. *J Virol* 2008; **82**: 7100-7110 [PMID: 18448545 DOI: 10.1128/JVI.00403-08]

75 **Kar-Roy A**, Korkaya H, Oberoi R, Lal SK, Jameel S. The hepatitis E virus open reading frame 3 protein activates ERK through binding and inhibition of the MAPK phosphatase. *J Biol Chem* 2004; **279**: 28345-28357 [PMID: 15096509 DOI: 10.1074/jbc.M400457200]

76 **Moin SM**, Chandra V, Arya R, Jameel S. The hepatitis E virus ORF3 protein stabilizes HIF-1alpha and enhances HIF-1-mediated transcriptional activity through p300/CBP. *Cell Microbiol* 2009; **11**: 1409-1421 [PMID: 19489936 DOI: 10.1111/j.1462-5822.2009.01340.x]

77 **Chandra V**, Holla P, Ghosh D, Chakrabarti D, Padigaru M, Jameel S. The hepatitis E virus ORF3 protein regulates the expression of liver-specific genes by modulating localization of hepatocyte nuclear factor 4. *PLoS One* 2011; **6**: e22412 [PMID: 21799848 DOI: 10.1371/journal.pone.0022412]

78 **Xu K**, Guo S, Zhao T, Zhu H, Jiao H, Shi Q, Pang F, Li Y, Li G, Peng D, Nie X, Cheng Y, Wu K, Du L, Cui K, Zhang W, Wang F. Transcriptome Analysis of HepG2 Cells Expressing ORF3 from Swine Hepatitis E Virus to Determine the Effects of ORF3 on Host Cells. *Biomed Res Int* 2016; **2016**: 1648030 [PMID: 27648443 DOI: 10.1155/2016/1648030]

79 **Pütz MM**, Midgley CM, Law M, Smith GL. Quantification of antibody responses against multiple antigens of the two infectious forms of Vaccinia virus provides a benchmark for smallpox vaccination. *Nat Med* 2006; **12**: 1310-1315 [PMID: 17086190 DOI: 10.1038/nm1457]

80 **Feng Z**, Hensley L, McKnight KL, Hu F, Madden V, Ping L, Jeong SH, Walker C, Lanford RE, Lemon SM. A pathogenic picornavirus acquires an envelope by hijacking cellular membranes. *Nature* 2013; **496**: 367-371 [PMID: 23542590 DOI: 10.1038/nature12029]

81 **Votteler J**, Sundquist WI. Virus budding and the ESCRT pathway. *Cell Host Microbe* 2013; **14**: 232-241 [PMID: 24034610 DOI: 10.1016/j.chom.2013.08.012]

82 **Takahashi M**, Tanaka T, Takahashi H, Hoshino Y, Nagashima S, Jirintai, Mizuo H, Yazaki Y, Takagi T, Azuma M, Kusano E, Isoda N, Sugano K, Okamoto H. Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation. *J Clin Microbiol* 2010; **48**: 1112-1125 [PMID: 20107086 DOI: 10.1128/JCM.02002-09]

83 **Wen X**, He X, Zhang X, Liu L, Guan Y, Zhang Y, Bu Z. Genome sequences derived from pig and dried blood pig feed samples provide important insights into the transmission of African swine fever virus in China in 2018. *Emerg Microbes Infect* 2019; **8**: 303-306 [PMID: 30866784 DOI: 10.1080/22221751.2019.1565915]

84 **Neilan JG**, Zsak L, Lu Z, Kutish GF, Afonso CL, Rock DL. Novel swine virulence determinant in the left variable region of the African swine fever virus genome. *J Virol* 2002; **76**: 3095-3104 [PMID: 11884534 DOI: 10.1128/jvi.76.7.3095-3104.2002]

85 **Wang N**, Zhao D, Wang J, Zhang Y, Wang M, Gao Y, Li F, Bu Z, Rao Z, Wang X. Architecture of African swine fever virus and implications for viral assembly. *Science* 2019; **366**: 640-644 [PMID: 31624094 DOI: 10.1126/science.aaz1439]

86 **Kenney SP**, Wentworth JL, Heffron CL, Meng XJ. Replacement of the hepatitis E virus ORF3 protein PxxP motif with heterologous late domain motifs affects virus release *via* interaction with TSG101. *Virology* 2015; **486**: 198-208 [PMID: 26457367 DOI: 10.1016/j.virol.2015.09.012]

87 **Kenney SP**, Pudupakam RS, Huang YW, Pierson FW, LeRoith T, Meng XJ. The PSAP motif within the ORF3 protein of an avian strain of the hepatitis E virus is not critical for viral infectivity *in vivo* but plays a role in virus release. *J Virol* 2012; **86**: 5637-5646 [PMID: 22438540 DOI: 10.1128/JVI.06711-11]

88 **Pasdeloup D**, McElwee M, Beilstein F, Labetoulle M, Rixon FJ. Herpesvirus tegument protein pUL37 interacts with dystonin/BPAG1 to promote capsid transport on microtubules during egress. *J Virol* 2013; **87**: 2857-2867 [PMID: 23269794 DOI: 10.1128/JVI.02676-12]

89 **Takahashi M**, Yamada K, Hoshino Y, Takahashi H, Ichiyama K, Tanaka T, Okamoto H. Monoclonal antibodies raised against the ORF3 protein of hepatitis E virus (HEV) can capture HEV particles in culture supernatant and serum but not those in feces. *Arch Virol* 2008; **153**: 1703-1713 [PMID: 18679765 DOI: 10.1007/s00705-008-0179-6]

90 **Gouttenoire J**, Pollán A, Abrami L, Oechslin N, Mauron J, Matter M, Oppliger J, Szkolnicka D, Dao Thi VL, van der Goot FG, Moradpour D. Palmitoylation mediates membrane association of hepatitis E virus ORF3 protein and is required for infectious particle secretion. *PLoS Pathog* 2018; **14**: e1007471 [PMID: 30532200 DOI: 10.1371/journal.ppat.1007471]

91 **Chatterjee SN**, Devhare PB, Pingle SY, Paingankar MS, Arankalle VA, Lole KS. Hepatitis E virus (HEV)-1 harbouring HEV-4 non-structural protein (ORF1) replicates in transfected porcine kidney cells. *J Gen Virol* 2016; **97**: 1829-1840 [PMID: 27072797 DOI: 10.1099/jgv.0.000478]

92 **Feagins AR**, Córdoba L, Sanford BJ, Dryman BA, Huang YW, LeRoith T, Emerson SU, Meng XJ. Intergenotypic chimeric hepatitis E viruses (HEVs) with the genotype 4 human HEV capsid gene in the backbone of genotype 3 swine HEV are infectious in pigs. *Virus Res* 2011; **156**: 141-146 [PMID: 21195119 DOI: 10.1016/j.virusres.2010.12.011]

93 **Tian D**, Yugo DM, Kenney SP, Lynn Heffron C, Opriessnig T, Karuppannan AK, Bayne J, Halbur PG, Meng XJ. Dissecting the potential role of hepatitis E virus ORF1 nonstructural gene in cross-species infection by using intergenotypic chimeric viruses. *J Med Virol* 2020 [PMID: 32589758 DOI: 10.1002/jmv.26226]

94 **Meng XJ**. Expanding Host Range and Cross-Species Infection of Hepatitis E Virus. *PLoS Pathog* 2016; **12**: e1005695 [PMID: 27490119 DOI: 10.1371/journal.ppat.1005695]

95 **Córdoba L**, Feagins AR, Opriessnig T, Cossaboom CM, Dryman BA, Huang YW, Meng XJ. Rescue of a genotype 4 human hepatitis E virus from cloned cDNA and characterization of intergenotypic chimeric viruses in cultured human liver cells and in pigs. *J Gen Virol* 2012; **93**: 2183-2194 [PMID: 22837416 DOI: 10.1099/vir.0.043711-0]

96 **Nguyen HT**, Shukla P, Torian U, Faulk K, Emerson SU. Hepatitis E virus genotype 1 infection of swine kidney cells *in vitro* is inhibited at multiple levels. *J Virol* 2014; **88**: 868-877 [PMID: 24198420 DOI: 10.1128/JVI.02205-13]

97 **Li L**, Zhang L, Hu Q, Zhao L, Nan Y, Hou G, Chen Y, Han X, Ren X, Zhao Q, Tao H, Sun Z, Zhang G, Wu C, Wang J, Zhou EM. MYH9 Key Amino Acid Residues Identified by the Anti-Idiotypic Antibody to Porcine Reproductive and Respiratory Syndrome Virus Glycoprotein 5 Involve in the Virus Internalization by Porcine Alveolar Macrophages. *Viruses* 2019; **12** [PMID: 31905776 DOI: 10.3390/v12010040]

98 **Zhao D**, Liu R, Zhang X, Li F, Wang J, Zhang J, Liu X, Wang L, Wu X, Guan Y, Chen W, Wang X, He X, Bu Z. Replication and virulence in pigs of the first African swine fever virus isolated in China. *Emerg Microbes Infect* 2019; **8**: 438-447 [PMID: 30898043 DOI: 10.1080/22221751.2019.1590128]

99 **Wang J**, Li J, Wang N, Ji Q, Li M, Nan Y, Zhou EM, Zhang Y, Wu C. The 40 kDa Linear Polyethylenimine Inhibits Porcine Reproductive and Respiratory Syndrome Virus Infection by Blocking Its Attachment to Permissive Cells. *Viruses* 2019; **11** [PMID: 31546799 DOI: 10.3390/v11090876]

100 **Nagashima S**, Takahashi M, Kobayashi T; Tanggis, Nishizawa T, Nishiyama T, Primadharsini PP, Okamoto H. Characterization of the Quasi-Enveloped Hepatitis E Virus Particles Released by the Cellular Exosomal Pathway. *J Virol* 2017; **91** [PMID: 28878075 DOI: 10.1128/JVI.00822-17]

101 **Ma H**, Song X, Harrison TJ, Li R, Huang G, Zhang H, Kong W, Wang Y. Immunogenicity and efficacy of a bacterially expressed HEV ORF3 peptide, assessed by experimental infection of primates. *Arch Virol* 2009; **154**: 1641-1648 [PMID: 19763777 DOI: 10.1007/s00705-009-0496-4]

102 **Syed SF**, Sun Y, Du T, Chen Y, Liu B, Wang X, Li H, Nan Y, Zhou EM, Zhao Q. Evaluation of recombinant Chinese avian hepatitis E virus (CaHEV) ORF2 and ORF3 proteins for protection of chickens against CaHEV infection. *Vaccine* 2017; **35**: 3482-3489 [PMID: 28528763 DOI:

10.1016/j.vaccine.2017.05.030]

103 **Zhao Q**, Sun YN, Hu SB, Wang XJ, Xiao YH, Hsu WH, Xiao SQ, Wang CB, Mu Y, Hiscox JA, Zhou EM. Characterization of antigenic domains and epitopes in the ORF3 protein of a Chinese isolate of avian hepatitis E virus. *Vet Microbiol* 2013; **167**: 242-249 [PMID: 24021883 DOI: 10.1016/j.vetmic.2013.08.006]

104 **Semiletov IuA**, Dement'evaEV, Iashina TL, Favorov MO, Shibnev VA. [Synthesis and antigenic activity of peptides from the ORF3 protein sequence of hepatitis E virus]. *Bioorg Khim* 1995; **21**: 156-157 [PMID: 7748209]

105 **Dement'eva EV**, Semiletov IuA, Iashina TL, Shibnev VA. [Analysis of the organization of immunodominant epitopes of hepatitis virus E orf2 and orf3 proteins using synthetic peptides]. *Vopr Virusol* 1997; **42**: 102-105 [PMID: 9297336]

106 **Yang Y**, Lin S, Nan Y, Ma Z, Yang L, Zhang Y. A Linear Surface Epitope in a Proline-Rich Region of ORF3 Product of Genotype 1 Hepatitis E Virus. *Viruses* 2016; **8**: 227 [PMID: 27548202 DOI: 10.3390/v8080227]

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REVIEW

Breakthroughs and challenges in the management of pediatric viral hepatitis

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Abstract

Chronic infections by hepatitis B virus (HBV) and hepatitis C virus (HCV) major causes of advanced liver disease and mortality worldwide. Although regarded as benign infections in children, their persistence through adulthood is undoubtedly of concern. Recent advances in HCV treatment have restored the visibility of these conditions and raised expectations for HBV treatment, which is currently far from being curative. Herein we describe direct-acting antivirals available for pediatric HCV (sofosbuvir/ledipasvir, sofosbuvir/velpatasvir, glecaprevir/pibrentasvir) and their real-world use. A critical review of the HBV pediatric classification is provided. Anti-HBV investigational compounds are reviewed in light of the pathophysiology in the pediatric population, including capsid assembly modulators, antigen secretion inhibitors, silencing RNAs, and immune modifiers. Recommendations for screening and management of immunosuppressed children or those with other risk factors or comorbidities are also summarized.

Key Words: Hepatitis C; Hepatitis B; Direct acting antivirals; Liver cirrhosis; Children

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Core Tip: Chronic hepatitis B and C account for substantial morbidity and mortality worldwide. Infection control in children has great clinical and epidemiological implications. This review discusses recent achievements and forthcoming opportunities in the management of pediatric viral hepatitis. Removing barriers to the access to novel direct-acting antivirals, and understanding the relevance of tolerance-breaking new

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INTRODUCTION

The World Health Organization has estimated that - in 2015 - hepatitis B virus (HBV) and hepatitis C virus (HCV) infections caused 1.34 million deaths worldwide, exceeding the death toll of human immunodeficiency virus (HIV) and malaria and reaching that of tuberculosis[1]. These epidemics now move into the spotlight for the forthcoming innovations in their treatment armamentarium. We are living in the aftermath of the breakthrough of direct-acting antivirals (DAAs) for HCV. Those drugs have radically changed the epidemiology of adult chronic liver disease in developed countries, but they are still poorly accessible in resource-limited settings, and their use in children is just beginning. Improvements in HBV treatment are just around the corner. Notably, most investigational compounds directed against HBV tackle the host immune response towards the virus.

The role of children in this scenario is very important. Treating children chronically infected by HBV and HCV is complementary to prevention of mother-to-child transmission, with the purpose of the epidemiological control. On the clinical side, treatments capable of restoring immune tolerance or exhaustion, and promoting viral clearance, are paramount to prevent advanced liver disease in adulthood. In this review, we identify and discuss the next challenges in treatment of pediatric viral hepatitis. Overcoming the barriers to the availability of treatment for all children is a major objective in HCV infection. Conversely, identifying homogeneous patients phenotypes of chronic infection, selecting timely and appropriate treatment, bearing in mind forthcoming therapeutic opportunities, are keys to meeting the current challenges in the management of pediatric HBV infection.

NATURAL HISTORY OF HCV INFECTION IN CHILDREN

HCV infection differs in children and adults in the mode of transmission, rate of clearance, progression of fibrosis, and duration of chronic infection when acquired at birth[2]. Vertical transmission of HCV from mother to child is reported to be the leading cause of pediatric infection worldwide. A recent review found that the risk of vertical HCV infection to the children of HCV antibody-positive and HCV-RNA-positive women was 5.8% [95% confidence interval (CI), 4.2-7.8] for children of HIV-negative women and 10.8% (95%CI, 7.6-15.2) for children of HIV-positive women[3].

Symptomatic acute HCV is rare in childhood, but can present with lethargy, fever, and myalgia[4]. Approximately 20%-25% of acute HCV infections can be cleared spontaneously. Spontaneous clearance of the virus following vertical transmission is reported in around 20% of infected patients, usually by 4 years of age[5]. Children who do not clear HCV spontaneously develop chronic infection that, is usually asymptomatic in pediatric patients[6]. During the chronic course, transaminase levels may be normal or intermittently elevated. Serum HCVRNA levels may considerably fluctuate as well, but without immediate prognostic relevance[7,8]. Histological findings are usually unremarkable, but the presence of cirrhosis is reported in 1%-4% of patients[7,9,10]. Overall, the risk of severe hepatic complications in pediatric patients is low[7].

Information on childhood to adult progression of liver is lacking, and the proportion of HCV-infected children who develop serious long-term liver disease is not clear[10]. Hepatic fibrosis is reported in less than 2% of pediatric cases, but the percentage is much higher in patients with long-term follow-up, suggesting that the development of fibrosis correlates with age and the duration of infection[11-14]. In a recent study from the United Kingdom including 1049 patients with a chronic hepatitis

C acquired during childhood, one-third developed liver disease, with a median of 33 years after infection. Patients who acquired the infection vertically developed cirrhosis at an earlier age (36 years) compared with patients who acquired HCV in childhood through drug abuse (48 years), blood transfusion (46 years), or with an unknown route of infection (52 years, $P < 0.0001$). In that cohort, the incidence of hepatocellular carcinoma (HCC) was 5%; 4% required a liver transplant, and death occurred in 3%[11]. Long-term liver complications included cirrhosis, HCC, or requirement for transplantation[11,12,14]. In adults, the natural history of chronic HCV infection is affected by associated medical and social factors including hepatic steatosis, alcohol consumption, malignancy, and viral HIV and HBV coinfection[11,15]. Extrahepatic manifestations of chronic HCV (*e.g.*, positive nonorgan-specific autoantibodies, autoimmune thyroiditis, glomerulonephritis) are rare in children[15,16]. In conclusion, early acquired hepatitis C infection is a clinically and histologically silent condition. Nevertheless, it may become insidious. Although the percentage of children developing liver cirrhosis in adulthood is low, progression beyond the second decade of life is likely. Thus, the main aim of therapeutic interventions in pediatric patients is not the treatment of an ongoing liver disease, but the prevention of progression by early eradication of the infection[17].

THE BREAKTHROUGH OF DIRECT-ACTING ANTIVIRALS

In 2011 boceprevir and telaprevir, two first-generation NS3/4A protease inhibitors, were approved for use in combination with pegylated interferon (peg-IFN) and ribavirin for the treatment of adults with chronic HCV infection[18,19]. The response rates to triple therapy with boceprevir or telaprevir was improved when compared with dual therapy with peg-IFN and ribavirin, but was accompanied by significant side effects. Since then, the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA) have approved 13 different DAAs and six fixed-dose combinations for the treatment of adults with chronic HCV infection (sofosbuvir/ledipasvir, ombitasvir/paritaprevir/ritonavir, elbasvir/grazoprevir, glecaprevir/pibrentasvir, sofosbuvir/velpatasvir, and sofosbuvir/ velpatasvir/ voxilaprevir)[20]. DAAs are classified into several categories by their molecular targets. Those agents were designed to inhibit specific viral proteins that have critical roles in HCV replication. They include NS3/4A protease inhibitors (*e.g.*, simeprevir, paritaprevir, grazoprevir, voxilaprevir, and glecaprevir), nucleotide (*e.g.*, sofosbuvir) and non-nucleotide (*e.g.*, dasabuvir) inhibitors of NS5B polymerase, and NS5A inhibitors (*e.g.*, daclatasvir, ledipasvir, ombitasvir, velpatasvir, elbasvir, and pibrentasvir). The development of combinations of DAAs is based on the concept that at least two drugs are needed to achieve the treatment goal of a virological response of > 95%) without selecting resistant mutants. When the backbone of the treatment is a nucleoside NS5B inhibitor like sofosbuvir, only one other drug, an NS3/4A protease inhibitor or a NS5A-inhibitor, is usually required. Conversely, a non-nucleoside NS5B inhibitor like dasabuvir should be used together with both NS3/4A proteases and NS5A inhibitors.

The introduction of DAAs changed the HCV treatment paradigm. These regimens are oral, patient-friendly, have treatment schedules as short as 8 wk, are highly effective, and have few side effects. Given these clear advantages over IFN-based therapies, DAAs have become the preferred treatment for adults with HCV. Remarkably, the latest generation of DAAs (glecaprevir/pibrentasvir, sofosbuvir/velpatasvir, and sofosbuvir/velpatasvir/voxilaprevir) have pan-genotypic activity, thus simplifying treatment decisions.

The aims of treating HCV with DAAs are multiple. The first is to prevent the complications of HCV-related liver disease, namely cirrhosis, liver failure, and HCC. Secondly, the treatment can improve the quality of life of patients with chronic infection and remove the social stigma of being HCV-positive. Finally, treatment effective for HCV elimination also prevents further transmission of the infection, which targets the goal of global HCV elimination. The excellent efficacy and safety profile of DAAs, have also made antiviral therapy possible for patients with advanced liver disease and for those on the waiting list for liver transplantation (LT), leading to remarkable clinical improvement, avoiding the infection of the graft, and even allowing the delisting of up to one third of the patients[21]. Unfortunately, the initial lack of clinical data specific to DAA treatment in adolescents and children with chronic HCV infection has delayed the introduction of those agents for pediatric use. Several recent studies have demonstrated the safety and efficacy of various DAA-based

regimens in pediatric patients[22], supporting their use in that setting (Table 1).

TREATING CHILDREN WITH HCV

IFN-based treatment regimens are no longer recommended as therapeutic options in adolescents and children with HCV, given the high toxicity and modest sustained virological response (SVR) rates[4,23,24]. In 2017, the United States. FDA and the EMA approved the use of sofosbuvir/ledipasvir for adolescents 12 years of age or older with HCV genotype 1, 4, 5, or 6 infection, and sofosbuvir in combination with ribavirin for those with HCV genotype 2 or 3. Since then, four different regimens with age-specific limitations have been approved based on the results of four phase II-III, open-label, multicenter, multicohort studies (Table 2).

Sofosbuvir/ledipasvir was tested in 100 adolescents 12-17 years of age, 92 children 6-11 years of age, and 34 toddlers 3-5 years of age with chronic HCV infections[25-27]. The efficacy of the combination across the three different age cohorts was high (Table 1). One adolescent discontinued treatment and one did not attend post-treatment follow-up visits after having achieved the end-of-treatment response. One cirrhotic child 6 years of age with HCV genotype 1a infection relapsed by the 4-wk follow-up visit, and one 3-year-old patient discontinued treatment after 5 days because of "abnormal drug taste" and vomiting. The treatment was well tolerated. No serious or grade 3-4 drug-related adverse events were reported, and no patient discontinued treatment because of an adverse event. The efficacy of this combination has been confirmed in real-world studies[28-32].

The combination of sofosbuvir and ribavirin was tested in 52 adolescents, 41 children 6-11 years of age and 13 toddlers 3-5 years of age with chronic HCV infection, showing high efficacy (Table 1)[33,34]. One adolescent with HCV genotype 3 infection who did not achieve SVR 12 wk after the end of treatment (SVR12), was lost to follow-up after achieving the end-of-treatment response and SVR4 (HCV-RNA negative 4 wk after the end of treatment). In the younger cohorts, a 4-year-old child who did not achieve the primary endpoint of SVR12 discontinued treatment after 3 d because of an "abnormal drug taste". No patients had a virologic nonresponse, breakthrough, or relapse. Treatment was well tolerated. One serious adverse event, accidental ribavirin overdose requiring hospitalization for monitoring was reported in a 3-year-old patient. The child completed treatment and achieved SVR12.

A sofosbuvir/velpatasvir fixed-dose formulation was tested in 102 adolescents and 73 children 6-11 years of age with chronic HCV infection[35]. Two treatment failures were reported, a 17-year-old girl with genotype 1a who became pregnant and discontinued therapy at week 4, and a 10-year-old girl with genotype 1a. Eight patients were lost to follow-up. The treatment was well tolerated. No serious or grade 3-4 drug-related adverse events were reported, and no patient discontinued treatment because of an adverse event. The combination of glecaprevir/pibrentasvir was tested in 47 adolescents[36], and an SVR12 of 100% was reported. No patients had virologic nonresponse, breakthrough infection, or relapse. The treatment was well tolerated. No serious adverse events were reported, and no patient discontinued treatment because of an adverse event.

Sofosbuvir/ledipasvir and sofosbuvir plus ribavirin are approved for children as young as 3 years of age. Sofosbuvir/velpatasvir and glecaprevir/pibrentasvir are approved for those older than 6 years older than 12 years of age, respectively. The results of the trials of those regimens for younger age cohorts (3-5 years of age for sofosbuvir/velpatasvir and 3-11 years of age for glecaprevir/pibrentasvir) are now available[35,37], and expansion of the indication is expected in late 2021. Remarkably, adherence to the treatment regimen seems to be the main factor impacting the effectiveness of DAA regimens. Noncompliance was responsible of most of the treatment failures across the different trials[22].

REAL-WORLD USE OF DAA IN CHILDREN

After the approval of DAAs by international regulatory agencies and their inclusion in clinical guidelines in the early 2010's, other barriers had to be overcome before they actually became available. In the adult setting, delay in private third-party payer authorization was an obstacle to access to DAA[38,39]. In the pediatric setting, the situation is paradoxical. DAA treatment has been approved by the EMA and the FDA, but the drugs are not available in many countries because of a lack of indications

Table 1 Pediatric studies of different combinations of direct-acting antivirals

Drug regimen	Age range in yr	Sample size	Genotype(s)	Number of patients with SVR12, %	Ref.
Sofosbuvir/ledipasvir	12-17	100	1	98/100 (98)	Balistreri *et al*[25]
	6-11	92	1, 3, 4	91/92 (99)	Murray *et al*[26]
	3-5	34	1, 4	33/34 (97)	Schwarz *et al*[27]
Sofosbuvir plus ribavirin	12-17	52	2, 3	51/52 (98)	Wirth *et al*[33]
	6-11	41		41/41 (100)	Rosenthal *et al*[34]
	3-5	13		12/13 (92)	Rosenthal *et al*[34]
Sofosbuvir/velpatasvir	12-17	102	1, 2, 3, 4	97/102 (95)	Sokal *et al*[35]
	6-11	73		68/73 (93)	Sokal *et al*[35]
	3-5	41		34/41 (83)	Sokal *et al*[35]
Glecaprevir/pibrentasvir	12-17	47	1, 2, 3, 4	47/47 (100)	Jonas *et al*[36]
	6-11	32		31/32 (97)	Jonas *et al*[37]
	3-5	16		15/16 (96)	Jonas *et al*[37]
Ombitasvir/paritaprevir/ritonavir ± dasabuvir ± ribavirin	12-17	38	1, 4	38/38 (100)	Leung *et al*[134]
	6-11	26	1	25/26 (95)	Rosenthal *et al*[135]
Elbasvir/grazoprevir	12-17	22	1, 4	22/22 (100)	Wirth *et al*[136]
	6-11	17		17/17 (100)	Wirth *et al*[136]
	3-5	18		18/18 (100)	Wirth *et al*[136]
Sofosbuvir/velpatasvir/voxilaprevir	12-17	21	1, 2, 3, 4	21/21 (100)	Bansal *et al*[137]
Sofosbuvir plus daclatasvir	15-17	13	4	13/13 (100)	El-Sayed *et al*[138]
	13-17	10		10/10 (100)	El-Shabrawi *et al*[139]
	12-17	30		29/29 (100)	Yakoot *et al*[140]
	8-17	40	1, 4	39/39 (100)	Abdel Ghaffar *et al*[141]
	7-13	14	3	14/14 (100)	Padhi *et al*[57]

SVR12: Sustained virological response 12 wk after the end of treatment.

approved by the national agencies[40]. Nevertheless, eradicating HCV infection in early life has been demonstrated to impact favorably on the quality of life and to be cost-effective[41,42]. In fact, reports of real-world experience with the pediatric use of DAAs are increasing, and have shown that the excellent results of the experimental trials can be replicated in adolescents. A study in a cohort of 78 Italian children 12-17 years of age with chronic genotype 1, 3, and 4 HCV infections treated with sofosbuvir/ledipasvir had an SVR12 of 98.7%[28]. Similar results were obtained in three cohorts of Egyptian children. Thirty adolescents with genotype 2 and 144 with genotype 3 infections achieved 99%-100% SVR12s[31,32], and 20 children 6-12 years of age had an SVR12 of 95% after fixed half-dose sofosbuvir/ledipasvir, with only one patient lost to follow-up[43]. Real-world experience has also confirmed that children are ideal candidates for a shortened DAA course, as they typically exhibit little or no fibrosis. In fact, noncirrhotic, treatment-naïve children with genotype 1 and 4 infections and low viremia below 6.8 $\log_{10}$ IU/mL HCV-RNA have been reported to achieve 100% SVR12 after only 8 wk of sofosbuvir/ledipasvir[29,44].

HCV TREATMENT OF HCV IN CHILDREN AT RISK

The advent of DAA has radically changed the approach to HCV eradication treatment

Table 2 Direct-acting antiviral regimens approved for treatment of chronic hepatitis C virus infection in children and adolescents

Regimen	Genotype and duration of treatment	Formulations	Dosage
Sofosbuvir/ledipasvir	GT 1, 4, 5, 6: 12 wk	Tablet (FDC) 400/90 mg	> 35 kg: 400/90 mg/d
	GT 1, treatment-experienced, cirrhosis: 24 wk		17-35 kg: 200/45 mg/d
		Tablet (FDC) 200/45 mg	< 17 kg, older than 3 yr of age: 150/33.75 mg/d
		Pellets 200/45 mg and 150/33.75	
Sofosbuvir + ribavirin	GT 2: 12 wk	Sofosbuvir: tablet 400 mg	Sofosbuvir: > 35 kg: 400 mg/d
	GT 3: 24 wk		17-35 kg: 200 mg/d
		Tablet 100 mg	< 17 kg, older than 3 yr of age: 200 mg if ≥ 17 kg
		Capsules 50 mg containing granules	150 mg/d if < 17 kg. Ribavirin: 15 mg/kg per day in two divided doses
Sofosbuvir/velpatasvir	All GTs: 12 wkdecompensated cirrhosis: 12 wk + ribavirin	Tablet (FDC) 400/100 mg	> 30 kg: 400/100 mg/d
		Tablet (FDC) 200/50 mg	17-30 kg, older than 6 yr of age: 200/50 mg/d. Ribavirin: 15 mg/kg per day in two divided doses
Glecaprevir/pibrentasvir	All GTs: 8 wk	Tablet (FDC) 100/40 mg/d	12-17 yr or > 45 kg: 300/120 mg/d
	All GTs, cirrhosis: 12 wk		
	GT 3 treatment-experienced: 16 wk		

FDC: Fixed-dose combination; GT: Genotype.

not only for otherwise healthy children but also for those with comorbidities. The high safety profile of DAA fostered wide experimental use for previously neglected clinical conditions for which IFN-based treatment strategy was proscribed[45]. HCV recurrence after LT is a well-known problem in the adult setting, and can progress rapidly to graft cirrhosis and early loss[46]. HCV eradication with DAA before and after LT brought a tremendous improvement in transplantation outcomes[47-49]. The type of post-LT immunosuppression regimen seems not to impact the DAA treatment response[50], but fluctuation of calcineurin inhibitor trough levels during treatment may account for graft immune-mediated dysfunction[51,52]. Although a similar DAA efficacy might be expected in pediatric LT recipients, no relevant pediatric experience has been published.

So far only seven children receiving DAAs after hemopoietic stem cell transplantation (HSCT) for hematological disease have been described in the literature. One had received HSCT for acute lymphoblastic leukemia (4 years of age, genotype 1a)[53], and another for sickle cell disease (15-year-old, genotype 4)[54], and were treated with a combination of sofosbuvir/simeprevir for 24 wk and 12 wk, respectively. They achieved stable viral clearance with calcineurin inhibitor-based regimens (cyclosporin + mycophenolate mofetil, and tacrolimus + sirolimus, respectively). The other five children were 5-12 years of age, and received haploidentical allogeneic HSCT for genotype 1b refractory lymphoblastic leukemia, and were under cyclosporin maintenance[55]. All of them were treated with 24 wk of sofosbuvir/velpatasvir after a median HSCT follow-up of 15 mo, and achieved SVR by 1 mo. El-Shabrawi *et al*[56] reported on a 12-wk course of sofosbuvir/daclatasvir in 20 genotype 4-infected children in full remission of a hematological malignancy for more than 18 mo. All patients achieved SVR24 without notable adverse events. Two teams of researchers have reported on DAA-driven HCV eradication in thalassemic patients. In total, 25 children were enrolled, 14 with genotype 3 received 12-wk sofosbuvir/daclatasvir and 11 with genotype 4 received 12-wk sofosbuvir/ledipasvir). All achieved SVR12 without complaining of any serious adverse reactions[57,58].

Due to their efficacy and safety profile, DAA use is expanding to cutting-edge therapeutic scenarios. Hematopoietic stem cell-gene therapy (HSC-GT)—a life-saving option for inborn error diseases—is contraindicated in HCV-infected children because of the infection risk of bone marrow cells used as starting material for the manufacture. A 12-wk course with sofosbuvir/ledipasvir was found to allow autologous HSC-GT for the correction of severe combined immunodeficiency caused

by adenosine deaminase deficiency as described in a pioneering study[59]. Pediatric studies have not yet replicated the encouraging results with DAA in adults with HIV/HCV coinfection[60-62], even when vertically transmitted[63], nor for the treatment of HCV in hemodialyzed[64] and kidney transplant patients[65,66].

NATURAL HISTORY OF HBV IN CHILDREN

Similar to HCV, childhood HBV infections occur mostly by mother-to-child transmission. In highly endemic countries, up to one third of the HBV incident transmissions are horizontal, caused by child-to-child, household or intrafamilial contacts[67]. Unlike adults, the prevalence of HBV infections in children reflects the extent of infant and HBV vaccination at birth, which is done with the goal of eradication in the absence of an effective antiviral treatment[68]. According to the last World Health Organization hepatitis report, global hepatitis B surface antigen (HBsAg) prevalence in preschool children dropped from approximately 4.7% between the 1980 and the early 2000s to 1.3% in 2015, following the widespread adoption of universal infant immunization[69]. The age at transmission determines the development of chronic HBV infection, which occurs in approximately 90% of infected newborns and infants compared with only 10% of infections acquired later in childhood or in adults[70,71]. Once chronic infection is established, the subsequent disease progression is the result of the adaptive immune response to HBV, immune-inflammatory liver injury, and the pathogen itself.

Until recently, patients have been classified by immune activity and control criteria, assuming that the clinical phenotype of HBV infection represented a clear-cut immunological state. Due to the lack of immunological data, the latest classification by the European Association for the Study of the Liver (EASL) replaces states with "phases", which focused more on the infection course in adults, but do not fit the pediatric scenario[72]. In fact, the natural history of chronic HBV infection across all ages has been described by few large, long-term, prospective studies[73-80]. After infection, the vast majority of children enter a state characterized by detectable HBsAg and hepatitis B e antigen (HBeAg), high viremia, and normal or near-normal transaminase levels. This "tolerant" phase can last for decades, and Asian children or those carrying genotypes C or D have a higher likelihood to remain so[81-83]. Eventually, at a median age of 30 years, 3%-6% of the patients achieve HBeAg/hepatitis B e antibody (HBeAb) seroconversion annually, and most of those patients succeed in suppressing viremia[73,75,81,82]. Spontaneous HBsAg/hepatitis B surface antibody (HBsAb) seroconversion, which potentially allows long-term infection control, rarely occurs, with a rate of approximately 0.5%/year[73,75].

The major concern in children with chronic pediatric HBV infection is the risk of cirrhosis and HCC, which occur in 1%-5% and 0.03%-2% of the patients, respectively[73,74]. As this risk increases in parallel with the activity and the duration of chronic hepatitis B (CHB) (*e.g.*, transaminase elevation in the presence of elevated viral load)[84], the ultimate long-term goal is the achievement of sustained viral suppression, either as a result of the immunologic control or with the use of antiviral drugs.

PHENOTYPE CHARACTERIZATION OF CHILDREN WITH CHRONIC HBV INFECTION

The current classification of chronic HBV infection recognizes different phases and considers their interchangeability[72]. The phases are identified by the presence or absence of HBeAg (*i.e.* HBeAg positive and HBeAg negative) and by increased transaminases, which distinguishes chronic hepatitis from chronic infection. These categories do not fit the pediatric scenario well, where HBeAg-positive chronic infection tends to persist and HBeAg-negative hepatitis is anecdotal[85].

Classifying patients with HBV infection by the clinical phenotype, which reflects the immune status against the virus, has some advantages. Phenotype classification has been progressively refined to harmonize population data, estimate the need for antiviral treatment, and possibly predict the outcome of the infection[81,82]. It describes three childhood phenotypes: (1) immune-active (HBeAg +/-, elevated transaminases, HBV-DNA > $\log_{10}$ 4 IU/mL); (2) immune-tolerant (HBeAg-positive, normal transaminases, HBV-DNA > $\log_{10}$ 4 IU/mL); and (3) inactive carriers (HBeAg

+/-, normal transaminases, HBV-DNA ≤ $\log_{10}$ 4 IU/mL); with the remaining being "indeterminant"[81-83]. So defined, the immune phenotype may help predict the fate towards infection control. Indeed, HBeAg-positive immune-active children are much more likely to achieve spontaneous HBeAg/HBeAb seroconversion (28% and 7.47%/year) compared with immune-tolerant children (11% and 2.29%/year)[81]. In a scenario characterized by the lack of clear treatment indications, and disappointing therapeutic outcomes, the definition of phenotypes has proven to bear prognostic value, and to help define homogeneous groups for patient selection in future trials.

CONTROVERSIES IN THE TREATMENT OF CHILDREN WITH HBV

The current pediatric guidelines recommend limiting treatment to children with prolonged (> 6 mo) active hepatitis B and evidence of fibrosis, and to observe those with the immune-tolerant phenotype[86]. The rationale for this approach, is that HBeAg-positive CHB, especially when protracted and beginning early, at < 3 years of age, has the highest risk of progression to cirrhosis, which is between 1% and 5% by the third decade[73,74]. Many centers tend to delay treatment based on the sound conception that CHB is harmless and that transaminase activity heralds spontaneous immune clearance[73]. Moreover, there is evidence that treatment only accelerates HBeAg/HBeAb seroconversion without influencing the proportion of patients who seroconvert over time[75]. On the other hand, treatment could be indicated to break tolerance. The EASL recommends nucleoside or nucleotide analogues (NAs) for long-term viral suppression in immune-tolerant patients 30 years of age or older[72], to reduce the risk of fibrosis and cirrhosis in those with delayed immune clearance [87,88]. In fact, studies highlight that high viremia in persistently HBeAg-positive patients is associated with the highest risk of cirrhosis, HCC, and liver-related mortality and that functional infection control following HBeAg/HBeAb seroconversion lowers those risks[87,89,90]. Thus, the question has arisen whether treatment should also be offered to immune-tolerant children.

The pediatric age has been regarded as a good time window to break viral tolerance. The first encouraging experience of treating immune-tolerant children was published in 2006. HBV-DNA clearance and HBsAg/HBsAb seroconversion were achieved in 5 of 22 children (23%) after 10 mo of sequential combination therapy with lamivudine and IFN-a[91]. That result was confirmed in a case-control study in which 6 of 22 immune-tolerant children achieved HBsAb-dependent functional control[92]. Another study reported a success rate of 33%[93]. Those studies reported rates of HBsAb seroconversion of 20%-25%. Conversely, a clinical trial using a combination of entecavir and peg-IFN-a 2a resulted in only 2 of 60 children achieving the primary endpoint of HBeAg loss and sustained HBV-DNA levels < 1000 IU/mL), with adverse events reported in more than 50% of the children[94].

In favor of treating immune-tolerant children is evidence that their spontaneous seroconversion rate is much lower than that seen in adults[87]. Also, immune-tolerant children treated with a combination of lamivudine and IFN achieved a high rate of loss of HBsAg. Moreover, unlike adults, immune tolerance in young patients is characterized by a high level of HBV DNA integration and clonal hepatocyte expansion with malignant potential despite a relatively preserved anti-HBV T-cell response[95]. Several new compounds in the pipeline that aim at increasing immune responses and overcoming immune exhaustion, with probably play a role in this specific group of children.

DRUGS APPROVED FOR CHILDREN WITH CHB

Seven different drugs have been approved for children and adolescents with chronic HBV infection by the United States FDA and the EMA (Table 3). IFN- and peg-IFN- are immune modulators that can be administered for a predefined duration with the aim of inducing immune-mediated control of HBV infection and achieving long-lasting suppression of off-treatment viral replication[68]. NA are potent HBV inhibitors that are used as long-term oral treatments to suppress viral replication, or as treatments of finite duration with or without IFN, to obtain a sustained off-treatment virological response. NAs are also classified as genetic barriers to resistance by the threshold of mutations required for clinically meaningful loss of drug susceptibility. Lamivudine, adefovir, and telbivudine have low and tenofovir, and entecavir have high, genetic barriers to resistance[68]. Treatment is indicated for children and adolescents with

Table 3 Antiviral drugs approved for adolescents and children with chronic hepatitis B virus infection

Drug	Use in children	Dose	Formulation
Interferon-α-2b	≥ 1 yr	6 million IU/m^2 3 times a week	Subcutaneous injections
Pegylated interferon-α-2a	≥ 3 yr	180 μg/1.73 m^2 once a week	Subcutaneous injections
Lamivudine	≥ 3 yr	3 mg/kg daily (max 100 mg)	Oral solution (5 mg/mL)
			tablets (100 mg)
Entecavir	≥ 2 yr	10-30 kg: 0.015 mg/kg daily (max 0 5 mg)	Oral solution (0.05 mg/mL)
			tablets (0.5 mg and 1 mg)
Adefovir	≥ 12 yr	10 mg daily	Tablets (10 mg)
Tenofovir disoproxil fumarate	≥ 12 yr (FDA)	300 mg daily	Oral powder (40 mg per 1 g)
	≥ 2 yr (EMA)		tablets (150, 200, 250 and 300 mg)
Tenofovir alafenamide	≥ 12 yr (EMA)	25 mg daily	Tablets (25 mg)

EMA: European Medicines Agency; FDA: Food and Drug Administration.

active viral replication (detectable HBV-DNA levels), prolonged (6-12 mo) active hepatitis (elevated serum transaminase levels) and/or inflammation or fibrosis on liver biopsy. Unless within a clinical trial, treatment is contraindicated when transaminases are normal[72,86,96].

IFN--2b, peg-IFN--2a, lamivudine, adefovir, tenofovir disoproxil fumarate, and entecavir were approved for the treatment of children and adolescents with chronic HBV infection following six randomized placebo-controlled trials[97-102]. Tenofovir alafenamide was approved on the basis of studies of its use in HIV infection (Table 4). IFN therapy may be associated with higher rates of HBsAg loss compared with NAs. In all studies, a good overall treatment response defined by reduction of serum HBV-DNA to undetectable concentrations, loss of serum HBeAg, and normalization of transaminases, was associated with and increased baseline histology activity index score, increased baseline transaminase concentrations, and decreased baseline HBV-DNA concentrations.

Peg-IFN, entecavir, and tenofovir disoproxil fumarate are considered the drugs of choice for the treatment of CHB in children by the major international societies [72,86,96]. The advantages of IFN and peg-IFN use in children, compared with NAs, are the absence of viral resistance and a predictable, finite duration of treatment. However, the use of IFN and peg-IFN is demanding for children because it requires subcutaneous injection and is associated with a nearly certain occurrence of flu-like symptoms.

DRUGS IN THE PIPELINE: RELEVANCE TO PEDIATRIC AGE

Currently available drugs against HBV have inherent limitations. NAs are safe and well tolerated and usually succeed in suppressing replication. They are not curative, as they act at a late stage of the viral cycle and do not prevent HBV-DNA persistence in episomal or integrated forms[96]. On the other hand, IFNs have limited efficacy and frequent side effects, but they are the most likely to achieve a definite "functional" cure with HBeAg or HBsAg loss, seroconversion, normal transaminases, and undetectable viremia)[103]. Various new classes of compounds are under investigation with the aim of achieving high rates of HBsAg seroconversion[104]. The candidate drugs are relevant to the pediatric field, as almost all act as immune modifiers to achieve tolerance breakthrough. The molecules currently in phase II and later trials are listed and summarized in Table 5.

Capsid assembly inhibitors are antiviral agents complementary to NAs. JNJ-56136379 is an oral compound with two distinct mechanisms, the inhibition of the encapsidation of the pregenomic RNA (pgRNA) and the formation of covalently closed circular (ccc)DNA. It has been studied in 57 subjects with CHB treated for 28 d. HBV-DNA and HBV-RNA decreased at all tested doses and HBV-DNA was undetectable at the end of the study in one third of the patients. Nonetheless, none achieved HBsAg/HBsAb seroconversion[105]. ABI-H0731 (Vebicorvir) is an oral

Table 4 Summary of results of clinical trials of hepatitis B antiviral therapy in children

	Interferon-α-2b	Lamivudine	Adefovir	Tenofovir DF	Entecavir	Pegylated interferon-α-2a
Number treated	144	191	173	52	120	101
Duration of treatment in wk	24	52	48	72	48	48
Age, median (range)	5 (1-17)	9 (2-17)	11	15.5 (12-17)	12 (2-17)	11 (3-7)
Virological response as HBeAg negative HBV DNA undetectable (% treated *vs* placebo)	26 (*vs* 11)	23 (*vs* 13)	10.6 (*vs* 0)	21.2 (*vs* 0)	24.2 (*vs* 3.3)	19.8 (*vs* 2)
HBsAg negative (% treated *vs* placebo)	10 (*vs* 1)	2 (*vs* 0)	0.8 (*vs* 0)	1.9 (*vs* 0)	5.8 (*vs* 0)	8.9 (0)
Ref.	Sokal *et al*[97]	Jonas *et al*[98]	Jonas *et al* [99]	Murray *et al* [100]	Jonas *et al* [101]	Wirth *et al*[102]

HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen.

Table 5 Investigational drugs for chronic hepatitis B infection

Class	Compound	Mechanism	Known side effects	Route	Status	Ref.
Entry inhibitors	Bulevirtide	Blocks entry *via* interaction with NTCP	Injection site reactions, cholestasis	Subcutaneous (iv)	Approved for CHD (HBV/HDV) Phase II for HBeAg-CHB	European Association for the Study of the Liver[117] and Bogomolov *et al*[118]
Capsid assembly modulators	JNJ-56136379	Prevents encapsidation of pgRNA Prevents formation of cccDNA	Hypertransaminasemia	Oral	Phase II for CHB ± NA	Zoulim *et al*[105]
Capsid assembly modulators	ABI-H0731 (Vebicorvir)	Prevents packaging of pgRNA into capsids	Skin rash, dizziness	Oral	Phase II for CHB ± NA	Yuen *et al*[106]
HBsAg secretion inhibitors	REP-2139	Inhibits the secretion of HBsAg subviral particles	Fever, chills, hypertransaminasemia, leukopenia	iv	Phase II for CHB + NA Phase II for CHD + peg-IFN-α	Bazinet *et al*[109,110]
HBsAg secretion inhibitors	REP-2165	Inhibits the secretion of HBsAg subviral particles	Fever, chills, hypertransaminasemia, leukopenia	iv	Phase II for CHD + peg-IFN-α	Bazinet *et al*[110]
RNA interference	JNJ-3989	Silences the mRNA viral transcripts reducing HBsAg	-	iv	Phase II for CHB	Yuen *et al*[111] and Gane *et al*[112]
RNA interference	VIR-2218	Silences the mRNA viral transcripts reducing HBsAg	-	iv	Phase II for CHB	VIR Biotechnology[113]
RIG-1 agonist	Inarigivir (SB9200)	Activates PRR RIG-1 and IFN-I response	Headache, dizziness, UTI, ILI, GI symptoms, hypertransaminasemia	Oral	Phase II for CHB + NA	Yuen *et al*[114]
Immune modifier	Selgantolimod (GS-9688)	TLR8 agonist	Nausea, vomiting, chills, headache, UTI	Oral	Phase II for CHB + NA	Gane *et al*[116]

CHB: Chronic hepatitis B; CHD: Chronic hepatitis D; GI: Gastrointestinal.; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; ILI: Influenza-like illness; NA: Nucleotide analogue; peg-IFN-: Pegylated interferon α; UTI: Urinary tract infection.

compound inhibiting encapsidation, binding the core protein, and thus blocking the packaging of pgRNA into nucleocapsids. A phase I study conducted in healthy volunteers and 38 patients with CHB reported good tolerability and a prompt but temporary drop in viremia[106]. Two phase II studies are ongoing, whose interim results show that in already NA-suppressed patients, the addition of Vebicorvir significantly suppressed HBV-RNA levels. In treatment-naïve patients, its association with standard care resulted in a greater decrease in HBV DNA levels[107].

Nucleic acid polymeric secretion inhibitors reduce the release of HBsAg small viral particles, considered crucial in immune system exhaustion, thus favoring HBsAg loss

and the seroconversion to HBsAb. The polymer REP-2139, administered intravenously once weekly, has been selected for its tolerability within this class[108]. The sequential use of REP-2139 and peg-IFN-a in chronic HBV/HDV coinfection over a period of 63 wk, resulted in sustained HBsAg loss and seroconversion to HBsAb in six of 12 patients. HBV-DNA and HDV-RNA were negative in seven of the patients at the end of the treatment and in nine after 1 year of follow-up[109]. In HBeAg-negative CHB, REP-2139 or its analogue REP-2165 were used in combination with tenofovir and peg-IFN-a and achieved sustained HBsAg/HBsAb seroconversion in 41% of the patients and functional control (undetectable HBV-DNA and normal transaminases) in 77%, an unprecedented result[110].

RNA interference is another promising strategy that aims at silencing the translation of viral transcripts and subsequently decreasing HBsAg. Preliminary reports of efficacy are available from ongoing phase I/II studies on CHB entailing 3 moly administration of the small interfering (si)RNA JNJ-3989 (ARO-HBV). Regardless of HBeAg status and previous treatment, HBsAg decreased by 97%-100% after one dose and the majority of participants achieved HBsAg loss and dramatically reduced HBV-DNA shortly after protocol completion[111,112]. This drug aims at breaking the immune stall toward the virus, as suggested by an ongoing trial in immune-tolerant adults. Similar interim results have been reported in a trial of the siRNA VIR-2218[113].

Immune modulators are a heterogeneous class of candidate antivirals that target different effectors of innate immunity. Inarigivir is an RIG-1 pattern recognition receptor agonist, whose binding activates the IFN-I response. Final results of the phase II ACHIEVE trial demonstrated dose-dependent HBV-DNA reduction after inarigivir monotherapy, and the endpoint of an HBsAg reduction > 0.5 $\log_{10}$ was achieved in 22% of the patients[114]. Selgantolimod (formerly GS-9688), is a potent, orally administered Toll-like-receptor (TLR8) agonist capable of inducing tumor necrosis factor-, IFN-γ, interleukin (IL)-12, and IL-18 expression[115]. Interim results of a phase II study show that it induced a significant HBsAg reduction in 16%-30% of CHB patients and occasional HBsAg loss 24 wk after treatment onset[116]. Bulevirtide is the only viral-entry inhibitor approved by the EMA in 2020 for HBV/HDV coinfection, while it is on a phase II study for HBeAg-negative CHB (NCT02881008). It binds sodium taurocholate cotransporting polypeptide (NTCP) to prevent HBV from entering hepatocytes. Combined with peg-IFN-a, it was shown to significantly decrease HBV-DNA and HDV-DNA compared with peg-IFN-a alone[117,118]. Figure 1 depicts the different mechanisms of action of the HBV investigational products. Although many steps remain to achieve availability in the pediatric population, these drugs will certainly change the burden of HBV in children as much as in adults. The perspective of feasible and curative treatments aiming to break tolerance will increase the efforts to eradicate HBV early in life.

SCREENING AND TREATMENT OF HBV IN CHILDREN AT RISK

Data on the risk of hepatitis B reactivation in children who need to start immunosuppressive treatment for concomitant diseases are extremely scarce. For that reason, the recommendations expressed in a recent position paper of the Hepatology committee of the ESPGHAN[119] are mostly derived from adult evidence[72,96,120]. Experts recommend HBV screening, with HBsAg, HBsAb, and hepatitis B core antibody (HBcAb) testing, of all patients at risk of HBV reactivation, including those who are going to start immunosuppressive treatment. The tests should be performed even if HBV vaccination is complete, because, as shown in inflammatory bowel diseases, immunosuppressed children have low serologic protection from childhood vaccines[121]. Patients with negative HBV screening should be vaccinated before starting immunosuppressive treatment. This statement was supported by a recent study of 580 children that demonstrated a high seroconversion rate after the vaccine booster even after immunosuppression initiation[122].

Reactivation risk is classified as mild, moderate, or severe depending on the administered immunosuppressive agents[119]. The risk classes are based exclusively on adult evidence, as no corresponding pediatric study results have been published. Children scheduled for moderate or high-risk drugs should start antiviral prophylaxis, while a preemptive approach is preferred for children starting on low-risk drugs[123,124]. Even in the absence of pediatric evidence, a robust network meta-analysis reported that entecavir or tenofovir should be preferred for HBV reactivation treatment in immunosuppressed patients[125].

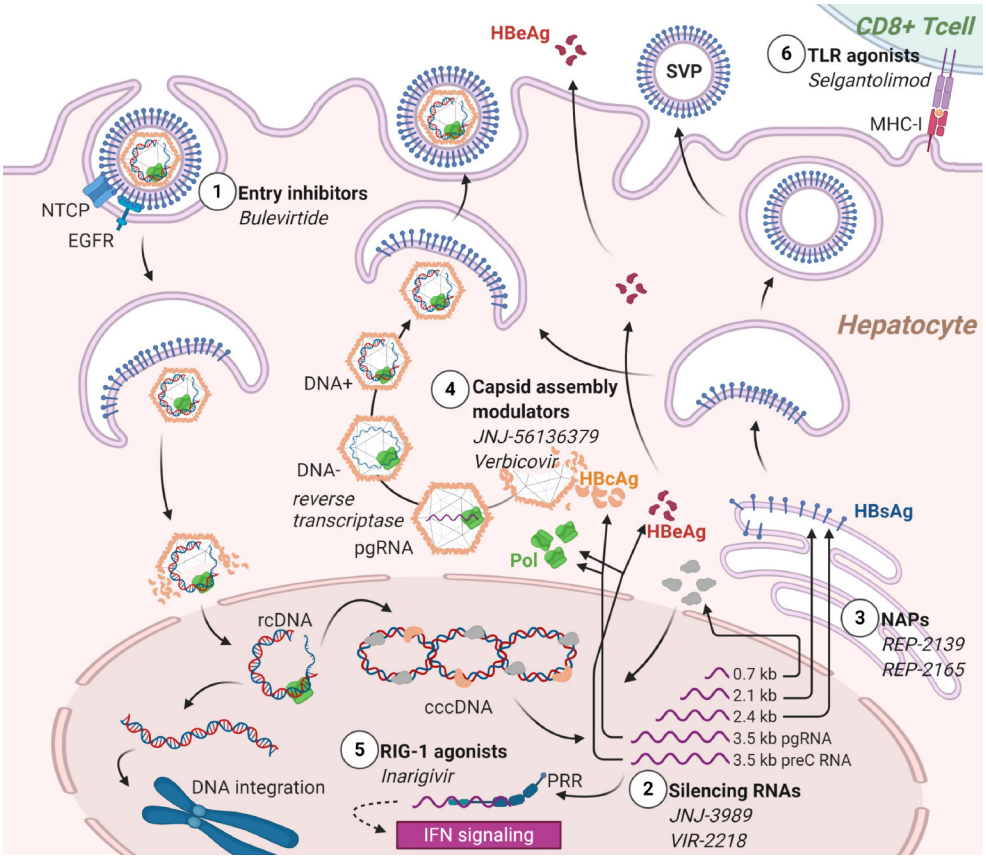


Figure 1 Novel investigational approaches to chronic hepatitis. cccDNA: Covalently closed circular DNA; DNA-: Negative-sense DNA filament synthesis; DNA+: Positive-sense DNA filament synthesis; EGFR: Epidermal growth factor receptor; NTCP: Sodium taurocholate cotransporting polypeptide; HBcAg: Hepatitis B core antigen; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; NAPs: Nucleic acid polymers; pgRNA: Pregenomic RNA; preC RNA: Pre-core RNA; PRR: Pattern recognition receptor; rcDNA: Relaxed circular DNA; SVP: Small viral particle; TLR: Toll-like receptor.

Currently, reactivation of HBV infection in pediatric solid organ transplant recipients is anecdotal, as HBcAb-positive donors are almost no longer used, and end-stage liver disease in HBsAg-positive recipients is exceptional. However, current recommendations replicate those for adults. In HBsAg-positive recipients, tenofovir or entecavir treatment should be started as soon as possible before transplant to achieve undetectable HBV-DNA[72,96]. NA treatment should be continued indefinitely, and HBV-specific immunoglobulins can be stopped after 5-7 d, unless there is a history of drug resistance or poor compliance[72,96,126]. The use of grafts from HBcAb positive/HBsAg negative donors might be acceptable in case of organ shortage and in highly endemic countries. In those situations, recipients with HBsAb titers > 200 IU/mL might be protected from infection. However, ultimately the overall risk of developing infection depends on multiple factors and implies that recipients of such organs receive NAs (*e.g.*, entecavir, tenofovir, or tenofovir alafenamide) for at least 1 year after transplant. Discontinuation might then be carefully evaluated on a case-by-case basis in HBsAb-positive children[127].

Another challenge for HBV treatment is represented by HBeAg-negative CHB, which is the most prevalent chronic hepatitis in many countries[128]. In adults, the clinical profile is characterized by wide fluctuations in viral replication and biochemical activity, with temporary spontaneous remissions[129]. The risk of cirrhosis in HBeAg-negative CHB is higher (8%-10%/year) than in HBeAg-positive (2%-5%/year)[130,131]. Infants with fulminant hepatitis caused by mother-to-child transmission of HBV e-minus variants are described[132]. This behavior is thought to result from absence of the tolerogenic effect of HBeAg. Adults are treated with long courses of NAs[133], but evidence of the benefits in children with HBeAg-negative hepatitis is lacking. However, pediatric HBeAg-negative and HBeAg-positive CHB have the same therapeutic approach, which is based on IFN- and indefinite use of NAs. The target should be decrease in HBsAg, HBV-DNA clearance, and normalization of transaminases.

CONCLUSION

HBV and HCV infections in childhood usually progress to chronic hepatitis through different mechanisms of immune tolerance and exhaustion. For HCV infection, different combinations of DAAs are increasingly available, including pan-genotypic combinations, with very few side effects and extremely high SVRs. For HBV infection, recent cohort studies have clarified that several factors including immune host phenotype, viral genotype, and ethnicity, contribute to spontaneous control. Viral hepatitis should not be a barrier to the use of immunosuppressive medications in the treatment of autoimmune conditions, nor to antineoplastic chemotherapy, provided that timely screening and appropriate pharmacological interventions are performed. Finally, new drugs in development for the treatment of HBV, mostly acting by fostering the breaking of tolerance, could dramatically improve the treatment outcome of CHB in children.

REFERENCES

1 **World Health Organization**. Viral hepatitis: a hidden killer gains visibility. [cited 2 March 2021]. In: World Health Organization [Internet]. Available from: http://www.who.int/publications/10-year-review/hepatitis/en/

2 **Robinson JL**, Doucette K. The natural history of hepatitis C virus infection acquired during childhood. *Liver Int* 2012; **32**: 258-270 [PMID: 22098487 DOI: 10.1111/j.1478-3231.2011.02633.x]

3 **Benova L**, Mohamoud YA, Calvert C, Abu-Raddad LJ. Vertical transmission of hepatitis C virus: systematic review and meta-analysis. *Clin Infect Dis* 2014; **59**: 765-773 [PMID: 24928290 DOI: 10.1093/cid/ciu447]

4 **Indolfi G**, Easterbrook P, Dusheiko G, El-Sayed MH, Jonas MM, Thorne C, Bulterys M, Siberry G, Walsh N, Chang MH, Meyers T, Giaquinto C, Wirth S, Chan PL, Penazzato M. Hepatitis C virus infection in children and adolescents. *Lancet Gastroenterol Hepatol* 2019; **4**: 477-487 [PMID: 30982721 DOI: 10.1016/S2468-1253(19)30046-9]

5 **Resti M**, Jara P, Hierro L, Azzari C, Giacchino R, Zuin G, Zancan L, Pedditzi S, Bortolotti F. Clinical features and progression of perinatally acquired hepatitis C virus infection. *J Med Virol* 2003; **70**: 373-377 [PMID: 12766999 DOI: 10.1002/jmv.10405]

6 **Seeff LB**. Natural history of chronic hepatitis C. *Hepatology* 2002; **36**: S35-S46 [PMID: 12407575 DOI: 10.1053/jhep.2002.36806]

7 **Bortolotti F**, Verucchi G, Cammà C, Cabibbo G, Zancan L, Indolfi G, Giacchino R, Marcellini M, Marazzi MG, Barbera C, Maggiore G, Vajro P, Bartolacci S, Balli F, Maccabruni A, Guido M; Italian Observatory for HCV Infection and Hepatitis C in Children. Long-term course of chronic hepatitis C in children: from viral clearance to end-stage liver disease. *Gastroenterology* 2008; **134**: 1900-1907 [PMID: 18439604 DOI: 10.1053/j.gastro.2008.02.082]

8 **Jara P**, Resti M, Hierro L, Giacchino R, Barbera C, Zancan L, Crivellaro C, Sokal E, Azzari C, Guido M, Bortolotti F. Chronic hepatitis C virus infection in childhood: clinical patterns and evolution in 224 white children. *Clin Infect Dis* 2003; **36**: 275-280 [PMID: 12539067 DOI: 10.1086/345908]

9 **Goodman ZD**, Makhlouf HR, Liu L, Balistreri W, Gonzalez-Peralta RP, Haber B, Jonas MM, Mohan P, Molleston JP, Murray KF, Narkewicz MR, Rosenthal P, Smith LJ, Robuck PR, Schwarz KB. Pathology of chronic hepatitis C in children: liver biopsy findings in the Peds-C Trial. *Hepatology* 2008; **47**: 836-843 [PMID: 18167062 DOI: 10.1002/hep.22094]

10 **Indolfi G**, Guido M, Azzari C, Resti M. Histopathology of hepatitis C in children, a systematic review: implications for treatment. *Expert Rev Anti Infect Ther* 2015; **13**: 1225-1235 [PMID: 26202832 DOI: 10.1586/14787210.2015.1070668]

11 **Modin L**, Arshad A, Wilkes B, Benselin J, Lloyd C, Irving WL, Kelly DA. Epidemiology and natural history of hepatitis C virus infection among children and young people. *J Hepatol* 2019; **70**: 371-378 [PMID: 30496763 DOI: 10.1016/j.jhep.2018.11.013]

12 **Jara P**, Hierro L. Treatment of hepatitis C in children. *Expert Rev Gastroenterol Hepatol* 2010; **4**: 51-61 [PMID: 20136589 DOI: 10.1586/egh.09.76]

13 **Guido M**, Bortolotti F, Leandro G, Jara P, Hierro L, Larrauri J, Barbera C, Giacchino R, Zancan L, Balli F, Crivellaro C, Cristina E, Pucci A, Rugge M. Fibrosis in chronic hepatitis C acquired in infancy: is it only a matter of time? *Am J Gastroenterol* 2003; **98**: 660-663 [PMID: 12650803 DOI: 10.1111/j.1572-0241.2003.07293.x]

14 **Abdel-Hady M**, Bunn SK, Sira J, Brown RM, Brundler MA, Davies P, Kelly DA. Chronic hepatitis C in children--review of natural history at a National Centre. *J Viral Hepat* 2011; **18**: e535-e540 [PMID: 21914074 DOI: 10.1111/j.1365-2893.2011.01456.x]

15 **Garazzino S**, Calitri C, Versace A, Alfarano A, Scolfaro C, Bertaina C, Vatrano S, Mignone F, Licciardi F, Gabiano C, Tovo PA. Natural history of vertically acquired HCV infection and associated autoimmune phenomena. *Eur J Pediatr* 2014; **173**: 1025-1031 [PMID: 24585099 DOI: 10.1007/s00431-014-2286-6]

16 **Sugiura T**, Yamada T, Kimpara Y, Fujita N, Goto K, Koyama N. Effects of pegylated interferon alpha-2a on hepatitis-C-virus-associated glomerulonephritis. *Pediatr Nephrol* 2009; **24**: 199-202 [PMID: 18696119 DOI: 10.1007/s00467-008-0948-1]

17 **Indolfi G**, Thorne C, El Sayed MH, Giaquinto C, Gonzalez-Peralta RP. The Challenge of Treating Children With Hepatitis C Virus Infection. *J Pediatr Gastroenterol Nutr* 2017; **64**: 851-854 [PMID: 28362694 DOI: 10.1097/MPG.0000000000001589]

18 **Sarrazin C**, Rouzier R, Wagner F, Forestier N, Larrey D, Gupta SK, Hussain M, Shah A, Cutler D, Zhang J, Zeuzem S. SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. *Gastroenterology* 2007; **132**: 1270-1278 [PMID: 17408662 DOI: 10.1053/j.gastro.2007.01.041]

19 **Reesink HW**, Zeuzem S, Weegink CJ, Forestier N, van Vliet A, van de Wetering de Rooij J, McNair L, Purdy S, Kauffman R, Alam J, Jansen PL. Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. *Gastroenterology* 2006; **131**: 997-1002 [PMID: 17030169 DOI: 10.1053/j.gastro.2006.07.013]

20 **Jakobsen JC**, Nielsen EE, Feinberg J, Katakam KK, Fobian K, Hauser G, Poropat G, Djurisic S, Weiss KH, Bjelakovic M, Bjelakovic G, Klingenberg SL, Liu JP, Nikolova D, Koretz RL, Gluud C. Direct-acting antivirals for chronic hepatitis C. *Cochrane Database Syst Rev* 2017; **9**: CD012143 [PMID: 28922704 DOI: 10.1002/14651858.CD012143.pub3]

21 **Belli LS**, Berenguer M, Cortesi PA, Strazzabosco M, Rockenschaub SR, Martini S, Morelli C, Donato F, Volpes R, Pageaux GP, Coilly A, Fagiuoli S, Amaddeo G, Perricone G, Vinaixa C, Berlakovich G, Facchetti R, Polak W, Muiesan P, Duvoux C; European Liver and Intestine Association (ELITA). Delisting of liver transplant candidates with chronic hepatitis C after viral eradication: A European study. *J Hepatol* 2016; **65**: 524-531 [PMID: 27212241 DOI: 10.1016/j.jhep.2016.05.010]

22 **Indolfi G**, Giometto S, Serranti D, Bettiol A, Bigagli E, De Masi S, Lucenteforte E. Systematic review with meta-analysis: the efficacy and safety of direct-acting antivirals in children and adolescents with chronic hepatitis C virus infection. *Aliment Pharmacol Ther* 2020; **52**: 1125-1133 [PMID: 32809230 DOI: 10.1111/apt.16037]

23 **Indolfi G**, Hierro L, Dezsofi A, Jahnel J, Debray D, Hadzic N, Czubkowski P, Gupte G, Mozer-Glassberg Y, van der Woerd W, Smets F, Verkade HJ, Fischler B. Treatment of Chronic Hepatitis C Virus Infection in Children: A Position Paper by the Hepatology Committee of European Society of Paediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2018; **66**: 505-515 [PMID: 29287014 DOI: 10.1097/MPG.0000000000001872]

24 **Indolfi G**, Fischler B, Gonzalez-Peralta RP, Ciocca M, Porta G, Neelam M, El-Guindi M, Kelly D, Ni YH, Sibal A, Leung DH, Chang MH; Hepatitis Expert Team of the Federation of International Societies of Pediatric Gastroenterology, Hepatology, and Nutrition (FISPGHAN). Comparison of Recommendations for Treatment of Chronic Hepatitis C Virus Infection in Children and Adolescents: A Position Paper of the Federation of International Societies of Pediatric Gastroenterology, Hepatology, and Nutrition. *J Pediatr Gastroenterol Nutr* 2020; **70**: 711-717 [PMID: 32205770 DOI: 10.1097/MPG.0000000000002710]

25 **Balistreri WF**, Murray KF, Rosenthal P, Bansal S, Lin CH, Kersey K, Massetto B, Zhu Y, Kanwar B, German P, Svarovskaia E, Brainard DM, Wen J, Gonzalez-Peralta RP, Jonas MM, Schwarz K. The safety and effectiveness of ledipasvir-sofosbuvir in adolescents 12-17 years old with hepatitis C virus genotype 1 infection. *Hepatology* 2017; **66**: 371-378 [PMID: 27997679 DOI: 10.1002/hep.28995]

26 **Murray KF**, Balistreri WF, Bansal S, Whitworth S, Evans HM, Gonzalez-Peralta RP, Wen J, Massetto B, Kersey K, Shao J, Garrison KL, Parhy B, Brainard DM, Arnon R, Gillis LA, Jonas MM, Lin CH, Narkewicz MR, Schwarz K, Rosenthal P. Safety and Efficacy of Ledipasvir-Sofosbuvir With or Without Ribavirin for Chronic Hepatitis C in Children Ages 6-11. *Hepatology* 2018; **68**: 2158-2166 [PMID: 30070726 DOI: 10.1002/hep.30123]

27 **Schwarz KB**, Rosenthal P, Murray KF, Honegger JR, Hardikar W, Hague R, Mittal N, Massetto B, Brainard DM, Hsueh CH, Shao J, Parhy B, Narkewicz MR, Rao GS, Whitworth S, Bansal S, Balistreri WF. Ledipasvir-Sofosbuvir for 12 Weeks in Children 3 to <6 Years Old With Chronic Hepatitis C. *Hepatology* 2020; **71**: 422-430 [PMID: 31220349 DOI: 10.1002/hep.30830]

28 **Serranti D**, Nebbia G, Cananzi M, Nicastro E, Di Dato F, Nuti F, Garazzino S, Silvestro E, Giacomet V, Forlanini F, Pinon M, Calvo PL, Riva S, Dodi I, Cangelosi AM, Antonucci R, Ricci S, Bartolini E, Mastrangelo G, Trapani S, Lenge M, Gaio P, Vajro P, Iorio R, D'Antiga L, Indolfi G. Efficacy of Sofosbuvir/Ledipasvir in Adolescents With Chronic Hepatitis C Genotypes 1, 3, and 4: A Real-world Study. *J Pediatr Gastroenterol Nutr* 2021; **72**: 95-100 [PMID: 32810039 DOI: 10.1097/MPG.0000000000002900]

29 **Serranti D**, Dodi I, Nicastro E, Cangelosi AM, Riva S, Ricci S, Bartolini E, Trapani S, Mastrangelo G, Vajro P, D'Antiga L, Resti M, Indolfi G. Shortened 8-Week Course of Sofosbuvir/Ledipasvir Therapy in Adolescents With Chronic Hepatitis C Infection. *J Pediatr Gastroenterol Nutr* 2019; **69**: 595-598 [PMID: 31335836 DOI: 10.1097/MPG.0000000000002449]

30 **El-Khayat H**, Kamal EM, Yakoot M, Gawad MA, Kamal N, El Shabrawi M, Sameh Y, Haseeb A, Fouad Y, Attia D. Effectiveness of 8-week sofosbuvir/Ledipasvir in the adolescent chronic hepatitis C-infected patients. *Eur J Gastroenterol Hepatol* 2019; **31**: 1004-1009 [PMID: 30676473 DOI: 10.1097/MEG.0000000000001360]

31 **El-Khayat HR**, Kamal EM, El-Sayed MH, El-Shabrawi M, Ayoub H, RizK A, Maher M, El

Sheemy RY, Fouad YM, Attia D. The effectiveness and safety of ledipasvir plus sofosbuvir in adolescents with chronic hepatitis C virus genotype 4 infection: a real-world experience. *Aliment Pharmacol Ther* 2018; **47**: 838-844 [PMID: 29349793 DOI: 10.1111/apt.14502]

32 **El-Karaksy H**, Mogahed EA, Abdullatif H, Ghobrial C, El-Raziky MS, El-Koofy N, El-Shabrawi M, Ghita H, Baroudy S, Okasha S. Sustained Viral Response in Genotype 4 Chronic Hepatitis C Virus-infected Children and Adolescents Treated With Sofosbuvir/Ledipasvir. *J Pediatr Gastroenterol Nutr* 2018; **67**: 626-630 [PMID: 30216203 DOI: 10.1097/MPG.0000000000002101]

33 **Wirth S**, Rosenthal P, Gonzalez-Peralta RP, Jonas MM, Balistreri WF, Lin CH, Hardikar W, Kersey K, Massetto B, Kanwar B, Brainard DM, Shao J, Svarovskaia E, Kirby B, Arnon R, Murray KF, Schwarz KB. Sofosbuvir and ribavirin in adolescents 12-17 years old with hepatitis C virus genotype 2 or 3 infection. *Hepatology* 2017; **66**: 1102-1110 [PMID: 28543053 DOI: 10.1002/hep.29278]

34 **Rosenthal P**, Schwarz KB, Gonzalez-Peralta RP, Lin CH, Kelly DA, Nightingale S, Balistreri WF, Bansal S, Jonas MM, Massetto B, Brainard DM, Hsueh CH, Shao J, Parhy B, Davison S, Feiterna-Sperling C, Gillis LA, Indolfi G, Sokal EM, Murray KF, Wirth S. Sofosbuvir and Ribavirin Therapy for Children Aged 3 to <12 Years With Hepatitis C Virus Genotype 2 or 3 Infection. *Hepatology* 2020; **71**: 31-43 [PMID: 31222783 DOI: 10.1002/hep.30821]

35 **Sokal EM**, Schwarz KB, Rosenthal, Philip, Verucchi G, Lin C-H, Balistreri W, Wen J, Whitworth S, Leung DH, Bansal S, Wikrom K, Mangia A, Rao GS, Shao J, Hsueh C-H, Parhy B, Gaggar A, Kersey K, Narkewicz MR, Gonzalez-Peralta RP, Murray KF, Romero R, Jonas MM. Safety and efficacy of sofosbuvir/velpatasvir for the treatment of chronic hepatitis c infection in adolescents and children aged 3 to 17 years old through 24 weeks post-treatment. *Hepatology* 2020; 570A

36 **Jonas MM**, Squires RH, Rhee SM, Lin CW, Bessho K, Feiterna-Sperling C, Hierro L, Kelly D, Ling SC, Strokova T, Del Valle-Segarra A, Lovell S, Liu W, Ng TI, Porcalla A, Gonzalez YS, Burroughs M, Sokal E. Pharmacokinetics, Safety, and Efficacy of Glecaprevir/Pibrentasvir in Adolescents With Chronic Hepatitis C Virus: Part 1 of the DORA Study. *Hepatology* 2020; **71**: 456-462 [PMID: 31254392 DOI: 10.1002/hep.30840]

37 **Jonas MM**, Lon HK, Rhee SM, Gilmour SM, Gonzalez-Peralta RP, Leung DH, Ling SC, Lobritto S, Narkewicz MR, Sabharwal V, Del Valle-Segarra A, Wen J, Lovell S, Porcalla A, Tripathi R, Sokal EM. Pharmacokinetics of glecaprevir/pibrentasvir in children with chronic hcv infection: interim analysis of part 2 of the Dora study. *Hepatology* 2019; 934-935

38 **Do A**, Mittal Y, Liapakis A, Cohen E, Chau H, Bertuccio C, Sapir D, Wright J, Eggers C, Drozd K, Ciarleglio M, Deng Y, Lim JK. Drug Authorization for Sofosbuvir/Ledipasvir (Harvoni) for Chronic HCV Infection in a Real-World Cohort: A New Barrier in the HCV Care Cascade. *PLoS One* 2015; **10**: e0135645 [PMID: 26312999 DOI: 10.1371/journal.pone.0135645]

39 **Torabi J**, Rocca JP, Ajaimy M, Melvin J, Campbell A, Akalin E, Liriano LE, Azzi Y, Pynadath C, Greenstein SM, Le M, Goldstein DY, Fox AS, Carrero J, Weiss JM, Powell T, Racine AD, Reinus JF, Kinkhabwala MM, Graham JA. Commercial insurance delays direct-acting antiviral treatment for hepatitis C kidney transplantation into uninfected recipients. *Transpl Infect Dis* 2021; **23**: e13449 [PMID: 32810315 DOI: 10.1111/tid.13449]

40 **Marascio N**, Mazzitelli M, Pavia G, Giancotti A, Barreca GS, Costa C, Pisani V, Greco G, Serapide F, Trecarichi EM, Casalinuovo F, Liberto MC, Matera G, Torti C. Clinical, Virological Characteristics, and Outcomes of Treatment with Sofosbuvir/Ledipasvir in Two Pediatric Patients Infected by HCV Genotype 4. *Cells* 2019; **8** [PMID: 31060315 DOI: 10.3390/cells8050416]

41 **Younossi ZM**, Stepanova M, Balistreri W, Schwarz K, Murray KF, Rosenthal P, Bansal S, Hunt S. Health-related Quality of Life in Adolescent Patients With Hepatitis C Genotype 1 Treated With Sofosbuvir and Ledipasvir. *J Pediatr Gastroenterol Nutr* 2018; **66**: 112-116 [PMID: 28957984 DOI: 10.1097/MPG.0000000000001754]

42 **Ruggeri M**, Romano F, Basile M, Coretti S, Rolli FR, Drago C, Cicchetti A. Cost-Effectiveness Analysis of Early Treatment of Chronic HCV with Sofosbuvir/Velpatasvir in Italy. *Appl Health Econ Health Policy* 2018; **16**: 711-722 [PMID: 30039348 DOI: 10.1007/s40258-018-0410-x]

43 **El-Shabrawi MHF**, Kamal NM, El-Khayat HR, Kamal EM, AbdElgawad MMAH, Yakoot M. A pilot single arm observational study of sofosbuvir/Ledipasvir (200 + 45 mg) in 6- to 12- year old children. *Aliment Pharmacol Ther* 2018; **47**: 1699-1704 [PMID: 29696674 DOI: 10.1111/apt.14677]

44 **Behairy BE**, El-Araby HA, El-Guindi MA, Basiouny HM, Fouad OA, Ayoub BA, Marei AM, Sira MM. Safety and Efficacy of 8 Weeks Ledipasvir/Sofosbuvir for Chronic Hepatitis C Genotype 4 in Children Aged 4-10 Years. *J Pediatr* 2020; **219**: 106-110 [PMID: 32008767 DOI: 10.1016/j.jpeds.2019.12.034]

45 **Suda G**, Ogawa K, Morikawa K, Sakamoto N. Treatment of hepatitis C in special populations. *J Gastroenterol* 2018; **53**: 591-605 [PMID: 29299684 DOI: 10.1007/s00535-017-1427-x]

46 **Berenguer M**, Schuppan D. Progression of liver fibrosis in post-transplant hepatitis C: mechanisms, assessment and treatment. *J Hepatol* 2013; **58**: 1028-1041 [PMID: 23262248 DOI: 10.1016/j.jhep.2012.12.014]

47 **Brown RS Jr**, O'Leary JG, Reddy KR, Kuo A, Morelli GJ, Burton JR Jr, Stravitz RT, Durand C, Di Bisceglie AM, Kwo P, Frenette CT, Stewart TG, Nelson DR, Fried MW, Terrault NA; Hepatitis C Therapeutic Registry Research Network Study Group. Interferon-free therapy for genotype 1 hepatitis C in liver transplant recipients: Real-world experience from the hepatitis C therapeutic registry and research network. *Liver Transpl* 2016; **22**: 24-33 [PMID: 26519873 DOI: 10.1002/lt.24366]

48 **Agarwal K**, Castells L, Müllhaupt B, Rosenberg WMC, McNabb B, Arterburn S, Camus G,

McNally J, Stamm LM, Brainard DM, Mani Subramanian G, Mariño Z, Dufour JF, Forns X. Sofosbuvir/velpatasvir for 12 wk in genotype 1-4 HCV-infected liver transplant recipients. *J Hepatol* 2018; **69**: 603-607 [PMID: 29886154 DOI: 10.1016/j.jhep.2018.05.039]

49 **Rupp C**, Hippchen T, Neuberger M, Sauer P, Pfeiffenberger J, Stremmel W, Gotthardt DN, Mehrabi A, Weiss KH. Successful combination of direct antiviral agents in liver-transplanted patients with recurrent hepatitis C virus. *World J Gastroenterol* 2018; **24**: 1353-1360 [PMID: 29599610 DOI: 10.3748/wjg.v24.i12.1353]

50 **El-Hamamsy M**, Montasser IF, Mansy AE, Nabet DE, El-Meteini M. Effect of cyclosporine A *vs* tacrolimus on the response to antiviral therapy after hepatitis C genotype-4 recurrence post-liver transplantation: A prospective cohort trial. *J Clin Pharm Ther* 2019; **44**: 447-453 [PMID: 30714175 DOI: 10.1111/jcpt.12807]

51 **Bixby AL**, Fitzgerald L, Leek R, Mellinger J, Sharma P, Tischer S. Impact of direct-acting antivirals for hepatitis C virus therapy on tacrolimus dosing in liver transplant recipients. *Transpl Infect Dis* 2019; **21**: e13078 [PMID: 30884055 DOI: 10.1111/tid.13078]

52 **Chan C**, Schiano T, Agudelo E, Paul Haydek J, Hoteit M, Laurito MP, Norvell JP, Terrault N, Verna EC, Yang A, Levitsky J. Immune-mediated graft dysfunction in liver transplant recipients with hepatitis C virus treated with direct-acting antiviral therapy. *Am J Transplant* 2018; **18**: 2506-2512 [PMID: 30075485 DOI: 10.1111/ajt.15053]

53 **Thomas P**, Santiago T, Dallas MH. Treatment of hepatitis C in a pediatric patient using simeprevir and sofosbuvir immediately after an umbilical cord blood transplantation. *Bone Marrow Transplant* 2016; **51**: 735-737 [PMID: 26752145 DOI: 10.1038/bmt.2015.309]

54 **Fischler B**, Priftakis P, Sundin M. Sofosbuvir and Simeprevir Treatment of a Stem Cell Transplanted Teenager With Chronic Hepatitis C Infection. *Pediatr Infect Dis J* 2016; **35**: 708-710 [PMID: 26928522 DOI: 10.1097/INF.0000000000001122]

55 **Jaiswal SR**, Bhakuni P, Soni M, Gupta M, Thatai A, Chakrabarti S. Safety and efficacy of Sofosbuvir and Velpatasvir in children with active hepatitis C virus infection undergoing haploidentical transplantation. *Transpl Infect Dis* 2020; e13490 [PMID: 33037697 DOI: 10.1111/tid.13490]

56 **El-Shabrawi MH**, Sherief LM, Yakoot M, Kamal NM, Almalky MA, AbdElgawad MM, Mahfouz AA, Helmy S, Kamal EM, Attia D, El-Khayat HR. Effects of dual sofosbuvir/daclatasvir therapy on, chronic hepatitis C infected, survivors of childhood malignancy. *World J Clin Cases* 2019; **7**: 2247-2255 [PMID: 31531319 DOI: 10.12998/wjcc.v7.i16.2247]

57 **Padhi S**, Maharshi S, Gupta GK, Garg K, Nijhawan S. Efficacy and Safety of Direct Acting Antiviral Therapy for Chronic Hepatitis C in Thalassemic Children. *J Pediatr Hematol Oncol* 2018; **40**: 511-514 [PMID: 29771862 DOI: 10.1097/MPH.0000000000001217]

58 **Makhlouf NA**, Abdelmalek MO, Ibrahim ME, Abu-Faddan NH, Kheila AE, Mahmoud AA. Ledipasvir/Sofosbuvir in Adolescents With Chronic Hepatitis C Genotype 4 With and Without Hematological Disorders: Virological Efficacy and Impact on Liver Stiffness. *J Pediatric Infect Dis Soc* 2021; **10**: 7-13 [PMID: 32060510 DOI: 10.1093/jpids/piaa006]

59 **Tucci F**, Calbi V, Barzaghi F, Migliavacca M, Ferrua F, Bernardo ME, Canarutto D, Consiglieri G, Recupero S, Calzatini F, Gabaldo M, Lucano C, Casiraghi M, Darin S, Dionisio F, Marktel S, Cestone E, Finazzi R, Mieli-Vergani G, Boeri E, Appleby J, Abd Elaziz D, Ciceri F, Aiuti A, Cicalese MP. Successful Treatment With Ledipasvir/Sofosbuvir in an Infant With Severe Combined Immunodeficiency Caused by Adenosine Deaminase Deficiency With HCV Allowed Gene Therapy with Strimvelis. *Hepatology* 2018; **68**: 2434-2437 [PMID: 30014500 DOI: 10.1002/hep.30160]

60 **Naggie S**, Cooper C, Saag M, Workowski K, Ruane P, Towner WJ, Marks K, Luetkemeyer A, Baden RP, Sax PE, Gane E, Santana-Bagur J, Stamm LM, Yang JC, German P, Dvory-Sobol H, Ni L, Pang PS, McHutchison JG, Stedman CA, Morales-Ramirez JO, Bräu N, Jayaweera D, Colson AE, Tebas P, Wong DK, Dieterich D, Sulkowski M; ION-4 Investigators. Ledipasvir and Sofosbuvir for HCV in Patients Coinfected with HIV-1. *N Engl J Med* 2015; **373**: 705-713 [PMID: 26196665 DOI: 10.1056/NEJMoa1501315]

61 **Molina JM**, Orkin C, Iser DM, Zamora FX, Nelson M, Stephan C, Massetto B, Gaggar A, Ni L, Svarovskaia E, Brainard D, Subramanian GM, McHutchison JG, Puoti M, Rockstroh JK; PHOTON-2 study team. Sofosbuvir plus ribavirin for treatment of hepatitis C virus in patients co-infected with HIV (PHOTON-2): a multicentre, open-label, non-randomised, phase 3 study. *Lancet* 2015; **385**: 1098-1106 [PMID: 25659285 DOI: 10.1016/S0140-6736(14)62483-1]

62 **Rockstroh JK**, Nelson M, Katlama C, Lalezari J, Mallolas J, Bloch M, Matthews GV, Saag MS, Zamor PJ, Orkin C, Gress J, Klopfer S, Shaughnessy M, Wahl J, Nguyen BY, Barr E, Platt HL, Robertson MN, Sulkowski M. Efficacy and safety of grazoprevir (MK-5172) and elbasvir (MK-8742) in patients with hepatitis C virus and HIV co-infection (C-EDGE CO-INFECTION): a non-randomised, open-label trial. *Lancet HIV* 2015; **2**: e319-e327 [PMID: 26423374 DOI: 10.1016/S2352-3018(15)00114-9]

63 **Carrasco I**, Sainz T, Frick MA, Jiménez de Ory S, Fortuny C, Burgos J, Montero M, Gavilán C, Falcón MD, Couceiro JA, Bernardino JI, Bisbal O, Guerrero C, Aldámiz-Echevarría MT, Berenguer J, Navarro ML; the Pediatric National AIDS Research Network of Spain (CoRISpe) integrated in the Translational Research Network in Pediatric Infectious Diseases (RITIP). Response to direct-acting antivirals for hepatitis C treatment in vertically HIV/HCV co-infected patients. *J Viral Hepat* 2020; **27**: 955-958 [PMID: 32347645 DOI: 10.1111/jvh.13308]

64 **Roth D**, Nelson DR, Bruchfeld A, Liapakis A, Silva M, Monsour H Jr, Martin P, Pol S, Londoño

MC, Hassanein T, Zamor PJ, Zuckerman E, Wan S, Jackson B, Nguyen BY, Robertson M, Barr E, Wahl J, Greaves W. Grazoprevir plus elbasvir in treatment-naive and treatment-experienced patients with hepatitis C virus genotype 1 infection and stage 4-5 chronic kidney disease (the C-SURFER study): a combination phase 3 study. *Lancet* 2015; **386**: 1537-1545 [PMID: 26456905 DOI: 10.1016/S0140-6736(15)00349-9]

65 **Fernández-Ruiz M**, Polanco N, García-Santiago A, Muñoz R, Hernández AM, González E, Mercado VR, Fernández I, Aguado JM, Praga M, Andrés A. Impact of anti-HCV direct antiviral agents on graft function and immunosuppressive drug levels in kidney transplant recipients: a call to attention in the mid-term follow-up in a single-center cohort study. *Transpl Int* 2018; **31**: 887-899 [PMID: 29356211 DOI: 10.1111/tri.13118]

66 **González-Corvillo C**, Beneyto I, Sánchez-Fructuoso A, Perelló M, Alonso A, Mazuecos A, Jiménez C, Zárraga S, Paul J, Lauzurica R, Hernández D, Guirado L, Franco A, Ruiz JC, Llorente S, Crespo M, Rodríguez-Benot A, de Gracia Guindo MDC, Díaz-Corte C, Gentil MÁ. Direct-acting Antivirals for the Treatment of Kidney Transplant Patients With Chronic Hepatitis C Virus Infection in Spain: A Long-term Prospective Observational Study. *Transplant Direct* 2019; **5**: e510 [PMID: 32095505 DOI: 10.1097/TXD.0000000000000954]

67 **Gupta S**, Gupta R, Joshi YK, Singh S. Role of horizontal transmission in hepatitis B virus spread among household contacts in north India. *Intervirology* 2008; **51**: 7-13 [PMID: 18309243 DOI: 10.1159/000118790]

68 **Indolfi G**, Easterbrook P, Dusheiko G, Siberry G, Chang MH, Thorne C, Bulterys M, Chan PL, El-Sayed MH, Giaquinto C, Jonas MM, Meyers T, Walsh N, Wirth S, Penazzato M. Hepatitis B virus infection in children and adolescents. *Lancet Gastroenterol Hepatol* 2019; **4**: 466-476 [PMID: 30982722 DOI: 10.1016/S2468-1253(19)30042-1]

69 **World Health Organization**. Global hepatitis report, 2017. [cited 2 March 2021]. In: World Health Organization [Internet]. Available from: https://www.who.int/publications-detail-redirect/global-hepatitis-report-2017

70 **McMahon BJ**, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP, Maynard JE. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. *J Infect Dis* 1985; **151**: 599-603 [PMID: 3973412 DOI: 10.1093/infdis/151.4.599]

71 **Stevens CE**, Beasley RP, Tsui J, Lee WC. Vertical transmission of hepatitis B antigen in Taiwan. *N Engl J Med* 1975; **292**: 771-774 [PMID: 1113797 DOI: 10.1056/NEJM197504102921503]

72 **European Association for the Study of the Liver**. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]

73 **Bortolotti F**, Guido M, Bartolacci S, Cadrobbi P, Crivellaro C, Noventa F, Morsica G, Moriondo M, Gatta A. Chronic hepatitis B in children after e antigen seroclearance: final report of a 29-year longitudinal study. *Hepatology* 2006; **43**: 556-562 [PMID: 16496323 DOI: 10.1002/hep.21077]

74 **Wen WH**, Chang MH, Hsu HY, Ni YH, Chen HL. The development of hepatocellular carcinoma among prospectively followed children with chronic hepatitis B virus infection. *J Pediatr* 2004; **144**: 397-399 [PMID: 15001956 DOI: 10.1016/j.jpeds.2003.11.022]

75 **Marx G**, Martin SR, Chicoine JF, Alvarez F. Long-term follow-up of chronic hepatitis B virus infection in children of different ethnic origins. *J Infect Dis* 2002; **186**: 295-301 [PMID: 12134225 DOI: 10.1086/341508]

76 **Wu JF**, Su YR, Chen CH, Chen HL, Ni YH, Hsu HY, Wang JL, Chang MH. Predictive effect of serial serum alanine aminotransferase levels on spontaneous HBeAg seroconversion in chronic genotype B and C HBV-infected children. *J Pediatr Gastroenterol Nutr* 2012; **54**: 97-100 [PMID: 21716132 DOI: 10.1097/MPG.0b013e31822a033e]

77 **Ni YH**, Chang MH, Wang KJ, Hsu HY, Chen HL, Kao JH, Yeh SH, Jeng YM, Tsai KS, Chen DS. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology* 2004; **127**: 1733-1738 [PMID: 15578511 DOI: 10.1053/j.gastro.2004.09.048]

78 **Roushan MR**, Bijani A, Ramzaninejad S, Roushan MH, Amiri MJ, Baiani M. HBeAg seroconversion in children infected during early childhood with hepatitis B virus. *J Clin Virol* 2012; **55**: 30-33 [PMID: 22727680 DOI: 10.1016/j.jcv.2012.05.007]

79 **Tseng YR**, Wu JF, Ni YH, Chen HL, Chen CC, Wen WH, Hsu HY, Chang MH. Long-term effect of maternal HBeAg on delayed HBeAg seroconversion in offspring with chronic hepatitis B infection. *Liver Int* 2011; **31**: 1373-1380 [PMID: 21745315 DOI: 10.1111/j.1478-3231.2011.02574.x]

80 **Wu JF**, Chiu YC, Chang KC, Chen HL, Ni YH, Hsu HY, Chang MH. Predictors of hepatitis B e antigen-negative hepatitis in chronic hepatitis B virus-infected patients from childhood to adulthood. *Hepatology* 2016; **63**: 74-82 [PMID: 26389515 DOI: 10.1002/hep.28222]

81 **Nicastro E**, Mangili B, Giacomet V, Benincaso AR, Di Giorgio A, Sansotta N, Callegaro A, D'Antiga L. Longitudinal Immune Phenotype Assessment and Serological Outcome in Foreign-born Children With Chronic Hepatitis B. *J Pediatr Gastroenterol Nutr* 2020; **71**: 381-387 [PMID: 32826716 DOI: 10.1097/MPG.0000000000002804]

82 **Schwarz KB**, Lombardero M, Di Bisceglie AM, Murray KF, Rosenthal P, Ling SC, Cloonan YK, Rodriguez-Baez N, Schwarzenberg SJ, Hoofnagle JH, Teckman J; Hepatitis B Research Network (HBRN). Phenotypes of Chronic Hepatitis B in Children From a Large North American Cohort. *J Pediatr Gastroenterol Nutr* 2019; **69**: 588-594 [PMID: 31436702 DOI:

10.1097/MPG.0000000000002467]
83 **Di Bisceglie AM**, Lombardero M, Teckman J, Roberts L, Janssen HL, Belle SH, Hoofnagle JH; Hepatitis B Research Network (HBRN). Determination of hepatitis B phenotype using biochemical and serological markers. *J Viral Hepat* 2017; **24**: 320-329 [PMID: 27917600 DOI: 10.1111/jvh.12643]
84 **Lee MH**, Yang HI, Liu J, Batrla-Utermann R, Jen CL, Iloeje UH, Lu SN, You SL, Wang LY, Chen CJ; R. E.V.E.A.L.-HBV Study Group. Prediction models of long-term cirrhosis and hepatocellular carcinoma risk in chronic hepatitis B patients: risk scores integrating host and virus profiles. *Hepatology* 2013; **58**: 546-554 [PMID: 23504622 DOI: 10.1002/hep.26385]
85 **Arnone OC**, Serranti D, Bartolini E, Mastrangelo G, Stinco M, Trapani S, Ricci S, Resti M, Indolfi G. Chronic hepatitis B in children, report of a single-centre longitudinal study on 152 children. *J Viral Hepat* 2020; **27**: 1344-1351 [PMID: 32853482 DOI: 10.1111/jvh.13382]
86 **Sokal EM**, Paganelli M, Wirth S, Socha P, Vajro P, Lacaille F, Kelly D, Mieli-Vergani G; European Society of Pediatric Gastroenterology, Hepatology and Nutrition. Management of chronic hepatitis B in childhood: ESPGHAN clinical practice guidelines: consensus of an expert panel on behalf of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition. *J Hepatol* 2013; **59**: 814-829 [PMID: 23707367 DOI: 10.1016/j.jhep.2013.05.016]
87 **Chu CM**, Hung SJ, Lin J, Tai DI, Liaw YF. Natural history of hepatitis B e antigen to antibody seroconversion in patients with normal serum aminotransferase levels. *Am J Med* 2004; **116**: 829-834 [PMID: 15178498 DOI: 10.1016/j.amjmed.2003.12.040]
88 **Wong GL**, Wong VW, Choi PC, Chan AW, Chim AM, Yiu KK, Chan HY, Chan FK, Sung JJ, Chan HL. Clinical factors associated with liver stiffness in hepatitis B e antigen-positive chronic hepatitis B patients. *Clin Gastroenterol Hepatol* 2009; **7**: 227-233 [PMID: 19121647 DOI: 10.1016/j.cgh.2008.10.023]
89 **Chen YC**, Chu CM, Liaw YF. Age-specific prognosis following spontaneous hepatitis B e antigen seroconversion in chronic hepatitis B. *Hepatology* 2010; **51**: 435-444 [PMID: 19918971 DOI: 10.1002/hep.23348]
90 **Chen CF**, Lee WC, Yang HI, Chang HC, Jen CL, Iloeje UH, Su J, Hsiao CK, Wang LY, You SL, Lu SN, Chen CJ; Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer in HBV (REVEAL–HBV) Study Group. Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. *Gastroenterology* 2011; **141**: 1240-1248, 1248.e1-1248. e2 [PMID: 21703214 DOI: 10.1053/j.gastro.2011.06.036]
91 **D'Antiga L**, Aw M, Atkins M, Moorat A, Vergani D, Mieli-Vergani G. Combined lamivudine/interferon-alpha treatment in "immunotolerant" children perinatally infected with hepatitis B: a pilot study. *J Pediatr* 2006; **148**: 228-233 [PMID: 16492434 DOI: 10.1016/j.jpeds.2005.09.020]
92 **Poddar U**, Yachha SK, Agarwal J, Krishnani N. Cure for immune-tolerant hepatitis B in children: is it an achievable target with sequential combo therapy with lamivudine and interferon? *J Viral Hepat* 2013; **20**: 311-316 [PMID: 23565612 DOI: 10.1111/jvh.12007]
93 **Zhu S**, Zhang H, Dong Y, Wang L, Xu Z, Liu W, Gan Y, Tang H, Chen D, Wang F, Zhao P. Antiviral therapy in hepatitis B virus-infected children with immune-tolerant characteristics: A pilot open-label randomized study. *J Hepatol* 2018; **68**: 1123-1128 [PMID: 29452204 DOI: 10.1016/j.jhep.2018.01.037]
94 **Rosenthal P**, Ling SC, Belle SH, Murray KF, Rodriguez-Baez N, Schwarzenberg SJ, Teckman J, Lin HS, Schwarz KB; Hepatitis B Research Network (HBRN). Combination of Entecavir/Peginterferon Alfa-2a in Children With Hepatitis B e Antigen-Positive Immune Tolerant Chronic Hepatitis B Virus Infection. *Hepatology* 2019; **69**: 2326-2337 [PMID: 30318613 DOI: 10.1002/hep.30312]
95 **Wong GL**. Management of chronic hepatitis B patients in immunetolerant phase: what latest guidelines recommend. *Clin Mol Hepatol* 2018; **24**: 108-113 [PMID: 29353469 DOI: 10.3350/cmh.2017.0068]
96 **Terrault NA**, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM, Brown RS Jr, Bzowej NH, Wong JB. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. *Hepatology* 2018; **67**: 1560-1599 [PMID: 29405329 DOI: 10.1002/hep.29800]
97 **Sokal EM**, Conjeevaram HS, Roberts EA, Alvarez F, Bern EM, Goyens P, Rosenthal P, Lachaux A, Shelton M, Sarles J, Hoofnagle J. Interferon alfa therapy for chronic hepatitis B in children: a multinational randomized controlled trial. *Gastroenterology* 1998; **114**: 988-995 [PMID: 9558288 DOI: 10.1016/s0016-5085(98)70318-x]
98 **Jonas MM**, Mizerski J, Badia IB, Areias JA, Schwarz KB, Little NR, Greensmith MJ, Gardner SD, Bell MS, Sokal EM; International Pediatric Lamivudine Investigator Group. Clinical trial of lamivudine in children with chronic hepatitis B. *N Engl J Med* 2002; **346**: 1706-1713 [PMID: 12037150 DOI: 10.1056/NEJMoa012452]
99 **Jonas MM**, Kelly D, Pollack H, Mizerski J, Sorbel J, Frederick D, Mondou E, Rousseau F, Sokal E. Safety, efficacy, and pharmacokinetics of adefovir dipivoxil in children and adolescents (age 2 to <18 years) with chronic hepatitis B. *Hepatology* 2008; **47**: 1863-1871 [PMID: 18433023 DOI: 10.1002/hep.22250]
100 **Murray KF**, Szenborn L, Wysocki J, Rossi S, Corsa AC, Dinh P, McHutchison J, Pang PS, Luminos LM, Pawlowska M, Mizerski J. Randomized, placebo-controlled trial of tenofovir disoproxil fumarate in adolescents with chronic hepatitis B. *Hepatology* 2012; **56**: 2018-2026

[PMID: 22544804 DOI: 10.1002/hep.25818]

101 **Jonas MM**, Chang MH, Sokal E, Schwarz KB, Kelly D, Kim KM, Ling SC, Rosenthal P, Oraseanu D, Reynolds L, Thiry A, Ackerman P. Randomized, controlled trial of entecavir *vs* placebo in children with hepatitis B envelope antigen-positive chronic hepatitis B. *Hepatology* 2016; **63**: 377-387 [PMID: 26223345 DOI: 10.1002/hep.28015]

102 **Wirth S**, Zhang H, Hardikar W, Schwarz KB, Sokal E, Yang W, Fan H, Morozov V, Mao Q, Deng H, Huang Y, Yang L, Frey N, Nasmyth-Miller C, Pavlovic V, Wat C. Efficacy and Safety of Peginterferon Alfa-2a (40KD) in Children With Chronic Hepatitis B: The PEG-B-ACTIVE Study. *Hepatology* 2018; **68**: 1681-1694 [PMID: 29689122 DOI: 10.1002/hep.30050]

103 **Smolders EJ**, Burger DM, Feld JJ, Kiser JJ. Review article: clinical pharmacology of current and investigational hepatitis B virus therapies. *Aliment Pharmacol Ther* 2020; **51**: 231-243 [PMID: 31840863 DOI: 10.1111/apt.15581]

104 **Hepatitis B Foundation**. Drug Watch. [cited 2 March 2021]. In: Hepatitis B Foundation [Internet]. Available from: https://www.hepb.org/treatment-and-management/drug-watch/

105 **Zoulim F**, Lenz O, Vandenbossche JJ, Talloen W, Verbinnen T, Moscalu I, Streinu-Cercel A, Bourgeois S, Buti M, Crespo J, Manuel Pascasio J, Sarrazin C, Vanwolleghem T, Shukla U, Fry J, Yogaratnam JZ. JNJ-56136379, an HBV Capsid Assembly Modulator, Is Well-Tolerated and Has Antiviral Activity in a Phase 1 Study of Patients With Chronic Infection. *Gastroenterology* 2020; **159**: 521-533. e9 [PMID: 32343960 DOI: 10.1053/j.gastro.2020.04.036]

106 **Yuen MF**, Agarwal K, Gane EJ, Schwabe C, Ahn SH, Kim DJ, Lim YS, Cheng W, Sievert W, Visvanathan K, Ruby E, Liaw S, Yan R, Huang Q, Colonno R, Lopatin U. Safety, pharmacokinetics, and antiviral effects of ABI-H0731, a hepatitis B virus core inhibitor: a randomised, placebo-controlled phase 1 trial. *Lancet Gastroenterol Hepatol* 2020; **5**: 152-166 [PMID: 31711752 DOI: 10.1016/S2468-1253(19)30346-2]

107 **Ma X**, Lalezari J, Nguyen T, Bae H, Schiff ER, Fung S, Yuen M-F, Hassanein T, Hann H-W, Elkhashab M, Dieterich D, Sulkowski PK, Nahass R, Agarwal K, Ramji A, Park J, Ravendhran N, Chan S, Weilert F, Han S-H, Ayoub W, Gane EJ, Jacobson I, Bennett M, Huang Q, Yan R, Huey V, Ruby E, Liaw S, Colonno R, Lopatin U. Interim safety and efficacy results of the ABI-H0731 phase 2a program exploring the combination of ABI-H0731 with Nuc therapy in treatment-naive and treatment-suppressed chronic hepatitis B patients. [cited 2 March 2021]. Available from: https://program.manage.com/ProgramSearch/DownloadAbstractOfPresentation/ilc2019?id=417422

108 **Al-Mahtab M**, Bazinet M, Vaillant A. Safety and Efficacy of Nucleic Acid Polymers in Monotherapy and Combined with Immunotherapy in Treatment-Naive Bangladeshi Patients with HBeAg+ Chronic Hepatitis B Infection. *PLoS One* 2016; **11**: e0156667 [PMID: 27257978 DOI: 10.1371/journal.pone.0156667]

109 **Bazinet M**, Pântea V, Cebotarescu V, Cojuhari L, Jimbei P, Albrecht J, Schmid P, Le Gal F, Gordien E, Krawczyk A, Mijočević H, Karimzadeh H, Roggendorf M, Vaillant A. Safety and efficacy of REP 2139 and pegylated interferon alfa-2a for treatment-naive patients with chronic hepatitis B virus and hepatitis D virus coinfection (REP 301 and REP 301-LTF): a non-randomised, open-label, phase 2 trial. *Lancet Gastroenterol Hepatol* 2017; **2**: 877-889 [PMID: 28964701 DOI: 10.1016/S2468-1253(17)30288-1]

110 **Bazinet M**, Pântea V, Placinta G, Moscalu I, Cebotarescu V, Cojuhari L, Jimbei P, Iarovoi L, Smesnoi V, Musteata T, Jucov A, Dittmer U, Krawczyk A, Vaillant A. Safety and Efficacy of 48 Weeks REP 2139 or REP 2165, Tenofovir Disoproxil, and Pegylated Interferon Alfa-2a in Patients With Chronic HBV Infection Naïve to Nucleos(t)ide Therapy. *Gastroenterology* 2020; **158**: 2180-2194 [PMID: 32147484 DOI: 10.1053/j.gastro.2020.02.058]

111 **Yuen MF**, Locarnini S, Huey Lim T, Strasser S, Sievert W, Cheng W, Thompson A, Given B, Schluep T, Hamilton J, Cloherty G, Wong D, Schwabe C, Jackson K, Ferrari C, Lai C-L, Gish R, Gane E. Short term RNA interference therapy in chronic hepatitis B using JNJ-3989 brings majority of patients to HBsAg < 100 IU/mL threshold. [cited 2 March 2021]. Available from: https://program.m-anage.com/ilc2019/en-GB/ProgramSearch/DownloadAbstract/151378

112 **Gane E**, Locarnini S, Huey Lim T, Strasser S, Sievert W, Cheng W, Thompson A, Given B, Schluep T, Hamilton J, Li Z, Cloherty G, Wong D, Schwabe C, Jackson K, Ferrari C, Lai C-L, Gish R, Yuen MF. First results with RNA interference (RNAi) in chronic hepatitis B (CHB) using ARO-HBV. The Liver Meeting; 2018 November; San Francisco, USA

113 **VIR Biotechnology**. VIR-2218 Demonstrates Dose-Dependent and Durable Reductions of Hepatitis B Surface Antigen in Phase 1/2 Trial. [cited 2 March 2021]. In: VIR Biotechnology [Internet]. Available from: https://investors.vir.bio/news-releases/news-release-details/vir-2218-demonstrates-dose-dependent-and-durable-reductions/

114 **Yuen MF**, Chen CY, Liu CJ, Jeng RWJ, Elkhashab M, Coffin C, Kim W, Greenbloom S, Ramji A, Lim YS, Kim YJ, Fung S, Kim DJ, Jang JW, Lee KS, Afdhal N, Radhakrishnan I, Macfarlane C, Jackson K, Locarnini S, Chan H. Ascending dose cohort study of inarigivir - A novel RIG I agonist in chronic HBV patients: Final results of the ACHIEVE trial. *J Hepatol* 2019; **70**: e47-E48 [DOI: 10.1016/S0618-8278(19)30084-2]

115 **Reyes M**, Lutz JD, Lau AH, Gaggar A, Grant EP, Joshi A, Mackman RL, Ling J, Tan SK, Ayithan N, Daffis S, Woo J, Wu P, Lam T, Fletcher SP, Kottilil S, Poonia B, Gane EJ, Mathias A, German P. Safety, pharmacokinetics and pharmacodynamics of selgantolimod, an oral Toll-like receptor 8 agonist: a Phase Ia study in healthy subjects. *Antivir Ther* 2020; **25**: 171-180 [PMID: 32667286 DOI: 10.3851/IMP3363]

116 **Gane E**, Zhao Y, Tan SK, Lau AH, Gaggar A, Subramanian M, Wallin J, Brooks AE, Dubar PR, Kottilil S, Tang L. Efficacy and Safety of Oral TLR8 Agonist Selgantolimod in Virally Suppressed Adult Patients With Chronic Hepatitis B: a Phase 2, Randomized, Double-Blind, Placebo-Controlled, Multicenter Study. *Hepatology* 2019

117 **European Association for The Study of The Liver**. Bulevirtide shows promise in the treatment of chronic hepatitis B/D (HBV/HDV) coinfection. [cited 2 March 2021]. In: European Association for The Study of The Liver [Internet]. Available from: https://2019.ilc-congress.eu/press-release/bulevirtide-shows-promise-in-the-treatment-of-chronic-hepatitis-b-d-hbv-hdv-coinfection/

118 **Bogomolov P**, Alexandrov A, Voronkova N, Macievich M, Kokina K, Petrachenkova M, Lehr T, Lempp FA, Wedemeyer H, Haag M, Schwab M, Haefeli WE, Blank A, Urban S. Treatment of chronic hepatitis D with the entry inhibitor myrcludex B: First results of a phase Ib/IIa study. *J Hepatol* 2016; **65**: 490-498 [PMID: 27132170 DOI: 10.1016/j.jhep.2016.04.016]

119 **Indolfi G**, Abdel-Hady M, Bansal S, Debray D, Smets F, Czubkowski P, van der Woerd W, Samyn M, Jahnel J, Gupte G, Zellos A, Mozer-Glassberg Y, Verkade HJ, Sokal E, Fischler B. Management of Hepatitis B Virus Infection and Prevention of Hepatitis B Virus Reactivation in Children With Acquired Immunodeficiencies or Undergoing Immune Suppressive, Cytotoxic, or Biological Modifier Therapies. *J Pediatr Gastroenterol Nutr* 2020; **70**: 527-538 [PMID: 31977956 DOI: 10.1097/MPG.0000000000002628]

120 **Sarin SK**, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS, Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int* 2016; **10**: 1-98 [PMID: 26563120 DOI: 10.1007/s12072-015-9675-4]

121 **deBruyn JCC**, Soon IS, Fonseca K, Feng S, Purtzki M, Goedhart C, Kuhn S, Vanderkooi OG, Wrobel I. Serologic Status of Routine Childhood Vaccines, Cytomegalovirus, and Epstein-Barr Virus in Children With Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2019; **25**: 1218-1226 [PMID: 30551205 DOI: 10.1093/ibd/izy366]

122 **Aljaberi N**, Ghulam E, Smitherman EA, Favier L, Dykes DMH, Danziger-Isakov LA, Brady RC, Huggins J. Maintaining Hepatitis B Protection in Immunocompromised Pediatric Rheumatology and Inflammatory Bowel Disease Patients. *J Rheumatol* 2020 [PMID: 32739895 DOI: 10.3899/jrheum.200283]

123 **Reddy KR**, Beavers KL, Hammond SP, Lim JK, Falck-Ytter YT; American Gastroenterological Association Institute. American Gastroenterological Association Institute guideline on the prevention and treatment of hepatitis B virus reactivation during immunosuppressive drug therapy. *Gastroenterology* 2015; **148**: 215-9; quiz e16 [PMID: 25447850 DOI: 10.1053/j.gastro.2014.10.039]

124 **Loomba R**, Liang TJ. Hepatitis B Reactivation Associated With Immune Suppressive and Biological Modifier Therapies: Current Concepts, Management Strategies, and Future Directions. *Gastroenterology* 2017; **152**: 1297-1309 [PMID: 28219691 DOI: 10.1053/j.gastro.2017.02.009]

125 **Zhang MY**, Zhu GQ, Shi KQ, Zheng JN, Cheng Z, Zou ZL, Huang HH, Chen FY, Zheng MH. Systematic review with network meta-analysis: Comparative efficacy of oral nucleos(t)ide analogues for the prevention of chemotherapy-induced hepatitis B virus reactivation. *Oncotarget* 2016; **7**: 30642-30658 [PMID: 27121321 DOI: 10.18632/oncotarget.8907]

126 **Fung J**, Wong T, Chok K, Chan A, Cheung TT, Dai JW, Sin SL, Ma KW, Ng K, Ng KT, Seto WK, Lai CL, Yuen MF, Lo CM. Long-term outcomes of entecavir monotherapy for chronic hepatitis B after liver transplantation: Results up to 8 years. *Hepatology* 2017; **66**: 1036-1044 [PMID: 28370215 DOI: 10.1002/hep.29191]

127 **Huprikar S**, Danziger-Isakov L, Ahn J, Naugler S, Blumberg E, Avery RK, Koval C, Lease ED, Pillai A, Doucette KE, Levitsky J, Morris MI, Lu K, McDermott JK, Mone T, Orlowski JP, Dadhania DM, Abbott K, Horslen S, Laskin BL, Mougdil A, Venkat VL, Korenblat K, Kumar V, Grossi P, Bloom RD, Brown K, Kotton CN, Kumar D. Solid organ transplantation from hepatitis B virus-positive donors: consensus guidelines for recipient management. *Am J Transplant* 2015; **15**: 1162-1172 [PMID: 25707744 DOI: 10.1111/ajt.13187]

128 **Hadziyannis SJ**, Papatheodoridis GV. Hepatitis B e antigen-negative chronic hepatitis B: natural history and treatment. *Semin Liver Dis* 2006; **26**: 130-141 [PMID: 16673291 DOI: 10.1055/s-2006-939751]

129 **Brunetto MR**, Oliveri F, Colombatto P, Coco B, Ciccorossi P, Bonino F. Treatment of HBeAg-negative chronic hepatitis B with interferon or pegylated interferon. *J Hepatol* 2003; **39** Suppl 1: S164-S167 [PMID: 14708697 DOI: 10.1016/s0168-8278(03)00329-5]

130 **Brunetto MR**, Oliveri F, Rocca G, Criscuolo D, Chiaberge E, Capalbo M, David E, Verme G, Bonino F. Natural course and response to interferon of chronic hepatitis B accompanied by antibody to hepatitis B e antigen. *Hepatology* 1989; **10**: 198-202 [PMID: 2663695 DOI: 10.1002/hep.1840100213]

131 **Hsu YS**, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002; **35**: 1522-1527 [PMID: 12029639 DOI: 10.1053/jhep.2002.33638]

132 **Ogunbosi B**, Smuts H, Eley B, Korsman S, De Lacy R, Hardie DR. Fulminant hepatitis B virus (HBV) infection in an infant following mother-to-child transmission of an e-minus HBV mutant: Time to relook at HBV prophylaxis in South African infants. *S Afr Med J* 2018; **108**: 389-392

[PMID: 29843851 DOI: 10.7196/SAMJ.2018.v108i5.13017]

133 **Lampertico P**, Viganò M, Di Costanzo GG, Sagnelli E, Fasano M, Di Marco V, Boninsegna S, Farci P, Fargion S, Giuberti T, Iannacone C, Regep L, Massetto B, Facchetti F, Colombo M; PegBeLiver Study Group. Randomised study comparing 48 and 96 wk peginterferon α-2a therapy in genotype D HBeAg-negative chronic hepatitis B. *Gut* 2013; **62**: 290-298 [PMID: 22859496 DOI: 10.1136/gutjnl-2011-301430]

134 **Leung DH**, Wirth S, Yao BB, Viani RM, Gonzalez-Peralta RP, Jonas MM, Lobritto SJ, Narkewicz MR, Sokal E, Fortuny C, Hsu EK, Del Valle-Segarra A, Zha J, Larsen L, Liu L, Shuster DL, Cohen DE, Rosenthal P. Ombitasvir/Paritaprevir/Ritonavir With or Without Dasabuvir and With or Without Ribavirin for Adolescents With HCV Genotype 1 or 4. *Hepatol Commun* 2018; **2**: 1311-1319 [PMID: 30411078 DOI: 10.1002/hep4.1250]

135 **Rosenthal P**, Narkewicz MR, Yao BB, Jolley CD, Lobritto SJ, Wen J, Molleston JP, Hsu EK, Jonas MM, Zha J, Liu L, Leung DH. Ombitasvir, Paritaprevir, Ritonavir, and Dasabuvir Mini-Tabs Plus Ribavirin for Children Aged 3-11 Years with Hepatitis C Genotype 1a. *Adv Ther* 2020; **37**: 3299-3310 [PMID: 32451952 DOI: 10.1007/s12325-020-01389-9]

136 **Wirth S**, Gonzalez-Peralta RP, Squires RH, Mutschler F, Lang T, Pawlowska M, Sluzewski W, Majda-Stanislawska E, Fischler B, Balistreri WF, Jonas MM, Blondet N, Rosenthal P, Alkhouri N, Romero R, Grandhi A, Castronuovo P, Caro L, Rosenblum D, Du L, Haber BH. Elbasvir/grazoprevir in children aged 3 to <18 years with chronic hepatitis c virus (hcv) genotype (gt) 1 or 4 infection: final results from an iterative pharmacokinetic (pk) modeling study. *Hepatology* 2020; 555-556

137 **Bansal S**, Pawlowska M, Nebbia G, Di Dato F, Sluzewski W, Giacomet V, Szenborn L, Shao J, Yue MS, Hsueh C-H, Parhy B, Gaggar A, Kersey K, Mangia A, Kelly DA, Indolfi G. Safety and efficacy of sofosbuvir/velpatasvir/voxilaprevir in adolescents with chronic hepatitis c virus infection. *Hepatology* 2020; 571

138 **El-Sayed MH**, Hassany M, Asem N. A pilot study for safety and efficacy of 12 wk sofosbuvir plus daclatasvir with or without ribavirin in Egyptian adolescents with chronic hepatitis C virus Infection. *J Hepatology* 2017; 178

139 **El-Shabrawi MH**, Abdo AM, El-Khayat HR, Yakoot M. Shortened 8 Weeks Course of Dual Sofosbuvir/Daclatasvir Therapy in Adolescent Patients, With Chronic Hepatitis C Infection. *J Pediatr Gastroenterol Nutr* 2018; **66**: 425-427 [PMID: 29135824 DOI: 10.1097/MPG.0000000000001838]

140 **Yakoot M**, El-Shabrawi MH, AbdElgawad MM, Mahfouz AA, Helmy S, Abdo AM, El-Khayat HR. Dual Sofosbuvir/Daclatasvir Therapy in Adolescent Patients With Chronic Hepatitis C Infection. *J Pediatr Gastroenterol Nutr* 2018; **67**: 86-89 [PMID: 29570560 DOI: 10.1097/MPG.0000000000001968]

141 **Abdel Ghaffar TY**, El Naghi S, Abdel Gawad M, Helmy S, Abdel Ghaffar A, Yousef M, Moafy M. Safety and efficacy of combined sofosbuvir/daclatasvir treatment of children and adolescents with chronic hepatitis C Genotype 4. *J Viral Hepat* 2019; **26**: 263-270 [PMID: 30380158 DOI: 10.1111/jvh.13032]

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MINIREVIEWS

Pancreatitis after endoscopic retrograde cholangiopancreatography: A narrative review

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Abstract

Acute post-endoscopic retrograde cholangiopancreatography pancreatitis (PEP) is a feared and potentially fatal complication that can be as high as up to 30% in high-risk patients. Pre-examination measures, during the examination and after the examination are the key to technical and clinical success with a decrease in adverse events. Several studies have debated on the subject, however, numerous topics remain controversial, such as the effectiveness of prophylactic medications and the amylase dosage time. This review was designed to provide an update on the current scientific evidence regarding PEP available in the literature.

Key Words: Endoscopic retrograde cholangiopancreatography; Pan-creatitis; Post-endoscopic retrograde cholangiopancreatography pancreatitis; Adverse events; Pancreatitis; Prevention

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Core Tip: Acute post-endoscopic retrograde cholangiopancreatography pancreatitis (PEP) is a feared and potentially fatal complication. Early diagnosis remains the key to the clinical success of these patients. Unfortunately, several topics remain controversial, especially early diagnosis with hyperamylasemia still being mistaken for PEP.

drafted the article, and final approval.

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The purpose of this review is to demonstrate the evidence in the current literature on PEP.

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INTRODUCTION

Starting in 1968, endoscopic retrograde cholangiopancreatography (ERCP) was a watershed in the diagnosis and treatment of biliopancreatic diseases. Since then, an accurate indication for this examination is very important given the potential adverse effects associated with the procedure[1].

Early recognition and proper management of potential adverse events are essential to reduce associated morbidity and mortality.

As in other endoscopic procedures, there are safety determinants for ERCP, in addition to the precise indication, the clinical condition of the patient, age, sex, the type of sedation used, what type of therapeutic procedure performed, the appropriate use of accessories and the training of the endoscopist and assistants are taken into consideration[2].

Acute pancreatitis is the most common serious complication after ERCP[3,4], often confused with an increase in serum amylase concentration that occurs in up to 75% of patients[5,6].

Acute clinical pancreatitis itself, defined as a clinical syndrome of abdominal pain and hyperamylasemia which requires hospitalization, is much less common than it appears. There are still some controversies in the literature on the subject. The purpose of this review is to provide an update on post-ERCP pancreatitis and its prevention.

PATHOGENESIS

The determinants of the inflammatory process in the pancreas are multifactorial. Several proposed factors can act independently or in combination to induce post-ERCP pancreatitis (PEP). The two most important are mechanical injury due to instrumentation in the pancreatic duct and hydrostatic injury due to contrast injection[7].

During ERCP and sphincterotomy, the pancreas is exposed to various forms of trauma: mechanical, chemical, hydrostatic, thermal, and even allergic[8].

It is also known that prolonged manipulation around the papillary orifice, inadvertent cannulation of the pancreatic duct and multiple injections into the pancreatic duct are common when selective cannulation of the bile duct is difficult[9,10]. This can result in mechanical damage to the duct or ampoule. Thermal injury to the electrocautery current can also produce edema of the pancreatic orifice, leading to obstruction of the duct, impairing the emptying of pancreatic secretions[11].

Hydrostatic injury due to excessive injection of contrast into the pancreatic duct is probably an important cause of PEP[12].

Either by allergy or chemical injury, contrast agents can lead to injuries. In a study by George *et al*[13], there was no statistically significant difference between the types of contrast in the analysis of randomized studies.

EPIDEMIOLOGY AND RISK FACTORS

Incidence

The incidence of pancreatitis post-ERCP can vary from 1% to 10%, reaching an alarming 30% in high-risk patients[14,15]. Stratification of the degree of post-examination pancreatitis shows incidence rates of 3.6% to 4% for mild acute pancre-

atitis, 1.8% to 2.8% for moderate acute pancreatitis, and 0.3% to 0.5% for severe acute pancreatitis[16,17] with a mortality rate of 0.2%[18]. Higher rates are observed in patients undergoing evaluation for possible sphincter of Oddi dysfunction[19].

Risk factors

According to the guidelines of the European Society for Gastrointestinal Endoscopy (ESGE)[20] and the American Society for Gastrointestinal Endoscopy (ASGE)[2]: History of pancreatitis, suspected sphincter of Oddi dysfunction, female gender, and young age are definitely "patient-related risk factors" for PEP. On the other hand, difficult cannulation, pancreatic injection, and pre-cut sphincterotomy are "risk factors related to the procedure[3,4].

Patient-related factors

There are several factors related to the patient, the most common factors are female gender, normal levels of bilirubin, young adults, history of recurrent pancreatitis, and patients with suspected sphincter of Oddi dysfunction. Patients with a history of chronic pancreatitis have a protective effect against PEP[2].

Unfortunately, risk factors are additive[7,21,22]. For example, the combination of female gender, patients with suspected sphincter of Oddi dysfunction, young age, difficult cannulation, bilirubin within the acceptable standard, and absence of bile duct stones are associated with a risk of the pancreatitis of more than 40%.

Operator-related factors

These are the most subjective factors. It is believed that the experience of the endoscopist, the presence of fellows and multiple operators is an independent risk factor for PEP[23,24].

Procedure-related factors

The factors related to the procedure are the best studied and discussed in the literature. Pre-cut sphincterotomy, often used in difficult ERCP, time and number of cannulation times, trauma, and edema of the major duodenal papillae due to the number of attempts are independent factors for PEP[25].

In a systematic review with a meta-analysis that included 25 randomized controlled trials (RCTs) evaluating the incidence of PEP in patients undergoing sphincterotomy, ballooning dilation of the major duodenal papilla without sphincterotomy and patients undergoing both procedures, it was concluded that the incidence of PEP was similar between the groups[26].

The risk factors can be divided into three groups and are shown in Table 1[7,27-29].

CLINICAL MANIFESTATIONS

The clinical manifestations of PEP are the same as those seen in patients with acute pancreatitis due to other causes.

These include epigastric or upper right quadrant pain, abdominal tenderness and high levels of amylase and lipase.

Post-ERCP acute pancreatitis can be classified as mild, moderate or severe based on the American Gastroenterology Association[30] and the American College of Gastroenterology[31]: (1) mild-amylase levels 24 h after the examination, remaining above up to three times the reference value with necessary hospitalization; (2) moderate-need for hospitalization of 4 to 10 d; and (3) severe-need for hospitalization over 10 d or need for invasive therapeutic intervention.

DIAGNOSIS

Most patients with PEP have an acute onset of severe and persistent epigastric abdominal pain and in approximately 50% of patients, the pain radiates to the back. Approximately 90% of patients experience nausea and vomiting that can persist for several hours[32].

Patients with severe acute pancreatitis may have dyspnea due to diaphragmatic inflammation secondary to pancreatitis, pleural effusions, or acute respiratory distress syndrome, and 5% to 10% of patients with severe acute pancreatitis may have painless disease and unexplained hypotension[33].

Table 1 Risk factors for post-endoscopic retrograde cholangiopancreatography pancreatitis related to the operator and the procedure

Operator related	Factors related to the procedure
Inadequate training	Extended procedure time (≥ 30 min)
Lack of experience	Difficult cannulation (≥ 15 min)
Patient-related risk factors	Injection of contrast into the pancreatic duct
Young age	Sphincter of Oddi manometry
Women	Pancreatic sphincterotomy
Normal serum bilirubin	Small papillary sphincterotomy
Recurrent pancreatitis	Biliary balloon sphincteroplasty
Previous ERCP-induced pancreatitis	Endoscopic papillectomy with loop
Sphincter of Oddi dysfunction	Pancreatic intraductal ultrasound
	Precut sphincterotomy

ERCP: Endoscopic retrograde cholangiopancreatography.

For diagnostic confirmation, radiological evidence with computed tomography may be necessary[34] but biochemical tests are more commonly used, as they are inexpensive and sensitive[35].

Early diagnosis of PEP is crucial as late diagnosis can be fatal[36,37].

Pancreatic enzymes

The diagnosis of PEP can be complicated, since elevations in pancreatic enzymes are common after the examination, but are generally not associated with clinical pancreatitis.

There is no consensus in the literature on the ideal time after examination to request serum amylase levels and their real meaning. Two prospective studies including 263 and 886 patients found that the 4-h post-ERCP amylase level proved useful in predicting PEP[38,39]. We suggest that the patient should fast for the next 12 h and amylase analysis should be requested for all patients.

In patients with suspected pancreatitis, the degree and speed of elevations in pancreatic enzymes may be a way of differentiating patients with PEP from those in pain due to other causes. Some studies state that patients with PEP often have serum amylase levels more than five times the upper limit of normal[40,41].

Patients undergoing a contrast study of the main pancreatic duct should be admitted if the 4-h amylase level is greater than 2.5 times the upper reference limit. Patients who have not undergone a contrast study should be admitted if the 4-h amylase level is greater than five times the upper limit of normal[38]. The 4-h post-ERCP amylase level was useful in predicting PEP in two prospective studies including 263 and 886 patients, respectively[38,39].

DIFFERENTIAL DIAGNOSIS

Not all patients with pain after ERCP have pancreatitis. Other causes of abdominal pain after ERCP include discomfort due to air insufflation[42-44] and perforation.

In patients with discomfort due to air insufflation, the pain is generally not as severe as that seen with PEP, and pancreatic enzyme levels may be normal or elevated, as pancreatic enzymes are elevated in most patients after ERCP[5].

If serum lipase is less than three times the upper limit of normal, pancreatitis is unlikely (specificity of 85 to 98%). However, it should be borne in mind that amylase and lipase start to increase several hours after the onset of pancreatitis; thus, blood tests taken soon after ERCP can show false negative results.

If the clinical suspicion of pancreatitis is high, tests should be repeated at least 4-6 h after ERCP. Perforated patients may experience diffuse abdominal pain, bloating, tachycardia, fever, and leukocytosis.

Symptoms can be immediate after the examination or hours later[45]. Many of the perforation symptoms overlap with those of acute pancreatitis and, if perforation is

suspected, an abdominal tomography should be performed immediately for intraperitoneal and retroperitoneal evaluation[46].

TREATMENT

Most of the patients who develop PEP requiring hospitalization are classified as mild. In severe cases, admission to an intensive care unit may be necessary[30-31]. Initial treatment should focus on the following:

Pain control

This usually manifests as abdominal pain and must be one of the main pillars in the treatment, since its non-control can lead to hemodynamic instability. There is still a lot of controversy in the use of opioids such as morphine as it has been shown to increase pressure in the sphincter of Oddi, but without clinical data that this has resulted in worsening of pancreatitis. Indicated: Meperidine, fentanyl, and morphine[47].

Particular attention should be given to patients who are dehydrated or who have not received an adequate amount of fluids since hypovolemia and hemoconcentration can cause ischemic pain and increased lactic acidosis.

Fluid replacement

Fluid replacement is one of the main items in the treatment of patients with PEP. The use of crystalloid solutions, mainly Ringer Lactate, from 5 to 10 mL/kg/h is recommended in patients without restrictions. In critically ill patients, with hemodynamic instability, 20 mL/kg is recommended in 30 min followed by 3 mL/kg/h in the next 8 to 12 h[48,49].

Monitoring

As these patients' condition may worsen in the next 24 h, it is recommended that they be monitored for at least 48 h. This surveillance includes vital signs, urine volume, electrolytes, and blood glucose[48].

Antibiotics

Prophylactic antibiotics are not recommended in patients with PEP regardless of the type or severity of the disease. Antibiotics should only be used in about 20% of patients who develop extrapancreatic infections[48,50].

Nutrition

Fasting is recommended for all patients with PEP. The time for restarting oral feeding is dependent on the severity of pancreatitis[51].

PREVENTION

Certain measures can reduce the incidence of PEP[7]: (1) adequate training and experience of endoscopists and assistants; (2) use of wire-guided techniques for biliary cannulation; (3) minimizing the number of cannulation attempts; (4) placement of a prophylactic pancreatic stent in patients at high risk of developing PEP; (5) placement of prophylactic pancreatic stents in patients who require the assistance of a pancreatic guidewire for biliary cannulation (double guidewire technique); (6) selective cannulation of the bile duct if an assessment of the pancreatic duct is not necessary; (7) minimizing the volume of contrast medium injected into the pancreatic duct, if necessary; (8) careful use of the electrocautery current during sphincterotomy; (9) high-risk patients should undergo ERCP in specialized centers; and (10) use of carbon dioxide for luminal insufflation to decrease post-procedure abdominal pain that can be mistaken for pancreatitis.

Effectiveness of preventive measures

Endoscopic techniques: The endoscopic technique is an important factor in the development of PEP. Cannulation guided by a hydrophilic-coated wire, careful use of electrocautery during sphincterotomy, and placement of a prophylactic pancreatic stent should be undertaken in patients at high risk of developing PEP.

Cannulation techniques: Various instruments such as guidewires are available and can decrease the risk of PEP as suggested by the ASGE and ESGE[52-55].

A systematic review that included only randomized trials, evaluating a total of 3450 patients, demonstrated that cannulation guided by a guidewire was superior to contrast-assisted cannulation technique[56]. Cannulation rates were higher for the wire-guided technique, and the risk of PEP was halved.

In a multicenter RCT, including 274 patients with naïve papilla undergoing ERCP using wire-guided cannulation in whom the guidewire was inadvertently inserted into the main pancreatic duct, the patients were randomized to undergo the double guidewire technique or a new cannulation attempt with a single wire. Conversion to the double guidewire technique did not facilitate selective bile duct cannulation and did not decrease the incidence of PEP compared to the new single guidewire cannulation attempt. However, double guidewire cannulation was more effective in patients with malignant biliary stenosis[57].

Electrocautery: In a recent systematic review evaluating 11 randomized studies involving 1791 patients, it was found that the performance of sphincterotomy with electrocautery in pure cut mode leads to a higher incidence of mild bleeding compared to endocut and blend. However, this modality may have a lower incidence of pancreatitis. Monopolar mode causes higher rates of pancreatitis compared to bipolar mode[11].

Pancreatic stent: Pancreatic stent placement can be performed as prophylaxis for PEP mainly in high-risk patients. We suggest the use in patients undergoing pancreatic sphincterotomy, a contrasting study of the main pancreatic duct when it is necessary to use the double guidewire technique, in patients with suspected sphincter of Oddi dysfunction, and in patients undergoing pre-cut sphincterotomy[7].

The possible benefit is believed to be related to a reduction in pancreatic intraductal pressure of papillary edema.

Studies have shown that in special situations, the passage of a pancreatic stent in the DPP may be necessary to prevent the evolution of pancreatitis after ERCP. This procedure must be performed 8 to 20 h after the start of PEP[58-60].

Pancreatic stents should be short (less than 5 cm and small in diameter (5 French), plastic, and not have flanges distally[7]. Non-flanged stents can lead to spontaneous migration to the gastrointestinal tract, which occurs in 95% of cases within 10 d[55]. If radiographs show evidence of persistent stent within 1 wk, a high endoscopy should be performed to remove the stent[55].

Intravenous hydration: ASGE guidelines suggest the use of periprocedural intravenous hydration with lactated Ringer to decrease the risk of PEP[2].

In a RCT of 150 patients, the PEP rate was lower in patients who received aggressive intravenous hydration compared to standard therapy[61].

In patients with contraindications to rectal non-steroidal anti-inflammatory drugs (NSAIDs), who are not at risk of fluid overload and a pancreatic stent has not been placed, the suggested alternative is aggressive hydration with lactated Ringer's solution (3 mL/kg/h during ERCP, 20 mL/kg bolus after ERCP and 3 mL/kg/h for 8 h after the examination)[25].

Chemoprevention

Since 1977, more than 35 different drugs have been evaluated for the prevention of PEP with variable results[62,63]. The available options are discussed below:

NSAIDs

Rectal NSAIDs: The ASGE and the ESGE recommend the administration of NSAIDs to reduce the incidence and severity of PEP (for example, 100 mg of indomethacin or diclofenac rectally immediately before or after ERCP)[2,20].

A systematic review with meta-analysis evaluating 21 RCTs with a total of 6854 patients, found that the rectal administration of NSAIDs in all patients adequately reduced the incidence of PEP and that mild pancreatitis was the only preventable result. In this context, both diclofenac and indomethacin are considered effective[64].

Rectal NSAIDs were also compared indirectly with stenting of the pancreatic duct. A meta-analysis showed that rectal NSAIDs were superior to pancreatic duct stenting for the prevention of PEP (OR 0.48, 95%CI: 0.26-0.87)[65].

Non-rectal NSAIDs: There are no data in the current literature to support the prophylactic use of any NSAIDs administered by any non-rectal route or in combination with

other agents.

In a multicenter study with 430 patients, oral diclofenac (50 mg) before and after ERCP showed no benefit compared to placebo[66].

Other agents in the prevention of PEP: There are several drugs potentially useful for the prevention of PEP although some drugs are difficult to access and few are used for this purpose.

Topical adrenaline

A systematic review with meta-analysis evaluating 6 randomized and 2 observational studies including 4123 patients found that topical adrenaline does not provide any additional advantage in combination with rectal indomethacin in the prevention of PEP in patients who underwent ERCP. However, topical adrenaline alone is associated with a lower risk of PEP compared to placebo and can be considered if rectal indomethacin is not available or if the patient has any contraindications to its use[67].

Nitrates

In a systematic review with 2000 patients, the use of nitroglycerin was compared to placebo and it was found that the intervention group demonstrated a 10% reduction in the development of PEP[68].

These data suggest that nitrates combined with rectal NSAIDs may provide more benefits than NSAIDs alone[69,70]. In a randomized trial including 886 patients undergoing ERCP, the risk of PEP was lower in patients treated with diclofenac suppositories and sublingual isosorbide dinitrate compared to patients receiving diclofenac suppositories alone (RR: 0.59, 95%CI: 0.37-0.95)[70].

PANCREATIC SECRETION INHIBITORS

Somatostatin

Somatostatin leads to a reduction in pancreatic exocrine secretion of basal origin and also when stimulated. A meta-analysis that included 9 studies concluded that somatostatin was ineffective in preventing PEP when administered in the short-term (< 6 h) or long-term (≥ 12 h)[71]. Another meta-analysis, which included 11 RCTs with a total of 2869 patients, found no benefit when somatostatin was administered as a short-term infusion, but showed a benefit when administered as a single bolus or as a long-term infusion[72].

Octreotide

Two systematic reviews with meta-analyses found no benefit of octreotide use in PEP prophylaxis[73,74].

INHIBITORS OF PROTEASE ACTIVATION

The most studied protease inhibitors include gabexate mesylate, nafamostat mesylate, and ulinastatin. As the activation of proteolytic enzymes can contribute to PEP, protease inhibitors have been investigated in the prevention of PEP. In a meta-analysis of 18 studies involving 4966 patients, there was a small benefit with the use of protease inhibitors[75].

Gabexate mesylate

Although controversial results have been observed, a meta-analysis of five studies concluded that gabexate mesylate was ineffective in reducing pancreatitis and post-ERCP pain[71].

Nafamostat mesylate

Although controversial results have been observed, a meta-analysis that included 7 RCTs with 2956 patients found that the incidence of PEP was reduced by 53% compared to patients in the control groups (RR: 0.47, 95%CI: 0.34-0.63)[76].

Another meta-analysis which included 26 studies, found that unlike gabexate mesylate and ulinastatin, nafamostat mesylate and NSAIDs were associated with decreased risk of PEP[77].

Ulinastatin

A systematic review with a meta-analysis that included 7 RCTs comparing ulinastatin with placebo or gabexate demonstrated a decreased risk of PEP in patients receiving ulinastatin[77].

MONITORING CARE AFTER ERCP

Many complications of ERCP are apparent during the first 6 h after the procedure, and others may take days to manifest. We suggest the following recommendations: (1) Serum amylase: Studies have shown that the 4-h serum amylase level is a useful measure in predicting PEP; (2) Clinical monitoring: The immediate post-examination period is critical and the patient must be monitored for signs and symptoms of adverse events; and (3) Diet: We recommend fasting the patient for 6 to 12 h after the examination and discharge only after the serum amylase results and clinical reassessment (patient without complaints of abdominal pain, for example).

CONCLUSION

Pancreatitis after ERCP is a feared, potentially fatal, and not entirely preventable complication. The correct and early diagnosis is a turning point in the outcome of the disease. Pre-examination measures such as a correct indication for the procedure, use of rectal NSAIDs, and well-trained staff are necessary. During the examination: Hyperhydration, examination with precision and speed with the correct technique and appropriate material, and prophylactic use of a pancreatic stent. After the examination, maintaining fasting and the appropriate amylase dosage are essential for the clinical and technical success of the procedure.

REFERENCES

1 **Thiruvengadam NR**, Kochman ML. Emerging Therapies to Prevent Post-ERCP Pancreatitis. *Curr Gastroenterol Rep* 2020; **22**: 59 [PMID: 33188441 DOI: 10.1007/s11894-020-00796-w]

2 **ASGE Standards of Practice Committee**, Chandrasekhara V, Khashab MA, Muthusamy VR, Acosta RD, Agrawal D, Bruining DH, Eloubeidi MA, Fanelli RD, Faulx AL, Gurudu SR, Kothari S, Lightdale JR, Qumseya BJ, Shaukat A, Wang A, Wani SB, Yang J, DeWitt JM. Adverse events associated with ERCP. *Gastrointest Endosc* 2017; **85**: 32-47 [PMID: 27546389 DOI: 10.1016/j.gie.2016.06.051]

3 **Feurer ME**, Adler DG. Post-ERCP pancreatitis: review of current preventive strategies. *Curr Opin Gastroenterol* 2012; **28**: 280-286 [PMID: 22450899 DOI: 10.1097/MOG.0b013e3283528e68]

4 **ASGE Standards of Practice Committee**, Anderson MA, Fisher L, Jain R, Evans JA, Appalaneni V, Ben-Menachem T, Cash BD, Decker GA, Early DS, Fanelli RD, Fisher DA, Fukami N, Hwang JH, Ikenberry SO, Jue TL, Khan KM, Krinsky ML, Malpas PM, Maple JT, Sharaf RN, Shergill AK, Dominitz JA. Complications of ERCP. *Gastrointest Endosc* 2012; **75**: 467-473 [PMID: 22341094 DOI: 10.1016/j.gie.2011.07.010]

5 **Pieper-Bigelow C**, Strocchi A, Levitt MD. Where does serum amylase come from and where does it go? *Gastroenterol Clin North Am* 1990; **19**: 793-810 [PMID: 1702756]

6 **Hormati A**, Alemi F, Mohammadbeigi A, Sarkeshikian SS, Saeidi M. Prevalence of Endoscopic Retrograde Cholangiopancreatography Complications and Amylase Sensitivity for Predicting Pancreatitis in ERCP Patients. *Gastroenterol Nurs* 2020; **43**: 350-354 [PMID: 32889967 DOI: 10.1097/SGA.0000000000000473]

7 **Boškoski I**, Costamagna G. How to Prevent Post-Endoscopic Retrograde Cholangiopancreatography Pancreatitis. *Gastroenterology* 2020; **158**: 2037-2040 [PMID: 32197979 DOI: 10.1053/j.gastro.2020.03.019]

8 **Funatsu E**, Masuda A, Takenaka M, Nakagawa T, Shiomi H, Yoshinaka H, Kobayashi T, Sakai A, Yagi Y, Yoshida M, Arisaka Y, Okabe Y, Kutsumi H, Azuma T. History of Post-Endoscopic Retrograde Cholangiopancreatography Pancreatitis and Acute Pancreatitis as Risk Factors for Post-ERCP Pancreatitis. *Kobe J Med Sci* 2017; **63**: E1-E8 [PMID: 29434167]

9 **Johnson GK**, Geenen JE, Johanson JF, Sherman S, Hogan WJ, Cass O. Evaluation of post-ERCP pancreatitis: potential causes noted during controlled study of differing contrast media. Midwest Pancreaticobiliary Study Group. *Gastrointest Endosc* 1997; **46**: 217-222 [PMID: 9378207 DOI: 10.1016/S0016-5107(97)70089-0]

10 **Proença IM**, dos Santos MEL, de Moura DTH, Ribeiro IB, Matuguma SE, Cheng S, McCarty TR, do Monte Junior ES, Sakai P, de Moura EGH. Role of pancreatography in the endoscopic management of encapsulated pancreatic collections – review and new proposed classification. *World J*

Gastroenterol 2020; 26: 7104-7117 [PMID: 33362371 DOI: 10.3748/wjg.v26.i45.7104]

11 **Funari MP**, Ribeiro IB, de Moura DTH, Bernardo WM, Brunaldi VO, Rezende DT, Resende RH, de Marco MO, Franzini TAP, de Moura EGH. Adverse events after biliary sphincterotomy: Does the electric current mode make a difference? *Clin Res Hepatol Gastroenterol* 2020; **44**: 739-752 [PMID: 32088149 DOI: 10.1016/j.clinre.2019.12.009]

12 **Sherman S**, Hawes RH, Troiano FP, Lehman GA. Pancreatitis following bile duct sphincter of Oddi manometry: utility of the aspirating catheter. *Gastrointest Endosc* 1992; **38**: 347-350 [PMID: 1376705 DOI: 10.1016/S0016-5107(92)70430-1]

13 **George S**, Kulkarni AA, Stevens G, Forsmark CE, Draganov P. Role of osmolality of contrast media in the development of post-ERCP pancreatitis: a metanalysis. *Dig Dis Sci* 2004; **49**: 503-508 [PMID: 15139506 DOI: 10.1023/B:DDAS.0000020511.98230.20]

14 **Zhao ZH**, Hu LH, Ren HB, Zhao AJ, Qian YY, Sun XT, Su S, Zhu SG, Yu J, Zou WB, Guo XR, Wang L, Li ZS, Liao Z. Incidence and risk factors for post-ERCP pancreatitis in chronic pancreatitis. *Gastrointest Endosc* 2017; **86**: 519-524. e1 [PMID: 28062312 DOI: 10.1016/j.gie.2016.12.020]

15 **Sun HL**, Han B, Zhai HP, Cheng XH, Ma K. Rectal NSAIDs for the prevention of post-ERCP pancreatitis: a meta-analysis of randomized controlled trials. *Surgeon* 2014; **12**: 141-147 [PMID: 24332479 DOI: 10.1016/j.surge.2013.10.010]

16 **Vandervoort J**, Soetikno RM, Tham TC, Wong RC, Ferrari AP Jr, Montes H, Roston AD, Slivka A, Lichtenstein DR, Ruymann FW, Van Dam J, Hughes M, Carr-Locke DL. Risk factors for complications after performance of ERCP. *Gastrointest Endosc* 2002; **56**: 652-656 [PMID: 12397271 DOI: 10.1067/mge.2002.129086]

17 **Ferreira Ade F**, Bartelega JA, Urbano HC, de Souza IK. Acute pancreatitis gravity predictive factors: which and when to use them? *Arq Bras Cir Dig* 2015; **28**: 207-211 [PMID: 26537149 DOI: 10.1590/S0102-67202015000300016]

18 **Kochar B**, Akshintala VS, Afghani E, Elmunzer BJ, Kim KJ, Lennon AM, Khashab MA, Kalloo AN, Singh VK. Incidence, severity, and mortality of post-ERCP pancreatitis: a systematic review by using randomized, controlled trials. *Gastrointest Endosc* 2015; **81**: 143-149. e9 [PMID: 25088919 DOI: 10.1016/j.gie.2014.06.045]

19 **Yaghoobi M**, Pauls Q, Durkalski V, Romagnuolo J, Fogel EL, Tarnasky PR, Aliperti G, Freeman ML, Kozarek RA, Jamidar PA, Wilcox CM, Elta GH, Hawes RH, Wood-Williams A, Cotton PB. Incidence and predictors of post-ERCP pancreatitis in patients with suspected sphincter of Oddi dysfunction undergoing biliary or dual sphincterotomy: results from the EPISOD prospective multicenter randomized sham-controlled study. *Endoscopy* 2015; **47**: 884-890 [PMID: 26165739 DOI: 10.1055/s-0034-1392418]

20 **Dumonceau JM**, Andriulli A, Elmunzer BJ, Mariani A, Meister T, Deviere J, Marek T, Baron TH, Hassan C, Testoni PA, Kapral C; European Society of Gastrointestinal Endoscopy. Prophylaxis of post-ERCP pancreatitis: European Society of Gastrointestinal Endoscopy (ESGE) Guideline - updated June 2014. *Endoscopy* 2014; **46**: 799-815 [PMID: 25148137 DOI: 10.1055/s-0034-1377875]

21 **Freeman ML**, Nelson DB, Sherman S, Haber GB, Herman ME, Dorsher PJ, Moore JP, Fennerty MB, Ryan ME, Shaw MJ, Lande JD, Pheley AM. Complications of endoscopic biliary sphincterotomy. *N Engl J Med* 1996; **335**: 909-918 [PMID: 8782497 DOI: 10.1056/NEJM199609263351301]

22 **Freeman ML**, DiSario JA, Nelson DB, Fennerty MB, Lee JG, Bjorkman DJ, Overby CS, Aas J, Ryan ME, Bochna GS, Shaw MJ, Snady HW, Erickson RV, Moore JP, Roel JP. Risk factors for post-ERCP pancreatitis: a prospective, multicenter study. *Gastrointest Endosc* 2001; **54**: 425-434 [PMID: 11577302 DOI: 10.1067/mge.2001.117550]

23 **Pan Y**, Zhao L, Leung J, Zhang R, Luo H, Wang X, Liu Z, Wan B, Tao Q, Yao S, Hui N, Fan D, Wu K, Guo X. Appropriate time for selective biliary cannulation by trainees during ERCP--a randomized trial. *Endoscopy* 2015; **47**: 688-695 [PMID: 25750038 DOI: 10.1055/s-0034-1391564]

24 **Morales SJ**, Sampath K, Gardner TB. A Review of Prevention of Post-ERCP Pancreatitis. *Gastroenterol Hepatol (N Y)* 2018; **14**: 286-292 [PMID: 29991936]

25 **Dumonceau JM**, Kapral C, Aabakken L, Papanikolaou IS, Tringali A, Vanbiervliet G, Beyna T, Dinis-Ribeiro M, Hritz I, Mariani A, Paspatis G, Radaelli F, Lakhtakia S, Veitch AM, van Hooft JE. ERCP-related adverse events: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. *Endoscopy* 2020; **52**: 127-149 [PMID: 31863440 DOI: 10.1055/a-1075-4080]

26 **Matsubayashi CO**, Ribeiro IB, de Moura DTH, Brunaldi VO, Bernardo WM, Hathorn KE, de Moura EGH. Is Endoscopic Balloon Dilation Still Associated With Higher Rates of Pancreatitis? *Pancreas* 2020; **49**: 158-174 [PMID: 32049951 DOI: 10.1097/MPA.0000000000001489]

27 **Badalov N**, Tenner S, Baillie J. The Prevention, recognition and treatment of post-ERCP pancreatitis. *JOP* 2009; **10**: 88-97 [PMID: 19287099]

28 **Testoni PA**, Mariani A, Giussani A, Vailati C, Masci E, Macarri G, Ghezzo L, Familiari L, Giardullo N, Mutignani M, Lombardi G, Talamini G, Spadaccini A, Briglia R, Piazzi L; SEIFRED Group. Risk factors for post-ERCP pancreatitis in high- and low-volume centers and among expert and non-expert operators: a prospective multicenter study. *Am J Gastroenterol* 2010; **105**: 1753-1761 [PMID: 20372116 DOI: 10.1038/ajg.2010.136]

29 **Mariani A**, Giussani A, Di Leo M, Testoni S, Testoni PA. Guidewire biliary cannulation does not reduce post-ERCP pancreatitis compared with the contrast injection technique in low-risk and high-risk patients. *Gastrointest Endosc* 2012; **75**: 339-346 [PMID: 22075192 DOI: 10.1016/j.gie.2011.09.002]

30 **Crockett SD**, Wani S, Gardner TB, Falck-Ytter Y, Barkun AN; American Gastroenterological

Association Institute Clinical Guidelines Committee. American Gastroenterological Association Institute Guideline on Initial Management of Acute Pancreatitis. *Gastroenterology* 2018; **154**: 1096-1101 [PMID: 29409760 DOI: 10.1053/j.gastro.2018.01.032]

31 **Tenner S**, Baillie J, DeWitt J, Vege SS; American College of Gastroenterology. American College of Gastroenterology guideline: management of acute pancreatitis. *Am J Gastroenterol* 2013; **108**: 1400-15; 1416 [PMID: 23896955 DOI: 10.1038/ajg.2013.218]

32 **Swaroop VS**, Chari ST, Clain JE. Severe acute pancreatitis. *JAMA* 2004; **291**: 2865-2868 [PMID: 15199038 DOI: 10.1001/jama.291.23.2865]

33 **Lee PJ**, Papachristou GI. Management of Severe Acute Pancreatitis. *Curr Treat Options Gastroenterol* 2020; 1-12 [PMID: 33230385 DOI: 10.1007/s11938-020-00322-x]

34 **Manoharan D**, Srivastava DN, Gupta AK, Madhusudhan KS. Complications of endoscopic retrograde cholangiopancreatography: an imaging review. *Abdom Radiol (NY)* 2019; **44**: 2205-2216 [PMID: 30809695 DOI: 10.1007/s00261-019-01953-0]

35 **Boxhoorn L**, Voermans RP, Bouwense SA, Bruno MJ, Verdonk RC, Boermeester MA, van Santvoort HC, Besselink MG. Acute pancreatitis. *Lancet* 2020; **396**: 726-734 [PMID: 32891214 DOI: 10.1016/S0140-6736(20)31310-6]

36 **Freeman ML**, Nelson DB, Sherman S, Haber GB, Fennerty MB, DiSario JA, Ryan ME, Kortan PP, Dorsher PJ, Shaw MJ, Herman ME, Cunningham JT, Moore JP, Silverman WB, Imperial JC, Mackie RD, Jamidar PA, Yakshe PN, Logan GM, Pheley AM. Same-day discharge after endoscopic biliary sphincterotomy: observations from a prospective multicenter complication study. The Multicenter Endoscopic Sphincterotomy (MESH) Study Group. *Gastrointest Endosc* 1999; **49**: 580-586 [PMID: 10228255 DOI: 10.1016/S0016-5107(99)70385-8]

37 **Ho KY**, Montes H, Sossenheimer MJ, Tham TC, Ruymann F, Van Dam J, Carr-Locke DL. Features that may predict hospital admission following outpatient therapeutic ERCP. *Gastrointest Endosc* 1999; **49**: 587-592 [PMID: 10228256 DOI: 10.1016/S0016-5107(99)70386-X]

38 **Sutton VR**, Hong MK, Thomas PR. Using the 4-hour Post-ERCP amylase level to predict post-ERCP pancreatitis. *JOP* 2011; **12**: 372-376 [PMID: 21737899]

39 **Thomas PR**, Sengupta S. Prediction of pancreatitis following endoscopic retrograde cholangiopancreatography by the 4-h post procedure amylase level. *J Gastroenterol Hepatol* 2001; **16**: 923-926 [PMID: 11555108 DOI: 10.1046/j.1440-1746.2001.02547.x]

40 **Testoni PA**, Bagnolo F, Caporuscio S, Lella F. Serum amylase measured four hours after endoscopic sphincterotomy is a reliable predictor of postprocedure pancreatitis. *Am J Gastroenterol* 1999; **94**: 1235-1241 [PMID: 10235200 DOI: 10.1111/j.1572-0241.1999.01072.x]

41 **Testoni PA**, Bagnolo F. Pain at 24 hours associated with amylase levels greater than 5 times the upper normal limit as the most reliable indicator of post-ERCP pancreatitis. *Gastrointest Endosc* 2001; **53**: 33-39 [PMID: 11154486 DOI: 10.1067/mge.2001.111390]

42 **Shi H**, Chen S, Swar G, Wang Y, Ying M. Carbon dioxide insufflation during endoscopic retrograde cholangiopancreatography: a review and meta-analysis. *Pancreas* 2013; **42**: 1093-1100 [PMID: 23867366 DOI: 10.1097/MPA.0b013e3182909da5]

43 **Bretthauer M**, Seip B, Aasen S, Kordal M, Hoff G, Aabakken L. Carbon dioxide insufflation for more comfortable endoscopic retrograde cholangiopancreatography: a randomized, controlled, double-blind trial. *Endoscopy* 2007; **39**: 58-64 [PMID: 17252462 DOI: 10.1055/s-2006-945036]

44 **Passos ML**, Ribeiro IB, de Moura DTH, Korkischko N, Silva GLR, Franzini TP, Bernando WM, de Moura EGH. Efficacy and safety of carbon dioxide insufflation versus air insufflation during endoscopic retrograde cholangiopancreatography in randomized controlled trials: a systematic review and meta-analysis. *Endosc Int Open* 2019; **7**: E487-E497 [PMID: 31041365 DOI: 10.1055/a-0854-3739]

45 **Olaiya B**, Adler DG. Intestinal perforations after endoscopic retrograde cholangiopancreatography in the United States: a 16-year study using the national inpatient sample. *Minerva Gastroenterol Dietol* 2020 [PMID: 32492997 DOI: 10.23736/S1121-421X.20.02718-X]

46 **Borazan E**, Konduk BT. Comparison of early and delayed diagnosis of mortality in ERCP perforations: A high-volume patient experience. *Ulus Travma Acil Cerrahi Derg* 2020; **26**: 746-753 [PMID: 32946098 DOI: 10.14744/tjtes.2020.61289]

47 **Basurto Ona X**, Rigau Comas D, Urrútia G. Opioids for acute pancreatitis pain. *Cochrane Database Syst Rev* 2013; CD009179 [PMID: 23888429 DOI: 10.1002/14651858.CD009179.pub2]

48 **Mederos MA**, Reber HA, Girgis MD. Acute Pancreatitis: A Review. *JAMA* 2021; **325**: 382-390 [PMID: 33496779 DOI: 10.1001/jama.2020.20317]

49 **Messallam AA**, Body CB, Berger S, Sakaria SS, Chawla S. Impact of early aggressive fluid resuscitation in acute pancreatitis. *Pancreatology* 2021; **21**: 69-73 [PMID: 33257225 DOI: 10.1016/j.pan.2020.11.006]

50 **Zhang J**, Zhu S, Tan D, Ma A, Yang Y, Xu J. A meta-analysis of early oral refeeding and quickly increased diet for patients with mild acute pancreatitis. *Saudi J Gastroenterol* 2019; **25**: 14-19 [PMID: 30226482 DOI: 10.4103/sjg.SJG_240_18]

51 **Lella F**, Bagnolo F, Colombo E, Bonassi U. A simple way of avoiding post-ERCP pancreatitis. *Gastrointest Endosc* 2004; **59**: 830-834 [PMID: 15173796 DOI: 10.1016/S0016-5107(04)00363-3]

52 **Artifon EL**, Sakai P, Cunha JE, Halwan B, Ishioka S, Kumar A. Guidewire cannulation reduces risk of post-ERCP pancreatitis and facilitates bile duct cannulation. *Am J Gastroenterol* 2007; **102**: 2147-2153 [PMID: 17581267 DOI: 10.1111/j.1572-0241.2007.01378.x]

53 **Bailey AA**, Bourke MJ, Williams SJ, Walsh PR, Murray MA, Lee EY, Kwan V, Lynch PM. A

prospective randomized trial of cannulation technique in ERCP: effects on technical success and post-ERCP pancreatitis. *Endoscopy* 2008; **40**: 296-301 [PMID: 18389448 DOI: 10.1055/s-2007-995566]

54 **Testoni PA**, Mariani A, Aabakken L, Arvanitakis M, Bories E, Costamagna G, Devière J, Dinis-Ribeiro M, Dumonceau JM, Giovannini M, Gyokeres T, Hafner M, Halttunen J, Hassan C, Lopes L, Papanikolaou IS, Tham TC, Tringali A, van Hooft J, Williams EJ. Papillary cannulation and sphincterotomy techniques at ERCP: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline. *Endoscopy* 2016; **48**: 657-683 [PMID: 27299638 DOI: 10.1055/s-0042-108641]

55 **Tse F**, Yuan Y, Moayyedi P, Leontiadis GI. Guide wire-assisted cannulation for the prevention of post-ERCP pancreatitis: a systematic review and meta-analysis. *Endoscopy* 2013; **45**: 605-618 [PMID: 23807804 DOI: 10.1055/s-0032-1326640]

56 **Sasahira N**, Kawakami H, Isayama H, Uchino R, Nakai Y, Ito Y, Matsubara S, Ishiwatari H, Uebayashi M, Yagioka H, Togawa O, Toda N, Sakamoto N, Kato J, Koike K. Early use of double-guidewire technique to facilitate selective bile duct cannulation: the multicenter randomized controlled EDUCATION trial. *Endoscopy* 2015; **47**: 421-429 [PMID: 25590186 DOI: 10.1055/s-0034-1391228]

57 **Madácsy L**, Kurucsai G, Joó I, Gódi S, Fejes R, Székely A. Rescue ERCP and insertion of a small-caliber pancreatic stent to prevent the evolution of severe post-ERCP pancreatitis: a case-controlled series. *Surg Endosc* 2009; **23**: 1887-1893 [PMID: 19057957 DOI: 10.1007/s00464-008-0199-z]

58 **Kerdsirichairat T**, Attam R, Arain M, Bakman Y, Radosevich D, Freeman M. Urgent ERCP with pancreatic stent placement or replacement for salvage of post-ERCP pancreatitis. *Endoscopy* 2014; **46**: 1085-1094 [PMID: 25216326 DOI: 10.1055/s-0034-1377750]

59 **Karjula H**, Nordblad Schmidt P, Mäkelä J, Liisanantti JH, Ohtonen P, Saarela A. Prophylactic pancreatic duct stenting in severe acute necrotizing pancreatitis: a prospective randomized study. *Endoscopy* 2019; **51**: 1027-1034 [PMID: 30895583 DOI: 10.1055/a-0865-1960]

60 **Shaygan-Nejad A**, Masjedizadeh AR, Ghavidel A, Ghojazadeh M, Khoshbaten M. Aggressive hydration with Lactated Ringer's solution as the prophylactic intervention for postendoscopic retrograde cholangiopancreatography pancreatitis: A randomized controlled double-blind clinical trial. *J Res Med Sci* 2015; **20**: 838-843 [PMID: 26759569 DOI: 10.4103/1735-1995.170597]

61 **Vadalà di Prampero SF**, Faleschini G, Panic N, Bulajic M. Endoscopic and pharmacological treatment for prophylaxis against postendoscopic retrograde cholangiopancreatography pancreatitis: a meta-analysis and systematic review. *Eur J Gastroenterol Hepatol* 2016; **28**: 1415-1424 [PMID: 27580214 DOI: 10.1097/MEG.0000000000000734]

62 **Kubiliun NM**, Adams MA, Akshintala VS, Conte ML, Cote GA, Cotton PB, Dumonceau JM, Elta GH, Fogel EL, Freeman ML, Lehman GA, Naveed M, Romagnuolo J, Scheiman JM, Sherman S, Singh VK, Elmunzer BJ; United States Cooperative for Outcomes Research in Endoscopy (USCORE). Evaluation of Pharmacologic Prevention of Pancreatitis After Endoscopic Retrograde Cholangiopancreatography: A Systematic Review. *Clin Gastroenterol Hepatol* 2015; **13**: 1231-9; quiz e70 [PMID: 25579870 DOI: 10.1016/j.cgh.2014.11.038]

63 **Serrano JPR**, de Moura DTH, Bernardo WM, Ribeiro IB, Franzini TP, de Moura ETH, Brunaldi VO, Salesse MT, Sakai P, De Moura EGH. Nonsteroidal anti-inflammatory drugs vs placebo for post-endoscopic retrograde cholangiopancreatography pancreatitis: a systematic review and meta-analysis. *Endosc Int Open* 2019; **07**: E477-E486 [PMID: 30957004 DOI: 10.1055/a-0862-0215]

64 **Akbar A**, Abu Dayyeh BK, Baron TH, Wang Z, Altayar O, Murad MH. Rectal nonsteroidal anti-inflammatory drugs are superior to pancreatic duct stents in preventing pancreatitis after endoscopic retrograde cholangiopancreatography: a network meta-analysis. *Clin Gastroenterol Hepatol* 2013; **11**: 778-783 [PMID: 23376320 DOI: 10.1016/j.cgh.2012.12.043]

65 **Ishiwatari H**, Urata T, Yasuda I, Matsusaki S, Hisai H, Kawakami H, Ono M, Iwashita T, Doi S, Kawakubo K, Hayashi T, Sonoda T, Sakamoto N, Kato J. No Benefit of Oral Diclofenac on Post-Endoscopic Retrograde Cholangiopancreatography Pancreatitis. *Dig Dis Sci* 2016; **61**: 3292-3301 [PMID: 27447477 DOI: 10.1007/s10620-016-4251-x]

66 **Iqbal U**, Siddique O, Khara HS, Khan MA, Haq KF, Siddiqui MA, Solanki S, Zuchelli TE, Shellenberger MJ, Birk JW. Post-endoscopic retrograde cholangiopancreatography pancreatitis prevention using topical epinephrine: systematic review and meta-analysis. *Endosc Int Open* 2020; **8**: E1061-E1067 [PMID: 32743060 DOI: 10.1055/a-1190-3777]

67 **Chen B**, Fan T, Wang CH. A meta-analysis for the effect of prophylactic GTN on the incidence of post-ERCP pancreatitis and on the successful rate of cannulation of bile ducts. *BMC Gastroenterol* 2010; **10**: 85 [PMID: 20673365 DOI: 10.1186/1471-230X-10-85]

68 **Sotoudehmanesh R**, Eloubeidi MA, Asgari AA, Farsinejad M, Khatibian M. A randomized trial of rectal indomethacin and sublingual nitrates to prevent post-ERCP pancreatitis. *Am J Gastroenterol* 2014; **109**: 903-909 [PMID: 24513806 DOI: 10.1038/ajg.2014.9]

69 **Tomoda T**, Kato H, Ueki T, Akimoto Y, Hata H, Fujii M, Harada R, Ogawa T, Wato M, Takatani M, Matsubara M, Kawai Y, Okada H. Combination of Diclofenac and Sublingual Nitrates Is Superior to Diclofenac Alone in Preventing Pancreatitis After Endoscopic Retrograde Cholangiopancreatography. *Gastroenterology* 2019; **156**: 1753-1760. e1 [PMID: 30772342 DOI: 10.1053/j.gastro.2019.01.267]

70 **Andriulli A**, Leandro G, Federici T, Ippolito A, Forlano R, Iacobellis A, Annese V. Prophylactic administration of somatostatin or gabexate does not prevent pancreatitis after ERCP: an updated meta-analysis. *Gastrointest Endosc* 2007; **65**: 624-632 [PMID: 17383459 DOI: 10.1016/j.gie.2006.10.030]

71 **Qin X**, Lei WS, Xing ZX, Shi F. Prophylactic effect of somatostatin in preventing Post-ERCP pancreatitis: an updated meta-analysis. *Saudi J Gastroenterol* 2015; **21**: 372-378 [PMID: 26655132

DOI: 10.4103/1319-3767.167187]
72 **Bai Y**, Gao J, Zou DW, Li ZS. Prophylactic octreotide administration does not prevent post-endoscopic retrograde cholangiopancreatography pancreatitis: a meta-analysis of randomized controlled trials. *Pancreas* 2008; **37**: 241-246 [PMID: 18815543 DOI: 10.1097/MPA.0b013e31816c90a1]
73 **Omata F**, Deshpande G, Tokuda Y, Takahashi O, Ohde S, Carr-Locke DL, Jacobs JL, Mine T, Fukui T. Meta-analysis: somatostatin or its long-acting analogue, octreotide, for prophylaxis against post-ERCP pancreatitis. *J Gastroenterol* 2010; **45**: 885-895 [PMID: 20373114 DOI: 10.1007/s00535-010-0234-4]
74 **Seta T**, Noguchi Y. Protease inhibitors for preventing complications associated with ERCP: an updated meta-analysis. *Gastrointest Endosc* 2011; **73**: 700-706. e1-2 [PMID: 21145053 DOI: 10.1016/j.gie.2010.09.022]
75 **Yu G**, Li S, Wan R, Wang X, Hu G. Nafamostat mesilate for prevention of post-ERCP pancreatitis: a meta-analysis of prospective, randomized, controlled trials. *Pancreas* 2015; **44**: 561-569 [PMID: 25822153 DOI: 10.1097/MPA.0000000000000310]
76 **Yuhara H**, Ogawa M, Kawaguchi Y, Igarashi M, Shimosegawa T, Mine T. Pharmacologic prophylaxis of post-endoscopic retrograde cholangiopancreatography pancreatitis: protease inhibitors and NSAIDs in a meta-analysis. *J Gastroenterol* 2014; **49**: 388-399 [PMID: 23720090 DOI: 10.1007/s00535-013-0834-x]
77 **Chen S**, Shi H, Zou X, Luo H. Role of ulinastatin in preventing post-endoscopic retrograde cholangiopancreatography pancreatitis: the Emperor's New Clothes or Aladdin's Magic Lamp? *Pancreas* 2010; **39**: 1231-1237 [PMID: 20531245 DOI: 10.1097/MPA.0b013e3181dc67e7]

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MINIREVIEWS

RON in hepatobiliary and pancreatic cancers: Pathogenesis and potential therapeutic targets

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Abstract

The receptor protein tyrosine kinase RON belongs to the c-MET proto-oncogene family. Research has shown that RON has a role in cancer pathogenesis, which places RON on the frontline of the development of novel cancer therapeutic strategies. Hepatobiliary and pancreatic (HBP) cancers have a poor prognosis, being reported as having higher rates of cancer-related death. Therefore, to combat these malignant diseases, the mechanism underlying the aberrant expression and signaling of RON in HBP cancer pathogenesis, and the development of RON as a drug target for therapeutic intervention should be investigated. Abnormal RON expression and signaling have been identified in HBP cancers, and also act as tumorigenic determinants for HBP cancer malignant behaviors. In addition, RON is emerging as an important mediator of the clinical prognosis of HBP cancers. Thus, not only is RON significant in HBP cancers, but also RON-targeted therapeutics could be developed to treat these cancers, for example, therapeutic monoclonal antibodies and small-molecule inhibitors. Among them, antibody-drug conjugates have become increasingly popular in current research and their potential as novel anti-cancer biotherapeutics will be determined in future clinical trials.

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Core Tip: The role of RON in cancer pathogenesis has received increasing research attention. Hepatobiliary and pancreatic (HBP) cancers have a poor prognosis, being reported as having higher rates of cancer-related death because of their high rates of recurrence, metastasis, and invasiveness, and their lack of sensitivity to chemotherapy. In this review, we discuss how RON functions in HBP cancer pathogenesis, as well as its potential role as a therapeutic target in HBP cancers.

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INTRODUCTION

RON receptor tyrosine kinase (RTK; also known as MST1R) was first identified in 1993 in a cDNA library from human epithelial cells[1]. RON belongs to the family of c-MET proto-oncogenes[2]. This RTK family only has two members, RON and Met, which share only 34% overall homology; however, the tyrosine kinase region of the receptors is quite similar at 80% homology[3]. In 1994, a mouse cDNA was cloned that encoded a homolog of RON, which was termed stem cell-derived tyrosine kinase receptor[4]. *RON* is located at human chromosome 3p21 and this gene shows high conservation in different species, including xenopus, zebrafish, chicken, cats, human, and mouse[4-11]. The RON receptor is initially synthesized as a biologically inactive single-chain precursor (pro-RON), then cleaved into a 145 kDa β-chain and a 35 kDa extracellular alpha chain, which are linked by a disulfide bond, forming the mature receptor. In 1994, the physiological ligand of RON was identified as macrophage-stimulating protein (MSP) [also called hepatocyte growth factor (HGF)-like protein], establishing the MSP-RON signaling system[12-15]. MSP is a member of the plasminogen-related kringle protein family[16,17]. The human *MSP* gene is also located at chromosome 3p21 and is evolutionarily conserved in different species, similar to RON. The main source of MSP is hepatocytes and MSP circulates in the blood as pro-MSP, which is a biologically inactive single-chain precursor. After subsequent proteolytic conversion, the active mature MSP consists of the disulfide-linked alpha subunit and β-chain. The RON receptor high affinity binding site is in the β-chain and RON activity is regulated by the alpha chain[18]. The binding of MSP to RON induces RON dimerization, which activates multiple downstream signaling pathways, leading to RON-mediated cell growth, survival, and invasiveness[19,20].

In the last two decades, increased research has focused on the tumorigenic and therapeutic roles of RON signaling. Although there have been few studies concerning pathology-related changes in MSP expression, numerous studies regarding aberrant RON activation in various types of tumors have been published, including RON protein overexpression[21-28], oncogenic variant generation[29-39], and persistent activation of downstream signaling pathways[21-39]. In addition, tumorigenic progression and malignancy are associated with functional crosstalk between signaling proteins and RON. In clinical application, increased RON expression can be used for prognostic evaluation of patient survival and disease progression. Hepatobiliary and pancreatic (HBP) cancers have a poor outcome, with high rates of cancer-related death because of their high incidences of recurrence, metastasis, and invasiveness, and their lack of sensitivity to chemotherapy[40]. Complete surgical resection remains the most effective treatment for HBP cancers[40]. Among these cancers, the 5-year survival rate of liver cancer is approximately 30%, whereas in biliary tract cancer and pancreatic cancer, it is less than 30% and less than 10%, respectively[41]. The high death rate of pancreatic cancer is caused by the lack of early

diagnosis and effective treatment. In pancreatic cancer, most cases are diagnosed when the disease is already at an advanced stage, and only 20% or less of patients present with potentially curable localized tumors amenable to surgical extirpation[42]. Thus, the identification of a novel potential therapeutic strategy is urgently required. Growing evidence suggests a close relationship between HBP cancers and RON dysregulation[24,43,44]. Thus, the present review primarily focuses on the role of RON in the pathogenesis of cancer, especially HBP cancers. Moreover, we summarize the latest progress in the development of strategies targeting RON as potential HBP cancer therapy.

ROLES OF RON AND C-MET IN CARCINOGENESIS

RON and c-MET, both of which are members of the semaphorin family of transmembrane receptor tyrosine kinases, share similar structural and biochemical properties[45]. The proteins exist as heterodimers comprising extracellular and transmembrane chains that are linked by disulfide bonds. The RON and c-MET extracellular sequences possess very similar functional domains, including SEMA, which regulates phosphorylation, receptor dimerization, and ligand binding. RON and c-MET are activated by their respective ligands: MSP for RON and HGF for c-MET. c-MET and HGF are expressed in a variety of cell and tissue types. Contrastingly, RON is restricted tightly to epithelial origin cells, whereas liver cells are the major source of its ligand, MSP[46]. Independent or ligand-dependent activation of RON and c-MET induces matrix invasion, cell migration, and cell proliferation, all of which are crucial for embryogenesis, wound healing, and tumorigenesis.

Increasing evidence has identified the role of RON and c-Met in the pathogenesis of cancer[47]. For example, c-MET and RON overexpression was observed in a variety of primary and metastatic tumors, leading to the activation of aberrant downstream signaling, which contributes to cancer development and progression. Moreover, clinical studies have validated that increased expression of RON and c-MET is a prognostic factor to predict the survival rate and disease progression in certain patients with cancer[48,49]. Moreover, activation of RON and c-MET promotes a cancer cell malignant phenotype. Increased RON and c-MET expression drives tumor cells to undergo epithelial to mesenchymal transition (EMT), which is characterized by epithelial feature loss and the gain of mesenchymal characteristics[12,50]. Increased c-MET and RON expression also contributes to acquired chemoresistance[51]. Given the above role of the increased expression of c-MET and RON in cancer pathogenesis, targeting RON and c-MET represents a promising cancer therapy strategy.

RON ACTIVATION AND SIGNALING PATHWAY MECHANISMS

Epithelial cells in the skin, adrenal gland, bone, brain, kidney, gut, lung, and liver express low levels of RON[12]. The action of RON plays a key role in the motility of epithelial cells, enhancement of adhesion, sperm motility in the epididymis, and embryonic development, as well as the regulation of inflammatory responses[52]. Under physiological conditions, the main cause of RON activation is stimulation of its ligand, MSP[12]. Moreover, three other biochemical events activate RON in tumors: RON overexpression, generation of oncogenic RON variants, and RON transactivation (Figure 1). The RON receptor consists of three essential regions: The extracellular domain that recognizes its ligand, the transmembrane domain that anchors the receptor to the membrane, and the intracellular domain that exerts the kinase activity (Figure 1)[53]. The first step for the activation of RON is dimerization at the cell surface, which is caused by the binding of MSP to the extracellular domain containing the specific ligand-binding site, likely resulting in a conformational change in the RON receptor. This activation leads to autophosphorylation at two tyrosine residues (Tyr1238 and Tyr1239) located in the A-loop (Phe1227-Pro1250) of the kinase domain. Phosphorylation of these regulatory residues leads to tyrosine kinase function activation, inducing further phosphorylation of residues Tyr1353 and Tyr1360 located in the C-terminal docking site. This then recruits the cytoplasmic molecules growth factor receptor-bound protein 2 (GRB2) and Son of Sevenless. In addition, the ubiquitin ligase, casitas B-lineage lymphoma (CBL), binds to the docking site to act as a negative modulator.

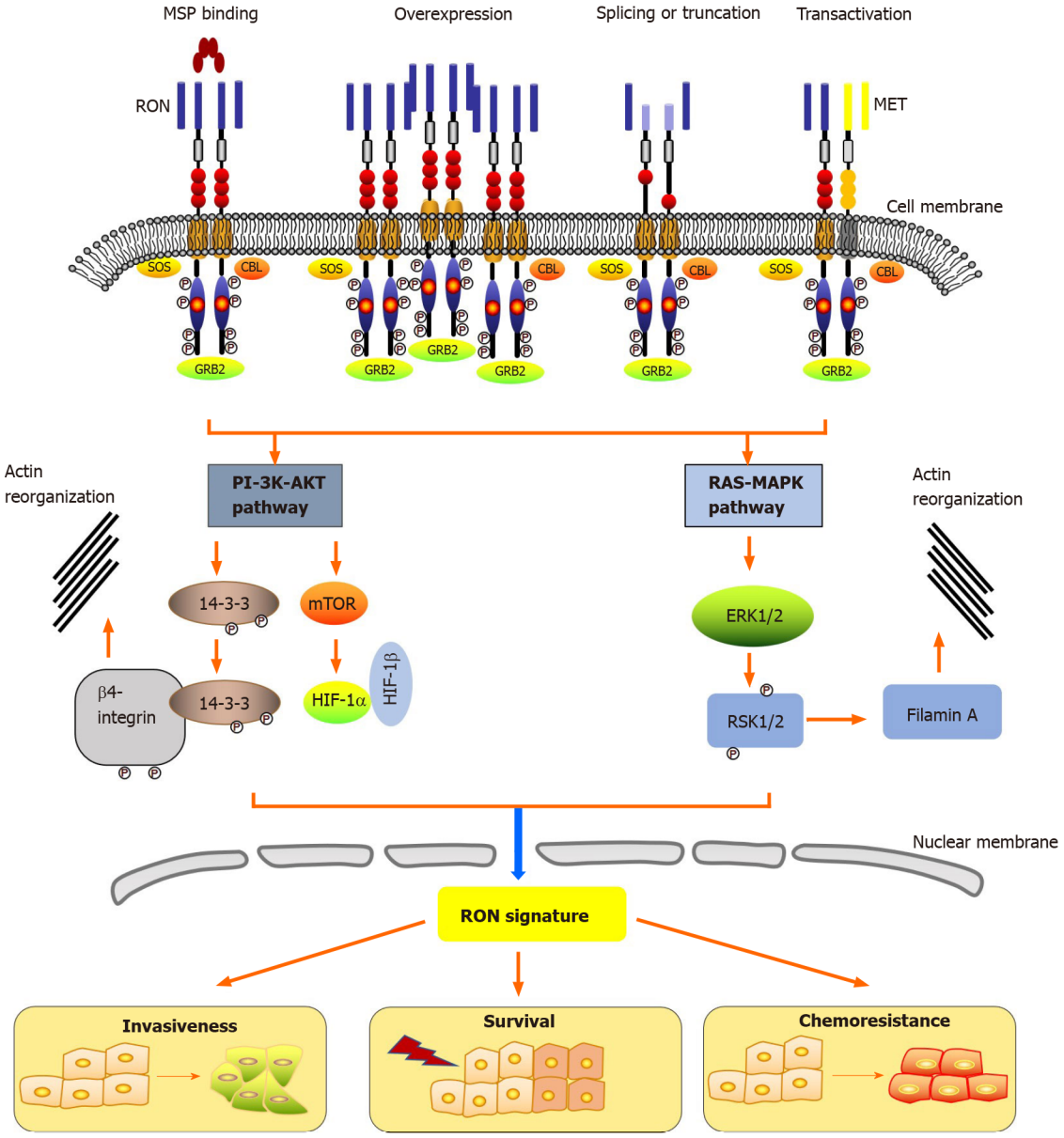


Figure 1 Mechanisms of RON activation and downstream signaling pathways. Classically, macrophage-stimulating protein (MSP) activates RON. In cancer, RON activation is induced by overexpression, splicing or truncation, and transactivation. The RON receptor consists of three regions including the extracellular domain, the transmembrane domain, as well as the intracellular domain. MSP binding to the extracellular domain leads to autophosphorylation of several tyrosine residues in the kinase activation loop or in the C-terminal tail, resulting in the activation of many biological activities, including increased proliferation/survival, motile-invasive activity, and chemoresistance. MSP: Macrophage-stimulating protein; SOS: Son of Sevenless; GRB2: Growth factor receptor-bound protein 2; CBL: Casitas B-lineage lymphoma; 14-3-3: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein; PI-3K-AKT: Phosphatidylinositol-4,5-Bisphosphate 3 kinase- protein kinase B; HIF: Hypoxia-inducible factor; RAS-MAPK: RAS-mitogen-activated protein kinase; ERK: Extracellular regulated kinase; RSK: Ribosomal protein S6 kinase; mTOR: Mechanistic target of rapamycin.

The interaction of RON with adaptor proteins, such as β-arrestin-1 and GRB2, represents the first step in the bridging of downstream signaling cascades and RON activation. *Via* its C-terminal docking site, RON interacts with a variety of cytoplasmic effector molecules, such as phospholipase C gamma, phosphatidylinositol-4,5-Bisphosphate 3-kinase (PI-3 kinase), Src (SRC proto-oncogene, non-receptor tyrosine kinase), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3), CBL protooncogene (c-Cbl), heat shock protein family A (Hsp70) member 8 (HSC70), integrin-β4, plectin, and protein phosphatase 1. The classical PI-3 kinase-protein kinase B (PI-3K-AKT) and RAS-mitogen-activated protein kinase (RAS-MAPK) pathways are triggered by the interaction of RON's docking site with downstream signaling proteins. PI-3K-AKT and RAS-MAPK pathways mediate many biological activities, including increased proliferation and survival, EMT, motile-invasive activity, and chemoresistance. The signaling pathways of RON also play a part in regulating tumorigenic activity. Among them, PI-3K-AKT and RAS-MAPK pathway

coordinated activation plays a crucial role in EMT *via* increased cellular motility[15,22,54,55]. Studies using the MDCK cell model showed that EMT mediated by RON is associated with decreased E-Cadherin expression and unregulated vimentin expression, under mediation by RAS-MAPK signaling[56,57]. The major protein that links EMT to RON signaling is ribosomal protein S6 kinase-2, which is an intermediate in the MAPK pathway. RON-mediated PI-3K-AKT signaling is also involved in invasive growth, including increased epithelial cell matrix invasion, migration, and adhesion *in vitro* and distant metastasis and tumor cell invasion *in vivo* [54,55].

ABERRANT RON SIGNALING AND EXPRESSION IN CANCER PATHOGENESIS

In general, normal epithelial cells, including those from the colon, lung, and breast, express low levels of RON; however, cells of a mesenchymal origin do not express RON[12,58]. RON activation in tumors is frequently the result of receptor overexpression, in contrast to classical MSP binding. Dysregulated RON activation and expression were detected in many types of cancers and have prognostic significance for patient survival. Results from the majority of published studies show that RON expression dysregulation is characterized mainly by elevated expression of wild-type RON and the production of active isoforms, ultimately leading to persistent activation of downstream signaling cascades[12]. There have been reports of *RON* amplification and point mutation; however, this kind of genetic alteration is observed rarely[26]. The relationships between cancer pathogenesis and dysregulated RON signaling and expression were proven *via* functional studies using immunohistochemical (IHC) staining of tumor specimens and cancer cell lines. The first report of wild-type RON overexpression in cancerous tissue was in primary breast cancer samples. Thereafter, IHC staining has further detected wild-type RON in thyroid, bladder, adrenal gland, head and neck, uterus, skin, lung, kidney, pancreatic, colorectal, and other tumors[59]. These findings are consistent with the results found in other cancers, such as human gliomas, melanoma, and Merkel cell carcinoma, suggesting that aberrant RON expression is also associated with both neurological and skin cancers[18]. In breast tissues, the expression of RON is relatively low in normal breast epithelial cells and even in cells from benign lesions (papilloma and adenoma), whereas its was highly expressed in 47% (35/75 cases) of histologically varied tumor specimens[25]. RON upregulation is associated strongly with its phosphorylation status and invasive activity, suggesting that dysregulated expression of RON functions in human breast carcinoma progression to invasive-metastatic phenotypes. Furthermore, in breast cancer unregulated RON expression was identified as an independent predictor of distant relapse[60]. By contrast, in certain tumors, such as hepatocellular carcinoma (HCC), the frequency of wild-type RON expression was relatively low[21]; however, its importance remains unknown, although this finding indicates that the wild-type RON is not expressed universally in different tumor types. Moreover, RON overexpression is related to oncogenic RON isoform production, for example RON△160, which comprises the deletion of exons 5 and 6, encoding 109 amino acids in the RON β-chain extracellular sequence[12]. RON variants are detected in primary cancer samples and cell lines relatively frequently, and are detected in 40% to 60% of cases. Cancer pathogenesis and clinical relevance are likely to be affected by the frequencies and levels of RON isoforms.

Increasing evidence has demonstrated the role of RON in regulating cancer cell invasiveness, which is related to the effects of RON activation on a variety of signaling mechanisms. The activation of complex downstream signaling networks including signal transducer and activator of transcription, β-catenin, JUN N-terminal kinase, MAPK, and PI3K/AKT pathways are key contributors to RON-mediated aggressive cancer phenotypes. In breast cancer, several signaling pathways that are vital for stemness, invasiveness, and proliferation are activated by RON. For example, the RON-cellular Abelson murine leukemia viral oncogene homolog (c-Abl)-proliferation cell nuclear antigen (PCNA) pathway was identified to contribute to RON-mediated cell growth in breast cancer. Dysregulated RON signaling results in c-Abl activation, consequently leading to PCNA phosphorylation[61]. Moreover, in breast cancer, RON signaling regulates the invasiveness of cancer cell *via* the activation of the DEK proto-oncogene (*DEK*), a DNA-binding non-histone nuclear phosphoprotein that induces closed circular DNA to form positive supercoils[62]. This process appears to function *via* a paracrine and autocrine canonical β-catenin signaling loop, which ultimately

influences breast cancer stemness. In addition, RON-mediated PI3K-dependent upregulation of methyl-CpG binding domain 4, DNA glycosylase (MBD4) increases the invasive growth and metastasis of breast cancer cell *via* the reprogramming of the DNA methylation of specific target genes[63]. Clinical data indicated that in patients with breast cancer, poor prognosis is related to the RON-MBD4 epigenetic pathway[63].

RON RECEPTOR AND HEPATOBILIARY CANCERS

In 2030, it is estimated that more than 1 million people will die because of liver cancer worldwide[64]. Primary malignancies of the liver and adjacent biliary tract include HCC, intrahepatic and extrahepatic cholangiocarcinoma (CCA), and gallbladder cancer (GBC). Among them, HCC and intrahepatic CCA account for 85% and 10%, respectively[65]. Abnormal RON expression has been observed in HCC, which may be related to pathological conditions of this cancer[43]. In an HCC cell line study, RON was shown to be associated with oncogenic and invasive phenotypes (*e.g.*, resistance to apoptosis, tumor cell migration, and tumor cell invasion) *via* AKT, c-Raf, and extracellular regulated kinase (ERK) signaling cascade modulation[66]. Clinically, RON and MET expression in patients with HCC after curative resection suggested no association of RON with overall survival and overall recurrence rates. However, patients with RON+/MET+ disease experienced higher overall recurrence rates compared with those displaying alternative expression patterns[67]. Similar to HCC, RON is emerging as an important mediator of CCA pathogenesis and clinical prognosis. Investigation of RON and MET expression in patients with perihilar CCA who underwent histologically curative resection revealed that patients with RON+/MET+ disease showed a worse overall survival rate than patients with other patterns[44]. In addition, in patients with extrahepatic CCA, the complete loss of MET, RON, or both (and their overexpression) was a factor for poor prognosis, likely due to the high rate of lymph-node metastasis[68]. Recently, Cheng and co-workers indicated that BMS-777607, a MET-RON dual inhibitor, inhibited HuCCT1 and KKU-100 human CCA cell growth, and decreased the growth of tumors in CCA rats. They further found that for patients with CCA who had previously undergone hepatectomies, upregulation of RON and MET was a predictor of poor survival[69]. Taken together, these studies suggest that the aberrant RON expression found in human hepatobiliary cancer samples and cell lines is closely related to pathological conditions and clinical outcome.

RON RECEPTOR AND PANCREATIC CANCER

The majority of malignant neoplasms of the pancreas are adenocarcinomas, among which pancreatic ductal adenocarcinoma is the most common malignancy, representing an excess of 95% of all pancreatic malignancies[70]. Pancreatic cancer presents a substantial health problem and is associated with an extremely poor prognosis because of the non-specific symptoms in patients, its aggressive and remarkable resistance to most conventional treatment options, and the fact that it harbors multiple genetic and epigenetic alterations[42]. Therefore, novel therapies to treat pancreatic cancer are urgently required. In recent years, the function of RON in pancreatic cancer has been identified extensively in a variety of model systems, such as animal, cellular, and clinical settings. To date, researchers have reported that RON is expressed in various pancreatic cancer cell lines, such as CFPAC-1, ASPC-1, Hs766.T, L3.6pl, HPAFII, HPAC, Capan-2, and BXPC-3. However, MIA-PACA-2 cells show minimal RON expression[71]. The association of RON with Kras-driven pancreatic carcinogenesis was investigated using genetically engineered mouse models. The results showed that overexpression of RON accelerated pancreatic intraepithelial neoplasia (PanIN) progression, enhanced acinar-ductal metaplasia, and promoted tumor progression towards invasive pancreatic cancer[72]. Moreover, the study proved that the initiation of PanIN was slowed by RON kinase domain genetic inactivation, resulting in smaller tumors, and eventually prolonging tumor-bearing mouse survival[72]. Great progress has been made in our understanding of the clinical relevance of RON in pancreatic cancer, which has focused mainly on RON expression status in pancreatic cancer samples and its possible utility as a prognostic biomarker for patient survival. IHC staining using anti-RON antibodies is a commonly used approach to evaluate RON expression in various experimental settings. Several studies

have identified positive sample rates in pancreatic cancer specimens such as 70%, 88%, 96%, 80%, and 86%, respectively[21,73]. Meanwhile, in pancreatic cancer samples, high RON expression has been detected, whereas minimal levels were detected in their corresponding normal epithelial cells. Notably, during pancreatic cell tumorigenic progression, the frequency and level of RON expression increased[22,74]. Among human pancreatic tissue samples, RON expression was detected in 83% of metastatic lesions, 79% of primary lesions, and 93% of high-grade PanIN using immunohistochemistry, with low expression being detected in low-grade PanIN and normal ducts (18% and 6%, respectively), suggesting that RON might function in pancreatic carcinogenesis and metastatic progression[22]. Moreover, RON expression levels were significantly related to overall survival in patients with pancreatic cancer, indicating that RON could be an important indicator of prognosis in pancreatic cancer[75]. Conflicting results between RON expression and pancreatic cancer prognosis were found in an early study[76], thus more research is needed to determine the utility of RON as a prognostic biomarker in patients with pancreatic cancer.

Primary and metastatic pancreatic tumor specimens and high grade PanIN lesions show increased RON expression[22]. Accumulating evidence suggests that dysregulated RON signaling and activation might function in tumor formation and metastasis. Generally, activation of RON results in increased pancreatic cancer tumorigenic stemness, chemoresistance, survival capability, angiogenesis, and cell invasiveness[73]. Among them, invasiveness occurs *via* a phenotype resembling EMT. A study found that MSP treatment of the pancreatic cancer cell line L3.6pl resulted in increased cell invasion, cell migration, and ERK phosphorylation[24]. Activation of RON resulted in decreased levels of membrane-bound E-cadherin together with β-catenin nuclear translocation, which resembled EMT. Treated L3.6pl cells acquired a spindle shape and lost their polarity, their intercellular separation increased, and more pseudopodia were formed[24]. Aberrant RON activation in collaboration with other growth factors, such as transforming growth factor-β, contributes to the phenotypic changes of pancreatic cancer cells towards EMT. Additionally, an investigation of RON signaling-mediated angiogenesis regulation in pancreatic cancer found that RON signaling leads to MAPK-mediated pancreatic cancer cell production of the well-characterized angiogenic protein, vascular endothelial growth factor. RON activation also caused the promotion of microtubule formation[77]. Finally, the RON signaling pathway also plays a part in chemoresistance, which is associated with enhanced survival capability[51,78]. Short hairpin RNA (shRNA)-mediated silencing of *RON* expression in pancreatic cancer xenografts resulted in increased sensitivity to gemcitabine therapy and susceptibility to apoptosis[51]. In the light of the above findings, it is clear that RON signaling is crucial for pancreatic cancer formation and metastasis.

RON AS A THERAPEUTIC TARGET FOR HBP CANCERS

Based on the pathogenic role of RON in cancers, including HBP cancers, efforts have focused predominantly on establishing RON as a drug target for therapeutic intervention[73]. A variety of techniques were proposed to effectively block RON signaling and expression. One approach is to inhibit RON expression using gene silencing with small interfering RNAs (siRNAs). In pancreatic cancer xenografts, *RON* silencing caused growth inhibition by enhancing their apoptosis susceptibility and *via* sensitization to gemcitabine therapy[51]. Thus, delivery of RON-specific siRNAs could have therapeutic potential. In addition, small-molecule kinase inhibitors (SMKIs), which block the receptor tyrosine kinase domain either *via* non-competitive inhibition or *via* ATP competition, have been proposed[45]. The structural similarities between the kinase domains of MET and RON resulted in the development of selective small molecule inhibitors targeting both the RON and MET kinase domains, with slightly different IC_{50} values. As described above, BMS-777607, a MET-RON dual inhibitor, has shown its effects in inhibiting the growth of human intra-hepatic CCA cell lines and also decreasing tumor growth in intrahepatic CCA rats[69]. However, preclinical studies to prove RON as a drug target showed unsatisfactory results when using RON-specific SMKIs[46,79-81]. The first reason for the above result is that HBP cancer cell survival does not depend on RON signaling. Second, an SMKI that specifically inhibits only RON kinase activities is not available. Synthetic SMKIs, including Tivantinib, BMS-777607, INCB28060, Compound-1, and PHA665752, all recognize both RON and MET, with similar kinase-binding affinities[73]. Thus, the characterized SMKI RON or MET-specific inhibitors are actually multiple RTK inhibitors and the

Table 1 Tyrosine kinase inhibitors and antibody drug conjugates specific to c-MET and RON

Therapeutic agents	Manufacturer	Target	*In vitro* effects	Effects in animal tumor models	Clinical trial information	Status	Ref.
TKIs							
Foretinib	GlaxoSmithKline	MET, RON, VEGFR2, and PDGFRβ	Inhibits MET and RON signaling and cell growth in various cancer cell lines	Attenuates MET- and RON-mediated tumor growth in mouse tumor xenograft models	Single agent and combination with erlotinib or lapatinib for various types of advanced cancers in Phase II/III clinical trials	Phase I/II/III	Eder *et al*[82]
MGCD265	MethylGene	MET, RON, VEGFR1, VEGFR2, VEGFR3, and TIE2	Inhibits MET and RON signaling and cell growth in cancer cell lines	Attenuates MET- and RON-mediated tumor growth in mouse tumor xenograft models	Single agent and combination with erlotinib or docetaxel for NSCLC in Phase II trials	Phase I/II	Belalcazar *et al*[83]
BMS-777607	Bristol-Myers Squibb	RON and MET	Inhibits MET and RON signaling, cell growth, and invasion in cancer cell lines	Inhibits MET- and RON-mediated tumor growth in mouse tumor xenograft models	Multiple ascending doses for metastatic cancers in Phase I trials	Phase I	Sharma *et al*[84]
MK-2461	Merck	MET, RON, FLT1, FLT3, FGFR1, FGFR2, and FGFR3	Inhibits MET and RON signaling, cell growth, and migration in cancer cell lines	Inhibits MET- and RON-mediated tumor growth in mouse tumor xenograft models	Antitumor efficacy is under evaluation in Phase II trials	Phase I/II	Pan *et al*[85]
MK-8033	Merck	MET and RON	Inhibits MET and RON signaling, cell growth, and migration in cancer cell lines	Causes tumor regression in mouse tumor xenograft models	Safety, tolerability, dose, clinical activity and pharmaco-dynamics are under evaluation in Phase I trials	Phase I	Northrup *et al*[86]
PHA665752	Pfizer	MET and RON	NA	NA	NA	Preclinical	Comoglio *et al*[87]
INC280	Novartis	MET	NA	NA	NA	Phase I/II	Qin *et al*[88]
Tivantinib	ArQule	MET	NA	NA	NA	Phase II/III	Rimassa *et al*[89]
Antibody drug conjugates							
Zt/g4-doxorubicin-immuoliposome	TTUHSC	RON	Moderately activates RON signaling and strongly induces RON endocytosis	No effect as naked antibody but completely inhibits tumors used as ADCs	NA	Preclinical	Guin *et al*[90]
Zt/g4-maytansinoid conjugate	TTUHSC	RON	Moderately activates RON signaling and strongly induces RON endocytosis	No effect as naked antibody but completely inhibits tumors used as ADCs	NA	Preclinical	Feng *et al*[91]
Zt/g4-MMAE	TTUHSC	RON	Moderately activates RON signaling and strongly induces RON endocytosis	No effect as naked antibody but completely inhibits tumors used as ADCs	NA	Preclinical	Yao *et al*[92]
H5B14-MMAE	TTUHSC	RON	NA	NA	NA	Preclinical	Yao *et al*[59]
SHR-A1403	HengRui	MET	Highly potent: 0.02 to 1.5 nmol/L for cell proliferation	Xenografts and PDXs, MET over-expressed and amplified	NA	Phase I	Yang *et al*[93]

TKIs: Tyrosine kinase inhibitors; VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; NSCLC: Non-small cell lung cancer; FGFR: Fibroblast growth factor receptor; ADC: Antibody-drug conjugate; MMAE: Monomethyl auristatin E; NA: Not available; PDX: Patient-derived xenografts.

development of SMKIs that exclusively target RON has been a challenge.

A more realistic approach is using anti-RON therapeutic monoclonal antibodies (TPABs) to treat HBP cancers. For instance, anti-RON antibody Zt/c9-directing doxorubicin-immunoliposomes was effective at killing purified pancreatic cancer stem cells *in vitro*. The underlying mechanism is that Zt/c9-directing doxorubicin-immunoliposomes specifically interact with pancreatic cancer stem cells and rapidly cause RON internalization, which leads to the uptake of liposome-coated Dox. In addition, preclinical models have been constructed using anti-RON TPABs, such as 7G8, 6D4, 6E6, narnatumab (or IMC-RON8), Zt/f2, and IMC-41A10, which either block MSP binding by recognizing RON's ligand-binding pocket or affect receptor dimerization by interacting with RON's extracellular domain (*e.g.*, SEMA), thereby attenuating signaling transduction[73]. However, previous studies concerning TPAB therapy revealed only partial inhibition of tumor growth, and there have been no reports of single anti-RON TPAB administration achieving complete inhibition. Thus, strategies to maximize anti-RON TPABs' therapeutic activity have moved on to an exciting new area. Anticancer therapeutic agents comprising antibody-drug conjugates (ADCs) combine the specificity of antibodies with the high potency of cytotoxins to enhance cell killing[12]. To generate RON-targeted ADCs, the anti-RON monoclonal antibodies PCM5B14 and Zt/g4 were selected to prepare immunotoxins. To generate Zt/g4 and PCM5B14-based ADCs, cytotoxic payloads with different mechanisms of action were conjugated, including pyrrolobenzodiazepine, duocarmycin (DCM), monomethyl auristatin E, and maytansinoid derivative 1, forming for example, Zt/g4-MME and PCM5B14-DCM[59]. Preclinical studies identified Zt/g4- and PCM5B14-based ADCs as lead candidates for clinical development and increased the chance of their entering into clinical trials (Table 1).

CONCLUSION

RON was identified over two decades ago, and since then, accumulating evidence has indicating RON's involvement in tumorigenesis, which has resulted in increased momentum for developing RON as a target for therapeutic drug intervention. As outlined in this review, the identification of dysregulated activation and expression of RON in various cancers has expanded our understanding of the mechanisms underlying cancer pathogenesis. Importantly, HBP cancers are characterized pathologically by the dysregulated signaling and expression of RON, which also act as tumorigenic determinants for the malignant behavior of HBP cancers. Moreover, abnormal RON expression is important to determine the clinical outcome of patients

with HBP cancers. The growing knowledge concerning the crucial role of RON in HBP cancers can be translated into promising cancer therapeutic strategies. Consequently, a number of clinical trials are underway to assess SMKIs and TPABs targeting RON as a molecular target, some of which have shown promising results. Furthermore, PCM5B14- and Zt/g4-based ADCs, as anti-RON ADCs, are receiving increased research interest and the striking advances in exploiting anti-RON ADCs will hopefully translate into clinical treatments for patients with HBP cancer in the future.

REFERENCES

1 **Ronsin C**, Muscatelli F, Mattei MG, Breathnach R. A novel putative receptor protein tyrosine kinase of the met family. *Oncogene* 1993; **8**: 1195-1202 [PMID: 8386824]
2 **Gherardi E**, Sharpe M, Lane K, Sirulnik A, Stoker M. Hepatocyte growth factor/scatter factor (HGF/SF), the c-met receptor and the behaviour of epithelial cells. *Symp Soc Exp Biol* 1993; **47**: 163-181 [PMID: 8165564]
3 **Wagh PK**, Peace BE, Waltz SE. Met-related receptor tyrosine kinase Ron in tumor growth and metastasis. *Adv Cancer Res* 2008; **100**: 1-33 [PMID: 18620091 DOI: 10.1016/S0065-230X(08)00001-8]
4 **Iwama A**, Okano K, Sudo T, Matsuda Y, Suda T. Molecular cloning of a novel receptor tyrosine kinase gene, STK, derived from enriched hematopoietic stem cells. *Blood* 1994; **83**: 3160-3169 [PMID: 8193352]
5 **De Maria R**, Maggiora P, Biolatti B, Prat M, Comoglio PM, Castagnaro M, Di Renzo MF. Feline STK gene expression in mammary carcinomas. *Oncogene* 2002; **21**: 1785-1790 [PMID: 11896610 DOI: 10.1038/sj.onc.1205221]
6 **Bassett DI**. Identification and developmental expression of a macrophage stimulating 1/ hepatocyte growth factor-like 1 orthologue in the zebrafish. *Dev Genes Evol* 2003; **213**: 360-362 [PMID: 12764615 DOI: 10.1007/s00427-003-0339-3]
7 **Huitema LF**, Renn J, Logister I, Gray JK, Waltz SE, Flik G, Schulte-Merker S. Macrophage-stimulating protein and calcium homeostasis in zebrafish. *FASEB J* 2012; **26**: 4092-4101 [PMID: 22787265 DOI: 10.1096/fj.11-202663]
8 **Théry C**, Sharpe MJ, Batley SJ, Stern CD, Gherardi E. Expression of HGF/SF, HGF1/MSP, and c-met suggests new functions during early chick development. *Dev Genet* 1995; **17**: 90-101 [PMID: 7554499 DOI: 10.1002/dvg.1020170110]
9 **Huff JL**, Jelinek MA, Jamieson TA, Parsons JT. Expression and maturation of the cellular sea receptor, a member of the hepatocyte growth factor (HGF) receptor family of protein tyrosine kinases. *Oncogene* 1996; **12**: 299-307 [PMID: 8570207]
10 **Wahl RC**, Hsu RY, Huff JL, Jelinek MA, Chen K, Courchesne P, Patterson SD, Parsons JT, Welcher AA. Chicken macrophage stimulating protein is a ligand of the receptor protein-tyrosine kinase Sea. *J Biol Chem* 1999; **274**: 26361-26368 [PMID: 10473593 DOI: 10.1074/jbc.274.37.26361]
11 **Nakamura T**, Aoki S, Takahashi T, Matsumoto K, Kiyohara T, Nakamura T. Cloning and expression of Xenopus HGF-like protein (HLP) and Ron/HLP receptor implicate their involvement in early neural development. *Biochem Biophys Res Commun* 1996; **224**: 564-573 [PMID: 8702427 DOI: 10.1006/bbrc.1996.1065]
12 **Yao HP**, Zhou YQ, Zhang R, Wang MH. MSP-RON signalling in cancer: pathogenesis and therapeutic potential. *Nat Rev Cancer* 2013; **13**: 466-481 [PMID: 23792360 DOI: 10.1038/nrc3545]
13 **Wang MH**, Iwama A, Skeel A, Suda T, Leonard EJ. The murine stk gene product, a transmembrane protein tyrosine kinase, is a receptor for macrophage-stimulating protein. *Proc Natl Acad Sci U S A* 1995; **92**: 3933-3937 [PMID: 7732008 DOI: 10.1073/pnas.92.9.3933]
14 **Wang MH**, Ronsin C, Gesnel MC, Coupey L, Skeel A, Leonard EJ, Breathnach R. Identification of the ron gene product as the receptor for the human macrophage stimulating protein. *Science* 1994; **266**: 117-119 [PMID: 7939629 DOI: 10.1126/science.7939629]
15 **Gaudino G**, Follenzi A, Naldini L, Collesi C, Santoro M, Gallo KA, Godowski PJ, Comoglio PM. RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. *EMBO J* 1994; **13**: 3524-3532 [PMID: 8062829]
16 **Han S**, Stuart LA, Degen SJ. Characterization of the DNF15S2 Locus on human chromosome 3: identification of a gene coding for four kringle domains with homology to hepatocyte growth factor. *Biochemistry* 1991; **30**: 9768-9780 [PMID: 1655021 DOI: 10.1021/bi00104a029]
17 **Yoshimura T**, Yuhki N, Wang MH, Skeel A, Leonard EJ. Cloning, sequencing, and expression of human macrophage stimulating protein (MSP, MST1) confirms MSP as a member of the family of kringle proteins and locates the MSP gene on chromosome 3. *J Biol Chem* 1993; **268**: 15461-15468 [PMID: 8393443]
18 **Benight NM**, Waltz SE. Ron receptor tyrosine kinase signaling as a therapeutic target. *Expert Opin Ther Targets* 2012; **16**: 921-931 [PMID: 22834780 DOI: 10.1517/14728222.2012.710200]
19 **Wang MH**, Julian FM, Breathnach R, Godowski PJ, Takehara T, Yoshikawa W, Hagiya M, Leonard EJ. Macrophage stimulating protein (MSP) binds to its receptor *via* the MSP beta chain. *J Biol Chem* 1997; **272**: 16999-17004 [PMID: 9202013 DOI: 10.1074/jbc.272.27.16999]

20 **Danilkovitch A**, Miller M, Leonard EJ. Interaction of macrophage-stimulating protein with its receptor. Residues critical for beta chain binding and evidence for independent alpha chain binding. *J Biol Chem* 1999; **274**: 29937-29943 [PMID: 10514476 DOI: 10.1074/jbc.274.42.29937]

21 **Wang MH**, Lee W, Luo YL, Weis MT, Yao HP. Altered expression of the RON receptor tyrosine kinase in various epithelial cancers and its contribution to tumourigenic phenotypes in thyroid cancer cells. *J Pathol* 2007; **213**: 402-411 [PMID: 17955509 DOI: 10.1002/path.2245]

22 **Thomas RM**, Toney K, Fenoglio-Preiser C, Revelo-Penafiel MP, Hingorani SR, Tuveson DA, Waltz SE, Lowy AM. The RON receptor tyrosine kinase mediates oncogenic phenotypes in pancreatic cancer cells and is increasingly expressed during pancreatic cancer progression. *Cancer Res* 2007; **67**: 6075-6082 [PMID: 17616662 DOI: 10.1158/0008-5472.CAN-06-4128]

23 **Kanteti R**, Krishnaswamy S, Catenacci D, Tan YH, EL-Hashani E, Cervantes G, Husain AN, Tretiakova M, Vokes EE, Huet H, Salgia R. Differential expression of RON in small and non-small cell lung cancers. *Genes Chromosomes Cancer* 2012; **51**: 841-851 [PMID: 22585712 DOI: 10.1002/gcc.21968]

24 **Camp ER**, Yang A, Gray MJ, Fan F, Hamilton SR, Evans DB, Hooper AT, Pereira DS, Hicklin DJ, Ellis LM. Tyrosine kinase receptor RON in human pancreatic cancer: expression, function, and validation as a target. *Cancer* 2007; **109**: 1030-1039 [PMID: 17311308 DOI: 10.1002/cncr.22490]

25 **Maggiora P**, Marchio S, Stella MC, Giai M, Belfiore A, De Bortoli M, Di Renzo MF, Costantino A, Sismondi P, Comoglio PM. Overexpression of the RON gene in human breast carcinoma. *Oncogene* 1998; **16**: 2927-2933 [PMID: 9671413 DOI: 10.1038/sj.onc.1201812]

26 **Catenacci DV**, Cervantes G, Yala S, Nelson EA, El-Hashani E, Kanteti R, El Dinali M, Hasina R, Brägelmann J, Seiwert T, Sanicola M, Henderson L, Grushko TA, Olopade O, Karrison T, Bang YJ, Kim WH, Tretiakova M, Vokes E, Frank DA, Kindler HL, Huet H, Salgia R. RON (MST1R) is a novel prognostic marker and therapeutic target for gastroesophageal adenocarcinoma. *Cancer Biol Ther* 2011; **12**: 9-46 [PMID: 21543897 DOI: 10.4161/cbt.12.1.15747]

27 **Ren X**, Daa T, Yada N, Kashima K, Fujitomi Y, Yokoyama S. Expression and mutational status of RON in neoplastic lesions of the breast: analysis of MSP/RON signaling in ductal carcinoma in situ and invasive ductal carcinoma. *APMIS* 2012; **120**: 358-367 [PMID: 22515290 DOI: 10.1111/j.1600-0463.2011.02841.x]

28 **Chou YC**, Chen CL, Yeh TH, Lin SJ, Chen MR, Doong SL, Lu J, Tsai CH. Involvement of recepteur d'origine nantais receptor tyrosine kinase in Epstein-Barr virus-associated nasopharyngeal carcinoma and its metastasis. *Am J Pathol* 2012; **181**: 1773-1781 [PMID: 22974584 DOI: 10.1016/j.ajpath.2012.07.014]

29 **Collesi C**, Santoro MM, Gaudino G, Comoglio PM. A splicing variant of the RON transcript induces constitutive tyrosine kinase activity and an invasive phenotype. *Mol Cell Biol* 1996; **16**: 5518-5526 [PMID: 8816464 DOI: 10.1128/mcb.16.10.5518]

30 **Santoro MM**, Collesi C, Grisendi S, Gaudino G, Comoglio PM. Constitutive activation of the RON gene promotes invasive growth but not transformation. *Mol Cell Biol* 1996; **16**: 7072-7083 [PMID: 8943362 DOI: 10.1128/mcb.16.12.7072]

31 **Zhou YQ**, He C, Chen YQ, Wang D, Wang MH. Altered expression of the RON receptor tyrosine kinase in primary human colorectal adenocarcinomas: generation of different splicing RON variants and their oncogenic potential. *Oncogene* 2003; **22**: 186-197 [PMID: 12527888 DOI: 10.1038/sj.onc.1206075]

32 **Liu X**, Zhao L, Derose YS, Lin YC, Bieniasz M, Eyob H, Buys SS, Neumayer L, Welm AL. Short-Form Ron Promotes Spontaneous Breast Cancer Metastasis through Interaction with Phosphoinositide 3-Kinase. *Genes Cancer* 2011; **2**: 753-762 [PMID: 22207901 DOI: 10.1177/1947601911421924]

33 **Eckerich C**, Schulte A, Martens T, Zapf S, Westphal M, Lamszus K. RON receptor tyrosine kinase in human gliomas: expression, function, and identification of a novel soluble splice variant. *J Neurochem* 2009; **109**: 969-980 [PMID: 19519771 DOI: 10.1111/j.1471-4159.2009.06027.x]

34 **Ma Q**, Zhang K, Guin S, Zhou YQ, Wang MH. Deletion or insertion in the first immunoglobulin-plexin-transcription (IPT) domain differentially regulates expression and tumorigenic activities of RON receptor Tyrosine Kinase. *Mol Cancer* 2010; **9**: 307 [PMID: 21114864 DOI: 10.1186/1476-4598-9-307]

35 **Wei X**, Hao L, Ni S, Liu Q, Xu J, Correll PH. Altered exon usage in the juxtamembrane domain of mouse and human RON regulates receptor activity and signaling specificity. *J Biol Chem* 2005; **280**: 40241-40251 [PMID: 16166096 DOI: 10.1074/jbc.M506806200]

36 **Ghigna C**, Giordano S, Shen H, Benvenuto F, Castiglioni F, Comoglio PM, Green MR, Riva S, Biamonti G. Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. *Mol Cell* 2005; **20**: 881-890 [PMID: 16364913 DOI: 10.1016/j.molcel.2005.10.026]

37 **Fialin C**, Larrue C, Vergez F, Sarry JE, Bertoli S, Mansat-De Mas V, Demur C, Delabesse E, Payrastre B, Manenti S, Roche S, Récher C. The short form of RON is expressed in acute myeloid leukemia and sensitizes leukemic cells to cMET inhibitors. *Leukemia* 2013; **27**: 325-335 [PMID: 22902361 DOI: 10.1038/leu.2012.240]

38 **Bardella C**, Costa B, Maggiora P, Patane' S, Olivero M, Ranzani GN, De Bortoli M, Comoglio PM, Di Renzo MF. Truncated RON tyrosine kinase drives tumor cell progression and abrogates cell-cell adhesion through E-cadherin transcriptional repression. *Cancer Res* 2004; **64**: 5154-5161 [PMID: 15289319 DOI: 10.1158/0008-5472.CAN-04-0600]

39 **Wang MH**, Kurtz AL, Chen Y. Identification of a novel splicing product of the RON receptor tyrosine kinase in human colorectal carcinoma cells. *Carcinogenesis* 2000; **21**: 1507-1512 [PMID:

10910951]
40 **Wu LM**, Zhang LL, Chen XH, Zheng SS. Is irreversible electroporation safe and effective in the treatment of hepatobiliary and pancreatic cancers? *Hepatobiliary Pancreat Dis Int* 2019; **18**: 117-124 [PMID: 30655073 DOI: 10.1016/j.hbpd.2019.01.001]
41 **Ueno M**, Morizane C, Ikeda M, Okusaka T, Ishii H, Furuse J. A review of changes to and clinical implications of the eighth TNM classification of hepatobiliary and pancreatic cancers. *Jpn J Clin Oncol* 2019; **49**: 1073-1082 [PMID: 31822900 DOI: 10.1093/jjco/hyz150]
42 **Kleeff J**, Korc M, Apte M, La Vecchia C, Johnson CD, Biankin AV, Neale RE, Tempero M, Tuveson DA, Hruban RH, Neoptolemos JP. Pancreatic cancer. *Nat Rev Dis Primers* 2016; **2**: 16022 [PMID: 27158978 DOI: 10.1038/nrdp.2016.22]
43 **Chen Q**, Seol DW, Carr B, Zarnegar R. Co-expression and regulation of Met and Ron proto-oncogenes in human hepatocellular carcinoma tissues and cell lines. *Hepatology* 1997; **26**: 59-66 [PMID: 9214452 DOI: 10.1002/hep.510260108]
44 **Watanabe H**, Yokoyama Y, Kokuryo T, Ebata T, Igami T, Sugawara G, Mizuno T, Shimoyama Y, Nagino M. Prognostic Value of Hepatocyte Growth Factor Receptor Expression in Patients with Perihilar Cholangiocarcinoma. *Ann Surg Oncol* 2015; **22**: 2235-2242 [PMID: 25586241 DOI: 10.1245/s10434-014-4170-z]
45 **Wang MH**, Padhye SS, Guin S, Ma Q, Zhou YQ. Potential therapeutics specific to c-MET/RON receptor tyrosine kinases for molecular targeting in cancer therapy. *Acta Pharmacol Sin* 2010; **31**: 1181-1188 [PMID: 20694025 DOI: 10.1038/aps.2010.106]
46 **Dussault I**, Bellon SF. From concept to reality: the long road to c-Met and RON receptor tyrosine kinase inhibitors for the treatment of cancer. *Anticancer Agents Med Chem* 2009; **9**: 221-229 [PMID: 19199866 DOI: 10.2174/187152009787313792]
47 **Chang K**, Karnad A, Zhao S, Freeman JW. Roles of c-Met and RON kinases in tumor progression and their potential as therapeutic targets. *Oncotarget* 2015; **6**: 3507-3518 [PMID: 25784650 DOI: 10.18632/oncotarget.3420]
48 **Lee CT**, Chow NH, Su PF, Lin SC, Lin PC, Lee JC. The prognostic significance of RON and MET receptor coexpression in patients with colorectal cancer. *Dis Colon Rectum* 2008; **51**: 1268-1274 [PMID: 18536971 DOI: 10.1007/s10350-008-9297-1]
49 **Ponzo MG**, Lesurf R, Petkiewicz S, O'Malley FP, Pinnaduwage D, Andrulis IL, Bull SB, Chughtai N, Zuo D, Souleimanova M, Germain D, Omeroglu A, Cardiff RD, Hallett M, Park M. Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. *Proc Natl Acad Sci USA* 2009; **106**: 12903-12908 [PMID: 19617568 DOI: 10.1073/pnas.0810402106]
50 **Comoglio PM**, Trusolino L, Boccaccio C. Known and novel roles of the MET oncogene in cancer: a coherent approach to targeted therapy. *Nat Rev Cancer* 2018; **18**: 341-358 [PMID: 29674709 DOI: 10.1038/s41568-018-0002-y]
51 **Logan-Collins J**, Thomas RM, Yu P, Jaquish D, Mose E, French R, Stuart W, McClaine R, Aronow B, Hoffman RM, Waltz SE, Lowy AM. Silencing of RON receptor signaling promotes apoptosis and gemcitabine sensitivity in pancreatic cancers. *Cancer Res* 2010; **70**: 1130-1140 [PMID: 20103639 DOI: 10.1158/0008-5472.CAN-09-0761]
52 **Zarei O**, Benvenuti S, Ustun-Alkan F, Hamzeh-Mivehroud M, Dastmalchi S. Strategies of targeting the extracellular domain of RON tyrosine kinase receptor for cancer therapy and drug delivery. *J Cancer Res Clin Oncol* 2016; **142**: 2429-2446 [PMID: 27503093 DOI: 10.1007/s00432-016-2214-4]
53 **Wang X**, Hankey PA. The ron receptor tyrosine kinase: a key regulator of inflammation and cancer progression. *Crit Rev Immunol* 2013; **33**: 549-574 [PMID: 24266348 DOI: 10.1615/critrevimmunol.2013007953]
54 **Xiao ZQ**, Chen YQ, Wang MH. Requirement of both tyrosine residues 1330 and 1337 in the C-terminal tail of the RON receptor tyrosine kinase for epithelial cell scattering and migration. *Biochem Biophys Res Commun* 2000; **267**: 669-675 [PMID: 10631120 DOI: 10.1006/bbrc.1999.2011]
55 **Wang J**, Rajput A, Kan JL, Rose R, Liu XQ, Kuropatwinski K, Hauser J, Beko A, Dominquez I, Sharratt EA, Brattain L, Levea C, Sun FL, Keane DM, Gibson NW, Brattain MG. Knockdown of Ron kinase inhibits mutant phosphatidylinositol 3-kinase and reduces metastasis in human colon carcinoma. *J Biol Chem* 2009; **284**: 10912-10922 [PMID: 19224914 DOI: 10.1074/jbc.M809551200]
56 **Wang D**, Shen Q, Chen YQ, Wang MH. Collaborative activities of macrophage-stimulating protein and transforming growth factor-beta1 in induction of epithelial to mesenchymal transition: roles of the RON receptor tyrosine kinase. *Oncogene* 2004; **23**: 1668-1680 [PMID: 15001985 DOI: 10.1038/sj.onc.1207282]
57 **Ma Q**, Guin S, Padhye SS, Zhou YQ, Zhang RW, Wang MH. Ribosomal protein S6 kinase (RSK)-2 as a central effector molecule in RON receptor tyrosine kinase mediated epithelial to mesenchymal transition induced by macrophage-stimulating protein. *Mol Cancer* 2011; **10**: 66 [PMID: 21619683 DOI: 10.1186/1476-4598-10-66]
58 **Faham N**, Welm AL. RON Signaling Is a Key Mediator of Tumor Progression in Many Human Cancers. *Cold Spring Harb Symp Quant Biol* 2016; **81**: 177-188 [PMID: 28057847 DOI: 10.1101/sqb.2016.81.031377]
59 **Yao HP**, Suthe SR, Tong XM, Wang MH. Targeting RON receptor tyrosine kinase for treatment of advanced solid cancers: antibody-drug conjugates as lead drug candidates for clinical trials. *Ther Adv Med Oncol* 2020; **12**: 1758835920920069 [PMID: 32426050 DOI: 10.1177/1758835920920069]
60 **Lee WY**, Chen HH, Chow NH, Su WC, Lin PW, Guo HR. Prognostic significance of co-expression

of RON and MET receptors in node-negative breast cancer patients. *Clin Cancer Res* 2005; **11**: 2222-2228 [PMID: 15788670 DOI: 10.1158/1078-0432.CCR-04-1761]

61 **Zhao H**, Chen MS, Lo YH, Waltz SE, Wang J, Ho PC, Vasiliauskas J, Plattner R, Wang YL, Wang SC. The Ron receptor tyrosine kinase activates c-Abl to promote cell proliferation through tyrosine phosphorylation of PCNA in breast cancer. *Oncogene* 2014; **33**: 1429-1437 [PMID: 23542172 DOI: 10.1038/onc.2013.84]

62 **Privette Vinnedge LM**, Benight NM, Wagh PK, Pease NA, Nashu MA, Serrano-Lopez J, Adams AK, Cancelas JA, Waltz SE, Wells SI. The DEK oncogene promotes cellular proliferation through paracrine Wnt signaling in Ron receptor-positive breast cancers. *Oncogene* 2015; **34**: 2325-2336 [PMID: 24954505 DOI: 10.1038/onc.2014.173]

63 **Bellacosa A**. Role of MED1 (MBD4) Gene in DNA repair and human cancer. *J Cell Physiol* 2001; **187**: 137-144 [PMID: 11267993 DOI: 10.1002/jcp.1064]

64 **Villanueva A**. Hepatocellular Carcinoma. *N Engl J Med* 2019; **380**: 1450-1462 [PMID: 30970190 DOI: 10.1056/NEJMra1713263]

65 **Nault JC**, Villanueva A. Biomarkers for Hepatobiliary Cancers. *Hepatology* 2021; **73** Suppl 1: 115-127 [PMID: 32045030 DOI: 10.1002/hep.31175]

66 **Cho SB**, Park YL, Song YA, Kim KY, Lee GH, Cho DH, Myung DS, Park KJ, Lee WS, Chung IJ, Choi SK, Kim KK, Joo YE. Small interfering RNA-directed targeting of RON alters invasive and oncogenic phenotypes of human hepatocellular carcinoma cells. *Oncol Rep* 2011; **26**: 1581-1586 [PMID: 21874262 DOI: 10.3892/or.2011.1435]

67 **Koh YW**, Park YS, Kang HJ, Shim JH, Yu E. MET is a predictive factor for late recurrence but not for overall survival of early stage hepatocellular carcinoma. *Tumour Biol* 2015; **36**: 4993-5000 [PMID: 25874493 DOI: 10.1007/s13277-015-3150-7]

68 **Hayashi Y**, Yamaguchi J, Kokuryo T, Ebata T, Yokoyama Y, Igami T, Sugawara G, Nagino M. The Complete Loss of Tyrosine Kinase Receptors MET and RON Is a Poor Prognostic Factor in Patients with Extrahepatic Cholangiocarcinoma. *Anticancer Res* 2016; **36**: 6585-6592 [PMID: 27919987 DOI: 10.21873/anticanres.11263]

69 **Cheng CT**, Chen YY, Wu RC, Tsai CY, Chiang KC, Yeh TS, Chen MH, Yeh CN. METRON dual inhibitor, BMS777607, suppresses cholangiocarcinoma cell growth, and METRON upregulation indicates worse prognosis for intrahepatic cholangiocarcinoma patients. *Oncol Rep* 2018; **40**: 1411-1421 [PMID: 30015968 DOI: 10.3892/or.2018.6543]

70 **Collisson EA**, Bailey P, Chang DK, Biankin AV. Molecular subtypes of pancreatic cancer. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 207-220 [PMID: 30718832 DOI: 10.1038/s41575-019-0109-y]

71 **Kang CM**, Babicky ML, Lowy AM. The RON receptor tyrosine kinase in pancreatic cancer pathogenesis and its potential implications for future targeted therapies. *Pancreas* 2014; **43**: 183-189 [PMID: 24518495 DOI: 10.1097/MPA.0000000000000088]

72 **Babicky ML**, Harper MM, Chakedis J, Cazes A, Mose ES, Jaquish DV, French RP, Childers B, Alakus H, Schmid MC, Foubert P, Miyamoto J, Holman PJ, Walterscheid ZJ, Tang CM, Varki N, Sicklick JK, Messer K, Varner JA, Waltz SE, Lowy AM. MST1R kinase accelerates pancreatic cancer progression *via* effects on both epithelial cells and macrophages. *Oncogene* 2019; **38**: 5599-5611 [PMID: 30967626 DOI: 10.1038/s41388-019-0811-9]

73 **Yao HP**, Hudson R, Wang MH. RON receptor tyrosine kinase in pancreatic ductal adenocarcinoma: Pathogenic mechanism in malignancy and pharmaceutical target for therapy. *Biochim Biophys Acta Rev Cancer* 2020; **1873**: 188360 [PMID: 32234337 DOI: 10.1016/j.bbcan.2020.188360]

74 **Li C**, Morvaridi S, Lam G, Chheda C, Kamata Y, Katsumata M, Edderkaoui M, Yuan X, Nissen N, Pandol SJ, Wang Q. MSP-RON Signaling Is Activated in the Transition From Pancreatic Intraepithelial Neoplasia (PanIN) to Pancreatic Ductal Adenocarcinoma (PDAC). *Front Physiol* 2019; **10**: 147 [PMID: 30863319 DOI: 10.3389/fphys.2019.00147]

75 **Hu CY**, Xu XM, Hong B, Wu ZG, Qian Y, Weng TH, Liu YZ, Tang TM, Wang MH, Yao HP. Aberrant RON and MET Co-overexpression as Novel Prognostic Biomarkers of Shortened Patient Survival and Therapeutic Targets of Tyrosine Kinase Inhibitors in Pancreatic Cancer. *Front Oncol* 2019; **9**: 1377 [PMID: 31867280 DOI: 10.3389/fonc.2019.01377]

76 **Tactacan CM**, Chang DK, Cowley MJ, Humphrey ES, Wu J, Gill AJ, Chou A, Nones K, Grimmond SM, Sutherland RL, Biankin AV, Daly RJ; Australian Pancreratic Genome Initiative. RON is not a prognostic marker for resectable pancreatic cancer. *BMC Cancer* 2012; **12**: 395 [PMID: 22958871 DOI: 10.1186/1471-2407-12-395]

77 **Thomas RM**, Jaquish DV, French RP, Lowy AM. The RON tyrosine kinase receptor regulates vascular endothelial growth factor production in pancreatic cancer cells. *Pancreas* 2010; **39**: 301-307 [PMID: 20358644 DOI: 10.1097/mpa.0b013e3181bb9f73]

78 **Zou Y**, Howell GM, Humphrey LE, Wang J, Brattain MG. Ron knockdown and Ron monoclonal antibody IMC-RON8 sensitize pancreatic cancer to histone deacetylase inhibitors (HDACi). *PLoS One* 2013; **8**: e69992 [PMID: 23922886 DOI: 10.1371/journal.pone.0069992]

79 **Kawada I**, Hasina R, Arif Q, Mueller J, Smithberger E, Husain AN, Vokes EE, Salgia R. Dramatic antitumor effects of the dual MET/RON small-molecule inhibitor LY2801653 in non-small cell lung cancer. *Cancer Res* 2014; **74**: 884-895 [PMID: 24305878 DOI: 10.1158/0008-5472.CAN-12-3583]

80 **Christensen JG**, Schreck R, Burrows J, Kuruganti P, Chan E, Le P, Chen J, Wang X, Ruslim L, Blake R, Lipson KE, Ramphal J, Do S, Cui JJ, Cherrington JM, Mendel DB. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes *in vitro* and exhibits cytoreductive antitumor activity in vivo. *Cancer Res* 2003; **63**: 7345-7355 [PMID: 14612533]

81 **Liu X**, Wang Q, Yang G, Marando C, Koblish HK, Hall LM, Fridman JS, Behshad E, Wynn R, Li Y, Boer J, Diamond S, He C, Xu M, Zhuo J, Yao W, Newton RC, Scherle PA. A novel kinase inhibitor, INCB28060, blocks c-MET-dependent signaling, neoplastic activities, and cross-talk with EGFR and HER-3. *Clin Cancer Res* 2011; **17**: 7127-7138 [PMID: 21918175 DOI: 10.1158/1078-0432.CCR-11-1157]

82 **Eder JP**, Shapiro GI, Appleman LJ, Zhu AX, Miles D, Keer H, Cancilla B, Chu F, Hitchcock-Bryan S, Sherman L, McCallum S, Heath EI, Boerner SA, LoRusso PM. A phase I study of foretinib, a multi-targeted inhibitor of c-Met and vascular endothelial growth factor receptor 2. *Clin Cancer Res* 2010; **16**: 3507-3516 [PMID: 20472683 DOI: 10.1158/1078-0432.CCR-10-0574]

83 **Belalcazar A**, Azaña D, Perez CA, Raez LE, Santos ES. Targeting the Met pathway in lung cancer. *Expert Rev Anticancer Ther* 2012; **12**: 519-528 [PMID: 22500688 DOI: 10.1586/era.12.16]

84 **Sharma S**, Zeng JY, Zhuang CM, Zhou YQ, Yao HP, Hu X, Zhang R, Wang MH. Small-molecule inhibitor BMS-777607 induces breast cancer cell polyploidy with increased resistance to cytotoxic chemotherapy agents. *Mol Cancer Ther* 2013; **12**: 725-736 [PMID: 23468529 DOI: 10.1158/1535-7163.MCT-12-1079]

85 **Pan BS**, Chan GK, Chenard M, Chi A, Davis LJ, Deshmukh SV, Gibbs JB, Gil S, Hang G, Hatch H, Jewell JP, Kariv I, Katz JD, Kunii K, Lu W, Lutterbach BA, Paweletz CP, Qu X, Reilly JF, Szewczak AA, Zeng Q, Kohl NE, Dinsmore CJ. MK-2461, a novel multitargeted kinase inhibitor, preferentially inhibits the activated c-Met receptor. *Cancer Res* 2010; **70**: 1524-1533 [PMID: 20145145 DOI: 10.1158/0008-5472.CAN-09-2541]

86 **Northrup AB**, Katcher MH, Altman MD, Chenard M, Daniels MH, Deshmukh SV, Falcone D, Guerin DJ, Hatch H, Li C, Lu W, Lutterbach B, Allison TJ, Patel SB, Reilly JF, Reutershan M, Rickert KW, Rosenstein C, Soisson SM, Szewczak AA, Walker D, Wilson K, Young JR, Pan BS, Dinsmore CJ. Discovery of 1-[3-(1-methyl-1H-pyrazol-4-yl)-5-oxo-5H-benzo[4,5]cyclohepta[1,2-b]pyridin-7-yl]-N-(pyridin-2-ylmethyl)methanesulfonamide (MK-8033): A Specific c-Met/Ron dual kinase inhibitor with preferential affinity for the activated state of c-Met. *J Med Chem* 2013; **56**: 2294-2310 [PMID: 23379595 DOI: 10.1021/jm301619u]

87 **Comoglio PM**, Giordano S, Trusolino L. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat Rev Drug Discov* 2008; **7**: 504-516 [PMID: 18511928 DOI: 10.1038/nrd2530]

88 **Qin S**, Chan SL, Sukeepaisarnjaroen W, Han G, Choo SP, Sriuranpong V, Pan H, Yau T, Guo Y, Chen M, Ren Z, Xu J, Yen CJ, Lin ZZ, Manenti L, Gu Y, Sun Y, Tiedt R, Hao L, Song W, Tanwandee T. A phase II study of the efficacy and safety of the MET inhibitor capmatinib (INC280) in patients with advanced hepatocellular carcinoma. *Ther Adv Med Oncol* 2019; **11**: 1758835919889001 [PMID: 31853265 DOI: 10.1177/1758835919889001]

89 **Rimassa L**, Assenat E, Peck-Radosavljevic M, Pracht M, Zagonel V, Mathurin P, Rota Caremoli E, Porta C, Daniele B, Bolondi L, Mazzaferro V, Harris W, Damjanov N, Pastorelli D, Reig M, Knox J, Negri F, Trojan J, López López C, Personeni N, Decaens T, Dupuy M, Sieghart W, Abbadessa G, Schwartz B, Lamar M, Goldberg T, Shuster D, Santoro A, Bruix J. Tivantinib for second-line treatment of MET-high, advanced hepatocellular carcinoma (METIV-HCC): a final analysis of a phase 3, randomised, placebo-controlled study. *Lancet Oncol* 2018; **19**: 682-693 [PMID: 29625879 DOI: 10.1016/S1470-2045(18)30146-3]

90 **Guin S**, Ma Q, Padhye S, Zhou YQ, Yao HP, Wang MH. Targeting acute hypoxic cancer cells by doxorubicin-immunoliposomes directed by monoclonal antibodies specific to RON receptor tyrosine kinase. *Cancer Chemother Pharmacol* 2011; **67**: 1073-1083 [PMID: 20658288 DOI: 10.1007/s00280-010-1408-8]

91 **Feng L**, Yao HP, Wang W, Zhou YQ, Zhou J, Zhang R, Wang MH. Efficacy of anti-RON antibody Zt/g4-drug maytansinoid conjugation (Anti-RON ADC) as a novel therapeutics for targeted colorectal cancer therapy. *Clin Cancer Res* 2014; **20**: 6045-6058 [PMID: 25294907 DOI: 10.1158/1078-0432.CCR-14-0898]

92 **Yao HP**, Feng L, Suthe SR, Chen LH, Weng TH, Hu CY, Jun ES, Wu ZG, Wang WL, Kim SC, Tong XM, Wang MH. Therapeutic efficacy, pharmacokinetic profiles, and toxicological activities of humanized antibody-drug conjugate Zt/g4-MMAE targeting RON receptor tyrosine kinase for cancer therapy. *J Immunother Cancer* 2019; **7**: 75 [PMID: 30871619 DOI: 10.1186/s40425-019-0525-0]

93 **Yang CY**, Wang L, Sun X, Tang M, Quan HT, Zhang LS, Lou LG, Gou SH. SHR-A1403, a novel c-Met antibody-drug conjugate, exerts encouraging anti-tumor activity in c-Met-overexpressing models. *Acta Pharmacol Sin* 2019; **40**: 971-979 [PMID: 30643210 DOI: 10.1038/s41401-018-0198-0]

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MINIREVIEWS

Evolving role of endoscopy in inflammatory bowel disease: Going beyond diagnosis

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Abstract

Inflammatory bowel disease, encompassing Crohn's disease (CD) and ulcerative colitis, are chronic immune-mediated inflammatory bowel diseases (IBD) that primarily affect the gastrointestinal tract with periods of activity and remission. Large body of evidence exist to strengthen the prognostic role of endoscopic evaluation for both disease activity and severity and it remains the gold standard for the assessment of mucosal healing. Mucosal healing has been associated with improved clinical outcomes with prolonged remission, decreased hospitalization, IBD-related surgeries and colorectal cancer risk. Therefore, endoscopic objectives in IBD have been incorporated as part of standard care. With the known increased risk of colorectal cancer in IBD, although prevention strategies continue to develop, regular surveillance for early detection of neoplasia continue to be paramount in IBD patients' care. It is thanks to evolving technology and visualization techniques that surveillance strategies are continuously advancing. Therapeutic endoscopic options in IBD have also been expanding, from surgery sparing therapies such as balloon dilation of fibrostenotic strictures in CD to endoscopic mucosal resection of neoplastic lesions. In this review article, we discuss the current evidence on the use of endoscopy as part of standard of care of IBD, its role in surveillance of neoplasia, and the role of interventional endoscopic therapies.

Key Words: Inflammatory bowel disease; Endoscopy; Crohn's disease; Ulcerative colitis; Therapeutic endoscopy; Surveillance

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Core Tip: Endoscopy is critical to the diagnosis of inflammatory bowel diseases (IBD) and is increasingly being used for disease monitoring and management to achieve the therapeutic goal of mucosa healing. In this review, we focus on the utility of endoscopy as a therapeutic objective, in disease monitoring, and in surveillance to detect and prevent neoplasia. We will also discuss the evolving role of endoscopic therapeutic interventions in IBD.

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INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) are immune-mediated inflammatory diseases that primarily affect the gastrointestinal tract with periods of activity and remission[1,2]. The diagnosis of IBD requires endoscopic and histological evaluation of the intestinal mucosa, combined with the proper clinical presentation and setting[3]. Endoscopy allows us to differentiate CD from UC and define its severity by visualization of the colonic mucosa, its vascular pattern, mucosal lesions, as well as patterns locations of inflammation including assessment of involvement of the terminal ileum and perianal region[4].

In the absence of effective treatment, intestinal inflammation is often progressive and cumulative, leading to complications such as strictures, fistulas that require surgery, and in the long term, the risk of dysplasia or colorectal cancer (CRC)[5]. There is also evidence that such cumulative damage may result in mucosal dysfunction, with dysmotility and hypersensitivity[6]. Therefore, endoscopic control has become part of the objective treatment pillar of IBD and mucosa healing is a preferred goal of treatment[7].

In this review we will focus on the usefulness of endoscopy as a therapeutic objective, in follow-up and surveillance to detect and to prevent the development of dysplasia and CRC, and its evolving utility in interventional therapies.

ENDOSCOPY TO ASSESS DISEASE ACTIVITY

Mucosal healing is recommended as a therapeutic objective in patients with IBD. This objective is associated with a better prognosis, lower rates of hospitalizations, lower risk of relapse and the need for surgery[8,9]. After the STRIDE consensus (selecting therapeutic targets in inflammatory bowel disease), it is recommended to evaluate the colonic mucosa looking for the resolution of ulcers and the friability of the mucosa 6-9 mo after starting therapy in CD and 3-6 mo in UC[10].

Other reviews have supported this approach as well[11,12]. It has been suggested that endoscopic remission in UC should be defined as a Ulcerative Colitis Endoscopic Index of Severity (UCEIS) of 0 or a Mayo Endoscopic Score (MES) of 0 or 1. However, in follow-up studies, a higher percentage/risk of relapse has been observed in patients who had reached a MES 1 index compared to the group with a MES 0 index (36.6% *vs* 9.4% with $P < 0.001$)[13]. On the other hand, an endoscopic response has been defined as a decrease in ≥ 1 degrees of the MES or a decrease in ≥ 2 points of the UCEIS[14,15].

The Crohn's Disease Endoscopic Index of Severity (CDEIS) and Simple Endoscopic Score for Crohn's Disease (SES-CD) are validated and reproducible indices in CD[16]. However, remission thresholds have been arbitrarily determined considering endoscopic remission with a SES-CD 0-2 index and after surgery a Rutgeerts i0-i1[17,18]. Endoscopic response is defined as a decrease greater than 50% in SES-CD or CDEIS[19] (Figure 1). STRIDE-II maintained endoscopic criteria for UC and CD[20].

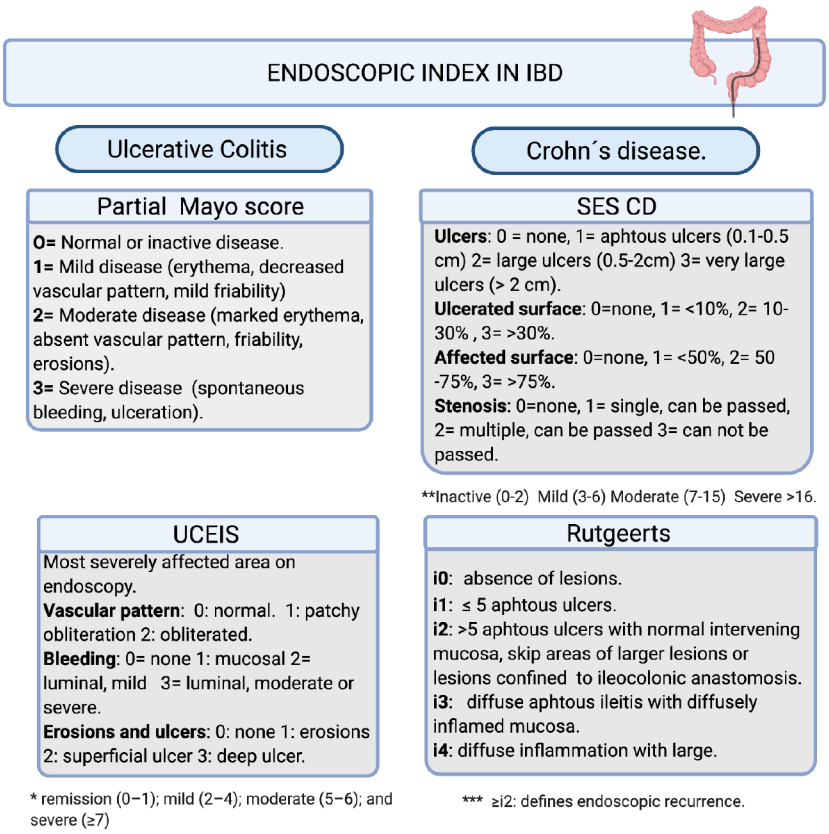


Figure 1 Endoscopic index in inflammatory bowel diseases[13-22]. The Simple endoscopic score Crohn´s disease: Sum of values of the four variables for the five bowel segments (rectum, left colon, transverse colon, right colon and ileum). UCEIS: Ulcerative colitis endoscopic index of severity; SES CD: Simple endoscopic score Crohn´s disease; IBD: Inflammatory bowel diseases.

One of the most important postoperative endoscopy studies was the multicenter POCER (postoperative Crohn's endoscopic recurrence), which included 17 centers in Australia and New Zealand[21]. Authors recommend performing a control endoscopy six months after surgery, regardless of the risk factors for recurrence, since approximately 60% may recur (80% if they have high risk factors *vs* 30% in those with low risk). In this control endoscopy the anastomosis and the terminal ileum are to be evaluated and assessed for recurrence. High risk factors for recurrence include the following: disease in those under 30 years of age, a penetrating phenotype, presence of perianal disease or having two or more surgeries[22]. The therapeutic objectives in endoscopy are fundamental to avoid future complications of the disease, and are summarized in Figure 2.

ENDOSCOPY FOR SCREENING AND SURVEILLANCE FOR COLORECTAL NEOPLASIA

CRC is the third most frequent neoplasm in the general population, with an incidence of 1.8 million new cases per year, being the second cause of cancer mortality in the world[23]. IBD presents a higher risk of CRC, and traditionally it was considered that patients with IBD had a 2% increase in CRC risk at 10 years of disease, 8% at 20 years and 18% after 30 years of evolution[24]. However, the estimated risk of neoplasia in recent years has been lower, possibly due to more effective therapies[25] as well as better prevention strategies. A subsequent meta-analysis identified a 2.4-fold risk of CRC in UC patients, with a higher risk in male patients, younger age at diagnosis and extensive disease. Cumulative absolute risk of developing CRC was 0.4 % at 10 years and 1.1%- 5.3% at 20 years[26].

Other risk factors to consider are a family history of CRC or its association with primary sclerosing cholangitis[27]. Studies in CD have identified risk factors similar to those in UC, including diagnosis at an early age, prolonged disease, distal location, and penetrating phenotype[28]. Perianal fistulas have also been identified to have a

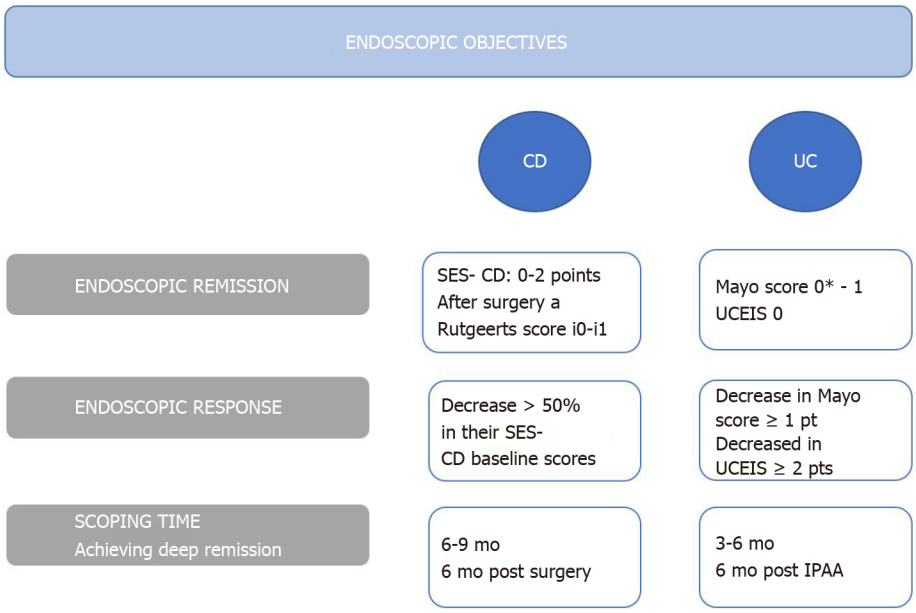


Figure 2 Endoscopic objectives in inflammatory bowel diseases treatment[8-22]. CCR: Colorectal cancer; UC: Ulcerative colitis; CD: Crohn's disease; IPAA: Ileal-pouch-anal anastomoses; SES-CD: Simple endoscopic score Crohn´s disease; UCEIS: Ulcerative colitis endoscopic index of severity.

risk of neoplastic transformation[29] (Table 1).

A screening endoscopic evaluation for dysplasia or colon cancer has been recommended 8 years after diagnosis in UC patients with an extension beyond the rectum or in CD patients with an extension over 30% of the colon or that compromises more than one segment[30]. In 2017, a Cochrane systematic review reported that those patients who were in endoscopic surveillance had a lower mortality associated with CRC compared to those who were not followed up [8.5% *vs* 22.3%, respectively; odds ratio (OR) 0.36, 95% confidence interval (CI): 0.19-0.69][31].

Endoscopic surveillance requires optimal control of the disease, including mucosal healing. This will allow for early recognition of neoplasia[3]. It has even been recommended that surveillance should be performed with fecal calprotectin levels under 100 μg/g[32] to improve visualization. It should not be forgotten that optimal bowel preparation is essential for the detection of lesions[33].

Dysplasia is defined as a neoplastic alteration of the intestinal epithelium that remains restricted within the basement membrane, without invasion of the lamina propria. These lesions can be low (LGD) or high grade (HGD). The distinction between these two depends on the distribution of nuclei within the mucosa. LGD maintains hyperchromatic nuclei located in the basal half of cells, whereas HGD presents nuclear stratification and loss of cell polarity[34].

Lesions found can be polypoid or flat, the latter being the most frequent. Flat lesions are associated with higher rate of neoplasia, common in regions that are or have been involved by IBD[35]. These lesions tend to be subtle and can be multifocal, requiring meticulous and trained endoscopists[36]. Furthermore, since dysplasia can be difficult to distinguish from epithelial regeneration secondary to inflammation[37], it is recommended that biopsies are evaluated by two expert pathologists[38].

DYSPLASIA DETECTION METHODS

Dye-based chromoendoscopy (DCE) is an image-enhanced endoscopic technique in which topical dyes such as methylene blue or indigo carmine are applied allowing a detailed view of the mucosa and a targeted evaluation of suspicious lesions[39]. It has reported a higher performance in detecting dysplasia in the analysis per patient OR 2.05 (95%CI: 1.26-3.35) and analysis by type of lesion OR 2.79 (95%CI: 2.08-3.73)[40]. On the other hand, Feuerstein *et al*[41] observed that DCE was more effective in identifying dysplasia compared to standard white light endoscopy (WLE), but without reaching significant differences compared to high-definition WLE (HD-WLE). Recently, a retrospective analysis, has shown no difference in the detection of dysplasia using DCE compared to HD-WLE, although withdrawal times were longer

Table 1 Risk factors for colitis associated neoplasia[26-29]

CRC risk		
Patient related factors	**Disease related factors**	
	UC	**CD**
Male sex; Longer duration of the disease or younger age of diagnosis; Prior History of colon neoplasia; History of CRC in first degree relative; Especially if < 50 yr old; Primary sclerosing colangitis	Extensive colitis; Severity of inflammation; Early onset of disease; Mass/stenosis; Pseudopolyps	Early onset of disease; Younger age of diagnosis; Perianal disease; Colonic extension; Bypassed segments of bowel strictures

UC: Ulcerative colitis; CD: Crohn's disease; CCR: Colorectal cancer.

with DCE (24.6 min *vs* 15.4 min, $P < 0.001$)[42].

SCENIC (Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients: International Consensus) recommends a surveillance study with high-definition colonoscopy or chromoendoscopy when standard white-light exam are performed[30]. The American College of Gastroenterology 2019 guidelines suggest targeted biopsies as an option with high-definition scopes. This approach is evolving as the technology is advancing. In the absence of augmenting imaging by high-definition or chromo, systematic sampling of the mucosa with random biopsies are recommended for every 10 cm of colon[3,37].

Virtual chromoendoscopy (VCE) [which includes narrow banding imaging (Olympus NBI), i-SCAN (Pentax) and Fujinon Fuji Intelligent Chromo-Endoscopy] is an optical imaging technique that uses filters to enhance the contrast of both the mucosa and the superficial vasculature, allowing a better evaluation of the mucosa. Bisschops *et al*[43], conducted a multicenter study with UC patients comparing DCE *vs* NBI. No significant difference was reported between these techniques in detecting neoplastic lesions (OR 1.02, 95%CI: 0.44-2.35, $P = 0.964$). The SCENIC consensus guidelines state that VCE should not replace DCE. Undoubtedly, larger studies are lacking to evaluate the usefulness of these techniques. The 2019 ACG guidelines recommend the use of DCE or NBI for the surveillance of dysplasia (conditional recommendation, low quality of evidence)[44].

MANAGEMENT OF DYSPLASIA

Once dysplasia has been identified, its respectability is essential to interrupt the carcinogenic sequence and thus reduce the incidence of CRC. The different management guidelines indicate that active surveillance and endoscopic follow-up should be performed according to the type of dysplasia found and the patient's risk factors. In Figures 3 and 4 the subsequent management is summarized. In selected patients, segmental resection without proctocolectomy is possible[45].

ENDOSCOPY FOR THERAPEUTIC INTERVENTIONS

Currently, therapeutic endoscopic interventions are considered in four areas: stenosis, fistulas, complications associated with surgery and neoplasms associated with colitis[46]. We will review the latter in a separate chapter.

Stenosis

For its diagnosis, an endoscopic evaluation and radiologic exams are required, which may be a magnetic resonance enterography or an abdomen-pelvis computed tomographic enterography[47]. It is important to record the number of stenoses, their location, their composition (inflammatory, fibrotic), morphology, size, as well as the detection of complications such as abscesses or fistulas[48].

There are three endoscopic therapeutic options in those stenoses smaller than 5 cm; endoscopic balloon dilation, endoscopic stricturotomy or stent placement, the latter with a very low level of evidence[49]. Endoscopic balloon dilation would have a lower risk of bleeding, but a higher risk of perforation. However, retrospective studies have shown it is a safe technique in patients with CD, with more than 40% of patients asymptomatic and without requiring surgery in a subsequent follow-up[50].

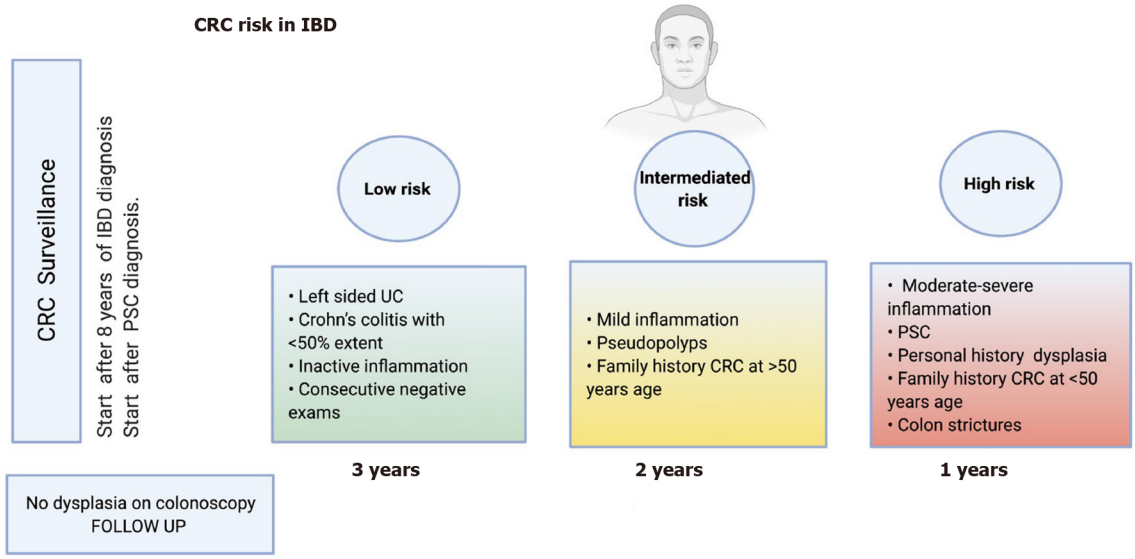


Figure 3 Inflammatory bowel diseases surveillance[3,24-28]. CCR: Colorectal cancer; IBD: Inflammatory bowel diseases; UC: Ulcerative colitis.

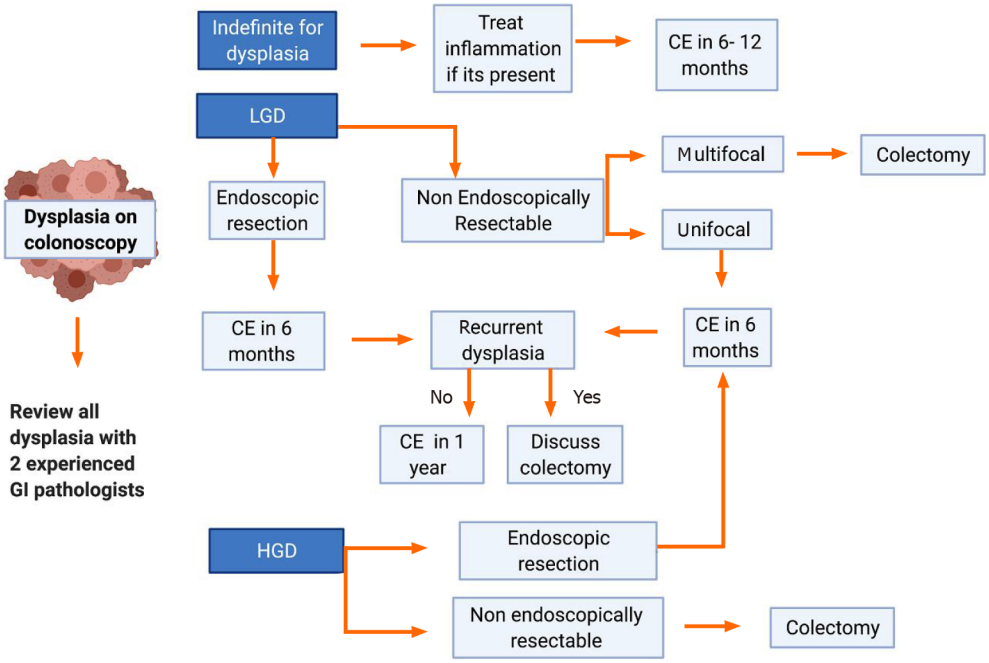


Figure 4 Algorithm for the management of dysplasia[3,37,42-46]. LGD: Low grade dysplasia; HGD: High grade dysplasia; CE: Chromoendoscopy.

Endoscopic stricturotomy is in evolution and may be an effective procedure in treating fibrotic, distal or anastomotic stenosis. The technique is based on an electro-incision, allowing control of the depth and location of the cut, and with a lower risk of perforation. In a small reported experience surgery free survival in IBD to non-IBD patients undergoing endoscopic stricturotomy, there was no statistically significant difference between the groups[51].

Endoscopic injections of steroids or anti-tumor necrosis factor agents in conjunction with endoscopic balloon dilation have been reported in series of cases with the intention of reducing the need for future dilation with inconsistent results[52,53].

In recurrent or refractory stenoses, self-expanding metal stents, covered metal stents or biodegradable metal stents have been used with good results[54,55]. These can be placed endoscopically with or without fluoroscopic guidance but must be maintained for at least 4 wk[56].

Fistulas

The penetrating phenotype may be primary or a result of a long-standing CD. The goals of endoscopic treatment are drainage, closure of the fistulas and preventing them from becoming complex[48]. In a cohort of 29 patients, about 90% achieved resolution of their fistulas by endoscopic fistulotomy[57]. This type of therapy might be performed in superficial, short and enteroenteric fistulas[56]. In addition, it is possible to close the fistula endoscopically by means of a clip, avoiding the formation of abscesses. This has been reported to be achieved with through-the-scope clips or over-the-scope clips, even with reports in perianal fistulas[48]. Further work is needed in this area, with clarification of risks, benefits, and approach to combination with medical interventions.

Post-operative complications

Post-surgery complications may present as dehiscence of the suture/staple line or present later with stenosis of the anastomosis, causing obstructive problems[58]. Endoscopic management of suture dehiscence has been described in clinical cases or case series, reporting lumen integrity in over 80%[59]. Approximately 11% of patients with UC require an ileal pouch anal anastomoses for their management[60], where strictures may develop in anastomosis, in the pouch or in the afferent loop. Postoperative strictures can be managed endoscopically with balloon dilations or stricturotomy. Endoscopic interventions should be avoided in periods of increased inflammation given the increased risk of perforation[21].

CONCLUSION

Endoscopic evaluation is essential to achieve current therapeutic goals in IBD, which focus on mucosal healing[61,62]. In cases in which the objectives are not achieved, endoscopic assessments direct therapeutic optimization in order to achieve them. Importantly, endoscopic assessment has a key role in prognostication and disease monitoring to control modifiable risk factors for worse clinical outcomes, with a focus on control of inflammation and prevention of complications. Among these, the incidence of CRC has a significant impact on patient morbidity and mortality, and its early diagnosis is a crucial element in IBD management. The evolution of endoscopic visualization techniques, such as the use of VCE and DCE, allow a better detection and characterization of premalignant endoscopic lesions, avoiding the risks of advanced stages[63]. Additionally, evolving endoscopic therapies allow greater minimally invasive therapeutic options for the treatment of stenosis and fistulas as an alternative to surgical management with lower morbidity.

REFERENCES

1 **Ungaro R**, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis. *Lancet* 2017; **389**: 1756-1770 [PMID: 27914657 DOI: 10.1016/S0140-6736(16)32126-2]

2 **Loftus EV Jr**. Crohn's Disease: Etiology, Complications, Assessment, Therapy, and Management. *Gastroenterol Clin North Am* 2017; **46**: xiii-xixv [PMID: 28838422 DOI: 10.1016/j.gtc.2017.06.001]

3 **Maaser C**, Sturm A, Vavricka SR, Kucharzik T, Fiorino G, Annese V, Calabrese E, Baumgart DC, Bettenworth D, Borralho Nunes P, Burisch J, Castiglione F, Eliakim R, Ellul P, González-Lama Y, Gordon H, Halligan S, Katsanos K, Kopylov U, Kotze PG, Krustinš E, Laghi A, Limdi JK, Rieder F, Rimola J, Taylor SA, Tolan D, van Rheenen P, Verstockt B, Stoker J; European Crohn's and Colitis Organisation [ECCO] and the European Society of Gastrointestinal and Abdominal Radiology [ESGAR]. ECCO-ESGAR Guideline for Diagnostic Assessment in IBD Part 1: Initial diagnosis, monitoring of known IBD, detection of complications. *J Crohns Colitis* 2019; **13**: 144-164 [PMID: 30137275 DOI: 10.1093/ecco-jcc/jjy113]

4 **Zilli A**, Capogreco A, Furfaro F, Allocca M, Roda G, Loy L, Fiorino G, Danese S. Improving quality of care in endoscopy of inflammatory bowel disease: can we do better? *Expert Rev Gastroenterol Hepatol* 2020; **14**: 819-828 [PMID: 32543983 DOI: 10.1080/17474124.2020.1780913]

5 **Vatn MH**. Natural history and complications of IBD. *Curr Gastroenterol Rep* 2009; **11**: 481-487 [PMID: 19903424 DOI: 10.1007/s11894-009-0073-8]

6 **Torres J**, Billioud V, Sachar DB, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis as a progressive disease: the forgotten evidence. *Inflamm Bowel Dis* 2012; **18**: 1356-1363 [PMID: 22162423 DOI: 10.1002/ibd.22839]

7 **Nuñez F P**, Mahadevan U, Quera R, Bay C, Ibañez P. Treat-to-target approach in the management of inflammatory Bowel disease. *Gastroenterol Hepatol* 2021; **44**: 312-319 [PMID: 33070988 DOI:

10.1016/j.gastrohep.2020.06.032]
8 **Colombel JF**, Rutgeerts P, Reinisch W, Esser D, Wang Y, Lang Y, Marano CW, Strauss R, Oddens BJ, Feagan BG, Hanauer SB, Lichtenstein GR, Present D, Sands BE, Sandborn WJ. Early mucosal healing with infliximab is associated with improved long-term clinical outcomes in ulcerative colitis. *Gastroenterology* 2011; **141**: 1194-1201 [PMID: 21723220 DOI: 10.1053/j.gastro.2011.06.054]
9 **Colombel JF**, Rutgeerts PJ, Sandborn WJ, Yang M, Camez A, Pollack PF, Thakkar RB, Robinson AM, Chen N, Mulani PM, Chao J. Adalimumab induces deep remission in patients with Crohn's disease. *Clin Gastroenterol Hepatol* 2014; **12**: 414-22. e5 [PMID: 23856361 DOI: 10.1016/j.cgh.2013.06.019]
10 **Peyrin-Biroulet L**, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant RV, D'Haens G, Dotan I, Dubinsky M, Feagan B, Fiorino G, Gearry R, Krishnareddy S, Lakatos PL, Loftus EV Jr, Marteau P, Munkholm P, Murdoch TB, Ordás I, Panaccione R, Riddell RH, Ruel J, Rubin DT, Samaan M, Siegel CA, Silverberg MS, Stoker J, Schreiber S, Travis S, Van Assche G, Danese S, Panes J, Bouguen G, O'Donnell S, Pariente B, Winer S, Hanauer S, Colombel JF. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for Treat-to-Target. *Am J Gastroenterol* 2015; **110**: 1324-1338 [PMID: 26303131 DOI: 10.1038/ajg.2015.233]
11 **Ungaro R**, Colombel JF, Lissoos T, Peyrin-Biroulet L. A Treat-to-Target Update in Ulcerative Colitis: A Systematic Review. *Am J Gastroenterol* 2019; **114**: 874-883 [PMID: 30908297 DOI: 10.14309/ajg.0000000000000183]
12 **Rutgeerts P**, Diamond RH, Bala M, Olson A, Lichtenstein GR, Bao W, Patel K, Wolf DC, Safdi M, Colombel JF, Lashner B, Hanauer SB. Scheduled maintenance treatment with infliximab is superior to episodic treatment for the healing of mucosal ulceration associated with Crohn's disease. *Gastrointest Endosc* 2006; **63**: 433-442; quiz 464 [PMID: 16500392 DOI: 10.1016/j.gie.2005.08.011]
13 **Barreiro-de Acosta M**, Vallejo N, de la Iglesia D, Uribarri L, Bastón I, Ferreiro-Iglesias R, Lorenzo A, Domínguez-Muñoz JE. Evaluation of the Risk of Relapse in Ulcerative Colitis According to the Degree of Mucosal Healing (Mayo 0 *vs* 1): A Longitudinal Cohort Study. *J Crohns Colitis* 2016; **10**: 13-19 [PMID: 26351390 DOI: 10.1093/ecco-jcc/jjv158]
14 **Vuitton L**, Peyrin-Biroulet L, Colombel JF, Pariente B, Pineton de Chambrun G, Walsh AJ, Panes J, Travis SP, Mary JY, Marteau P. Defining endoscopic response and remission in ulcerative colitis clinical trials: an international consensus. *Aliment Pharmacol Ther* 2017; **45**: 801-813 [PMID: 28112419 DOI: 10.1111/apt.13948]
15 **Negreanu L**, Voiosu T, State M, Voiosu A, Bengus A, Mateescu BR. Endoscopy in inflammatory bowel disease: from guidelines to real life. *Therap Adv Gastroenterol* 2019; **12**: 1756284819865153 [PMID: 31384307 DOI: 10.1177/1756284819865153]
16 **Daperno M**, Comberlato M, Bossa F, Biancone L, Bonanomi AG, Cassinotti A, Cosintino R, Lombardi G, Mangiarotti R, Papa A, Pica R, Rizzello F, D'Incà R, Orlando A. Inter-observer agreement in endoscopic scoring systems: preliminary report of an ongoing study from the Italian Group for Inflammatory Bowel Disease (IG-IBD). *Dig Liver Dis* 2014; **46**: 969-973 [PMID: 25154049 DOI: 10.1016/j.dld.2014.07.010]
17 **Rutgeerts P**, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology* 1990; **99**: 956-963 [PMID: 2394349 DOI: 10.1016/0016-5085(90)90613-6]
18 **Rivière P**, Vermeire S, Irles-Depe M, Van Assche G, Rutgeerts P, de Buck van Overstraeten A, Denost Q, Wolthuis A, D'Hoore A, Laharie D, Ferrante M. No Change in Determining Crohn's Disease Recurrence or Need for Endoscopic or Surgical Intervention With Modification of the Rutgeerts' Scoring System. *Clin Gastroenterol Hepatol* 2019; **17**: 1643-1645 [PMID: 30291910 DOI: 10.1016/j.cgh.2018.09.047]
19 **Vuitton L**, Marteau P, Sandborn WJ, Levesque BG, Feagan B, Vermeire S, Danese S, D'Haens G, Lowenberg M, Khanna R, Fiorino G, Travis S, Mary JY, Peyrin-Biroulet L. IOIBD technical review on endoscopic indices for Crohn's disease clinical trials. *Gut* 2016; **65**: 1447-1455 [PMID: 26353983 DOI: 10.1136/gutjnl-2015-309903]
20 **Turner D**, Ricciuto A, Lewis A, D'Amico F, Dhaliwal J, Griffiths AM, Bettenworth D, Sandborn WJ, Sands BE, Reinisch W, Schölmerich J, Bemelman W, Danese S, Mary JY, Rubin D, Colombel JF, Peyrin-Biroulet L, Dotan I, Abreu MT, Dignass A; International Organization for the Study of IBD. STRIDE-II: An Update on the Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE) Initiative of the International Organization for the Study of IBD (IOIBD): Determining Therapeutic Goals for Treat-to-Target strategies in IBD. *Gastroenterology* 2021; **160**: 1570-1583 [PMID: 33359090 DOI: 10.1053/j.gastro.2020.12.031]
21 **De Cruz P**, Kamm MA, Hamilton AL, Ritchie KJ, Krejany EO, Gorelik A, Liew D, Prideaux L, Lawrance IC, Andrews JM, Bampton PA, Gibson PR, Sparrow M, Leong RW, Florin TH, Gearry RB, Radford-Smith G, Macrae FA, Debinski H, Selby W, Kronborg I, Johnston MJ, Woods R, Elliott PR, Bell SJ, Brown SJ, Connell WR, Desmond PV. Crohn's disease management after intestinal resection: a randomised trial. *Lancet* 2015; **385**: 1406-1417 [PMID: 25542620 DOI: 10.1016/S0140-6736(14)61908-5]
22 **Bhattacharya A**, Shen B, Regueiro M. Endoscopy in Postoperative Patients with Crohn's Disease or Ulcerative Colitis. Does It Translate to Better Outcomes? *Gastrointest Endosc Clin N Am* 2019; **29**: 487-514 [PMID: 31078249 DOI: 10.1016/j.giec.2019.02.013]
23 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018:

GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

24 **Eaden JA**, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; **48**: 526-535 [PMID: 11247898 DOI: 10.1136/gut.48.4.526]

25 **Herrinton LJ**, Liu L, Levin TR, Allison JE, Lewis JD, Velayos F. Incidence and mortality of colorectal adenocarcinoma in persons with inflammatory bowel disease from 1998 to 2010. *Gastroenterology* 2012; **143**: 382-389 [PMID: 22609382 DOI: 10.1053/j.gastro.2012.04.054]

26 **Jess T**, Rungoe C, Peyrin-Biroulet L. Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies. *Clin Gastroenterol Hepatol* 2012; **10**: 639-645 [PMID: 22289873 DOI: 10.1016/j.cgh.2012.01.010]

27 **Beaugerie L**, Itzkowitz SH. Cancers complicating inflammatory bowel disease. *N Engl J Med* 2015; **372**: 1441-1452 [PMID: 25853748 DOI: 10.1056/NEJMra1403718]

28 **Scaringi S**, Di Martino C, Zambonin D, Fazi M, Canonico G, Leo F, Ficari F, Tonelli F. Colorectal cancer and Crohn's colitis: clinical implications from 313 surgical patients. *World J Surg* 2013; **37**: 902-910 [PMID: 23381673 DOI: 10.1007/s00268-013-1922-z]

29 **Connell WR**, Sheffield JP, Kamm MA, Ritchie JK, Hawley PR, Lennard-Jones JE. Lower gastrointestinal malignancy in Crohn's disease. *Gut* 1994; **35**: 347-352 [PMID: 8150345 DOI: 10.1136/gut.35.3.347]

30 **Laine L**, Kaltenbach T, Barkun A, McQuaid KR, Subramanian V, Soetikno R; SCENIC Guideline Development Panel. SCENIC international consensus statement on surveillance and management of dysplasia in inflammatory bowel disease. *Gastroenterology* 2015; **148**: 639-651. e28 [PMID: 25702852 DOI: 10.1053/j.gastro.2015.01.031]

31 **Bye WA**, Nguyen TM, Parker CE, Jairath V, East JE. Strategies for detecting colon cancer in patients with inflammatory bowel disease. *Cochrane Database Syst Rev* 2017; **9**: CD000279 [PMID: 28922695 DOI: 10.1002/14651858.CD000279.pub4]

32 **Iacucci M**, Cannatelli R, Tontini GE, Panaccione R, Danese S, Fiorino G, Matsumoto T, Kochhar GS, Shen B, Kiesslich R, Ghosh S. Improving the quality of surveillance colonoscopy in inflammatory bowel disease. *Lancet Gastroenterol Hepatol* 2019; **4**: 971-983 [PMID: 31696831 DOI: 10.1016/S2468-1253(19)30194-3]

33 **Megna B**, Weiss J, Ley D, Saha S, Pfau P, Grimes I, Li Z, Caldera F. Clear liquid diet before bowel preparation predicts successful chromoendoscopy in patients with inflammatory bowel disease. *Gastrointest Endosc* 2019; **89**: 373-379. e2 [PMID: 30339950 DOI: 10.1016/j.gie.2018.09.039]

34 **Itzkowitz SH**, Harpaz N. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. *Gastroenterology* 2004; **126**: 1634-1648 [PMID: 15168373 DOI: 10.1053/j.gastro.2004.03.025]

35 **Vieth M**, Behrens H, Stolte M. Sporadic adenoma in ulcerative colitis: endoscopic resection is an adequate treatment. *Gut* 2006; **55**: 1151-1155 [PMID: 16423892 DOI: 10.1136/gut.2005.075531]

36 **Lakatos PL**, Lakatos L. Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies. *World J Gastroenterol* 2008; **14**: 3937-3947 [PMID: 18609676 DOI: 10.3748/wjg.14.3937]

37 **Mattar MC**, Lough D, Pishvaian MJ, Charabaty A. Current management of inflammatory bowel disease and colorectal cancer. *Gastrointest Cancer Res* 2011; **4**: 53-61 [PMID: 21673876]

38 **Cairns SR**, Scholefield JH, Steele RJ, Dunlop MG, Thomas HJ, Evans GD, Eaden JA, Rutter MD, Atkin WP, Saunders BP, Lucassen A, Jenkins P, Fairclough PD, Woodhouse CR; British Society of Gastroenterology; Association of Coloproctology for Great Britain and Ireland. Guidelines for colorectal cancer screening and surveillance in moderate and high risk groups (update from 2002). *Gut* 2010; **59**: 666-689 [PMID: 20427401 DOI: 10.1136/gut.2009.179804]

39 **American Society for Gastrointestinal Endoscopy Standards of Practice Committee**. , Shergill AK, Lightdale JR, Bruining DH, Acosta RD, Chandrasekhara V, Chathadi KV, Decker GA, Early DS, Evans JA, Fanelli RD, Fisher DA, Fonkalsrud L, Foley K, Hwang JH, Jue TL, Khashab MA, Muthusamy VR, Pasha SF, Saltzman JR, Sharaf R, Cash BD, DeWitt JM. The role of endoscopy in inflammatory bowel disease. *Gastrointest Endosc* 2015; **81**: 1101-21. e1-13 [PMID: 25800660 DOI: 10.1016/j.gie.2014.10.030]

40 **Har-Noy O**, Katz L, Avni T, Battat R, Bessissow T, Yung DE, Engel T, Koulaouzidis A, Eliakim R, Ben-Horin S, Kopylov U. Chromoendoscopy, Narrow-Band Imaging or White Light Endoscopy for Neoplasia Detection in Inflammatory Bowel Diseases. *Dig Dis Sci* 2017; **62**: 2982-2990 [PMID: 28965228 DOI: 10.1007/s10620-017-4772-y]

41 **Feuerstein JD**, Rakowsky S, Sattler L, Yadav A, Foromera J, Grossberg L, Cheifetz AS. Meta-analysis of dye-based chromoendoscopy compared with standard- and high-definition white-light endoscopy in patients with inflammatory bowel disease at increased risk of colon cancer. *Gastrointest Endosc* 2019; **90**: 186-195. e1 [PMID: 31009609 DOI: 10.1016/j.gie.2019.04.219]

42 **Clarke K**, Kang M, Gorrepati VS, Stine JG, Tinsley A, Williams E, Moyer M, Coates M. Dysplasia detection is similar between chromoendoscopy and high-definition white-light colonoscopy in inflammatory bowel disease patients: a US-matched case-control study. *Int J Colorectal Dis* 2020; **35**: 2301-2307 [PMID: 32812090 DOI: 10.1007/s00384-020-03719-3]

43 **Bisschops R**, Bessissow T, Joseph JA, Baert F, Ferrante M, Ballet V, Willekens H, Demedts I, Geboes K, De Hertogh G, Vermeire S, Rutgeerts P, Van Assche G. Chromoendoscopy *vs* narrow band imaging in UC: a prospective randomised controlled trial. *Gut* 2018; **67**: 1087-1094 [PMID: 28698230 DOI: 10.1136/gutjnl-2016-313213]

44 **Rubin DT**, Ananthakrishnan AN, Siegel CA, Sauer BG, Long MD. ACG Clinical Guideline: Ulcerative Colitis in Adults. *Am J Gastroenterol* 2019; **114**: 384-413 [PMID: 30840605 DOI: 10.14309/ajg.0000000000000152]

45 **Krugliak Cleveland N**, Ollech JE, Colman RJ, Rodriquez D, Hirsch A, Cohen RD, Hanauer SB, Hart J, Hurst R, Rubin DT. Efficacy and Follow-up of Segmental or Subtotal Colectomy in Patients With Colitis-Associated Neoplasia. *Clin Gastroenterol Hepatol* 2019; **17**: 205-206 [PMID: 29751167 DOI: 10.1016/j.cgh.2018.04.061]

46 **Shen B**, Kochhar G, Hull TL. Bridging Medical and Surgical Treatment of Inflammatory Bowel Disease: The Role of Interventional IBD. *Am J Gastroenterol* 2019; **114**: 539-540 [PMID: 30464306 DOI: 10.1038/s41395-018-0416-x]

47 **Fletcher JG**, Fidler JL, Bruining DH, Huprich JE. New concepts in intestinal imaging for inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1795-1806 [PMID: 21530746 DOI: 10.1053/j.gastro.2011.02.013]

48 **Gu YB**, Zhong J; Chinese IBD Endoscopic Club. Endoscopic management of stricturing Crohn's disease. *J Dig Dis* 2020; **21**: 351-354 [PMID: 32537911 DOI: 10.1111/1751-2980.12914]

49 **Shen B**, Kochhar G, Navaneethan U, Liu X, Farraye FA, Gonzalez-Lama Y, Bruining D, Pardi DS, Lukas M, Bortlik M, Wu K, Sood A, Schwartz DA, Sandborn WJ; Global Interventional Inflammatory Bowel Disease Group. Role of interventional inflammatory bowel disease in the era of biologic therapy: a position statement from the Global Interventional IBD Group. *Gastrointest Endosc* 2019; **89**: 215-237 [PMID: 30365985 DOI: 10.1016/j.gie.2018.09.045]

50 **Tilmant M**, Serrero M, Poullenot F, Bouguen G, Pariente B, Altwegg R, Basile P, Filippi J, Vanelslander P, Buisson A, Desjeux A, Laharie D, Le Balch E, Nachury M, Boivineau L, Savoye G, Hebuterne X, Poincloux L, Vuitton L, Brazier F, Yzet C, Lamrani A, Peyrin-Biroulet L, Fumery M. Endoscopic balloon dilation of colorectal strictures complicating Crohn's disease: a multicenter study. *Clin Res Hepatol Gastroenterol* 2020; 101561 [PMID: 33214090 DOI: 10.1016/j.clinre.2020.10.006]

51 **Zhang LJ**, Lan N, Wu XR, Shen B. Endoscopic stricturotomy in the treatment of anastomotic strictures in inflammatory bowel disease (IBD) and non-IBD patients. *Gastroenterol Rep (Oxf)* 2020; **8**: 143-150 [PMID: 32280474 DOI: 10.1093/gastro/goz051]

52 **Swaminath A**, Lichtiger S. Dilation of colonic strictures by intralesional injection of infliximab in patients with Crohn's colitis. *Inflamm Bowel Dis* 2008; **14**: 213-216 [PMID: 18022870 DOI: 10.1002/ibd.20318]

53 **Di Nardo G**, Oliva S, Passariello M, Pallotta N, Civitelli F, Frediani S, Gualdi G, Gandullia P, Mallardo S, Cucchiara S. Intralesional steroid injection after endoscopic balloon dilation in pediatric Crohn's disease with stricture: a prospective, randomized, double-blind, controlled trial. *Gastrointest Endosc* 2010; **72**: 1201-1208 [PMID: 20951986 DOI: 10.1016/j.gie.2010.08.003]

54 **Matsuhashi N**, Nakajima A, Suzuki A, Yazaki Y, Takazoe M. Long-term outcome of non-surgical strictureplasty using metallic stents for intestinal strictures in Crohn's disease. *Gastrointest Endosc* 2000; **51**: 343-345 [PMID: 10699786 DOI: 10.1016/s0016-5107(00)70366-x]

55 **Rodrigues C**, Oliveira A, Santos L, Pires E, Deus J. Biodegradable stent for the treatment of a colonic stricture in Crohn's disease. *World J Gastrointest Endosc* 2013; **5**: 265-269 [PMID: 23678382 DOI: 10.4253/wjge.v5.i5.265]

56 **Chen M**, Shen B. Endoscopic therapy for Kock pouch strictures in patients with inflammatory bowel disease. *Gastrointest Endosc* 2014; **80**: 353-359 [PMID: 24852104 DOI: 10.1016/j.gie.2014.03.039]

57 **Kochhar G**, Shen B. Endoscopic fistulotomy in inflammatory bowel disease (with video). *Gastrointest Endosc* 2018; **88**: 87-94 [PMID: 29501418 DOI: 10.1016/j.gie.2018.02.034]

58 **Chen M**, Shen B. Endoscopic Therapy in Crohn's Disease: Principle, Preparation, and Technique. *Inflamm Bowel Dis* 2015; **21**: 2222-2240 [PMID: 26284298 DOI: 10.1097/MIB.0000000000000433]

59 **Verlaan T**, Bartels SA, van Berge Henegouwen MI, Tanis PJ, Fockens P, Bemelman WA. Early, minimally invasive closure of anastomotic leaks: a new concept. *Colorectal Dis* 2011; **13** Suppl 7: 18-22 [PMID: 22098512 DOI: 10.1111/j.1463-1318.2011.02775.x]

60 **Prudhomme M**, Dozois RR, Godlewski G, Mathison S, Fabbro-Peray P. Anal canal strictures after ileal pouch-anal anastomosis. *Dis Colon Rectum* 2003; **46**: 20-23 [PMID: 12544517 DOI: 10.1007/s10350-004-6491-7]

61 **Agrawal M**, Colombel JF. Treat-to-Target in Inflammatory Bowel Diseases, What Is the Target and How Do We Treat? *Gastrointest Endosc Clin N Am* 2019; **29**: 421-436 [PMID: 31078245 DOI: 10.1016/j.giec.2019.02.004]

62 **Gupta A**, Yu A, Peyrin-Biroulet L, Ananthakrishnan AN. Treat to Target: The Role of Histologic Healing in Inflammatory Bowel Diseases: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2020 [PMID: 33010406 DOI: 10.1016/j.cgh.2020.09.046]

63 **Scoville EA**, Schwartz DA. Endoscopy in inflammatory bowel disease: advances in disease management. *Gastrointest Endosc* 2017; **86**: 952-961 [PMID: 28886962 DOI: 10.1016/j.gie.2017.08.034]

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MINIREVIEWS

Deep learning for diagnosis of precancerous lesions in upper gastrointestinal endoscopy: A review

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Abstract

Upper gastrointestinal (GI) cancers are the leading cause of cancer-related deaths worldwide. Early identification of precancerous lesions has been shown to minimize the incidence of GI cancers and substantiate the vital role of screening endoscopy. However, unlike GI cancers, precancerous lesions in the upper GI tract can be subtle and difficult to detect. Artificial intelligence techniques, especially deep learning algorithms with convolutional neural networks, might help endoscopists identify the precancerous lesions and reduce interobserver variability. In this review, a systematic literature search was undertaken of the Web of Science, PubMed, Cochrane Library and Embase, with an emphasis on the deep learning-based diagnosis of precancerous lesions in the upper GI tract. The status of deep learning algorithms in upper GI precancerous lesions has been systematically summarized. The challenges and recommendations targeting this field are comprehensively analyzed for future research.

Key Words: Artificial intelligence; Deep learning; Convolutional neural network; Precancerous lesions; Endoscopy

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Core Tip: Artificial intelligence techniques, especially deep learning algorithms with convolutional neural networks, have revolutionized upper gastrointestinal endoscopy. In recent years, several deep learning-based artificial intelligence systems have emerged in the gastrointestinal community for endoscopic detection of precancerous lesions. The current review provides an analysis and status of the deep learning-based

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diagnosis of precancerous lesions in the upper gastrointestinal tract and identifies future challenges and recommendations.

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INTRODUCTION

Upper gastrointestinal (GI) cancers, mainly including gastric cancer (8.2% of total cancer deaths) and esophageal cancer (5.3% of total cancer deaths), are the leading cause of cancer-related deaths worldwide[1]. Previous studies have shown that upper GI cancers always go through the stages of precancerous lesions, which can be defined as common conditions associated with a higher risk of developing cancers over time[2-4]. The detection of the precancerous lesions before cancer occurs could significantly reduce morbidity and mortality rates[5,6]. Currently, the main approach for the diagnosis of disorders or issues in the upper GI tract is endoscopy[7,8]. Compared with GI cancers, which usually show typical morphological characteristics, the precancerous lesions often appear in flat mucosa and exhibit few morphological changes. Manual screening through endoscopy is labor-intensive, time-consuming and relies heavily on clinical experience. Computer-assisted diagnosis based on artificial intelligence (AI) can overcome these dilemmas.

Over the past few decades, AI techniques such as machine learning (ML) and deep learning (DL) have been widely used in endoscopic imaging to improve the diagnostic accuracy and efficiency of various GI lesions[9-13]. The exact definition of AI, ML and DL can be misunderstood by physicians. AI, ML and DL are overlapping disciplines (Figure 1). AI is a hierarchy that encompasses ML and DL; it describes a computerized solution to address the issues of human cognitive defined by McCarthy in 1956[14]. ML is a subset of AI in which algorithms can execute complex tasks, but it needs handcrafted feature extraction. ML originated around the 1980s and focuses on patterns and inference[15]. DL is a subset of ML and became feasible in the 2010s; it is focused specifically on deep neural networks. A convolutional neural network (CNN) is the primary DL algorithm for image processing[16,17].

The diagnostic process of an AI model is similar to the human brain. We take our previous research as an example to illustrate the diagnostic process of ML, DL and human experts. When an input image with gastric intestinal metaplasia (GIM), a precancerous lesion of gastric cancer, is fed into the ML system, it usually needs a manual feature extraction step, while the handcrafted features are unable to discern slight variations in the endoscopic image (Figure 2)[15]. Unlike conventional ML algorithms, CNNs can automatically learn representative features from the endoscopic images[17]. When we apply a CNN model to detect GIM, it performs better than conventional ML models and is comparable to experienced endoscopists[18]. For a broad variety of image processing activities in endoscopy, CNNs also show excellent results, and some CNN-based algorithms have been used in clinical practice[19-21]. However, DL, especially CNN, has some limitations. First, DL requires a lot of data and easily leads to overfitting. Second, the diagnostic accuracy of DL relies on the training data, but the clinical data of different types of diseases are always imbalanced, which easily causes diagnosis bias. In addition, a DL model is complex and requires a huge calculation, so most researchers can only use the ready-made model.

Despite the above limitations, DL-based AI systems are revolutionizing GI endoscopy. While there are several surveys on DL for GI cancers[9-13], no specific review on the application of DL in the endoscopic diagnosis of precancerous lesions is available in the literature. Therefore, the performance of DL on gastroenterology is summarized in this review, with an emphasis on the automatic diagnosis of precancerous lesions in the upper GI tract. GI cancers are out of the scope of this review. Specifically, we review the status of intelligent diagnoses of esophageal and gastric precancerous lesions. The challenges and recommendations based on the findings of the review are comprehensively analyzed to advance the field.

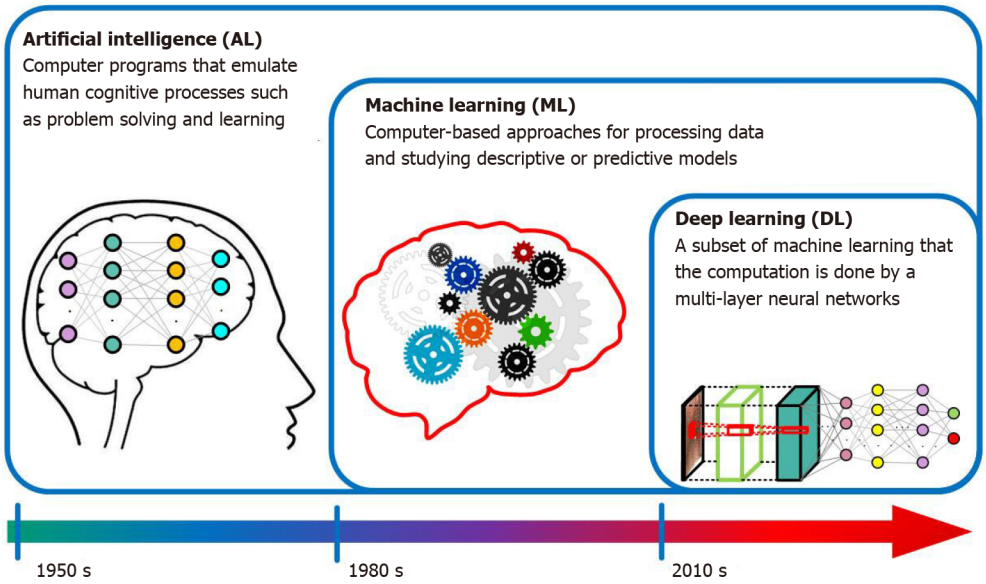


Figure 1 Infographic with icons and timeline for artificial intelligence, machine learning and deep learning.

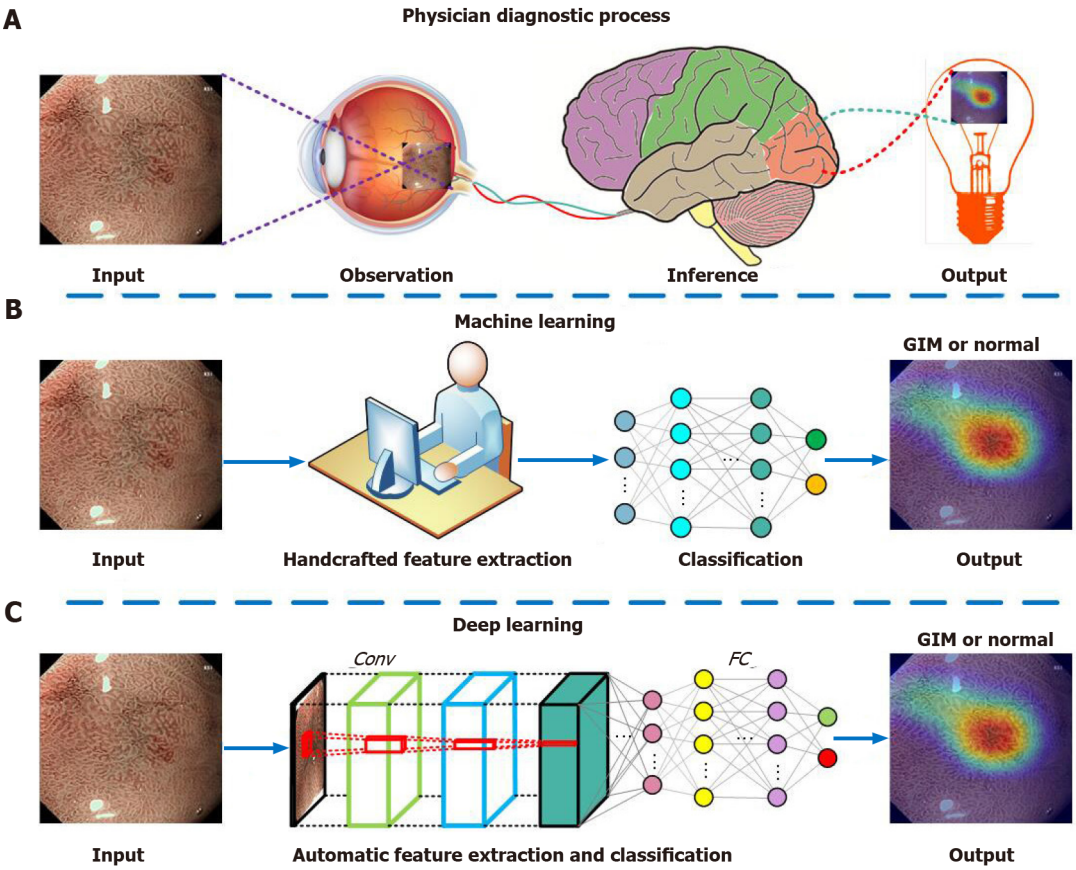


Figure 2 Illustration of the diagnostic process of physician, machine learning and deep learning. A: Physician diagnostic process; B: Machine learning; C: Deep learning. Conv: Convolutional layer; FC: Fully connected layer; GIM: gastric intestinal metaplasia.

DL IN ENDOSCOPIC DETECTION OF PRECANCEROUS LESIONS IN ESOPHAGEAL MUCOSA

Esophageal cancer is the eighth most prevalent form of cancer and the sixth most lethal cancer globally[1]. There are two major subtypes of esophageal cancer: Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC)[22]. Esophageal squamous dysplasia (ESD) is believed to be the precancerous

lesion of ESCC[23-25], and Barrett's esophagus (BE) is the identifiable precancerous lesion associated with EAC[4,26]. Endoscopic surveillance is recommended by GI societies to enable early detection of the two precancerous lesions of esophageal cancer[25,27].

However, the current endoscopic diagnosis methods for patients with BE, such as random 4-quadrant biopsy, laser-induced endomicroscopy, image enhanced endoscopy, *etc*., have disadvantages concerning the learning curve, cost, interobserver variability and time-consuming problems[28]. The current standard for identifying ESD is Lugol's chromoendoscopy, but it shows poor specificity[29]. Besides, iodine staining often presents a risk of allergic reactions. To overcome these challenges, DL-based AI systems have been established to help endoscopists identify ESD and BE.

DL in ESD

Low-grade and high-grade intraepithelial neoplasms, collectively referred to as ESD, are deemed as precancerous lesions of ESCC. Early and accurate detection of ESD is essential but also full of challenges[23-25]. DL is reliably able to depict ESD in real-time upper endoscopy. Cai *et al*[30] designed a novel computer-assisted diagnosis system to localize and identify early ESCC, including low-grade and high-grade intraepithelial neoplasia, through real-time white light endoscopy (WLE). The system achieved a sensitivity, specificity and accuracy of 97.8%, 85.4% and 91.4%, respectively. They also demonstrated that when referring to the results of the system, the overall diagnostic capability of the endoscopist has been increased. This research paved the way for the real-time diagnosis of ESD and ESCC. Following this work, Guo *et al*[31] applied 6473 narrow band (NB) images to train a real-time automated computer-assisted diagnosis system to support non-experts in the detection of ESD and ESCC. The system serves as a "second observer" in an endoscopic examination and achieves a sensitivity of 98.04% and specificity of 95.30% on NB images. The per-frame sensitivity was 96.10% for magnifying narrow band imaging (M-NBI) videos and 60.80% for non-M-NBI videos. The per lesion sensitivity was 100% in M-NBI videos.

DL in BE

BE is a disorder in which the lining of the esophagus is damaged by gastric acid. The critical purpose of endoscopic Barrett's surveillance is early detection of BE-related dysplasia[4,26-28]. Recently, there have been many studies on the DL-based diagnosis of BE, and we review some representative studies. de Groof *et al*[32] performed one of the first pilot studies to assess the performance of a DL-based system during live endoscopic procedures of patients with or without BE dysplasia. The system demonstrated 90% accuracy, 91% sensitivity and 89% specificity in a per-level analysis. Following up this work, they improved this system using stepwise transfer learning and five independent endoscopy data sets. The enhanced system obtained higher accuracy than non-expert endoscopists and with comparable delineation performance[33]. Furthermore, their team also demonstrated the feasibility of a DL-based system for tissue characterization of NBI endoscopy in BE, and the system achieved a promising diagnostic accuracy[34].

Hashimoto *et al*[35] borrowed from the Inception-ResNet-v2 algorithm to develop a model for real-time classification of early esophageal neoplasia in BE, and they also applied YOLO-v2 to draw localization boxes around regions classified as dysplasia. For detection of neoplasia, the system achieved a sensitivity of 96.4%, specificity of 94.2% and accuracy of 95.4%. Hussein *et al*[36] built a CNN model to diagnose dysplastic BE mucosa with a sensitivity of 88.3% and specificity of 80.0%. The results preliminarily indicated that the diagnostic performance of the CNN model was close to that of experienced endoscopists. Ebigbo *et al*[37] exploited the use of a CNN-based system to classify and segment cancer in BE. The system achieved an accuracy of 89.9% in 14 patients with neoplastic BE.

DL has also achieved excellent results in distinguishing multiple types of esophageal lesions, including BE. Liu *et al*[38] explored the use of a CNN model to distinguish esophageal cancers from BE. The model was trained and evaluated on 1272 images captured by WLE. After pre-processing and data augmentation, the average sensitivity, specificity and accuracy of the CNN model were 94.23%, 94.67% and 85.83%, respectively. Wu *et al*[39] developed a CNN-based framework named ELNet for automatic esophageal lesion (*i.e.* EAC, BE and inflammation) classification and segmentation, the ELNet achieved a classification sensitivity of 90.34%, specificity of 97.18% and accuracy of 96.28%. The segmentation sensitivity, specificity and accuracy were 80.18%, 96.55% and 94.62%, respectively. A similar study was proposed by Ghatwary *et al*[40], who applied a CNN algorithm to detect BE, EAC and ESCC from

endoscopic videos and obtained a high sensitivity of 93.7% and a high F-measure of 93.2%.

The studies exploring the creation of DL algorithms for the diagnosis of precancerous lesions in esophageal mucosa are summarized in Table 1.

DL IN ENDOSCOPIC DETECTION OF PRECANCEROUS LESIONS IN GASTRIC MUCOSA

Gastric cancer is the fifth most prevalent form of cancer and the third most lethal cancer globally[1]. Even though the prevalence of gastric cancer has declined during the last few decades, gastric cancer remains a significant clinical problem, especially in developing countries. This is because most patients are diagnosed in late stages with poor prognosis and restricted therapeutic choices[41]. The pathogenesis of gastric cancer involves a series of events starting with *Helicobacter pylori*-induced (*H. pylori*-induced) chronic inflammation, progressing towards atrophic gastritis, GIM, dysplasia and eventually gastric cancer[42]. Patients with the precancerous lesions (*e.g.*, *H. pylori*-induced chronic inflammation, atrophic gastritis, GIM and dysplasia) are at considerable risk of gastric cancer[3,6,43]. It has been argued that the detection of such precancerous lesions may significantly reduce the incidence of gastric cancer. However, endoscopic examination is difficult to identify these precancerous lesions, and the diagnostic result also has high interobserver variability due to their subtle morphological changes in the mucosa and lack of experienced endoscopists[44,45]. Currently, many researchers are trying to use DL-based methods to detect gastric precancerous lesions; here, we review these studies in detail.

DL in H. pylori infection

Most of the gastric precancerous lesions are correlated with long-term infections with *H. pylori*[46]. Shichijo *et al*[47] performed one of the pioneering studies to apply CNNs in the diagnosis of *H. pylori* infection. The CNNs were built on GoogLeNet and trained on 32208 WLE images. One of their CNN models has higher accuracy than endoscopists. The study showed the feasibility of using CNN to diagnose *H. pylori* from endoscopic images. After this study, Itoh *et al*[48] developed a CNN model to detect *H. pylori* infection in WLE images and showed a sensitivity of 86.7% and specificity of 86.7% in the test dataset. A similar model was developed by Zheng *et al*[49] to evaluate *H. pylori* infection status, and the per-patient sensitivity, specificity and accuracy of the model were 91.6%, 98.6% and 93.8%, respectively. Besides WLE, blue laser imaging-bright and linked color imaging systems were prospectively applied by Nakashima *et al*[50] to collect endoscopic images. With these images, they fine-tuned a pre-trained GoogLeNet to predict *H. pylori* infection status. As compared with linked color imaging, the model achieved the highest sensitivity (96.7%) and specificity (86.7%) when using blue laser imaging-bright. Nakashima *et al*[51] also did a single center prospective study to build a CNN model to identify the status of *H. pylori* in uninfected, currently infected and post-eradication patients. The area under the receiver operating characteristic curve for the uninfected, currently infected and post-eradication categories was 0.90, 0.82 and 0.77, respectively.

DL in atrophic gastritis

Atrophic gastritis is a form of chronic inflammation of the gastric mucosa; accurate endoscopic diagnosis is difficult[52]. Guimarães *et al*[53] reported the application of CNN to detect atrophic gastritis; the system achieved an accuracy of 93% and performed better than expert endoscopists. Recently, another CNN-based system for detecting atrophic gastritis was reported by Zhang *et al*[54]. The CNN model was trained and tested on a dataset containing 3042 images with atrophic gastritis and 2428 without atrophic gastritis. The diagnostic accuracy, sensitivity and specificity of the model were 94.2%, 94.5% and 94.0%, respectively, which were better than those of the experts. More recently, Horiuchi *et al*[55] explored the diagnostic ability of the CNN model to distinguish early gastric cancer and gastritis through M-NBI; the 22-layer CNN was built on GoogleNet and pretrained using 2570 endoscopic images, and the sensitivity, specificity and accuracy on 258 images were 95.4%, 71.0% and 85.3%, respectively. Except for high sensitivity, the CNN model also showed an overall test speed of 0.02 s *per* image, which was faster than human experts.

Table 1 Summary of studies using deep learning for detection of esophageal precancerous lesions

Ref.	Year	Imaging	Study design	Study aim	DL model	Dataset	Outcomes
Cai *et al*[30]	2019	WLE	Retrospective	Detection of precancerous lesions and early ESCC	--	2615 images	Sensitivity: 97.8%. Specificity: 85.4%. Accuracy: 91.4%
Guo *et al*[31]	2020	NBI, M-NBI	Retrospective	Detection of precancerous lesions and early ESCC	SegNet	13144 images and 168865 video frames	Sensitivity: 96.10% for M-NBI videos, 60.80% for non-M-NBI videos, 98.04% for images. Specificity: 99.90% for non-M-NBI/M-NBI videos, 95.30% for images
de Groof *et al*[32]	2020	WLE	Retrospective	Detection of Barrett's neoplasia	ResNet/U-Ne	1544 images	Sensitivity: 91%. Specificity: 89%. Accuracy: 90%
de Groof *et al*[33]	2020	WLE	Retrospective	Detection of Barrett's neoplasia	ResNet/U-Ne	494364 unlabeled images and 1704 labeled images	Sensitivity: 90%. Specificity: 88%. Accuracy: 89%
Struyvenberg *et al*[34]	2021	NBI	Retrospective	Detection of Barrett's neoplasia	ResNet/U-Ne	2677 images	Sensitivity: 88%. Specificity: 78%. Accuracy: 84%
Hashimoto *et al*[35]	2020	WLE, NBI	Retrospective	Recognition of early neoplasia in BE	Inception-ResNet-v2, YOLO-v2	2290 images	Sensitivity: 96.4%. Specificity: 94.2%. Accuracy: 95.4%
Hussein *et al*[36]	2020	WLE	Retrospective	Diagnosis of early neoplasia in BE	Resnet101	266930 video frames	Sensitivity: 88.26%. Specificity: 80.13%
Ebigbo *et al*[37]	2020	WLE	Retrospective	Diagnosis of early EAC in BE	DeepLab V.3+, Resnet101	191 images	Sensitivity: 83.7%. Specificity: 100%. Accuracy: 89.9%
Liu *et al*[38]	2020	WLE	Retrospective	Detection of esophageal cancer from precancerous lesions	Inception-ResNet	1272 images	Sensitivity: 94.23%. Specificity: 94.67%. Accuracy: 85.83%
Wu *et al*[39]	2021	WLE	Retrospective	Automatic classification and segmentation for esophageal lesions	ELNet	1051 images	Classification sensitivity: 90.34%. Classification specificity: 97.18%. Classification accuracy: 96.28%. Segmentation sensitivity: 80.18%. Segmentation Specificity: 96.55%, Segmentation accuracy: 94.62%
Ghatwary *et al*[40]	2021	WLE	Retrospective	Detection of esophageal abnormalities from endoscopic videos	DenseConvLstm, Faster R-CNN	42425 video frames	Sensitivity: 93.7%. F-measure: 93.2%

BE: Barrett's esophagus; DL: Deep learning; EAC: Esophageal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; M-NBI: Magnifying narrow band imaging; NBI: Narrow band imaging; WLE: White light endoscopy.

DL in GIM

GIM is the replacement of gastric-type mucinous epithelial cells with intestinal-type cells, which is a precancerous lesion with a worldwide prevalence of 25%[56]. The morphological characteristics of GIM are subtle and difficult to observe, so the manual diagnosis of GIM is full of challenges. Wang *et al*[57] reported the first instance of an AI system for localizing and identifying GIM from WLE images. The system achieved a high classification accuracy and a satisfactory segmentation result. A recent study developed a CNN-based diagnosis system that can detect atrophic gastritis and GIM from WLE images[58], and the detection sensitivity and specificity for atrophic gastritis were 87.2% and 91.1%, respectively. For detection of GIM, the system also achieved a sensitivity of 90.3% and a specificity of 93.7%. Recently, our team also developed a novel DL-based diagnostic system for detection of GIM in endoscopic images[18]. The difference from the previous research is that our system is composed of three independent CNNs, which can identify GIM from either NBI or M-NBI. The per-patient sensitivity, specificity and accuracy of the system were 91.9%, 86.0% and 88.8%, respectively. The diagnostic performance showed no significant differences as compared with human experts. Our research showed that the integration of NBI and M-NBI into the DL system could achieve satisfactory diagnostic performance for GIM.

DL in gastric dysplasia

Gastric dysplasia is the penultimate step of gastric carcinogenesis, and accurate diagnosis of this lesion remains controversial[59]. To accurately classify advanced low-grade dysplasia, high-grade dysplasia, early gastric cancer and gastric cancer, Cho *et al*[60] established three CNN models based on 5017 endoscopic images. They found that the Inception-Resnet-v2 model performed the best, while it showed lower five-class accuracy compared with the endoscopists (76.4% *vs* 87.6%). Inoue *et al*[61] constructed a detection system using the Single-Shot Multibox Detector, which can automatically detect duodenal adenomas and high-grade dysplasia from WLE or NBI. The system detected 94.7% adenomas and 100% high-grade dysplasia on a dataset containing 1080 endoscopic images within only 31 s. Although most of the AI-assisted system can achieve high accuracy on endoscopic diagnosis, no study has investigated the role of AI in the training of junior endoscopists. To evaluate the role of AI in the training of junior endoscopists in predicting histology of endoscopic gastric lesions, including dysplasia, Lui *et al*[62] designed and validated a CNN classifier based on 3000 NB images. The classifier achieved an overall accuracy of 91.0%, sensitivity of 97.1% and specificity of 85.9%, which was superior to all junior endoscopists. They also demonstrated that with the feedback from the CNN classifier, the learning curve of junior endoscopists was improved in predicting histology of gastric lesions.

The studies exploring the creation of DL algorithms for the diagnosis of precancerous lesions in gastric mucosa are summarized in Table 2.

CHALLENGES AND RECOMMENDATIONS

AI has gained much attention in recent years. In the field of GI endoscopy, DL is also a promising innovation in the identification and characterization of lesions[9-13]. Many successful studies have focused on GI cancers. Accurate detection of precancerous lesions such as ESD, BE, *H. pylori*-induced chronic inflammation, atrophic gastritis, GIM and gastric dysplasia can greatly reduce the incidence of cancers and require less cancer treatment. DL-assisted detection of these precancerous lesions has increasingly emerged in the last 5 yrs. To perform a systematic review of the status of DL for diagnosis of precancerous lesions of the upper GI tract, we conducted a comprehensive search for all original publications on this target between January 1, 2017 and December 30, 2020. A variety of published papers has verified the outstanding performance of DL-assisted systems, several challenges remain from the viewpoint of physicians and algorithm engineers. The challenges and our recommendations on future research directions are outlined below.

Prospective studies and clinical verification

The current literature reveals that most studies were designed in a retrospective manner with a strong probability of bias. In these retrospective studies, researchers tended to collect high-quality endoscopic images that showed typical characteristics of the detected lesions from a single medical center, while they excluded common low-quality images. This kind of selection bias may jeopardize the precision and lead to lower generalization of the DL models. Thus, data collected from multicenter studies with uninformative frames are necessary to build robust DL models, and prospective studies are needed to properly verify the accuracy of AI in clinical practice.

Handling of overfitting

Overfitting means an AI model performs well on the training set but has high error on unseen data. The deep CNN architectures usually contain several convolutional layers and fully connected layers, which produce millions of parameters that easily lead to strong overfitting[16,17]. Training these parameters needs large-scale well-annotated data. However, well-annotated data are costly and hard to obtain in the clinical community. Possible solutions for overcoming the lack of well-annotated data to avoid overfitting mainly include data augmentation[63], transfer learning[17,64], semi-supervised learning[65] and data synthesis using generative adversarial networks[66].

Data augmentation is a common method to train CNNs to reduce overfitting[63]. According to current literature, almost all studies use data augmentation. Data augmentation is performed by using several image transformations such as random image rotation, flipping, shifting, scaling and their combinations are shown in Figure 3. Transfer learning involves transfer knowledge learned from a large source domain to a target domain[17,64]. This technique is usually performed by initializing the CNN using the weights pretrained on ImageNet dataset. As there are many

Table 2 Summary of studies using deep learning for detection of gastric precancerous lesions

Ref.	Year	Imaging	Study design	Study aim	DL model	Dataset	Outcomes
Shichijo *et al*[47]	2017	WLE	Retrospective	Diagnosis of *H. pylori* infection	GoogLeNet	43689 images	Sensitivity: 88.9%; Specificity: 87.4%; Accuracy: 87.7%
Itoh *et al*[48]	2018	WLE	Retrospective	Analysis of *H. pylori* infection	GoogLeNet	179 images	Sensitivity: 86.7%; Specificity: 86.7%
Zheng *et al*[49]	2019	WLE	Retrospective	Evaluation of *H. pylori* infection status	ResNet-50	15484 images	Sensitivity: 91.6%; Specificity: 98.6%; Accuracy: 93.8%
Nakashima *et al*[50]	2018	BLI-bright, LCI	Prospective	Prediction of *H. pylori* infection status	GoogLeNet	666 images	Sensitivity: 96.7%; Specificity: 86.7%
Nakashima *et al*[51]	2020	WLE, LCI	Prospective	Diagnosis of *H. pylori* infection	--	13127 images	For currently infected patients, the sensitivity and specificity are 62.5% and 92.5%, respectively
Guimarães *et al*[53]	2020	WLE	Retrospective	Diagnosis of atrophic gastritis	VGG16	270 images	Accuracy: 93%
Zhang *et al*[54]	2020	WLE	Retrospective	Diagnosis of atrophic gastritis	DenseNet121	5470 images	Sensitivity: 94.5%; Specificity: 94.0%; Accuracy: 94.2%
Horiuchi *et al*[55]	2020	M-NBI	Retrospective	Differentiation between early gastric cancer and gastritis	GoogLeNet	2826 images	Sensitivity: 95.4%; Specificity: 71.0%; Accuracy: 85.3%
Wang *et al*[57]	2019	WLE	Retrospective	Localization and identification of GIM	DeepLab V.3+	200 images	Accuracy: 89.51%
Zheng *et al*[58]	2020	WLE	Retrospective	Detection of atrophic gastritis and GIM	ResNet-50	3759 images	Sensitivity for atrophic gastritis: 87.2%; Specificity for atrophic gastritis: 91.1%; Sensitivity for GIM: 90.3%; Specificity for GIM: 93.7%
Yan *et al*[18]	2020	NBI, M-NBI	Retrospective	Diagnosis of GIM	EfficientNetB4	2357 images	Sensitivity: 91.9%; Specificity: 86.0%; Accuracy: 88.8%
Cho *et al*[60]	2019	WLE	Prospective	Classification of multiclass gastric neoplasms	Inception-Resnet-v2	5217 images	Accuracy: 84.6%
Inoue *et al*[61]	2020	WLE, NBI	Retrospective	Detection of duodenal adenomas and high-grade dysplasias	Single-Shot Multibox Detector	1511 images	For high-grade dysplasia, the sensitivity and specificity are all 100%
Lui *et al*[62]	2020	NBI	Retrospective	Classification of gastric lesions	ResNet	3000 images	Sensitivity: 97.1%; Specificity: 85.9%; Accuracy: 91.0%

BLI-bright: Blue laser imaging-bright; DL: Deep learning; GIM: Gastric intestinal metaplasia; *H. pylori*: *Helicobacter pylori*; LCI: Linked color imaging; M-NBI: Magnifying narrow band imaging; NBI: Narrow band imaging; WLE: White light endoscopy.

imaging modalities such as WLE, NBI and M-NBI and the images share the common features of the detected lesions, Struyvenberg *et al*[34] applied a stepwise transfer learning approach to tackle the shortage of NB images. Their CNN model was first trained on many WLE images, which are easy to acquire as compared with NB images. Then, the weights were further trained and optimized using NB images. In the upper endoscopy, although well-annotated data are limited, the unlabeled data are abundant and easily available in most situations. Semi-supervised learning, which utilizes limited labeled data and large-scale unlabeled data to train and improve the CNN model, is useful in the GI tract[65,67,68]. Generative adversarial networks are widely used in the field of medical image synthesis; the synthetic images can be used to provide auxiliary training to improve the performance of the CNN models[66]. de Souza *et al*[69] introduced generative adversarial networks to generate high-quality endoscopic images for BE, and these synthetic images were used to provide auxiliary training. The detection results suggested that with the help of these synthetic images the CNN model outperformed the ones over the original datasets.

Although the above techniques were used to reduce overfitting to a certain extent, the single use of one type did not guarantee resolution of the overfitting problem. Integration of these techniques is a promising strategy, and it was verified in our research[18,70].

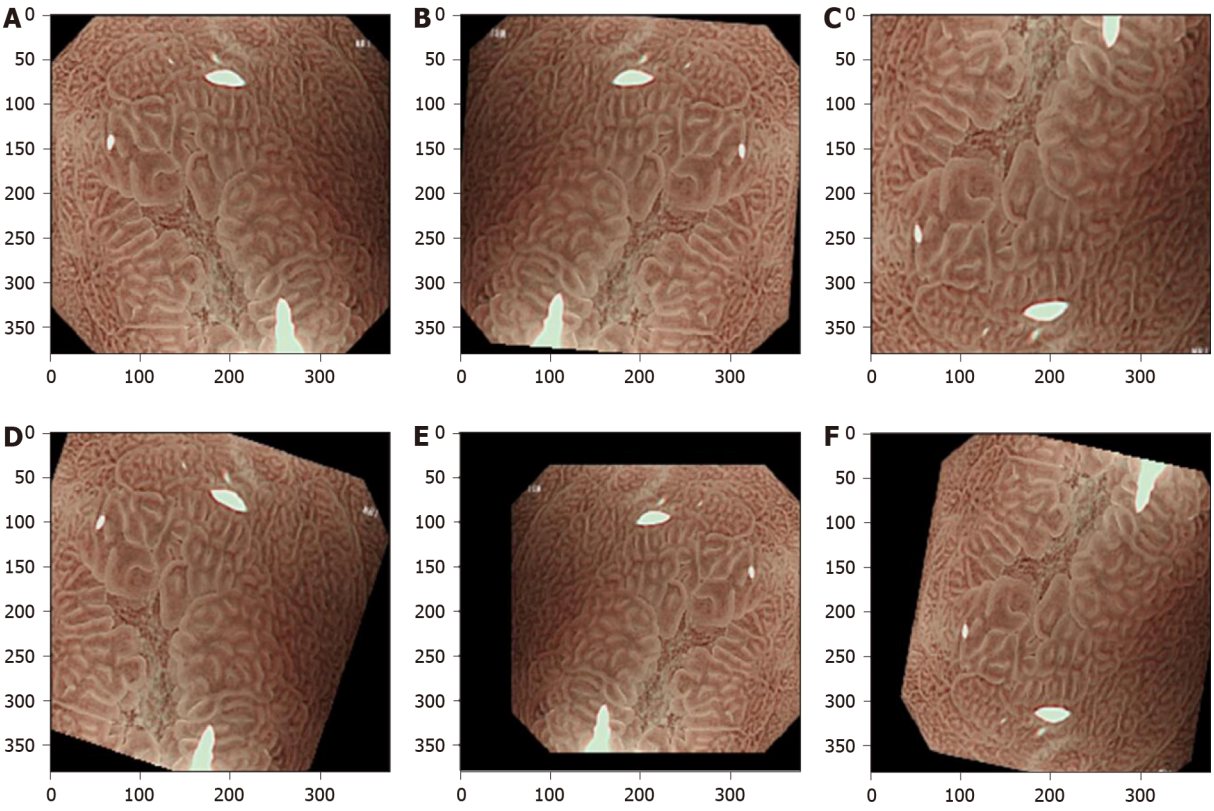


Figure 3 Data augmentation for a typical magnifying narrow band image for training a convolutional neural network model. This is performed by using a variety of image transformations and their combinations. A: Original image; B: Flip horizontal and random rotation; C: Flip vertical and magnification; D: Random rotation and shift; E: Flip horizontal, minification and shift; F: Flip vertical, rotation and shift.

Improvement on interpretability

Lack of interpretability (*i.e.* the "black box" nature), which is the nature of DL technology, is another gap between studies and clinical applications in the field of precancerous lesion detection from endoscopic images. The black box nature means that the decision-making process by the DL model is not clearly demonstrated, which may reduce the willingness of doctors to use it. Although attention maps can help explain the dominant areas by highlighting them, they are constrained in that they do not thoroughly explain how the algorithm comes to its final decision[71]. The attention maps are displayed as heat maps overlaid upon the original images, where warmer colors mean higher contributions to the decision making, which usually correspond to lesions. However, the attention maps also have some defects such as inaccurate display of lesions as shown in Figure 4, where the attention maps only cover partial areas associated with BE and GIM. This is the inherent shortcoming of attention maps. Therefore, understanding the mechanism used by the DL model for prediction is a hot research topic. The network dissection[72], an empirical method to identify the semantics of individual hidden nodes in the DL model, may be a feasible solution to improve interpretability.

Network design

In this review, we analyzed the DL model used in the detection of precancerous lesions in the upper GI tract. The literature shows that almost all the DL-based AI systems are developed based on state-of-the-art CNN architectures. These CNNs can only handle a single task, such as GoogLeNet for disease classification[47,48,50,55], YOLO for lesion identification[36] and SegNet for lesion segmentation[31]. Few networks can handle multiple tasks simultaneously. Thus, networks must be designed for multitask learning, which is valuable in clinical applications. Networks designed for handling high-resolution images can help detect micropatterns, which is profitable for small precancerous lesions. Moreover, attempts should be guided to exploit the use of videos rather than images to minimize the processing time and keep DL algorithms working at almost real-time level. Therefore, as suggested by Mori *et al*[73] and Thakkar *et al*[74], the AI systems may be treated as an extra pair of eyes to prevent the absence of subtle lesions.

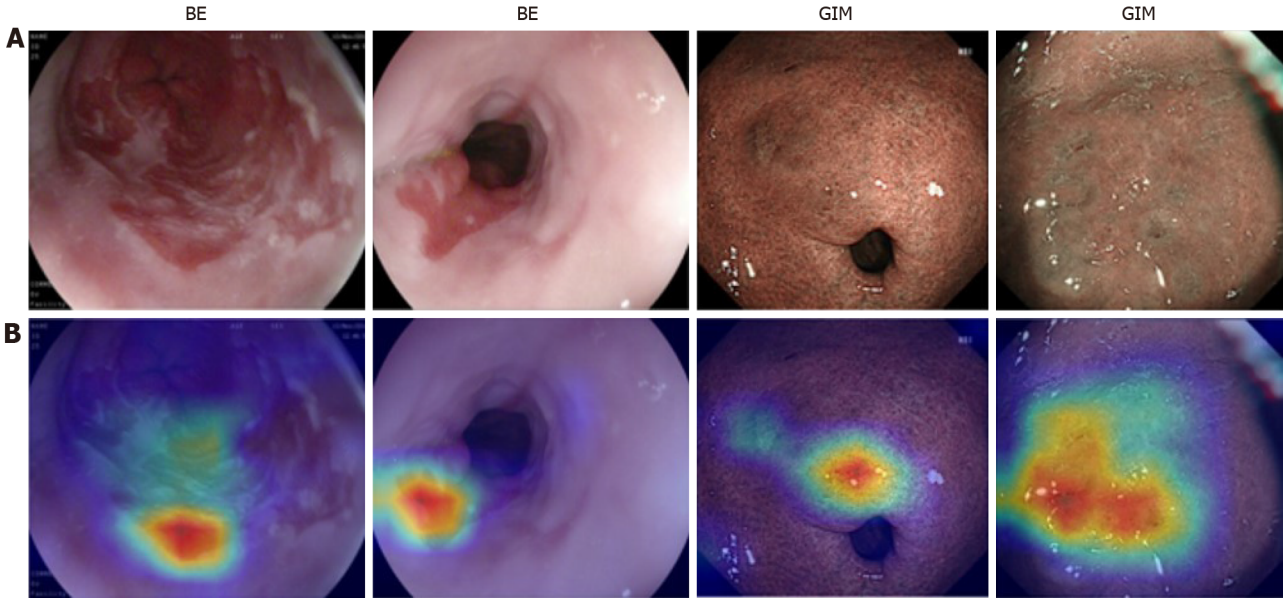


Figure 4 Informative features (partially related to lesions areas) acquired by the convolutional neural networks, where warmer colors mean higher contributions to decision making. A: Original endoscopic images; B: Corresponding attention. BE: Barrett's esophagus; GIM: Gastric intestinal metaplasia.

CONCLUSION

Upper GI cancers are a major cause of cancer-related deaths worldwide. Early detection of precancerous lesions could significantly reduce cancer incidence. Upper GI endoscopy is a gold standard procedure for identifying precancerous lesions in the upper GI tract. DL-based endoscopic systems can provide an easier, faster and more reliable endoscopic method. We have conducted a thorough review of detection of precancerous lesions of the upper GI tract using DL approaches since 2017. This is the first review on the DL-based diagnosis of precancerous lesions of the upper GI tract. The status, challenges and recommendations summarized in this review can provide guidance for intelligent diagnosis of other GI tract diseases, which can help engineers develop perfect AI products to assist clinical decision making. Despite the success of DL algorithms in upper GI endoscopy, prospective studies and clinical validation are still needed. Creation of large public databases, adoption of comprehensive overfitting prevention strategies and application of more advanced interpretable methods and networks are also necessary to encourage clinical application of AI for medical diagnosis.

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REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Morson BC**. Precancerous lesions of upper gastrointestinal tract. *JAMA* 1962; **179**: 311-315 [PMID: 14476362 DOI: 10.1001/jama.1962.03050050001001]

3 **Rugge M**, Capelle LG, Cappellesso R, Nitti D, Kuipers EJ. Precancerous lesions in the stomach: from biology to clinical patient management. *Best Pract Res Clin Gastroenterol* 2013; **27**: 205-223 [PMID: 23809241 DOI: 10.1016/j.bpg.2012.12.007]

4 **Coron E**, Robaszkiewicz M, Chatelain D, Svrcek M, Fléjou JF. Advanced precancerous lesions in the lower oesophageal mucosa: high-grade dysplasia and intramucosal carcinoma in Barrett's oesophagus. *Best Pract Res Clin Gastroenterol* 2013; **27**: 187-204 [PMID: 23809240 DOI:

10.1016/j.bpg.2013.03.011]
5 **Fassan M**, Croce CM, Rugge M. miRNAs in precancerous lesions of the gastrointestinal tract. *World J Gastroenterol* 2011; **17**: 5231-5239 [PMID: 22219591 DOI: 10.3748/wjg.v17.i48.5231]
6 **Pimentel-Nunes P**, Libânio D, Marcos-Pinto R, Areia M, Leja M, Esposito G, Garrido M, Kikuste I, Megraud F, Matysiak-Budnik T, Annibale B, Dumonceau JM, Barros R, Fléjou JF, Carneiro F, van Hooft JE, Kuipers EJ, Dinis-Ribeiro M. Management of epithelial precancerous conditions and lesions in the stomach (MAPS II): European Society of Gastrointestinal Endoscopy (ESGE), European Helicobacter and Microbiota Study Group (EHMSG), European Society of Pathology (ESP), and Sociedade Portuguesa de Endoscopia Digestiva (SPED) guideline update 2019. *Endoscopy* 2019; **51**: 365-388 [PMID: 30841008 DOI: 10.1055/a-0859-1883]
7 **Berci G**, Forde KA. History of endoscopy: what lessons have we learned from the past? *Surg Endosc* 2000; **14**: 5-15 [PMID: 10653228 DOI: 10.1007/s004649900002]
8 **Kavic SM**, Basson MD. Complications of endoscopy. *Am J Surg* 2001; **181**: 319-332 [PMID: 11438266 DOI: 10.1016/s0002-9610(01)00589-x]
9 **Niu PH**, Zhao LL, Wu HL, Zhao DB, Chen YT. Artificial intelligence in gastric cancer: Application and future perspectives. *World J Gastroenterol* 2020; **26**: 5408-5419 [PMID: 33024393 DOI: 10.3748/wjg.v26.i36.5408]
10 **Parasher G**, Wong M, Rawat M. Evolving role of artificial intelligence in gastrointestinal endoscopy. *World J Gastroenterol* 2020; **26**: 7287-7298 [PMID: 33362384 DOI: 10.3748/wjg.v26.i46.7287]
11 **Hussein M**, González-Bueno Puyal J, Mountney P, Lovat LB, Haidry R. Role of artificial intelligence in the diagnosis of oesophageal neoplasia: 2020 an endoscopic odyssey. *World J Gastroenterol* 2020; **26**: 5784-5796 [PMID: 33132634 DOI: 10.3748/wjg.v26.i38.5784]
12 **Huang LM**, Yang WJ, Huang ZY, Tang CW, Li J. Artificial intelligence technique in detection of early esophageal cancer. *World J Gastroenterol* 2020; **26**: 5959-5969 [PMID: 33132647 DOI: 10.3748/wjg.v26.i39.5959]
13 **Yang YJ**, Bang CS. Application of artificial intelligence in gastroenterology. *World J Gastroenterol* 2019; **25**: 1666-1683 [PMID: 31011253 DOI: 10.3748/wjg.v25.i14.1666]
14 **McCarthy J**, Minsky ML, Rochester N, Shannon CE. A proposal for the dartmouth summer research project on artificial intelligence, August 31, 1955. *AI Mag* 2006; **27**: 12-14 [DOI: 10.1609/aimag.v27i4.1904]
15 **Jordan MI**, Mitchell TM. Machine learning: Trends, perspectives, and prospects. *Science* 2015; **349**: 255-260 [PMID: 26185243 DOI: 10.1126/science.aaa8415]
16 **LeCun Y**, Bengio Y, Hinton G. Deep learning. *Nature* 2015; **521**: 436-444 [PMID: 26017442 DOI: 10.1038/nature14539]
17 **Shin HC**, Roth HR, Gao M, Lu L, Xu Z, Nogues I, Yao J, Mollura D, Summers RM. Deep Convolutional Neural Networks for Computer-Aided Detection: CNN Architectures, Dataset Characteristics and Transfer Learning. *IEEE Trans Med Imaging* 2016; **35**: 1285-1298 [PMID: 26886976 DOI: 10.1109/TMI.2016.2528162]
18 **Yan T**, Wong PK, Choi IC, Vong CM, Yu HH. Intelligent diagnosis of gastric intestinal metaplasia based on convolutional neural network and limited number of endoscopic images. *Comput Biol Med* 2020; **126**: 104026 [PMID: 33059237 DOI: 10.1016/j.compbiomed.2020.104026]
19 **Wang P**, Berzin TM, Glissen Brown JR, Bharadwaj S, Becq A, Xiao X, Liu P, Li L, Song Y, Zhang D, Li Y, Xu G, Tu M, Liu X. Real-time automatic detection system increases colonoscopic polyp and adenoma detection rates: a prospective randomised controlled study. *Gut* 2019; **68**: 1813-1819 [PMID: 30814121 DOI: 10.1136/gutjnl-2018-317500]
20 **Gong D**, Wu L, Zhang J, Mu G, Shen L, Liu J, Wang Z, Zhou W, An P, Huang X, Jiang X, Li Y, Wan X, Hu S, Chen Y, Hu X, Xu Y, Zhu X, Li S, Yao L, He X, Chen D, Huang L, Wei X, Wang X, Yu H. Detection of colorectal adenomas with a real-time computer-aided system (ENDOANGEL): a randomised controlled study. *Lancet Gastroenterol Hepatol* 2020; **5**: 352-361 [PMID: 31981518 DOI: 10.1016/S2468-1253(19)30413-3]
21 **An P**, Yang D, Wang J, Wu L, Zhou J, Zeng Z, Huang X, Xiao Y, Hu S, Chen Y, Yao F, Guo M, Wu Q, Yang Y, Yu H. A deep learning method for delineating early gastric cancer resection margin under chromoendoscopy and white light endoscopy. *Gastric Cancer* 2020; **23**: 884-892 [PMID: 32356118 DOI: 10.1007/s10120-020-01071-7]
22 **Rustgi AK**, El-Serag HB. Esophageal carcinoma. *N Engl J Med* 2014; **371**: 2499-2509 [PMID: 25539106 DOI: 10.1056/NEJMra1314530]
23 **Taylor PR**, Abnet CC, Dawsey SM. Squamous dysplasia--the precursor lesion for esophageal squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev* 2013; **22**: 540-552 [PMID: 23549398 DOI: 10.1158/1055-9965.EPI-12-1347]
24 **Raghu Subramanian C**, Triadafilopoulos G. Diagnosis and therapy of esophageal squamous cell dysplasia and early esophageal squamous cell cancer. *Gastroenterol Rep* 2017; **5**: 247-257 [DOI: 10.1093/gastro/gox022]
25 **Auld M**, Srinath H, Jeyarajan E. Oesophageal Squamous Dysplasia. *J Gastrointest Cancer* 2018; **49**: 385-388 [PMID: 29806061 DOI: 10.1007/s12029-018-0122-3]
26 **Hvid-Jensen F**, Pedersen L, Drewes AM, Sørensen HT, Funch-Jensen P. Incidence of adenocarcinoma among patients with Barrett's esophagus. *N Engl J Med* 2011; **365**: 1375-1383 [PMID: 21995385 DOI: 10.1056/NEJMoa1103042]
27 **Eluri S**, Shaheen NJ. Barrett's esophagus: diagnosis and management. *Gastrointest Endosc* 2017; **85**: 889-903 [PMID: 28109913 DOI: 10.1016/j.gie.2017.01.007]

28 **Tavakkoli A**, Appelman HD, Beer DG, Madiyal C, Khodadost M, Nofz K, Metko V, Elta G, Wang T, Rubenstein JH. Use of Appropriate Surveillance for Patients With Nondysplastic Barrett's Esophagus. *Clin Gastroenterol Hepatol* 2018; **16**: 862-869. e3 [PMID: 29432922 DOI: 10.1016/j.cgh.2018.01.052]

29 **Dawsey SM**, Fleischer DE, Wang GQ, Zhou B, Kidwell JA, Lu N, Lewin KJ, Roth MJ, Tio TL, Taylor PR. Mucosal iodine staining improves endoscopic visualization of squamous dysplasia and squamous cell carcinoma of the esophagus in Linxian, China. *Cancer* 1998; **83**: 220-231 [PMID: 9669803]

30 **Cai SL**, Li B, Tan WM, Niu XJ, Yu HH, Yao LQ, Zhou PH, Yan B, Zhong YS. Using a deep learning system in endoscopy for screening of early esophageal squamous cell carcinoma (with video). *Gastrointest Endosc* 2019; **90**: 745-753. e2 [PMID: 31302091 DOI: 10.1016/j.gie.2019.06.044]

31 **Guo L**, Xiao X, Wu C, Zeng X, Zhang Y, Du J, Bai S, Xie J, Zhang Z, Li Y, Wang X, Cheung O, Sharma M, Liu J, Hu B. Real-time automated diagnosis of precancerous lesions and early esophageal squamous cell carcinoma using a deep learning model (with videos). *Gastrointest Endosc* 2020; **91**: 41-51 [PMID: 31445040 DOI: 10.1016/j.gie.2019.08.018]

32 **de Groof AJ**, Struyvenberg MR, Fockens KN, van der Putten J, van der Sommen F, Boers TG, Zinger S, Bisschops R, de With PH, Pouw RE, Curvers WL, Schoon EJ, Bergman JJGHM. Deep learning algorithm detection of Barrett's neoplasia with high accuracy during live endoscopic procedures: a pilot study (with video). *Gastrointest Endosc* 2020; **91**: 1242-1250 [PMID: 31926965 DOI: 10.1016/j.gie.2019.12.048]

33 **de Groof AJ**, Struyvenberg MR, van der Putten J, van der Sommen F, Fockens KN, Curvers WL, Zinger S, Pouw RE, Coron E, Baldaque-Silva F, Pech O, Weusten B, Meining A, Neuhaus H, Bisschops R, Dent J, Schoon EJ, de With PH, Bergman JJ. Deep-Learning System Detects Neoplasia in Patients With Barrett's Esophagus With Higher Accuracy Than Endoscopists in a Multistep Training and Validation Study With Benchmarking. *Gastroenterology* 2020; **158**: 915-929. e4 [PMID: 31759929 DOI: 10.1053/j.gastro.2019.11.030]

34 **Struyvenberg MR**, de Groof AJ, van der Putten J, van der Sommen F, Baldaque-Silva F, Omae M, Pouw R, Bisschops R, Vieth M, Schoon EJ, Curvers WL, de With PH, Bergman JJ. A computer-assisted algorithm for narrow-band imaging-based tissue characterization in Barrett's esophagus. *Gastrointest Endosc* 2021; **93**: 89-98 [PMID: 32504696 DOI: 10.1016/j.gie.2020.05.050]

35 **Hashimoto R**, Requa J, Dao T, Ninh A, Tran E, Mai D, Lugo M, El-Hage Chehade N, Chang KJ, Karnes WE, Samarasena JB. Artificial intelligence using convolutional neural networks for real-time detection of early esophageal neoplasia in Barrett's esophagus (with video). *Gastrointest Endosc* 2020; **91**: 1264-1271. e1 [PMID: 31930967 DOI: 10.1016/j.gie.2019.12.049]

36 **Hussein M**, Juana Gonzales-Bueno P, Brandao P, Toth D, Seghal V, Everson MA, Lipman G, Ahmad OF, Kader R, Esteban JM, Bisschops R, Banks M, Mountney P, Stoyanov D, Lovat L, Haidry R. Deep Neural Network for the detection of early neoplasia in Barrett's oesophagus. *Gastrointest Endosc* 2020; **91**: AB250 [DOI: 10.1016/j.gie.2020.03.1826]

37 **Ebigbo A**, Mendel R, Probst A, Manzeneder J, Prinz F, de Souza LA Jr, Papa J, Palm C, Messmann H. Real-time use of artificial intelligence in the evaluation of cancer in Barrett's oesophagus. *Gut* 2020; **69**: 615-616 [PMID: 31541004 DOI: 10.1136/gutjnl-2019-319460]

38 **Liu G**, Hua J, Wu Z, Meng T, Sun M, Huang P, He X, Sun W, Li X, Chen Y. Automatic classification of esophageal lesions in endoscopic images using a convolutional neural network. *Ann Transl Med* 2020; **8**: 486 [PMID: 32395530 DOI: 10.21037/atm.2020.03.24]

39 **Wu Z**, Ge R, Wen M, Liu G, Chen Y, Zhang P, He X, Hua J, Luo L, Li S. ELNet:Automatic classification and segmentation for esophageal lesions using convolutional neural network. *Med Image Anal* 2021; **67**: 101838 [PMID: 33129148 DOI: 10.1016/j.media.2020.101838]

40 **Ghatwary N**, Zolgharni M, Janan F, Ye X. Learning Spatiotemporal Features for Esophageal Abnormality Detection From Endoscopic Videos. *IEEE J Biomed Health Inform* 2021; **25**: 131-142 [PMID: 32750901 DOI: 10.1109/JBHI.2020.2995193]

41 **Van Cutsem E**, Sagaert X, Topal B, Haustermans K, Prenen H. Gastric cancer. *Lancet* 2016; **388**: 2654-2664 [PMID: 27156933 DOI: 10.1016/S0140-6736(16)30354-3]

42 **Correa P**, Piazuelo MB. The gastric precancerous cascade. *J Dig Dis* 2012; **13**: 2-9 [PMID: 22188910 DOI: 10.1111/j.1751-2980.2011.00550.x]

43 **de Vries AC**, van Grieken NC, Looman CW, Casparie MK, de Vries E, Meijer GA, Kuipers EJ. Gastric cancer risk in patients with premalignant gastric lesions: a nationwide cohort study in the Netherlands. *Gastroenterology* 2008; **134**: 945-952 [PMID: 18395075 DOI: 10.1053/j.gastro.2008.01.071]

44 **Chung CS**, Wang HP. Screening for precancerous lesions of upper gastrointestinal tract: from the endoscopists' viewpoint. *Gastroenterol Res Pract* 2013; **2013**: 681439 [PMID: 23573079 DOI: 10.1155/2013/681439]

45 **Menon S**, Trudgill N. How commonly is upper gastrointestinal cancer missed at endoscopy? *Endosc Int Open* 2014; **2**: E46-E50 [PMID: 26135259 DOI: 10.1055/s-0034-1365524]

46 **Matysiak-Budnik T**, Mégraud F. Helicobacter pylori infection and gastric cancer. *Eur J Cancer* 2006; **42**: 708-716 [PMID: 16556496 DOI: 10.1016/j.ejca.2006.01.020]

47 **Shichijo S**, Nomura S, Aoyama K, Nishikawa Y, Miura M, Shinagawa T, Takiyama H, Tanimoto T, Ishihara S, Matsuo K, Tada T. Application of Convolutional Neural Networks in the Diagnosis of Helicobacter pylori Infection Based on Endoscopic Images. *EBioMedicine* 2017; **25**: 106-111 [PMID:

29056541 DOI: 10.1016/j.ebiom.2017.10.014]
48 **Itoh T**, Kawahira H, Nakashima H, Yata N. Deep learning analyzes Helicobacter pylori infection by upper gastrointestinal endoscopy images. *Endosc Int Open* 2018; **6**: E139-E144 [PMID: 29399610 DOI: 10.1055/s-0043-120830]
49 **Zheng W**, Zhang X, Kim JJ, Zhu X, Ye G, Ye B, Wang J, Luo S, Li J, Yu T, Liu J, Hu W, Si J. High Accuracy of Convolutional Neural Network for Evaluation of Helicobacter pylori Infection Based on Endoscopic Images: Preliminary Experience. *Clin Transl Gastroenterol* 2019; **10**: e00109 [PMID: 31833862 DOI: 10.14309/ctg.0000000000000109]
50 **Nakashima H**, Kawahira H, Kawachi H, Sakaki N. Artificial intelligence diagnosis of *Helicobacter pylori* infection using blue laser imaging-bright and linked color imaging: a single-center prospective study. *Ann Gastroenterol* 2018; **31**: 462-468 [PMID: 29991891 DOI: 10.20524/aog.2018.0269]
51 **Nakashima H**, Kawahira H, Kawachi H, Sakaki N. Endoscopic three-categorical diagnosis of Helicobacter pylori infection using linked color imaging and deep learning: a single-center prospective study (with video). *Gastric Cancer* 2020; **23**: 1033-1040 [PMID: 32382973 DOI: 10.1007/s10120-020-01077-1]
52 **Okamura T**, Iwaya Y, Kitahara K, Suga T, Tanaka E. Accuracy of Endoscopic Diagnosis for Mild Atrophic Gastritis Infected with Helicobacter pylori. *Clin Endosc* 2018; **51**: 362-367 [PMID: 29695148 DOI: 10.5946/ce.2017.177]
53 **Guimarães P**, Keller A, Fehlmann T, Lammert F, Casper M. Deep-learning based detection of gastric precancerous conditions. *Gut* 2020; **69**: 4-6 [PMID: 31375599 DOI: 10.1136/gutjnl-2019-319347]
54 **Zhang Y**, Li F, Yuan F, Zhang K, Huo L, Dong Z, Lang Y, Zhang Y, Wang M, Gao Z, Qin Z, Shen L. Diagnosing chronic atrophic gastritis by gastroscopy using artificial intelligence. *Dig Liver Dis* 2020; **52**: 566-572 [PMID: 32061504 DOI: 10.1016/j.dld.2019.12.146]
55 **Horiuchi Y**, Aoyama K, Tokai Y, Hirasawa T, Yoshimizu S, Ishiyama A, Yoshio T, Tsuchida T, Fujisaki J, Tada T. Convolutional Neural Network for Differentiating Gastric Cancer from Gastritis Using Magnified Endoscopy with Narrow Band Imaging. *Dig Dis Sci* 2020; **65**: 1355-1363 [PMID: 31584138 DOI: 10.1007/s10620-019-05862-6]
56 **Lam SK**, Lau G. Novel treatment for gastric intestinal metaplasia, a precursor to cancer. *JGH Open* 2020; **4**: 569-573 [PMID: 32782940 DOI: 10.1002/jgh3.12318]
57 **Wang C**, Li Y, Yao J, Chen B, Song J, Yang X. Localizing and Identifying Intestinal Metaplasia Based on Deep Learning in Oesophagoscope. 2019 8th International Symposium on Next Generation 2019; 1-4 [DOI: 10.1109/ISNE.2019.8896546]
58 **Zheng W**, Yu T, Lin N, Ye G, Zhu X, Shen Y, Zhang X, Liu J, Hu W, Cao Q, Si J. Tu1075 deep convolutional neural networks for recognition of atrophic gastritis and intestinal metaplasia based on endoscopy images. *Gastrointest Endosc* 2020; **91**: AB533-AB534 [DOI: 10.1016/j.gie.2020.03.3247]
59 **Sung JK**. Diagnosis and management of gastric dysplasia. *Korean J Intern Med* 2016; **31**: 201-209 [PMID: 26932397 DOI: 10.3904/kjim.2016.021]
60 **Cho BJ**, Bang CS, Park SW, Yang YJ, Seo SI, Lim H, Shin WG, Hong JT, Yoo YT, Hong SH, Choi JH, Lee JJ, Baik GH. Automated classification of gastric neoplasms in endoscopic images using a convolutional neural network. *Endoscopy* 2019; **51**: 1121-1129 [PMID: 31443108 DOI: 10.1055/a-0981-6133]
61 **Inoue S**, Shichijo S, Aoyama K, Kono M, Fukuda H, Shimamoto Y, Nakagawa K, Ohmori M, Iwagami H, Matsuno K, Iwatsubo T, Nakahira H, Matsuura N, Maekawa A, Kanesaka T, Yamamoto S, Takeuchi Y, Higashino K, Uedo N, Ishihara R, Tada T. Application of Convolutional Neural Networks for Detection of Superficial Nonampullary Duodenal Epithelial Tumors in Esophagogastroduodenoscopic Images. *Clin Transl Gastroenterol* 2020; **11**: e00154 [PMID: 32352719 DOI: 10.14309/ctg.0000000000000154]
62 **Lui TKL**, Wong KKY, Mak LLY, To EWP, Tsui VWM, Deng Z, Guo J, Ni L, Cheung MKS, Leung WK. Feedback from artificial intelligence improved the learning of junior endoscopists on histology prediction of gastric lesions. *Endosc Int Open* 2020; **8**: E139-E146 [PMID: 32010746 DOI: 10.1055/a-1036-6114]
63 **Shorten C**, Khoshgoftaar TM. A survey on image data augmentation for deep learning. *J Big Data* 2019; **6**: 60 [DOI: 10.1186/s40537-019-0197-0]
64 **Pan SJ**, Yang Q. A survey on transfer learning. *IEEE* 2009; **22**: 1345-1359 [DOI: 10.1109/TKDE.2009.191]
65 **Zhu X**, Goldberg AB. Introduction to Semi-Supervised Learning. Synthesis Lectures on Artificial Intelligence and Machine Learning 2009; 3: 1-130 [DOI: 10.2200/S00196ED1V01Y200906AIM006]
66 **Goodfellow I**, Pouget-Abadie J, Mirza M, Xu B, Warde-Farley D, Ozair S, Courville A, Bengio, Y. Generative adversarial nets. Advances in neural information processing systems 2014; 27: 2672-2680.
67 **Guo X**, Yuan Y. Semi-supervised WCE image classification with adaptive aggregated attention. *Med Image Anal* 2020; **64**: 101733 [PMID: 32574987 DOI: 10.1016/j.media.2020.101733]
68 **Borgli H**, Thambawita V, Smedsrud PH, Hicks S, Jha D, Eskeland SL, Randel KR, Pogorelov K, Lux M, Nguyen DTD, Johansen D, Griwodz C, Stensland HK, Garcia-Ceja E, Schmidt PT, Hammer HL, Riegler MA, Halvorsen P, de Lange T. HyperKvasir, a comprehensive multi-class image and video dataset for gastrointestinal endoscopy. *Sci Data* 2020; **7**: 283 [PMID: 32859981 DOI: 10.1038/s41597-020-00622-y]
69 **de Souza LA Jr**, Passos LA, Mendel R, Ebigbo A, Probst A, Messmann H, Palm C, Papa JP. Assisting Barrett's esophagus identification using endoscopic data augmentation based on Generative Adversarial Networks. *Comput Biol Med* 2020; **126**: 104029 [PMID: 33059236 DOI:

10.1016/j.compbiomed.2020.104029]
70 **Yan T**, Wong PK, Ren H, Wang H, Wang J, Li Y. Automatic distinction between COVID-19 and common pneumonia using multi-scale convolutional neural network on chest CT scans. *Chaos Solitons Fractals* 2020; **140**: 110153 [PMID: 32834641 DOI: 10.1016/j.chaos.2020.110153]
71 **Selvaraju**, RR, Cogswell M, Das A, Vedantam R, Parikh D, Batra D. Grad-cam: Visual explanations from deep networks via gradient-based localization. In: Proceedings of the IEEE International Conference on Computer Vision 2017; 618-626 [DOI: 10.1109/ICCV.2017.74]
72 **Bau D**, Zhu JY, Strobelt H, Lapedriza A, Zhou B, Torralba A. Understanding the role of individual units in a deep neural network. *Proc Natl Acad Sci USA* 2020; **117**: 30071-30078 [PMID: 32873639 DOI: 10.1073/pnas.1907375117]
73 **Mori Y**, Kudo SE, Mohmed HEN, Misawa M, Ogata N, Itoh H, Oda M, Mori K. Artificial intelligence and upper gastrointestinal endoscopy: Current status and future perspective. *Dig Endosc* 2019; **31**: 378-388 [PMID: 30549317 DOI: 10.1111/den.13317]
74 **Thakkar SJ**, Kochhar GS. Artificial intelligence for real-time detection of early esophageal cancer: another set of eyes to better visualize. *Gastrointest Endosc* 2020; **91**: 52-54 [PMID: 31865996 DOI: 10.1016/j.gie.2019.09.036]

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MINIREVIEWS

State of machine and deep learning in histopathological applications in digestive diseases

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Abstract

Machine learning (ML)- and deep learning (DL)-based imaging modalities have exhibited the capacity to handle extremely high dimensional data for a number of computer vision tasks. While these approaches have been applied to numerous data types, this capacity can be especially leveraged by application on histopathological images, which capture cellular and structural features with their high-resolution, microscopic perspectives. Already, these methodologies have demonstrated promising performance in a variety of applications like disease classification, cancer grading, structure and cellular localizations, and prognostic predictions. A wide range of pathologies requiring histopathological evaluation exist in gastroenterology and hepatology, indicating these as disciplines highly targetable for integration of these technologies. Gastroenterologists have also already been primed to consider the impact of these algorithms, as development of real-time endoscopic video analysis software has been an active and popular field of research. This heightened clinical awareness will likely be important for future integration of these methods and to drive interdisciplinary collaborations on emerging studies. To provide an overview on the application of these methodologies for gastrointestinal and hepatological histopathological slides, this review will discuss general ML and DL concepts, introduce recent and emerging literature using these methods, and cover challenges moving forward to further advance the field.

Key Words: Artificial intelligence; Machine learning; Deep learning; Gastroenterology; Hepatology; Histopathology

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Core Tip: Machine learning- and deep learning-based imaging approaches have been increasingly applied to histopathological slides and hold much potential in areas spanning diagnosis, disease grading and characterizations, academic research, and clinical decision support mechanisms. As these studies have entered into translational applications, tracking the current state of these methodologies and the clinical areas in which impact is most likely is of high importance. This review will thus provide a background of major concepts and terminologies while highlighting emerging literature regarding histopathological applications of these techniques and challenges and opportunities moving forward.

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INTRODUCTION

The past decade has seen the growing popularity of machine learning (ML) and deep learning (DL) applications across numerous domains, and the medical field has been no exception. A search for DL publications in the domains of Medical Informatics, Sensing, Bioinformatics, Imaging, and Public Health shows a 5-fold to 6-fold increase in publication counts from 2010-2015[1], and this trend continues today. Applications of DL in healthcare have been particularly wide ranged, covering proteomics, genomics and expression data, electronic health records for patient characterizations, as well as image analysis for histopathology, magnetic resonance images (MRI) scans, positron emission topography scans, computerized topography (CT) scans, and endoscopy videos. DL image analysis methodologies have the potential to automate and speed up pathologists' tasks with high accuracy and precision. Recent applications have also illustrated the capacity for DL methodologies to extract information from histopathological images unseen to the human eye, such as expression data. Importantly, these ML and DL image analysis applications have the benefit of requiring no additional sample collection from patients, as inputs are typically biomedical images already collected within the clinical workflow.

The majority of ML and DL focus in the gastroenterology and hepatology communities has been in endoscopy, and this is highlighted by the recent Breakthrough Device Designation granted by the United States Food and Drug Administration (FDA) for a DL-based endoscopic, real-time diagnostic software for gastric cancer[2]. However, the application of ML and DL methodologies on histopathological images is a blossoming field with significant potential for clinical impact. Imaging modalities like hematoxylin and eosin (HE)- or immunohistochemistry (IHC)-stained slides, unlike others such as CT, MRIs, or endoscopies, provide microscopic perspectives into tissue sections, allowing for the algorithms to utilize cellular and nuclear features like shape, size, color, and texture. Hence, the goal of this review is: (1) To cover major terminology and trainable tasks by ML and DL; (2) To briefly review the history of digital pathology; (3) To provide an overview of the current ML and DL histopathological imaging-based approaches in gastroenterology and hepatology; and (4) To discuss challenges and opportunities moving forward.

ML AND DL OVERVIEW

The FDA defines ML as "an artificial intelligence technique that can be used to design and train software algorithms to learn from and act on data"[3], where artificial intelligence is the development of computer systems capable of tasks deemed to require human intelligence. ML involves representation of samples or inputs by a fixed, user-determined set of features, then the application of a classifier that can

distinguish and separate classes or types within the set of samples based off those selected features. There have historically been a range of popular ML techniques. Some examples include logic-based approaches, such as decision tree-based methods like Random Forest (RF) classifiers, statistic-based approaches, such as Bayesian networks or nearest neighbor algorithms, and support vector machines (SVMs), which aim to find optimal hyperplanes to separate classes on high dimensional data feature spaces[4]. DL represents a modern, specific subset of ML that uses deep neural network architectures for feature extraction and predictions. A schematic of a deep neural network is shown in Figure 1.

The general goal of a DL algorithm is to connect an input, such as an image, to a desired output. The hidden layers in the network act as feature extractors, and a final layer aggregates and utilizes these extracted features to generate the desired output. Specifically, deep neural networks have an input layer that is followed by successive hidden layers, each containing nodes. Starting at the input layer containing data, nodes in each hidden layer compute weighted sums from outputs in the previous layer. Within each node, these weighted sums are then passed into activation functions, which are critical for neural networks as they introduce non-linear transformations onto data. Each hidden layer thus introduces additional mathematical complexity in an effort to transform the input into new, informative representations within a new feature space. This process of defining representations for inputs in this new feature space is called embedding, and the representations are deemed informative when they can be effectively utilized by the final output layer in the network to carry out desired predictions. Some popular activation functions include the sigmoid, tanh, and Rectified Linear Unit functions.

To train the model, gradient descent, a popular optimization method, is utilized to minimize the "loss function", which quantifies model performance. Specifically, gradient descent minimizes the "loss function" by adjusting algorithmic weights at layer nodes, which directly affect the weighted sum calculations. As a result, the embedding process is iteratively improved to gradually tune and train the model for the task at hand. More detail is provided in the Model Training and Gradient Descent subsection below.

Common trainable tasks

The three most common tasks for DL approaches in imaging applications is in classification, segmentation, and detection (Figure 2). Classification involves the prediction of a label for an input image, such as "Normal" *vs* "Cancer". Segmentation involves the identification and localization of objects within a single image and outputs pixel-level designation of classes. Therefore, output segmentation maps will commonly have objects in the image colored or shaded based on their predicted class type. Lastly, detection, which is not a focus in this review, involves the identification of object classes in an image with a bounding box placed around it, such as in facial detection.

Levels of supervision

An important aspect of these studies is image annotation of correct class labels. Due to the tremendous file size of these high-resolution images upon digitization of histological slides into whole-slide images (WSIs), analysis over an entire WSI at once is computationally infeasible. As such, WSIs are typically broken up into equally sized patches and require training patch-level models. Labeling therefore can occur at the level of the WSI and at the level of the patches.

When labelling the classes of individual patches from a WSI, this can be done in a fully supervised, weakly supervised, or unsupervised manner. This section will cover these levels of supervision in the context of classification tasks. An overview of these approaches is covered in Figure 3A.

The fully supervised approach involves dataset-wide annotation at the patch-level. For example, this may involve a dataset of patches extracted from WSIs with pathologist-annotated labels for each patch as "cancer" or "normal". Thus, cancer positive WSIs will likely contain both types of patch classes. Though training iterations, the model will eventually learn to correctly predict the patch labels from just input patch images.

Weakly supervised methods concern annotations provided only at the WSI-level. Extracted patches from these WSIs are then run through algorithms that determine which patches were most important for the WSI-level label. Some possible approaches involve expectation-maximization methods[5] or multiple instance learning (MIL)[6-8]. In the context of the cancer positive WSIs, this would mean that the model eventually learns that the cancerous patches were most responsible for the WSI label, while healthy patches were not.

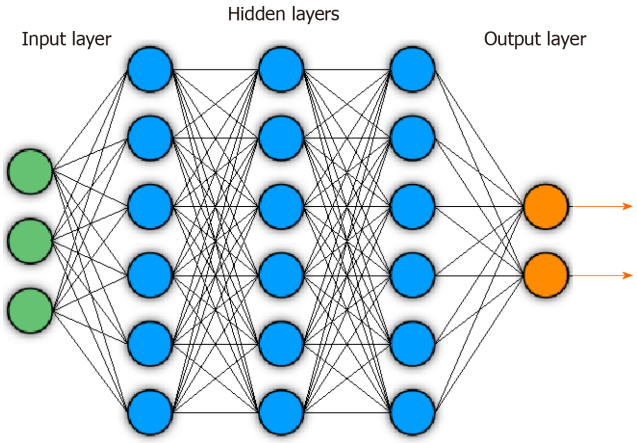


Figure 1 Example of general deep neural network architecture. Circles indicate nodes. Lines indicate feeding of layer node outputs into next layer nodes.

Unsupervised methods require no annotations to generate patch classes. While WSI-level labels may be provided, they are not utilized in patch class definition. These methods typically involve feature extraction from patches across the training dataset, followed by clustering approaches to define patch classes. For example, analysis of a dataset of cancer positive and negative WSIs may reveal dataset-wide patch-level classes for tumor, healthy, and fibrosis, although not all types may be present in each WSI. These approaches identify implicit patterns in the data to define these classes.

Fully supervised approaches require a tedious annotation process to provide correct output labels. As such, weakly supervised and unsupervised approaches hold the additional benefit of circumventing this labeling process and may be important in increasing throughput by decreasing annotation-related load. Another possible solution that is an active field of research is the generation of synthetic data that is indistinguishable from real world data. An example of this is general adversarial networks (GANs). GANs create synthetic data then have a 'discriminator' module that attempts to determine whether generated data is synthetic or real. The worse this discriminator performs, the better the GAN is at generating synthetic data. As such, computational approaches that effectively generate synthetic data across different classes may help develop labeled training datasets at high throughput.

Although the above methods discuss patch-level classifications, many biomedical imaging studies require a prediction at the WSI-level, such as a diagnosis. Often, this patch level information is aggregated by an additional classifier, and an overview of general approaches is provided in Figure 3B. This can be a ML classifier, such as an SVM or RF classifier that takes as input the relative counts of the different patch types per WSI to output a WSI-level prediction. This classifier can also be in the form of neural networks like recurrent neural networks (RNNs) and long short-term memory (LSTM) networks, covered later in this section, that take in variable length sequences of patches or patch representations as inputs to generate a WSI-level prediction. As the WSI-level label is typically clinically or biologically-informed, such as a diagnosis, prognosis, or grading, this part of the process typically receives supervision.

A variety of studies encompassing these approaches will be covered in this review in the two sections "Emulating the Pathologist" and "Beyond the Pathologist – Features Invisible to the Human Eye?". A general diagrammatic overview of the approaches used in provided in Figure 4.

Model training and gradient descent

In practice, DL is performed in response to the quantification by a "loss function" of how well the neural network performed across the training dataset. As loss functions quantify model performance, they require knowledge of the correct output for each sample and are easiest to introduce with supervised learning concepts. For classification, the output involves patch-level labels, and, for segmentation, the output will be images of the same dimensions as the inputs, where object classes in the image are distinguished by pixel-level color designation of classes (*e.g.*, shading cancerous areas with one color and shading healthy areas with another).

For classification, on each epoch, or iteration, of training, the algorithm attempts to predict the label of every image, then calculates, from the loss function, a scalar loss value that captures the degree to which the model-predicted output labels were

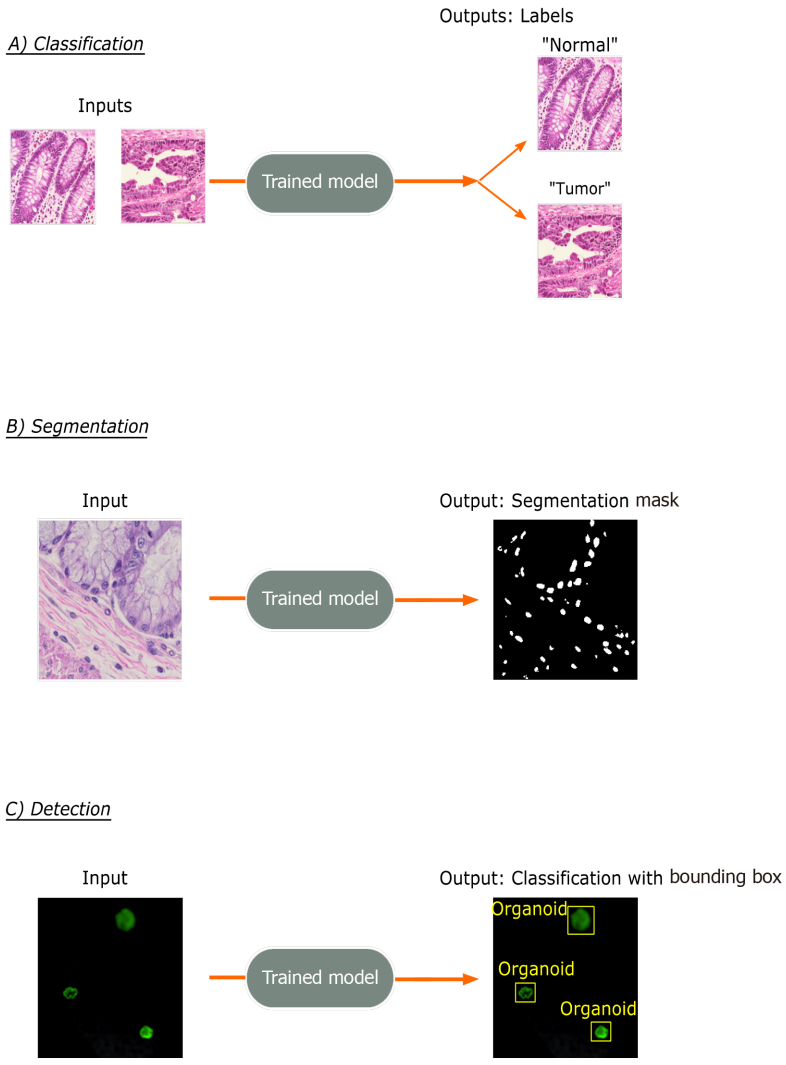


Figure 2 Common trainable tasks by deep learning. A: Classification involves designation of a class label to an image input. Image patches for the figure were taken from colorectal cancer and normal adjacent intestinal samples obtained *via* an IRB-approved protocol; B: Segmentation tasks output a mask with pixel-level color designation of classes. Here, white indicates nuclei and black represents non-nuclear areas; C: Detection tasks generate bounding boxes with object classifications. Immunofluorescence images of mouse-derived organoids with manually inserted classifications and bounding boxes in yellow are included for illustrative purposes.

different from the correct, user-designated output labels across the dataset. A lower loss value would therefore indicate better performance of the model in predicting the correct image labels. As segmentation involves correct, human-designated outputs at the pixel-level, the loss function quantifies correct class predictions across every pixel in a segmentation map output.

Gradient descent is an optimization method that iteratively moves in the direction of the steepest slope to approach minimums and is utilized in DL to minimize the loss functions. Gradient descent starts from the loss function and propagates through previous layers to the first, identifying the gradients for each algorithmic weight at every network layer node, then incrementally adjusting these weights according to the gradients. This process occurs every epoch with the overall goal of improving model performance by minimizing the loss function. These weights affect the non-linear mathematical operations performed at each hidden layer node and thus serves as a way for the network to tweak these operations to eventually determine a feature space and sample representations most effective for the task at hand. As opposed to ML techniques that depend on human-designated features for classification, the DL-based sample representations can be interpreted as an extraction of features deemed best and driven by the neural network's gradient descent optimization with respect to the loss function. This therefore introduces the common "black box" issue, where the meanings of these final representations, or extracted features, cannot be defined due to the high amount of mathematical complexity introduced onto the input image tensor by each of the layers in the network.

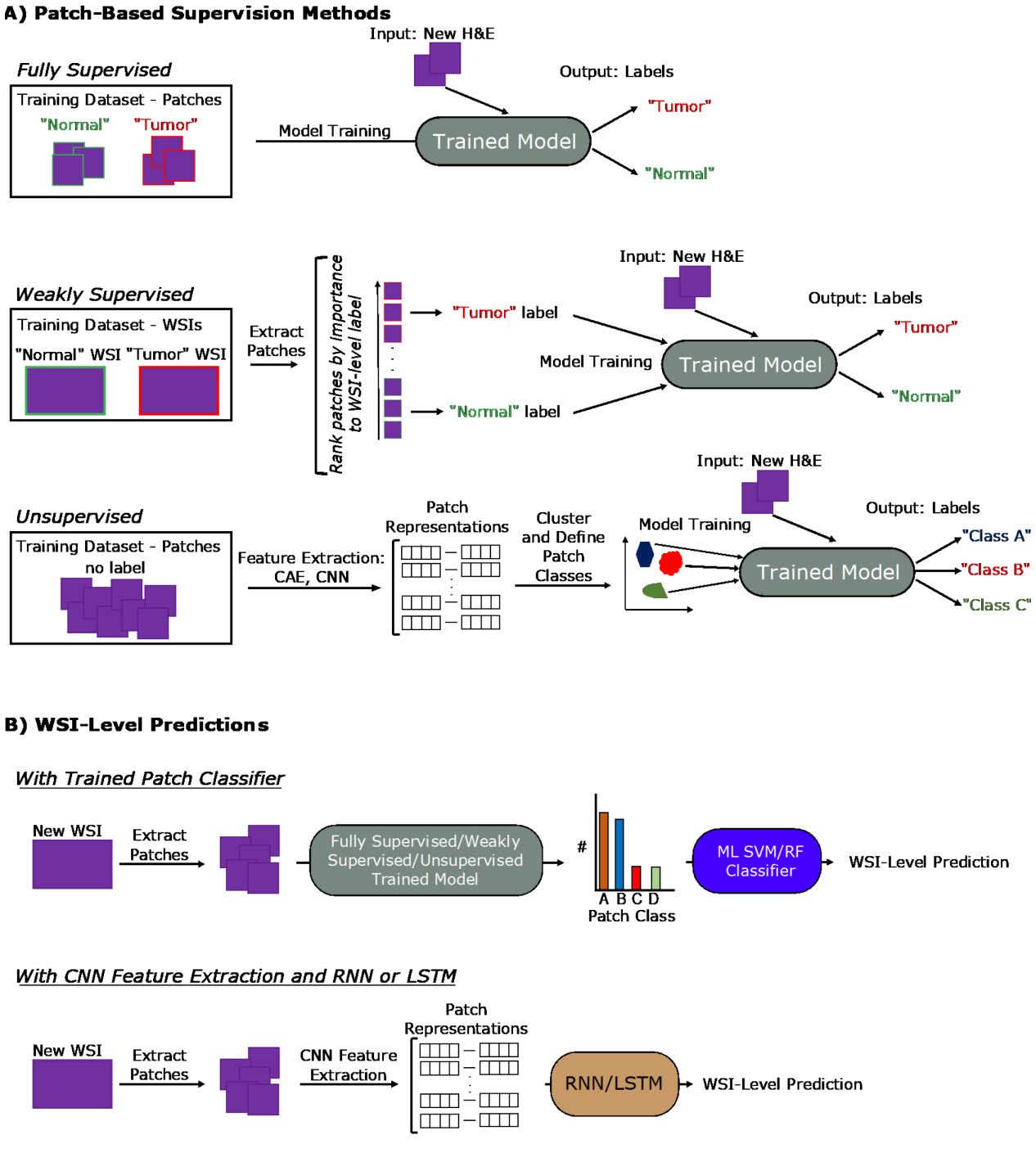


Figure 3 General deep learning training and prediction approaches. A: Examples pipelines for fully supervised, weakly supervised, and unsupervised learning methods for training patch classifiers are shown; B: Two pipelines translating patch-level information into whole-slide image-level predictions are shown. The top approach utilizes a patch classifier trained by one of the approaches in (A). The bottom approach uses a convolutional neural network feature extractor to generate patch representations that are fed into a long short-term memory or recurrent neural network. H&E: Hematoxylin and eosin; WSI: Whole-slide image; CNN: Convolutional neural network; RNN: Recurrent neural network; LSTM: Long short-term memory; CAE: Convolutional autoencoder.

Although gradient descent and loss functions are covered here, these are basic descriptions. For instance, the introduction of more complex loss functions that incorporate different learning constraints, study of approaches to take in multimodal inputs, and the development of novel network layers are all examples of highly active fields of research that add complexity to these concepts. Additionally, subcategories of gradient descent exist based on batch size, the number of images inputted before updating weights, such as stochastic gradient descent and mini-batch gradient descent. Other optimization algorithms like Adam optimization exist as well. Finally, various hyperparameters that affect model learning typically need to be tested over a range of values and each can affect different portions of training. Some major hyperparameters include learning rate, momentum, batch size, and number of epochs.

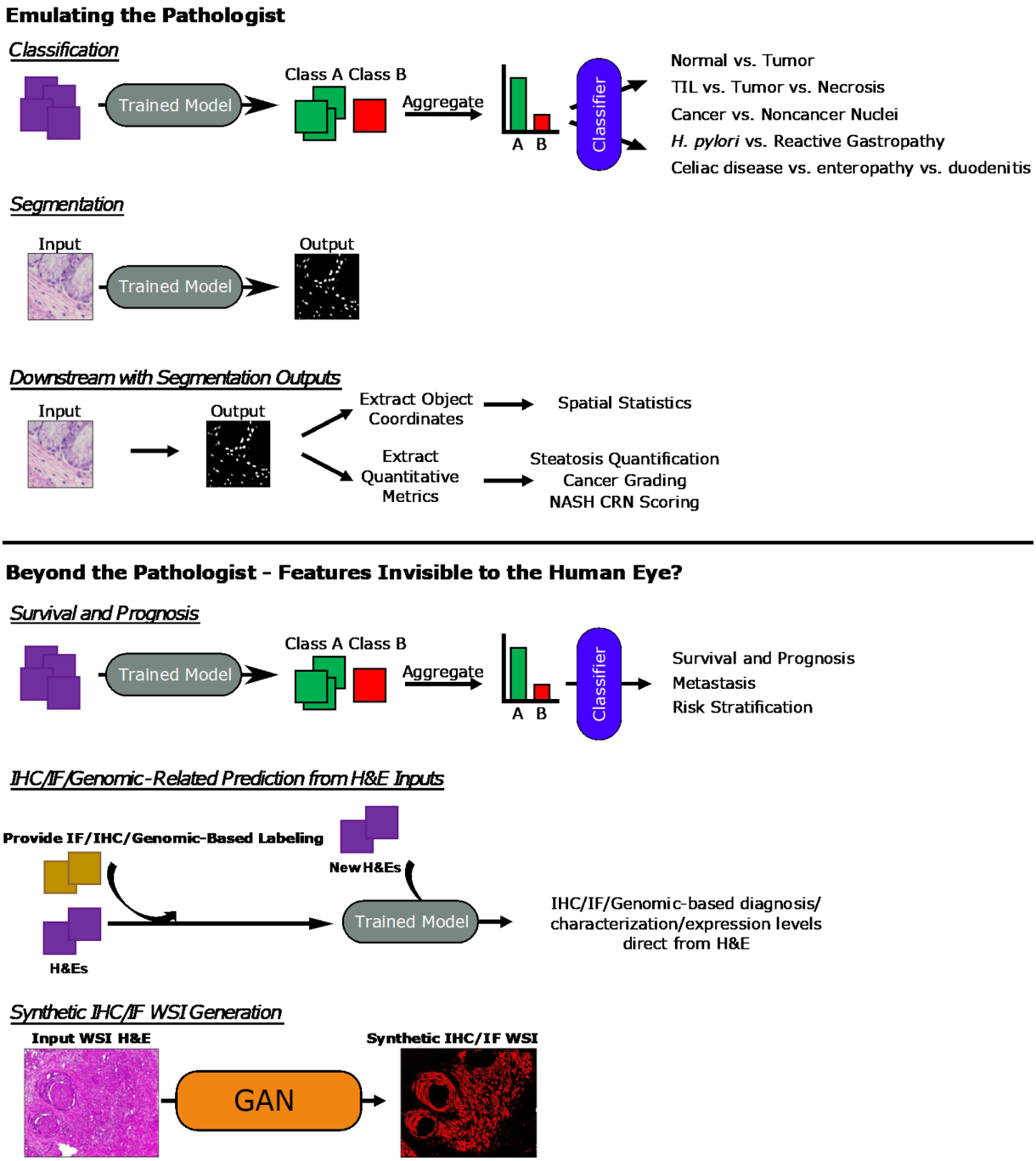


Figure 4 General overview of approaches. Overview of general machine learning - and deep learning-based approaches covered in the sections of this review are presented here. Whole-slide image (WSI) hematoxylin and eosin and Synthetic immunofluorescence (IF) WSI images in the Synthetic immunohistochemistry/IF Generation pipeline. Citation: Burlingame EA, McDonnell M, Schau GF, Thibault G, Lanciault C, Morgan T, Johnson BE, Corless C, Gray JW, Chang YH. SHIFT: speedy histological-to-immunofluorescent translation of a tumor signature enabled by deep learning. *Sci Rep* 2020; 10: 17507. Copyright© The Authors 2020. Published by Springer Nature. TIL: Tumor-infiltrating lymphocyte; *H. pylori*: *Helicobacter pylori*; NASH CRN: Nonalcoholic Steatohepatitis Clinical Research Network; IHC: Immunohistochemistry; IF: Immunofluorescence; H&E: Hematoxylin and eosin; WSI: Whole-slide image; GAN: General adversarial network.

Many of the common alternatives and hyperparameters are covered in this review by Shrestha and Mahmood[9].

Imaging data and convolutional neural networks

For imaging data, a specific type of neural network, called convolutional neural networks (CNNs), need to be utilized as inputs are in the form of 3-dimensional matrices, or tensors. A key concept is that any image can be represented by its numerical, pixel intensity values. For example, a 224 × 224 pixels grayscale image will be a 224 × 224 tensor with all values on a range from 0-1. A 224 × 224 RGB image, however, will be a 224 × 224 × 3 tensor with all values on a range from 0-255, though values are typically normalized to a range from 0-1.

To work with these tensors, convolutions need to be implemented in the form of convolutional network layers. Convolutions can be interpreted as the sliding of another tensor, or filter, typically of much smaller size than the input, over the input tensor. The filter slides from left to right of the input tensor, then moves down and repeats the process from left to right again. The mathematical operations can be considered as an expansion of the weighted sum and activation function approaches described earlier in this section. As the filter slides over the input, a weighted sum is calculated incorporating every cell overlap between the two tensors and generates a new output tensor that is then passed to an activation function. Like the layer nodes, convolution filters have weights that are trainable by gradient descent. Each hidden layer will perform similar operations on outputs from the previous layer but may have varying filter sizes or activation functions. Since the non-linearities introduced by the various layers sequentially add complexity, the earlier layers are believed to encode simpler features like edges, while the latter layers capture even more abstract features.

Analysis of images requires consideration of relationships between adjacent regions to capture spatial information. Though the weighted sum calculation in convolutions does account for neighboring pixels, the receptive fields are still quite small. Pooling layers are also carried out by filters and further aggregate local information from previous layers. The two major types are max and average pooling. For a 4 × 4 pooling filter, this would mean selecting the maximum value within the 4 × 4 receptive field in max pooling or averaging the 16 values for average pooling, as opposed to performing the weighted sum calculations that would occur in convolutions. Importantly, pooling layers do not have any trainable weights and represent fixed operations.

Convolutional layers typically reduce tensor height or width while increasing number of channel dimensions. Pooling layers do not affect channel dimensions but reduce tensor height and width. Thus, a series of convolutional and pooling layers will serve to reduce tensor height and width and increase channel dimensions relative to the original input.

The outputs of convolutional and pooling layers are often 3-dimensional and need to be flattened into a 1-dimensional vector towards the end of the network. The flattened 1-dimensional vectors then feed into a full-connected layer, a feed forward layer where nodes calculate weighted sums from the flattened vector input and pass values to an activation function. Finally, these outputs are utilized for the final classification layer, which typically uses a softmax activation function in classification tasks. The softmax layer will have the same number of nodes as the number of possible classes to predict. The outputs of this layer will sum up to 1 and can be interpreted as the relative probabilities for each class prediction with each class corresponding to one softmax node.

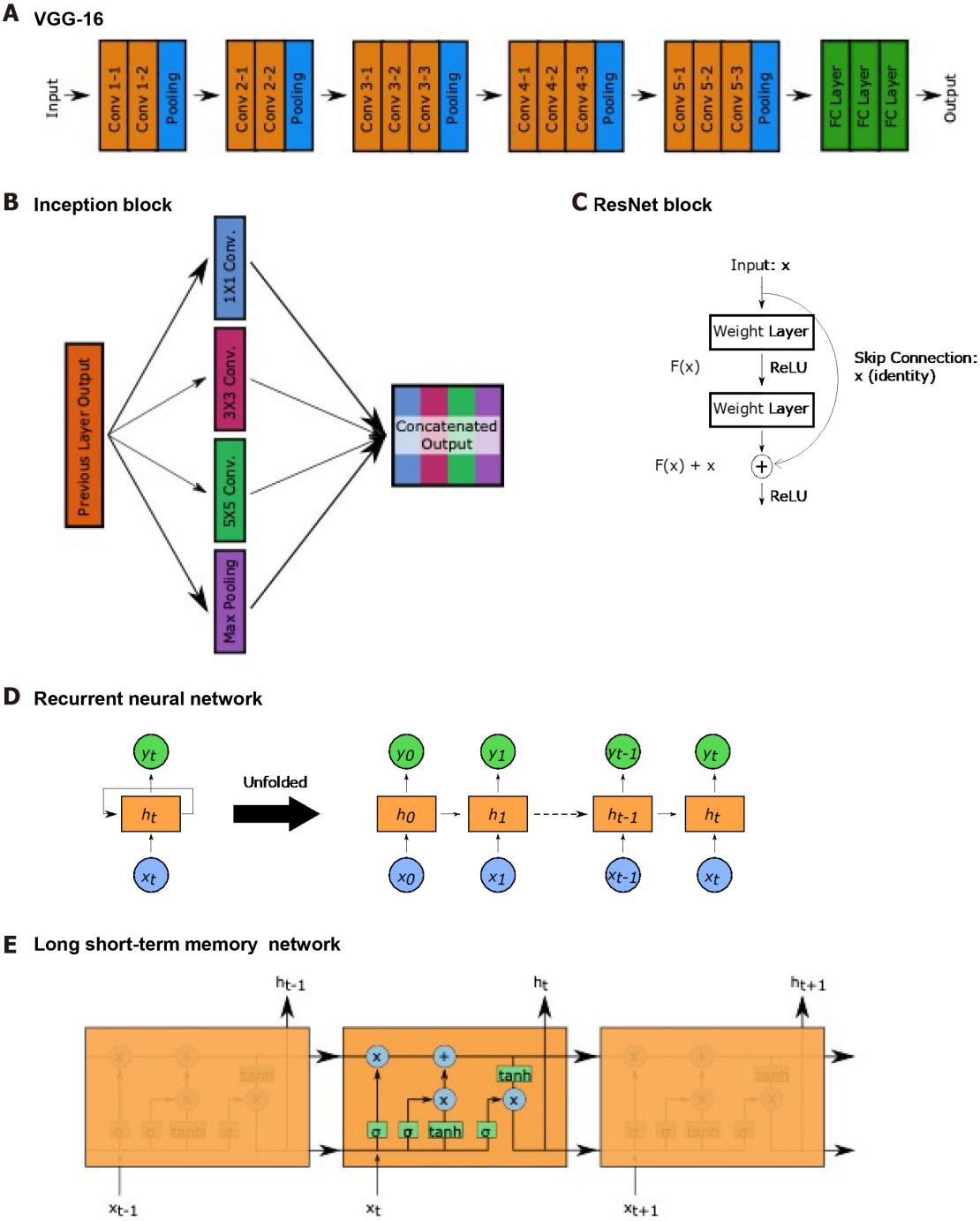
Common landmark neural network architectures

Although the focus of this review is not to delve deeply into the different types of neural network architectures, those that appear will be covered briefly here to provide background. An overview of the network structures is shown in Figure 5.

The visual geometry group (VGG)-16 and VGG-19 networks published by Simonyan and Zisserman[10] consist of sequences of convolutions and pooling operations followed by fully connected layers for a total of 16 or 19 layers, respectively. The authors incorporated very small 3 × 3 convolutional filters and demonstrated the capacity to create a network that had a lot of layers relative to other networks at time of publication.

The Inception network was initially published in 2015 by Szegedy *et al*[11], though several improved versions, such as the Inception-v3 used by some studies in this review, have since been developed. The major contribution of these networks is the introduction of the inception module that performs 1 × 1 convolutions, 3 × 3 convolutions, 5 × 5 convolutions, and max pooling at the same layer. An $n \times n$ convolution refers to a convolutional layer with an $n \times n$ dimension filter. The general concept is that predicting the optimal convolution filter size may depend on the image at hand. Instead of selecting a single filter size, more may be learned by incorporating information from convolutions with different receptive fields along with max pooling.

He *et al*[12] introduced ResNets which contain a the residual block with a skip connection. Deep neural networks with many layers often experience the issue of vanishing or exploding gradients. With the high amount of mathematical complexity introduced by many layers, backprogating these gradients can approach local minima and maxima and impede training. Since calculated gradient values are used to update layer node weights, a value of zero means the weight barely shifts, while infinity causes too significant of a change. Without getting into much detail, these residual blocks allow for skipping of portions of the network where this occurs to allow for


A VGG-16
Input
Conv 1-1
Conv 1-2
Pooling
Conv 2-1
Conv 2-2
Pooling
Conv 3-1
Conv 3-2
Conv 3-3
Pooling
Conv 4-1
Conv 4-2
Conv 4-3
Pooling
Conv 5-1
Conv 5-2
Conv 5-3
Pooling
FC Layer
FC Layer
FC Layer
Output
B Inception block
Previous Layer Output
1X1 Conv.
3X3 Conv.
5X5 Conv.
Max Pooling
Concatenated Output
C ResNet block
Input: x
Weight Layer
F(x)
ReLU
Weight Layer
Skip Connection: x (identity)
F(x) + x
ReLU
D Recurrent neural network
Unfolded
E Long short-term memory network
tanh
σ
F
Input
Conv/Pool
Conv/Pool
Conv/Pool
Conv/Pool
Conv
Conv
Output: Pixel-Wise Segmentation Map
Upsample

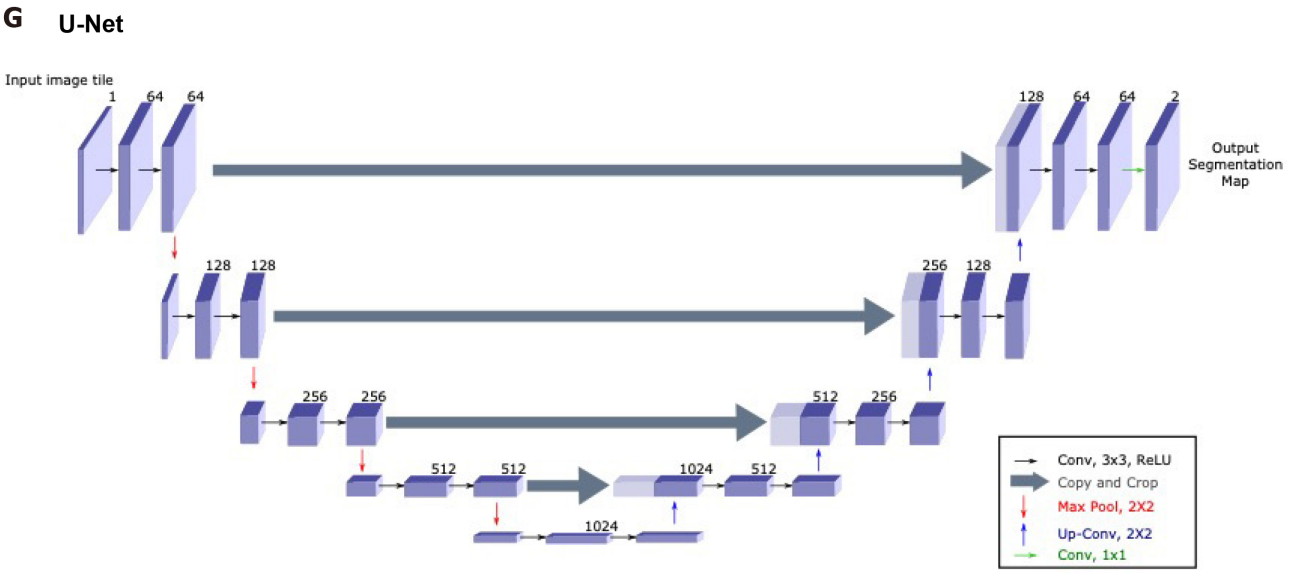


Figure 5 Common landmark network architectures. Overviews of landmark network architectures utilized in this paper are presented. A: The visual geometry group network incorporates sequential convolutional and pooling layers into fully connected layers for classification; B: The inception block utilized in the inception networks incorporates convolutions with multiple filter sizes and max pooling onto inputs entering the same layer and concatenates to generate an output; C: The residual block used in ResNet networks incorporates a skip connection; D: Recurrent neural networks (RNNs) have repeating, sequential blocks that take previous block outputs as input. Predictions at each block are dependent on earlier block predictions; E: Long short-term memory network that also has a sequential format similar to RNN. The horizontal arrow at the top of the cell represents the memory component of these networks; F: Fully convolutional networks perform a series of convolution and pooling operations but have no fully-connected layer at the end. Instead, convolutional layers are added and deconvolution operations are performed to upsample and generate a segmentation map output of same dimensions as the input. Nuclear segmentation images are included for illustration purposes; G: U-Net exhibits a U-shape from the contraction path that does convolutions and pooling and from the decoder path that performs deconvolutions to upsample dimensions. Horizontal arrows show concatenation of feature maps from convolutional layers to corresponding deconvolution outputs. VGG: Visual geometry group.

continued training. This has allowed for networks like the ResNet-34 and ResNet-50, which have 34 and 50 layers, respectively, and for extraction of even higher dimensional features.

Though typically for sequential or temporal data, RNNs and LSTM networks are utilized by a few studies covered in this review. RNNs were developed earlier and process sequences of data. Each layer performs the same task; however, the decisions made at each layer is dependent on previous outputs. This capacity has been important for speech data as words typically have a relationship with the previous word in a sentence. LSTMs have similar use cases but with a superior ability to identify longer term dependencies and relationships than RNNs. In the context of this review, RNNs and LSTMs are useful in being able to take in a variable length sequences of inputs to provide one output. As WSIs are composed of varying numbers of patches, these networks are implemented to aggregate patch information into a WSI-level output. In these studies, patch sequences are typically shuffled to ensure input patch ordering has no effect on the output and focus on leveraging the capacity to take in variable length inputs as opposed to the temporal component. While not utilized in studies covered in this review, bidirectional encoder representations from transformers is a more modern technique that, instead of only reading sequences left-to-right or right-to-left, considers bidirectional contexts when making predictions[13].

Segmentation tasks, which generate a segmentation map output of the same dimensions as the input, require specific types of networks. Long *et al*[14] introduced the fully convolutional network (FCN), which replaces the fully connected layers described earlier in the CNN description, with additional convolutional layers. Forgoing the flattening operation and fully connected layers maintains the spatial relationships in the 3-dimensional tensors. The FCN then performs deconvolutions, also known as transpose convolutions, which in practice perform the opposite function of convolutions. Deconvolutions increase the height and width dimensions of inputs, allowing for eventual generation of an output with the same dimensions as input. FCNs are able to generate probability heatmaps of possible segmentation classes.

Ronneberg *et al*[15] built upon the FCN by developing the U-Net, named due to its U-shaped network structure. U-Net has 4 convolutional layers to generate a bottleneck tensor, then 4 deconvolutional layers to up-sample the bottleneck tensor back to the

original input dimensions. Additionally, each deconvolutional layer receives input feature maps from the corresponding convolutional layer. As the convolutional layers occur earlier and encode spatial relationships more, concatenating these feature maps to the deconvolutional layer outputs helps the network with localization, which is important for segmentation tasks.

Quantitative performance metrics

Lastly, it is important to understand the quantitative metrics used to assess model performance. The most popular are accuracy, precision, recall, and F1 score. These metrics are all calculated based off the total number of true positives, true negatives, false positives, and false negatives, and their formulas are shown in Figure 6. Area under the curves (AUCs) are also a common metric and involve calculating this metric from a plot of sensitivity *vs* (1-specificity).

RISE OF DIGITAL PATHOLOGY

While ML and DL approaches have been applied to many input data types, the fields of computer vision and image analysis owe much of its popularity to the emergence of digital pathology. An early milestone for digital pathology was the development of software to view histology images, such as the Virtual Microscope developed from 1996 to 1998 that had to take advantage of existing methods for handling satellite and earth science data[16]. The Virtual Microscope was further refined to allow for capabilities like data caching, support for simultaneous queries from multiple users, and precomputed image pyramids. Modern viewers have continued to grow capabilities and allow for collaborative, multi-user work on the same images, annotations, the ability to zoom and inspect WSIs, and construction of imaging datasets and cohorts.

At the time the Virtual Microscope was being developed, WSI scanners were not yet available, so histology sections had to be digitally tiled up before uploading into viewing systems. Today, many commercially available WSI scanners that can scan entire slides exist, and this issue can be avoided. Furthermore, there are now two FDA-approved digital pathology platforms: the Phillips IntelliSite Pathology Solution[17] and the Sectra Digital Pathology Module[18].

Digital pathology comes with some clear benefits, including the ease of sample storage and access through software and the capacity to perform image analysis directly on digitized WSIs. However, the utilization of WSIs comes with its own set of quality concerns, which are covered nicely in the review by Kothari *et al*[19]. In brief, these methods can introduce image artifacts and batch effects. Image artifacts can occur both from scanning or preparation of samples. Some examples include blurring of tissue regions due to microscope autofocusing mechanisms, shadows in the image, pen marks from pathologists, or folding of tissue. In these cases, care needs to be taken to remove the artifacts, such as in the case of pen marks, or to filter out image areas with issues like blurring. Batch effects can occur due to the individual preparing the sample, the specific reagents used, the site of acquisition, or the microscope type. To address some of these concerns, studies frequently apply methods to normalize the color or pixel intensity values across their images. However, certain factors into batch effect exist that cannot be addressed computationally, such as varying patient population demographics based on location. As such, there is a strong need to incorporate multicenter data sources to develop more generalizable models, or a realization that certain models may only be used within specific demographics.

So long as these quality control concerns are recognized, however, digitized WSIs have the potential to be further adopted within practices. They are high-resolution gigapixel scale images that can be stored digitally and distributed for research. Furthermore, Al-Janabi *et al*[20] examined the feasibility of utilizing WSIs instead of classic light microscopy for diagnosis of gastrointestinal tract pathologies. For 100 cases of biopsies and resections along the entire gastrointestinal tract that had been diagnosed by light microscopy a year earlier, the authors recruited the same pathologists to re-diagnose their own cases using digitized WSIs. The study showed a 95% concordance between light microscopy- and WSI-based diagnoses with the discordant 5% of cases showing no clinical implications, highlighting the potential for the adoption of WSIs into the diagnostic workflow.

Finally, the growing popularity of DL imaging approaches owes itself to the development of computational hardware and software[21]. Graphical Processing Units (GPUs) were primarily used in the setting of video games, but their high capacities for

$$precision = \frac{TP}{TP + FP}$$

$$recall = \frac{TP}{TP + FN}$$

$$F1\ Score = \frac{2 \times precision \times recall}{precision + recall}$$

$$accuracy = \frac{TP + TN}{TP + FN + TN + FP}$$

$$specificity = \frac{TN}{TN + FP}$$

Figure 6 Quantitative performance metric equations. Quantitative metrics are based off of true positive, true negative, false positive, and false negative counts from results. Equations for precision, recall, F1 score, accuracy, and specificity. Precision is also known as the true positive rate, and recall is also known as sensitivity. TP: True positive; TN: True negative; FP: False positive; FN: False negative.

parallel computation were found to be ideal for DL methodologies. GPUs significantly increased DL model training speeds relative to Central Processing Units and played a strong part in the popularity growth of these methodologies. In addition, the DL community has been aided by the presence of open-source libraries for efficient DL GPU implementation. Some examples include PyTorch, Caffe, and Tensorflow. These libraries can easily load up and train major, landmark network architectures, simplify design of new networks relative to manual coding, and encourage cohesion amongst researchers by having standardized coding styles and pre-defined functions for common operations within each library. The emergence of digital pathology viewers, WSI scanners, GPUs, and open-source DL libraries has led to Pathomics, defined as the generation of quantitative imaging features to describe the diverse phenotypes found in tissue sample WSIs[22]. The foundations for DL-based imaging fields have thus been set and have welcomed a new influx of applications and studies within the biomedical disciplines.

EMULATING AND AUTOMATING THE PATHOLOGIST

One clear application of these ML- and DL-based methodologies is to replicate the tasks of pathologists. A well-trained model has the benefits of eliminating interobserver variability amongst pathologists and of achieving a level of throughput impossible to humans. This section will cover research in classification for both cancer and non-cancer pathologies and in segmentation tasks aimed at the identification of structures or cell types within images.

Cancer classification

The most popular histopathological application of these methods in gastroenterology and hepatology occurs in the classification of cancers. The general concept is that if these classifications can be made with the human eye, then the models should be able to learn to make such distinctions themselves.

Colorectal: Thakur *et al*[23] recently published a comprehensive review of artificial intelligence applications in colorectal cancer pathology image analysis, but several papers will still be highlighted in this review.

In an earlier study, Yoon *et al*[24] trained a customized VGG-based network architecture on 28 normal and 29 colorectal cancer HE-stained slides that were tiled up into 256 × 256 pixel patches. After testing several, custom VGG-based networks, the best model had an accuracy of 93.5% with a sensitivity and specificity of about 95% and 93%, respectively, in determining if an image patch was cancer *vs* healthy. This study showed promise in the relatively simpler binary classification task of tumor *vs* normal.

In a study published since, Sena *et al*[25] took the classification task another step further to train a model to classify between normal mucosa, early neoplastic lesion, adenoma, and cancer in HE-stained samples. The authors used a custom network architecture similar to VGG with four sequential convolutional and pooling layers followed by dense layers. Even with this relatively simple network architecture, the model achieved about a 95% accuracy in predicting the exact label for its larger 864x648 pixel patches across the four classes.

While the above patch-level performances are encouraging, clinical diagnoses are typically at the slide level. To address this, researchers often train additional classifiers, in addition to the patch-level ones, that can make a prediction at the WSI-level by aggregating patch-level information. An example of this is the study by Iizuka *et al*[26], which initially trained the Inception-v3 network to classify between non-neoplastic, adenoma, and adenocarcinoma for 512 × 512 pixel patches from HE-stained colorectal and gastric biopsy WSIs. The authors utilized the trained Inception-v3 classifier as a feature extractor to generate 715-length feature vectors, the representations, for each patch. The sequence of feature vectors of every patch in a WSI are used as the input and the WSI-label as the output in training a subsequent RNN. Though RNNs are typically used in temporal data, they have the advantage of being able to take in variable length, sequential inputs in generating final output labels. This is an important feature considering that every WSI has a varying number of total patches sampled. The trained RNN can thus predict the WSI diagnosis by aggregating extracted feature vectors of all patches in that sample. The study achieved WSI-level prediction AUCs of up to 0.97 and 0.99 for gastric adenocarcinoma and adenoma, respectively, and 0.96 and 0.99 for colonic adenocarcinoma and adenoma, respectively. Of note, the gastric classifier model outperformed pathologists in classification accuracy when pathologists were given a 30 s time limit, which is the average amount of time the model takes per WSI. The gastric model achieved an accuracy of 95.6% compared to the 85.89% ± 1.401% (n = 23) for the pathologists.

Russakovsky *et al*[27] utilized the AlexNet architecture pretrained on ImageNet, a large collection of non-biomedical, natural images with 1000 classes, as a feature extractor for classification and patch-based segmentation tasks on brain and colorectal HE datasets[28]. To address the common lack of annotated training datasets for these DL methodologies, the authors took this approach to demonstrate the potential of CNNs pretrained on non-biomedical images as feature extractors in biomedical applications. For the classification task, CNN-extracted patch representations for each WSI were pooled and condensed by feature selection methods before input into a SVM classifier to generate a WSI prediction. In colorectal cancer classification, the network was trained for a binary classification task to recognize tumor *vs* normal, and a multiclass classification task to recognize between adenocarcinoma, mucinous carcinoma, serrated carcinoma, papillary carcinoma, cribriform comedo-type adenocarcinoma, and normal. SVM with these CNN features as inputs outperformed SVM with a set of manually extracted feature as inputs in both classification tasks, achieving a 98.0% accuracy in binary and 87.2% in multiclass classification compared to 90.1% and 75.75%, respectively. The segmentation task involved no feature pooling as a SVM was trained to utilize patch-level CNN features to generate a patch classification prediction. By utilizing overlapping patches, pixel-level class predictions can be designated based off an ensemble method aggregating overlapping patch predictions. Again, the SVM with CNN prediction inputs outperformed the SVM with manually extracted feature inputs, showing an overall accuracy of 93.2% compared to 77.0%.

Although the above studies demonstrate the value of patch-level classifications in determining a WSI-level prediction, the annotations required for such a training dataset are highly time-consuming. Additionally, clinically archived tissue specimens are typically accompanied only by the WSI or patient-level diagnosis. MIL encompasses approaches to obtain insight into patches or patch-level features most critical for designation of the WSI-level label. MIL thus represents a possible way to generate effective patch classifier models utilizing only WSI-level annotations.

In MIL, each WSI is considered a bag in which multiple instances, or patches, are contained. If any one of these patches are positive for cancer presence, then the WSI can be determined to be cancer positive. While the instance-level patches have their own classes, these are unprovided or unknown. As such, the goal of MIL is to train an instance-level classifier based on the WSI-level labels to determine these unknown patch labels.

Xu *et al*[7] applied the MIL-Boost algorithm for HE colorectal slides for binary cancer *vs* non-cancer classification. In brief, the MIL-Boost algorithm trains the instance-level classifier by "boosting". "Boosting" refers to the successive training of weak classifiers, where each classifier improves by adding weights to incorrect predictions made by the previous classifier. Here, weak classifier weights are iteratively updated by gradient descent on the bag-level classifier loss function. Backpropagation occurs along patch instances that most negatively affected the predicted bag-level cancer positivity relative to the true WSI-level label and adjusts algorithmic weights to reduce these errors on the next iteration of the weak, instance level classifiers. This process with weak classifiers is repeated until the loss function is

minimized and an effective instance-level classifier is developed. The authors demonstrated superior performance of this approach (96.30% accuracy) as opposed to a fully supervised, patch-annotated approach (95.40%) in the binary cancer *vs* normal classification task.

As patch instances make up a WSI bag, a MIL-type bag representation can be considered to be a collection of patch instance representations where positive instances are provided a higher weight. To this end, Ilse *et al*[6] utilized a CNN to extract patch feature representations, then incorporated an attention mechanism to output a weighted average of all instances in a bag. Notably, the attention mechanism weights are determined by a two-layer neural network, meaning they are trainable unlike conventional MIL pooling operators that calculate maxes and means. These weighted bag representations can also be used to identify the most important instances for the bag prediction. The authors utilized a published HE colorectal cancer dataset[29] with annotated nuclear patches for epithelial, inflammatory, fibroblast, and miscellaneous and formed the MIL problem so that a bag is considered positive if at least one epithelium-positive patch exists in a WSI. This MIL approach trained an epithelial patch classifier with an accuracy and F1 score of approximately 90% and AUC of 96.8%. Furthermore, the authors could threshold for only instances with high weights, leading to the visualization of epithelial regions in the original HE WSI. Although the focus of this paper was on superior performance of the neural network tunable attention-mechanism relative to fixed alternatives, the final performance metrics lend support to the capacity of MIL approaches in training patch-level classifiers from WSI-level annotations.

Another major part of the colorectal cancer field is in the histopathological evaluation of HE-stained polyps to determine cancerous potential. In 2017, Korbar *et al*[30,31] trained a ResNet-based network to detect between hyperplastic polyps, sessile serrated polyps, traditional serrated adenoma, tubular adenoma, and tubulovillous/villous adenoma. The authors trained a patch-based classifier, then designated WSI-level predictions according to the patch-level class prediction that was most prevalent in the sample, given that at least 5 patches outputted that prediction[31]. This model achieved a 93.0% overall accuracy [95% confidence interval (CI): 89.0-95.9]. In another study, the authors utilized the same network architecture to identify the 5 classes but focused on implementing Gradient-weighted Class Activation Mapping (Grad-CAM) approaches to address model interpretability[30]. Grad-CAM can backpropagate from a patch's predicted class label to identify the regions in the input image that contributed most to the prediction. Though this was an early approach, the study showed promising potential for these Grad-CAM approaches to help identify regions of interests (ROIs) that were most influential in the patch-level polyp classification.

Esophageal: While different from Grad-CAM, Tomita *et al*[32] utilized a related concept in implementing attention-based mechanisms for weakly supervised training to detect 4 classes—normal, Barrett's esophagus without dysplasia, Barrett's esophagus with dysplasia, and esophageal adenocarcinoma—from HE-stained esophageal and gastroesophageal junction biopsies. The approach involved breaking up a WSI into patches, from which a CNN would extract features. Each WSI could then be represented as a feature map that is an aggregated patch grid of extracted feature vectors. These feature maps serve as inputs to the attention-based model, the goal of which is to identify the regions of the input feature maps most important for the output label classifications. Therefore, a concept is shared with Grad-CAM in identifying input image regions most influential to the class predictions. Unlike Grad-CAM, the attention-based model will learn to add weights to influential areas in the feature map to aid in final model classification performance. Of note, this process is considered weakly supervised because image output labels are only provided at the WSI-level, as opposed to the patch-level, yet the most influential patch types can be distinguished. The model manages to learn on its own the most salient image features and regions that were most important for the WSI label. The approach here achieved an overall accuracy of 83.0% (95%CI: 80-86) in identifying the 4 classes, outperforming the supervised baseline with an overall accuracy of 76% (95%CI: 73-80) that depends upon extraction of patches from ROI tediously annotated by pathologists. It should be noted, however, that the model achieves an F1 score of 0.59 (95%CI: 0.52-0.66) and the supervised baseline an F1 score of 0.50 (95%CI: 0.43-0.56) possibly indicating a high rate of false positives and negatives.

Moving even further away from supervised learning, Sali *et al*[33] demonstrated superior performance of unsupervised approaches in classifying HE-stained WSIs to be dysplastic Barrett's esophagus, non-dysplastic Barrett's esophagus, and squamous

tissue relative to supervised methods. The supervised approach was analogous to Iizuka *et al*[26]. Training patches labeled by pathologists were used to train the model, then an SVM or RF classifier aggregated the patch-level information for the WSI-level prediction.

The unsupervised feature extraction approach involved a deep convolutional autoencoder (CAE). Deep CAEs are broken up into an encoder and decoder branch. The encoder branch typically applies a series of convolution and pooling operations to act as a feature extractor that outputs a bottleneck feature vector. The decoder branch upsamples back from the bottleneck feature vector and reproduces the original image. Here, the loss function minimizes the differences between the input image and reproduced version, thereby enforcing that the bottleneck feature vector is an effective representation of the input. A helpful analogy is when one zips files on the computer. The process compresses the original file to a smaller memory size (encoding), but then still allows one to re-generate the full-size, original file (decoding). As one knows the zipping mechanism works, he or she can confidently share zipped file versions to others, instead of the larger, original file.

Once the deep CAE is trained, it can be utilized as a feature extractor for all patches in one's training dataset. Then, by performing clustering approaches, such as k-nearest neighbors (k-NN) or Gaussian mixture models (GMM), on all feature vector-transformed patches, patch types or classes across the dataset can be defined. A SVM or RF classifier can be trained to predict the WSI class by using the relative proportions of the different patch class types in the sample. For WSI-level inference, the deep CAE extracts feature vectors from all patches in the WSI, bins and counts the number of patches per clustering-defined patch type, then utilizes the trained SVM or RF classifier to generate the WSI prediction. This process is called unsupervised because the different types of patches in the WSI are determined by the algorithm independent of any labelling. This is in contrast to the supervised approach, where a CNN was trained to classify between human-defined Barrett's esophagus, non-dysplastic Barrett's esophagus, and squamous tissue patch types. The unsupervised GMM method showed good performance with weighted averages for accuracy, AUC, F1, precision, and recall all above 90%. In contrast, the metrics for the supervised approaches ranged from 50%-80%.

Gastric: Though gastric pathologies and cancers will be covered further in other sections of this review, not a tremendous amount of literature exists regarding just classification of gastric cancers. Leon *et al*[34] demonstrated that, in gastric cancer classification, inputting image patches as a whole into a custom, Keras sequential model shows superior performance than utilizing nuclei extracted from these image patches as input. This may be explained by the fact that the whole image patch contains morphological features that might be important for classification, while the cell input approach sacrifices those portions of the image. The other major study to note is the one by Iizuka *et al*[26] mentioned earlier, which showed impressive performance in classifying gastric and colorectal adenomas and adenocarcinomas.

Liver: As in the mentioned studies by Iizuka *et al*[26] and Ilse *et al*[6], CNNs can be used to extract patch feature representations. These representations are 1-dimensional vectors comprised of numerical, float values, and higher values can be interpreted as features most important, or highly activated nodes, for the prediction at hand, while lower values may be interpreted as important for the other non-predicted class.

Since these feature values can be reflective of their relative importance in the predicted class, Sun *et al*[8] used a CNN to extract patch representations from HE-stained WSIs, performed a pooling operation to aggregate patch features at the image level, then sorted the representations to organize activation values from high to low importance in terms of liver cancer prediction. The authors selected a range of top-k and bottom-k features from this sorted list to use in patch representations, driven by the idea that high activations should indicate features important for cancer classifications, while the lower activations should correspond to normal. The variable length representations dependent on k were tested to generate condensed patch representations in training a binary cancer *vs* normal classifier. A value of 100 for k was deemed optimal, and the authors used the patch classifications to predict WSI cancer *vs* normal status. The approach achieved an accuracy of 98%, a recall of 1.0, and an F1 score of 0.99.

In addition to the design of effective image classification algorithms, the incorporation of these methodologies into clinical workflow is important to consider. Kiani *et al*[35] trained a DenseNet CNN to classify between hepatocellular carcinoma and cholangiocarcinoma from HE image patches and developed a diagnostic support

tool that outputs predicted classes with probabilities and class activation maps (CAMs) to highlight areas of the input patch important for the prediction. The effects of the diagnostic support tool were analyzed and revealed that, while correct classifier predictions significantly improved accuracy, incorrect classifier predictions significantly decreased accuracy of diagnosing pathologists. Thus, this study highlights the important notion that the damaging effects of incorrect and misleading classifiers need to be strongly considered before clinical implementations.

Pancreatic neuroendocrine: IHC stains are another common technique applied to histopathological samples. The ability to detect specific antigens can be important for the characterization of certain cancer types. In pancreatic neoplasms, for example, the Ki67 stain is used to define proliferative rate and assign grades to pancreatic neuroendocrine tumors (NETs). However, this process is complicated by Ki67 stain positivity in both tumor and non-tumor regions. To address this issue, Niazi *et al*[36] trained an Inception-v3 network pretrained on ImageNet in a transfer learning setting to detect tumor and non-tumor regions on Ki67-stained pancreatic NET WSIs. As with Xu *et al*[28], the concept is that learned features from training on ImageNet should be beneficial within the biomedical setting. By freezing weights on all layers except for the final classification layer, the authors ensured that the feature extraction portion of the network remains unchanged. Training thus affects only the manner in which the classification layer utilizes patch representations instead of affecting the feature extraction itself. The trained model was used to create probability maps for tumor and non-tumor predictions for every pixel in the WSI, then thresholded by 0.5 to generate masks for each class. As each pixel in the image was then assigned to its most probable class, the output generated a segmentation map-type output that is shaded by predicted classes. In identifying tumor and non-tumor regions on a Ki67-stained IHC slide, the model showed about 96%-99% overall accuracy with 97.8% sensitivity and 88.8% specificity.

Cancer lymphocyte interactions: In addition to the cancer itself, other cell types exist within the microenvironment. To address this, Saltz *et al*[37] trained a VGG-16 network to identify tumor-infiltrating lymphocyte (TIL) containing patches across 13 The Cancer Genome Atlas (TCGA) HE-stained tumor types. The study identified four types of TIL infiltration patterns: Brisk Diffuse, Brisk Band-like, Non-Brisk Multifocal, and Non-Brisk Focal. The study also found associations between TIL infiltration patterns, cancer type, inflammatory response subtype, and molecular cancer subtypes and supports the notion that spatial phenotypes have the exciting potential to correlate with molecular findings.

Cancer nuclei classification: Another avenue of classification tasks in cancer applications has been in the study of nuclei. Pathologists are able to utilize visual, nuclear information, such as aberrant chromatin structures, to identify cancerous cells. Thus, groups have worked on replication this task of nuclei classification.

Chang *et al*[38] extracted HE-stained nuclei, used immunofluorescence (IF) pancytokeratin (panCK) stains aligned to the HE slide by image registration methods to label the HE-extracted nuclei as cancerous or non-cancerous, then trained a CNN to make these distinctions from just an HE input. The panCK-defined cancer positivity approach eliminated the need for tedious, pathologist annotations on the HE images and achieved a 91.3% accuracy with 89.9% sensitivity, 92.8% specificity, and 92.6% precision in classifying cancerous *vs* non-cancerous nuclei on the independent test set.

Sirinukunwattana *et al*[29] implemented a spatially constrained CNN to identify pixels most likely to represent the center of nuclei, then trained a subsequent CNN classifier to predict whether the nuclei came from an epithelial, inflammatory, fibroblast, or miscellaneous cell in colon cancer. The authors also implement a Neighboring Ensemble Predictor in the nuclei classifications, which, when predicting the class of a nuclei, incorporated the predictions from all neighboring patches. This approach achieved a weighted average F1 score of 0.784 and AUC of 0.917 in the nucleus classification tasks and a weighted average F1 score of 0.692 in the combined nucleus detection and classification tasks. In a follow up study since, Shapcott *et al*[39] utilized this nuclei classification algorithm to quantify the four cell types to correlate cellular proportions with different clinical variables in TCGA colorectal cancer patients. This led to findings such as samples with metastasis having more fibroblasts with fewer epithelial and inflammatory cells, samples with residual tumor having more fibroblasts and fewer epithelial and inflammatory cells, and that both venous and vascular invasion were associated with more fibroblasts.

Non-cancer classification

Though much focus in image classification has been in cancers, other image classification applications exist and are highlighted here.

Celiac disease, environmental enteropathy, and nonspecific duodenitis: Wei *et al*[40] trained a ResNet-based model to classify between celiac disease, normal tissue, and nonspecific duodenitis on HE-stained WSIs with accuracies of 95.3%, 91.0%, and 89.2%, respectively. This was a supervised, patch-based approach for training, and WSIs were predicted to be nonspecific duodenitis if more than 5 patches were classified as such or predicted to be the dominant patch class otherwise.

In a similar supervised fashion, Srivastava *et al*[41] trained a ResNet model on duodenal HE biopsies to classify between celiac disease, environmental enteropathy, and normal tissue. Patch classifications were aggregated for the WSI prediction and returned an overall 97.6% accuracy.

Sali *et al*[42] also trained a ResNet model, but for the task of Marsh Score-based grading of celiac disease severity using HE-stained duodenal biopsies. The authors utilized a CAE to generate patch representations, then performed a 2-class k-NN clustering to filter out useless, non-tissue containing patches. The tissue-containing patches were then used for supervised training of the ResNet model to recognize between Marsh scores of I, IIIa, IIIb, and IIIC. Again, patch predictions were aggregated for a WSI-level prediction. The model showed an accuracy and F1 score of around 80-90% for all classes and also implemented CAM approaches to localize certain cell subsets contributing to some of these Marsh Score categories.

In another study, Sali *et al*[43] took a novel, hierarchical approach towards training a VGG classifier to detect 7 classes: Duodenum-celiac disease, Duodenum-Environmental enteropathy, Duodenum-normal, Ileum-Crohn's, Ileum-normal, Esophagus-eosinophilic esophagitis, and Esophagus-normal. In addition to having the classifier predict the disease type with the final classification layer, the approach incorporated another output branch in the VGG network to predict anatomic location. The loss function combined outputs of the two branches and enforced the network to learn both anatomic origin and specific disease type. Additionally, the anatomic origin branch occurs before the final classification layer, meaning that the network needs to correctly determine the anatomic origin first, before homing in on the specific diagnosis. Across all 7 classes, the model exhibited F1 scores ranging from 0.714 for Duodenum-normal to 0.950 for Duodenum- Environmental enteropathy.

***Helicobacter pylori* gastritis and reactive gastropathy:** Similar to the other examples, these represent diagnoses that can be made from HE-stained specimens. Martin *et al*[44] trained the commercially available HALO-AI CNN to classify between *Helicobacter pylori*, reactive gastropathy, and normal in gastric biopsies. The model achieved sensitivity/specificity pairings of 73.7%/79.6%, 95.7%/100%, 100%/62.5% for normal, *Helicobacter pylori*, and reactive gastropathy, respectively.

Klein *et al*[45] developed a model that combines image processing techniques with DL. The authors utilized image processing techniques on both Giemsa- and HE-stained slides to identify potential *Helicobacter pylori* regions, then had experts review these as being positive or negative for *Helicobacter pylori* presence. These could then be utilized as input-output pairs to train a VGG-style network. The main goal of this paper, however, was to create a clinical decision support system that utilized the trained model and directs pathologists to *Helicobacter pylori* hotspots using Grad-CAM-style methodologies. Although this clinical decision support approach showed higher sensitivity than just microscopic diagnosis (100% *vs* 68.4%), specificity was lower than with just microscopic diagnosis (66.2% *vs* 92.6%).

Segmentation

Segmentation generally refers to operations that localize and detect cells and structures within a WSI. As pathologists can detect these objects within a sample, the goal is to train models to replicate these tasks.

The gland segmentation in colon histology images challenge contest challenge: A key contributor to the progression of computer vision disciplines has been the presence of challenges that provide a dataset and rank submitted models based off of performance-related quantitative metrics such as F1 scores or AUC values. One example of this is the gland segmentation in colon histology images challenge contest (GlaS) that was held in 2015[46]. These challenges help to stimulate computational disciplines. For one, the announcement of the challenge itself encourages researchers worldwide to address and tackle the problem. Compared to standalone papers, these

challenges also have the advantage of pitting the best models against each other to generate a clear benchmark for state-of-the-art performance.

Furthermore, even after completion of the challenge, groups will continue to optimize their algorithms and will have the ability to compare performance to previous high rankers in the challenge. Even since the GlaS challenge, numerous groups have continued to work on gland segmentation models by incorporating novel mechanisms. In 2016, Xu *et al*[47] added multichannel feature extractions for region and edge probability maps that were then fed into the final CNN for instance segmentation. Also in 2016, BenTaieb *et al*[48] applied topological and geometric loss functions into their FCN-based model. In 2019, Graham *et al*[49] introduced a new network component, the minimal information loss unit, that re-introduces resized versions of the original input image to combat the loss of information that accompanies downsampling from the successive convolution and max-pooling operations that occur in neural networks. Most recently in 2020, Zhao *et al*[50] incorporated spatial attention to weight important spatial locations and channel attention to weight important features to improve gland segmentation performance.

Non-colon gland segmentations: In general, segmentation methodologies require an additional step of development compared to classification tasks. For example, identifying glands in colonic mucosa is an important task but needs additional interpretation to be useful in the clinic. Some possibilities include quantifying the total number of glands or extracting shape-based glandular information to feed into a colorectal cancer classifier. Classification tasks like "Tumor" *vs* "Healthy", on the other hand, often already have a clear path towards clinical integration within the pathologist diagnostic workflow.

Reflective of this, many histopathological segmentation studies in gastroenterology and hepatology tend to be focused on optimizing segmentation results themselves, as opposed to continuing onto the translational application. However, high performance segmentations are critical in developing the downstream, clinically impactful algorithms. While some studies have continued onto the next step, the next few years will likely see some more of these segmentation studies bridging into more translational studies.

To highlight some examples, Xiao *et al*[51] segment out liver portal area components for eventual hepatitis grading. Extraction of features from these segmented structures to train a classifier to grade hepatitis will likely be the next step of this process. Xu *et al*[52] used a patch-based segmentation approach to identify epithelial and stromal regions in HE-stained breast and epithelial growth factor receptor-stained colon cancer slides as tumor-stroma ratios are recognized to have prognostic value. Here, the next step would be to assess the impact of algorithm-derived epithelium and stroma ratios in patient prognosis or cancer classification. Similarly, to address the eventual use case of segmenting tumors to assess pre-surgical tumor burden, Wang *et al*[53] used multitask and ensemble learning techniques for pixel-wise HE hepatocellular carcinoma segmentation. For eventual use in computer-assisted diagnosis systems, Qaiser *et al*[54] develop a fast HE colorectal segmentation algorithm that defines persistent homology profiles to capture morphological differences between normal and cancer nuclei. The emergence of more directly translational follow up studies and validations should be exciting and will be important to monitor.

Moving downstream with segmentation outputs: Some studies have entered this second phase and will be highlighted in this section. Awan *et al*[55] utilized a modified version of U-Net to perform colon gland segmentation on HE-stained colorectal adenocarcinoma patches, then extracted quantitative measures of glandular aberrance to train a SVM classifier for normal *vs* tumor classification and for normal *vs* low grade *vs* high grade classification. Glandular aberrance correlated with tumor grade, and this method achieved an accuracy of 97% and 91% for the two-class and three-class classifications, respectively. Thus, application of segmentation outputs in this manner can allow for the definition and extraction of novel quantitative features to aid in classification tasks and may provide a look into how these segmentation algorithms will be clinically implemented in the future.

Multiplex IHC (mIHC) involves concurrent histological staining of 6 cell markers or more, and Abousamra *et al*[56] developed an autoencoder-based color deconvolution algorithm to segment these different stains within a WSI. In a follow-up study, Fassler *et al*[57] utilized this algorithm on mIHC-stained pancreatic ductal adenocarcinoma (PDAC) WSIs to detect and perform spatial analyses on the cell types. Results indicated that CD16+ myeloid cells dominated the immune microenvironment and on average were of closer distance to tumor cells than CD3+, CD4+, CD8+, or CD20+

lymphocyte populations. In contrast to the study by Awan *et al*[55] that used segmentation outputs to inform a clinical task, Fassler *et al*[57] targeted a research application. A pipeline to detect all cell types from mIHC-stained WSIs, quantify, and perform special statistics would serve a wide audience of basic and translational researchers, and, in elevating analytical capacities, may stimulate research output.

A popular translational application of segmentation outputs has been in the field of hepatic steatosis quantification, which is important in the assessment of patients with fatty liver disease or to assess donor liver-quality for transplantation. In an earlier study, Lee *et al*[58] demonstrated correlation of steatosis quantification by image processing methods on WSIs with MRI measurements, pathologist visual scoring, and several clinical parameters, serving to validate the potential of image feature extraction from WSIs for these applications.

Forlano *et al*[59] took a ML-based approach to quantify the four histological features used in the Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) Scoring System, in an effort to automate the process and assess how their computationally extracted, quantitative histological metrics correlate with the semi-quantitative, categorial metrics of the NASH CRN Scoring System. The authors used image processing techniques to segment out and calculate percentages of fat, inflammation, ballooning, and collagen proportionate area, then fed the values into a binary logistic regression classifier to predict the presence of NASH. The authors argued that the traditional, semiquantitative approaches are outdated, due to their categorical nature and unavoidable interobserver variability, and demonstrated an AUC of 0.802 for their pipeline's capacity to predict NASH.

Sun *et al*[60] took a modified VGG-16 patch-based segmentation approach to quantify macrovesicular steatosis in HE-stained frozen, donor liver biopsies. The network was trained on patches extracted from WSIs with steatosis regions annotated by pathologists. As such, the final portion of their network could be trained against the pathologist-annotated steatosis maps to output pixel-wise steatosis prediction maps from HE patch inputs. Steatosis percent could then be calculated by summing steatosis probabilities from the predictions maps and dividing by total tissue area. Overall, the model had a sensitivity of 71.4% and specificity of 97.3% in predicting samples with over 30% steatosis, which is the threshold used by some centers for donor rejection.

Roy *et al*[61] trained a network to segment foreground steatosis droplet pixels from background, a network to recognize steatosis droplet boundaries, and a third neural network that took both of those outputs as input to generate the final segmentation map. Their segmentation results allowed for the calculation of steatosis pixel percentage (DSP%) and steatosis droplet count percentage (DSC%). DSC% most strongly correlated with histologically determined macrovesicular steatosis percentage (rho = 0.90, $P < 0.001$) and total steatosis percentage (rho = 0.90, $P < 0.001$). DSP% showed the best correlation with MRI fat quantification (rho = 0.85, $P < 0.001$).

Lastly, Salvi *et al*[62] gained the capacity to quantify both micro- and macrosteatosis on HE-stained liver WSIs. The algorithm achieved an overall accuracy of 97.27% on the test set for steatosis segmentation and showed the lowest average error of 1.07% when comparing automated steatosis quantification with manual quantification methods.

BEYOND THE PATHOLOGIST—FEATURES INVISIBLE TO THE HUMAN EYE ?

While the emulation and automation of pathologist tasks is a clear and exciting application of these methodologies, recent studies have shown that extraction of information that typically requires other sources of data or that are not obvious to the human eye are possible. This section will cover emerging research that utilizes histopathological specimens to extract such information.

Cancer survival and prognosis

Although pathologists and physicians can estimate cancer patient prognosis, these determinations often require more than microscopically examining a histological slide. For example, the tumor-node-metastasis (TNM) staging system, though informative, can require information like tumor size, typically gathered from CT scans, or nodal and distal metastases status, which is not evident from a single histopathological slide. In other cases, pathologists may perform genetic testing or IHC-staining for further molecular characterization and subtyping of cancers. Recent work shows that these ML- and DL-based methodologies may be able to learn to predict such information from just histopathological samples.

Bychkov *et al*[63] developed an approach to predict 5-year survival from HE-stained tumor microarrays from colorectal cancer patients. As with Xu *et al*[28] and Niazi *et al*[36], the authors took the VGG-16 network pretrained on the ImageNet dataset[27] as a feature extractor. Each WSI's collection of extracted patch features were then used to train a three-layer 1D LSTM network, since, similar to the RNN approach used by Iizuka *et al*[26], LSTM networks can take in a sequence of patch feature inputs. The LSTM model in this study was trained to generate a WSI-level 5-year prognosis probability. While the model's capacity to predict disease-specific survival was not extremely high (AUC = 0.69), it outperformed histological grade (AUC = 0.57) and Visual Risk Score (AUC = 0.58).

Yue *et al*[64] incorporated an unsupervised patch clustering method to define patch types, trained a VGG-16 network to recognize the patch types, then implemented an SVM classifier to predict 5-year disease-specific survival. For the unsupervised patch clustering, patch features were extracted by a CNN and pooled, dimensionality reduction was performed by principal component analysis, and the k-means clustering was performed in this lower-dimensional feature space to define patch clusters. While the best performing model generated an accuracy and F1 score of 100%, the approach needs to be validated given the small dataset of 75 WSIs. However, this study is another example of how these unsupervised patch clustering methodologies might be effective in determining patch classes.

To generate a more interpretable model for colorectal cancer survival, Kather *et al*[65] developed a prognostically predictive "deep stromal score" that utilizes outputs from a CNN trained to recognize adipose, background (glass slide), colorectal adenocarcinoma epithelium, debris, lymphocyte, mucus, smooth muscle, normal colon mucosa, and cancer-associated stroma. The authors used their NCT-CRC-HE-100K dataset that contains 100000 image patches covering these nine tissue classes to train and compare several models in classification performance. The top performing VGG-19 model was then applied to a held-out portion of their dataset. When fitting univariate Cox proportional hazard models to each of these 9 classes across the held-out dataset, the authors found that higher activation of five of the nine classes correlated with poor survival, though three were not significant (NS): Adipose [hazard ratio (HR) = 1.150 (NS)]; debris [HR = 5.967 (P = 0.004)]; lymphocytes [HR = 1.226 (NS)]; muscle [HR = 3.761 (P= 0.025)]; stroma [R = 1.154 (NS)] These five class activations were combined to form the prognostic deep stromal score and validated independently on colorectal adenocarcinoma cases from TCGA program. Multivariate analysis should that the deep stromal score was significant as a prognostic metric for overall survival [HR = 1.63 (P = 0.008)], disease-specific survival [HR = 2.29 (P = 0.0004)], and relapse-free survival [HR = 1.92 (P = 0.0004)].

Focusing on Stage III colon cancer patients, Jiang *et al*[66] used the NCT-CRC-HE-100K dataset generated by Kather *et al*[65] to train a classifier to determine the proportion of these tissue types in WSIs, then predict prognosis. Like Kather *et al*[65], the authors tested several networks on the classification task. They identified InceptionResNetV2 as their top performing model, which was utilized to extract proportions of the nine different tissue types from their own colorectal Stage III cancer dataset. The tissue proportions were fed into several ML classifiers, and the Gradient Boosting Decision Tree was identified as the top performer for prognostic predictions. On Stage III colorectal adenocarcinoma cases from TCGA, this top-performing approach correctly allocated patients into high- and low-risk recurrence groups to predict disease-free survival risk by both univariate and multivariate Cox regression analysis [univariate: HR = 4.324 (P = 0.004); multivariate: HR = 10.273 (P = 0.003)]. In addition, this approach also showed the capacity to predict overall survival risk on the TCGA dataset [univariate: HR = 5.766 (P = 0.000); multivariate: HR = 5.033 (P = 0.002)]. These results highlight a potential avenue for more interpretable ML- and DL-based algorithms and also are evidence of the importance of groups like Kather *et al*[65] making datasets publicly available to help advance the field as a whole.

For prediction of survival after hepatocellular carcinoma resection, Saillard *et al*[67] compared an weakly approach with and without an additional, supervised attention mechanism. In both approaches, a pre-trained CNN first extracts features from all patches in the WSI. For the weakly supervised approach (CHOWDER), these patch features are fed into the network along with WSI-level survival data to eventually determine the patches most influential to the survival outcome in an iterative learning process. For the approach with additional supervision (SCHOWDER), the weakly supervised mechanism in CHOWDER is further coupled by an attention mechanism that localizes to tumoral slide regions annotated by pathologists to identify the most influential patches. The SCHOWDER and CHOWDER surprisingly generated highly similar c-indices for survival prediction on the discovery set with 0.78 and 0.75,

respectively, further supporting the potential of these weakly supervised methodologies to rival more supervised ones. As CHOWDER assigns risk scores to tiles, the authors could also re-extract then visually inspect types of tiles indicated to be most high and low risk. This involved tumor presence, macrotrabecular architectural tumoral pattern, and vascular spaces in the tumor as high-risk, and tumoral and non-tumoral fibrosis and non-tumoral immune cells as low-risk.

Also in a weakly supervised fashion, Wulczyn *et al*[68] developed a DL system (DLS) to predict disease specific survival across 10 cancer types, including colon and stomach adenocarcinoma, from TCGA using only WSI-level survival data. Assuming the frequency of informative patches for cancer diagnosis on a slide is P, the probability of a randomly sampled patch being uninformative is 1-P. As such, as one samples n patches, the probability of not sampling any informative patches exponentially approaches zero as $(1\text{-}P)^n$. The authors leverage this property by randomly sampling multiple patches per slide, extracting features from each with CNNs that share weights, and performing average pooling of the extracted features. The outputs can then be fed into a fully-connected layer before the final logistic regression layer. Here, logistic regression is used as the authors found that discretizing time-to-event periods into specific intervals improved performance. In a combined cohort of all 10 cancers, the DLS was significantly associated with disease specific survival [HR = 1.58 ($P < 0.0001$)] after adjusting for cancer type, stage, age, and sex in multivariable Cox regression analysis. The DLS also outputs a risk score, allowing for stratification of stage II ($P = 0025$) and stage III ($P < 0.001$) patients. Finally, as with Saillard *et al*[67], high- and low-risk patches can be extracted and visualized for qualitative evaluation.

Although more of an intermediate endpoint, cancer metastasis is a specific event that correlates with reduced survival. Takamatsu *et al*[69] utilized image processing techniques to extract features from cytokeratin IHC-stained endoscopic resection samples and trained a RF ML classifier to predict lymph node metastasis. Their method demonstrated comparable performance to the predictive capacity of conventional histological features extracted from HE-stained slides. On the cross-validation approach, the ML achieved an average AUC of 0.822, compared to 0.855 for the conventional method. Although ML performance was not superior, comparable results by these algorithms are accompanied with the additional benefit of reduced interobserver variability. Furthermore, given the fact that the conventionally extracted HE features showed decent predictive power for lymph node metastasis, it will be interesting to see if a predictive classifier can be built off the HE images directly.

Circumventing staining methodologies

IF and IHC methods are often applied for further characterization of samples. However, these methodologies are time-consuming, can be costly, and require an unstained portion of the tissue. This section will thus focus on recent literature that trains using HE-stained images as input with output information that typically requires these additional staining techniques.

With the introduction of targeted molecular therapies for human epidermal growth factor receptor 2 (HER2) in gastric cancer, determining patient HER2 status has become increasingly important[70]. As this process requires IHC staining for HER2, automated extraction of such information from routinely collected HE samples has advantages in time, cost, and consistency. Sharma *et al*[71] utilized HER2 IHC-stained sections to define HER2 positive and negative regions on HE-stained sections from the same patient. HER2-stained IHC WSIs and HE-stained WSIs were aligned *via* semi-atuomatic image-registration approaches to define HER2+ and HER2- tumor regions on the HE based off of corresponding IHC positivity. Training on just the HE patches, the authors trained a custom CNN with three sequential convolution and pooling layers to classify between HER2+ tumor (69.6% accuracy), HER2- tumor (58.1% accuracy), and non-tumor patches (82.0%). Although the performance was modest in predicting IHC-defined output labels from HE, this was one of the earliest studies exploring this type of multimodal approach and used a relatively simple network architecture.

IHC can also be applied to metastatic NET samples to identify primary sites of origin. Redemann *et al*[72] trained the commercially available HALO-AI CNN on HE-stained samples of metastatic NET with known sites of origin to compare the algorithm's capacity to make such predictions against IHC-based approaches. While the algorithm achieved a worse overall accuracy of 72% compared to 82% for IHC-based diagnosis, the results are promising given the relatively comparable performance to the gold standard IHC approach and the author's training of an off-the-shelf, commercial algorithm. A comprehensive comparison of classification performance across multiple models may identify one with superior performance.

Govind *et al*[73] developed a DL-based pipeline to automate gastrointestinal NET grading, which classically involves IHC detection of a Ki-67-positive tumor hotspot region, then manual counting to obtain the percentage of Ki-67-positive tumor cells. The authors trained one model to detect Ki-67 hotpots and calculate a Ki-67 index from those hotspots similar to pathologists' workflow, and another model that generates Ki-67 index-based heat maps to classify hot-spot-sized tiles in the WSI as background, non-tumor, G1 tumor, or G2 tumor. Importantly, both models used synaptophysin- and Ki-67-double-stained (DS) WSIs as input. As DS WSIs are not common in clinical practice, the authors computationally merged synaptophysin- and Ki-67-single-stained IHC WSIs to generate DS WSIs to be used for this study.

The first model, SKIE, detects synaptophysin-positive, Ki-67-dense hotspots from these DS WSIs and emulates current pathologist workflow by calculating Ki-67 indices that capture proportional Ki-67 tumor positivity within these hotspots. The second model, Deep-SKIE, was trained by extracting hot-spot sized patches, then assigning correct, output labels according to SKIE outputs on those patches. Specifically, the four classes were background (class 0: if the tile has > 70% background pixels), non-tumor (class 1: < 20% synaptophysin stain), tumor grade 1 (class 2: Ki-67 index < 3%) and tumor grade 2 (class 3: 3% < Ki-67 index < 20%). In predicting these hot-spot patch labels, Deep-SKIE trained on DS WSIs exhibited an overall accuracy of 90.98% compared to 84.84% when trained on SS WSIs, indicating that the additional information from multiple markers aids in classification performance.

The most interesting contribution of this paper with respect to this section, however, is the authors' decision to train a cycle GAN to generate DS WSIs from SS WSI inputs. In brief, GANs attempt to generate synthetic imaging data that is indistinguishable from the real samples. There is typically a 'discriminator' module that attempts to correctly distinguish between the GAN-generated synthetic data and the real-world data. The worse your discriminator performs, the better your GAN is at generating synthetic data.

Here, the authors used a cycle GAN to generate synthetic DS WSIs from SS WSIs that are highly similar to the real DS WSIs already in the dataset. This approach has the ability to generate highly informative DS WSIs in the clinic without the required time and costs associated with additional stains. The authors showed that Deep-SKIE when trained on the GAN-generated DS WSIs showed an accuracy of 87.08% in predicting the four patch classes that was still significantly higher than 84.84% when trained on the SS WSIs.

IF staining is the other major staining technique. Unlike IHC, IF comes with an additional disadvantage regarding sample stability. As signal is carried by fluorophores, signals are often lost within a week, and samples are thus typically imaged immediately after staining. As such, DL approaches outputting IF-related data is not only informative and cost-efficient but may simplify acquisition of such data.

Burlingame *et al*[74] developed an experimental protocol allowing for HE and panCK IF staining in the same section of tissue, then trained a conditional GAN to output virtual panCK IF WSIs from HE PDAC WSI inputs. Similar to the cycle GAN used by Govind *et al*[73], the conditional GAN here depends upon a discriminator attempting to distinguish between real and virtual IF WSIs. As the protocol allows for HE and panCK IF staining on the same tissue, the authors have HE-IF WSI input-output pairs to train the conditional GAN. The virtual IF WSIs showed high similarity to the real IF WSIs in terms of structural similarity metrics, and the authors also present preliminary data on virtual IF generation for alpha-smooth muscle actin, a stromal marker.

This section has covered two categories of methodologies that circumvent the need for staining. The first category involves, directly from HE-stained inputs, the extraction of information that typically necessitates additional staining. These approaches may one day assist pathologists in quicker, cheaper molecular characterizations of patients. The second category involves the generation of synthetic staining outputs directly from HE inputs. These have the potential to complement the first category of these methods in outputting a virtual staining for pathologists to reference and may improve model interpretability. The other exciting avenue for this second category is research. Currently, many HE-stained imaging databases exist. A reliable methodology to generate high-quality, synthetic IF or IHC WSIs can augment these datasets for researchers worldwide. The addition of these new types of WSI data to existing datasets allows for the application of methods not previously utilized on these HE-only datasets. For example, synthetic IHC WSIs allow for segmentation approaches to identify cell types by marker positivity and allow for types of additional characterizations previously impossible with just the original HE WSIs.

Prediction of expression and genomic data

A string of recent studies has begun to explore the capacity of these approaches in extracting genomic or expression data from histopathological samples. As molecular subtypes affect underlying biology, the concept is that morphological shifts occur in the image and can be detected by algorithms.

Mutational panels are commonly used to further subtype cancers in the clinic. A method to detect these mutations directly from HE will allow for rapid subtyping without the need for additional genetic testing. Chen *et al*[75] developed an Inception-v3 model to detect hepatocellular carcinoma and predict mutational status for *CTNNB1*, *FMN2*, *TP53*, and *ZFX4* from frozen sections stained by HE. The mutational status prediction model was trained on the ten most significantly mutated genes in liver cancers. To validate the model, patients were split into mutated and wild-type groups for all of the ten genes, then the mutation prediction probabilities were examined across these cohorts. The four mentioned genes showed significant differences between the mutated and wild-type cohorts, indicating the model's ability to predict these mutations. As with the cancer classification tasks, mutational subtyping from HE has a clear path to clinical integration, and these results are encouraging in supporting the value of imaging-based molecular phenotypes.

The consensus molecular subtypes (CMS) transcriptionally distinguish four groups of colorectal cancer with different clinical behaviors and biology, so Sirinukunwattana *et al*[76] trained a model to designate image-based CMS (imCMS) classes to HE-stained slides. The authors used two resection cohorts (TCGA, FOCUS) and one biopsy cohort (GRAMPIAN), and patches were extracted from WSI regions annotated by pathologists to be tumor. The FOCUS dataset was used for training, as their associated transcriptional data could be utilized to provide CMS labels to the extracted tumoral patches. ImCMS predictions were thus CMS predictions made on a patch-level from histology, and WSI-level subtypes were assigned according to the most prevalent patch subtype prediction. On external validation, the trained model achieved a macro-average classification AUC ranging from 0.80 to 0.83 across the TCGA and GRAMPIAN datasets.

Interestingly, to improve model generalizability, the authors implemented domain-adversarial training. Similar to the GANs mentioned earlier, the goal is to reduce the discriminative ability of this domain-adversarial module in determining whether the input data came from the TCGA, FOCUS, or GRAMPIAN datasets. In practice, the network learns to identify input features important to determine imCMS, while lessening the importance of features that simply vary based off of dataset origin. This improved macro average AUCs in TCGA to 0.84 and in GRAMPIAN to 0.85.

This study by Sirinukunwattana *et al*[76] offered two additional novelties in CMS classification. First, patches with high prediction confidence for imCMS subtypes could be extracted to examine histological patterns. imCMS1 was associated with mucinous differentiation and lymphocytic infiltration, imCMS2 with cribriform growth patterns and comedo-like necrosis, imCMS3 with ectatic, mucin-filled glandular structures, and imCMS4 with prominent desmoplasia. Secondly, samples with tumoral heterogeneity are currently considered unclassifiable by CMS. The authors here compared agreement between the second most prominent CMS, determined transcriptionally, and the second most prominent imCMS, predicted through this pipeline. The authors noted a high degree of significant, cosine similarity between all four CMS-imCMS pairs. This ability to identify imCMS tumor heterogeneity may improve colorectal cancer classifications and is a nice example of how current molecular subtyping approaches may be augmented by improved spatial granularity.

Microsatellite instability (MSI) is another prognostic indicator in colorectal cancers that can be diagnosed *via* genetic analyses. Kather *et al*[77] thus trained a classifier to recognize MSI and microsatellite stability (MSS) from HE-stained TCGA slides. The approach involved training an initial ResNet-18 model to recognize tumor *vs* normal to eventually extract only tumor-containing patches from the WSIs. Patches were then assigned MSI or MSS labels based on the patient's TCGA-recorded MSI status or as MSI-positive if patients have an unknown status but a mutation count over 1000. This labeled data was then used to train another ResNet-18 model to predict MSI and MSS. In external validation on colorectal cases, the model exhibited a patient-level AUC of 0.84 (95%CI: 0.72-0.92). Interestingly, the gastric MSI-detection model achieved a lower AUC of 0.69 (95%CI: 0.52-0.82) on a Japanese cohort, likely reflective of the TCGA stomach adenocarcinoma training cohort being composed of 80% non-Asians and indicates the necessity of multi-center training data for more generalizable models. Finally, patient MSI-levels could be correlated with transcriptomic data. Higher MSI-levels correlated with lymphocyte gene expression in gastric cancer and with PD-L1

expression and interferon- γ signal in colorectal cancer.

This model was further refined by Kather *et al*[78] in a follow-up study. Specifically, the authors trained the ShuffleNet network, a more lightweight architecture that performed comparably to the more complex ones. After validating on the capacity to classify MSI in colorectal cancer, the model was trained on other tasks and found to be able detect the mutation of at least one clinically actionable mutation in 13 of 14 cancers from TCGA.

To take the next step of validation, Echle *et al*[79] applied this refined model to a multicenter dataset across Europe. This required the authors to form the MSIDETECT consortium and led to the generation of an 8000-patient dataset with molecular alterations. The algorithm was trained using data from multiple sources, including the TCGA, a German, a United Kingdom, and a Netherlands cohort. The study achieved an impressive AUC of 0.96 for detecting MSI in a large, international validation cohort and exemplifies the importance of multi-source training data. Compared to the study from[77], where authors showed an inability of the model trained on an 80% non-Asian TCGA dataset to perform well on a Japanese dataset, this study improved model generalizability by incorporating a more global patient distribution during training.

While the above studies targeted a specific molecular phenotype in MSI, Schmauch *et al*[80] set to explore whether general RNA-sequencing expression could be inferred from HE-stained tumor WSIs. The authors extracted up to 8000 tissue-containing patches per WSI from all TCGA cancer types and utilized an ImageNet-pre-trained ResNet-50 to extract 2048-length feature vectors from each. By utilizing k-means clustering, the authors generated 100 supertiles per WSI from these patches on the basis of location. The supertile representation consisted of averaged values for all contained patches over the 2048 ResNet-50 features. For each slide, the feature-extracted supertiles could be fed into another network where the output classification layer contains nodes corresponding to every gene. Thus, the inputs represent supertile feature vectors for every supertile in a WSI, and the network deconvolutes TCGA patient-level transcriptional levels across the WSI supertiles. The network is thus able to detect relationships between supertile features and WSI expression levels to identify the supertile-features most important for certain gene expressions. This is then translated into gene expression outputs at the supertile-level, which could be aggregated to generate gene expression heatmaps at the WSI-level.

Results were validated by comparing predicted expression of CD3 and CD20 with actual IHC-stained sections. For both markers, expression predicted by the model highly correlated with the percentage of cells positively stained in the IHC sections ($P < 0.0001$ for both). Furthermore, this predictive ability for expression data allowed the authors to implement lists of genes involved in major cancer pathways, including angiogenesis, hypoxia, deregulation of DNA repair, cell-cycling, B-cell responses, and T-cell responses for Gene Set Enrichment Analysis. The authors were able to assess the activation of these pathways across a wide range of cancers.

Finally, as MSI should be defined in part by some changes in expression levels, the authors tested whether the transcriptomic representations learned in their approach can improve the MSI detection performance relative to Kather *et al*[77]. When applied to HE-stained TCGA colorectal cancer slides, the model achieved a superior AUC of 0.81 compared to 0.68 for the method by Kather *et al*[77] in predicting MSI. The takeaway message is that, since their model can generate transcriptomic representations to the level of predicting supertile-level, gene-specific expression data, using this model as a feature extractor will generate sets of feature inputs superior in the subsequent MSI *vs* MSS classification compared to simply inputting the patches themselves. A method to detect expression levels with a patch-level resolution can impact not only these sorts of expression-based molecular characterizations, but also serves to provide the research community with a powerful tool to further leverage existing HE WSI datasets.

The above studies all occurred within the last few years and represent a growing application of these approaches. Historically in public datasets like TCGA, WSIs have been underutilized relative to the genomic data. These recent advances demonstrate promise in proving direct connection of imaging features with underlying genomic and expression data. Furthermore, even with the gradual decrease in sequencing costs, finances still inhibit widespread adoption and availability of these technologies. Thus, these sorts of algorithms may eventually alter clinical landscapes by providing access to genomic and expression characterizations worldwide at a fraction of the cost.

CHALLENGES MOVING FORWARD

Although the field is in an exciting, fast-moving period, several challenges exist moving forward. In terms of clinical integration, the highly discussed "black box" problem still persists. Deep neural networks and the representations they learn in making their predictions lack in interpretability. This issue is magnified in healthcare where discussion of clinical decisions between patient and physician is critical and is therefore a field of highly active research. For imaging-based studies, CAM visualizations like the one utilized by Kiani *et al*[35] can direct pathologists' attention the image locations utilized for generating final predictions. In addition, the decision of Kiani *et al*[35] to output prediction probabilities and confidences for every possible class provided pathologists with more transparency. This allowed for a clinical decision support system that operated as a tool to help inform pathologists in their decisions and may be more easily integrable into the clinic than algorithms that simply generate a prediction. The study importantly identified, however, the detrimental effects on pathologist performance when the classifier outputs an incorrect prediction. Future care must be taken to fully consider the ramifications of incorrect predictions on patient care and to manage liability of pathologists in these situations.

Another general feature of ML- and DL-based algorithms as a whole is their highly specialized nature. As might be evident from this review, models are typically focused on specific pathologies within a specific organ site. For example, there is no current study that attempts to tackle classification of gastric carcinoma, nonspecific duodenitis, and *Helicobacter pylori* gastritis at once. As algorithms for specific pathologies get adopted into clinical care, the feasibility of this approach will likely be tested. Special care will likely need to be taken for cataloguing a wide range of models per healthcare system, keeping up with library updates and decisions to keep or update code with deprecated support of certain functions, and maintaining constant quality control mechanisms to ensure high model performance. Each of these will be amplified by the addition of more models into a healthcare system.

As many of these studies rely upon sample annotation for certain use cases, models often become highly specialized. However, more generalizable approaches may become necessary. Hosseini *et al*[81] addressed this issue by establishing an "Atlas of Digital Pathology" that contains around 18000 annotated images of different tissue types across the human body. The dataset contains images across three levels, with the top level addressing the general tissue types and subsequent levels addressing subtypes. An example from top to lowest level would be Epithelium - Simple Epithelium - Simple Squamous Epithelium. Using this Atlas of Digital Pathology, Chan *et al*[82] then trained a model that can segment out 31 of the tissue types in the database across over more than 10 organ types. The generalizability of the model may be attributed to the non-organ-specific nature of the tissue types in the Atlas of Digital Pathology.

Binder *et al*[83] developed a gland segmentation algorithm for colon adenocarcinoma and breast invasive cancer by utilizing stromal masks. Here, this may be due to stroma appearing more similar across breast and colon than the glands themselves. Analogous approaches leveraging shared features across organ sites may thus help for future multi-organ models.

These research fields are highly interdisciplinary, requiring collaboration between the more quantitative computer scientists and the more biological physicians and academics. While the strength of these fields derives from the complementary nature of the two sides' highly specialized skillsets, efforts should be made to further increase their cohesion. To illustrate a difference between the two sides, physicians and academics may be surprised to find that a large number of high impact computational studies are in the forms of conference papers or open-access online publications. This is in contrast to biomedical conferences typically being restricted to abstracts, posters, and oral presentations, and the emphasis on peer-reviewed journals.

The computer vision challenges, such as the GlaS challenge[46], may represent an avenue to introduce more cohesion. As mentioned earlier, these challenges serve an important role of generating excitement for an application and leads to submissions of high performing models from a multitude of groups. At present, however, endpoints tend to be metrics like F1 score that focus on the computational performance for the task at hand. Introduction of challenges with more biomedical endpoints may invite design and competition of pipelines that incorporate these methodologies for more directly translational tasks. An example of this would be hepatic steatosis quantification. As opposed to having a challenge to only improve lipid droplet segmentation, a challenge that provides an HE dataset and evaluates submissions by ability to translate segmentation outputs into highly accurate steatosis quantification might fast

track the development of methods for specific clinical tasks. Furthermore, these sorts of challenges would require collaboration of computer scientists and physicians during submission, standardizing the concept of interdisciplinary workflows at a more preclinical point.

In a similar vein, Louis *et al*[84] argue the field of computational pathology faces an important need to "create a culture that considers the computer and computation as being as central to pathology as the microscope". Integral to this, the authors posit, is the early exposure to computational concepts ideally during medical school. A certain level of computational awareness and literacy on the physicians' side is integral to perpetuate excitement of these methodologies and for clinical integration. A similar need, however, exists on the computational side. Emerging computational scientists should be provided the opportunity and made aware of the various biomedical applications of their methodologies. This exposure benefits both sides by instilling experience of collaboration at an early stage, recognition of constraints on the other side, and a cultural adoption of the notion that the two sides should be integrated.

Overall, computational pathology is in an exciting time with rapid advancements. The imaging applications have advanced to the point of defining imaging phenotypes and correlating with some clinical variables. As covered in the NCI workshop report by Colen *et al*[85], the integration of imaging approaches with -omics information will be a powerful strategy to further characterize and direct clinical care but necessitates the definition of imaging standards. Though the workshop focused on radiological phenotypes, the ideas translate similarly to histopathology. Standardization of methods for data analysis, feature extraction, data integration, and data acquisition will likely be important for robust comparison of methodologies for clinical evaluation. These steps will allow for decreased uncertainties when comparing across different clinical sites and provide a confidence in the imaging phenotypes that will be necessary when beginning to correlate with other -omic phenotypes. While some studies in this review excitingly extracted -omic information directly from histopathological slides, future clinical decision support systems will likely still need to aggregate histopathological information with -omic data to a degree to inform users.

Lastly, these ML- and DL-based technologies are unique in their capacity to continuously learn in response to new data. The FDA has recognized this and published a discussion paper soliciting feedback for a potential premarket review approach for these technologies[86]. Specifically, the FDA has proposed a "Total Product Lifecycle Regulatory Approach" that covers not only premarket review and methods for transparent real-world monitoring upon rollout, but also required proposals for any anticipated changes and steps to be taken for model alterations. These changes include retraining based on new data, incorporation of new target demographics based on new data, increasing capability to different input types but with same intended use (being able to take in MRI in addition to CT for a particular diagnosis), or changing intended use (now able to diagnose an additional type of cancer). As indicated by the steps the FDA has already taken, these algorithms will need to be regulated in a unique way that maximizes the capacity for continued improvement.

In summary, these ML- and DL-based imaging methodologies are rapidly expanding and being increasingly applied in the biomedical domains. Even at this point in time, we are seeing studies that are focused on optimizing the computational tasks, on bridging into translational applications, and on integrating these technologies into clinical decision support tools. In addition to the exciting performance and potential over a wide range of topics, this field also represents an opportunity to further bring together computational scientists with their physician and academic counterparts. Future adoption of these technologies into the clinic will likely be accompanied by increased dependence of healthcare systems on computer scientists who can understand and manage the software and will hopefully encourage cultural standardization of these interdisciplinary workflows.

CONCLUSION

The application ML- and DL-based methodologies on histopathological slides in the context of gastroenterology and hepatology continues to rise. Though still largely preclinical, recent studies have exhibited the exciting performance of these models in classification and segmentation tasks and in the extraction of features unseen to the human eye, such as prognosis, information that typically necessitates additional stains, or genomic and expression data. The field is in a time rife with studies demonstrating

these potential applications, and the FDA has already taken steps to begin considering the adoption of these technologies into healthcare systems. As such, it will be of importance and interest to monitor not only the methodologies themselves, but the considerations necessary in developing clinical tools.

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REFERENCES

1 **Ravi D**, Wong C, Deligianni F, Berthelot M, Andreu-Perez J, Lo B, Yang GZ. Deep Learning for Health Informatics. *IEEE J Biomed Health Inform* 2017; **21**: 4-21 [PMID: 28055930 DOI: 10.1109/JBHI.2016.2636665]

2 **Cision PR Newswire**. AI Medical Service Inc. announces FDA Breakthrough Device Designation for endoscopic AI system. [cited 22 January 2021]. In: Cision PR Newswire [Internet]. Available from: https://www.prnewswire.com/news-releases/ai-medical-service-inc-announces-fda-breakthrough-device-designation-for-endoscopic-ai-system-300953301.html

3 **U.S. Food and Drug Administration**. Artificial Intelligence and Machine Learning in Software as a Medical Device. [cited 20 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/medical-devices/software-medical-device-samd/artificial-intelligence-and-machine-learning-software-medical-device

4 **Kotsiantis SB**, Zaharakis ID, Pintelas PE. Machine learning: a review of classification and combining techniques. *Artif Intell Rev* 2006; **26**: 159-190 [DOI: 10.1007/s10462-007-9052-3]

5 **Hou L**, Samaras D, Kurc TM, Gao Y, Davis JE, Saltz JH. Patch-based Convolutional Neural Network for Whole Slide Tissue Image Classification. *Proc IEEE Comput Soc Conf Comput Vis Pattern Recognit* 2016; **2016**: 2424-2433 [PMID: 27795661 DOI: 10.1109/CVPR.2016.266]

6 **Ilse M**, Tomczak J, Welling M. Attention-based deep multiple instance learning. International conference on machine learning. Proceedings of Machine Learning Research; 2018

7 **Xu Y**, Mo T, Feng Q, Zhong P, Lai M, Eric I, Chang C. Deep learning of feature representation with multiple instance learning for medical image analysis. *IEEE* 2014; 1626-1630 [DOI: 10.1109/ICASSP.2014.6853873]

8 **Sun C**, Xu A, Liu D, Xiong Z, Zhao F, Ding W. Deep Learning-Based Classification of Liver Cancer Histopathology Images Using Only Global Labels. *IEEE J Biomed Health Inform* 2020; **24**: 1643-1651 [PMID: 31670686 DOI: 10.1109/JBHI.2019.2949837]

9 **Shrestha A**, Mahmood A. Review of deep learning algorithms and architectures. *IEEE Access* 2019; **7**: 53040-53065 [DOI: 10.1109/ACCESS.2019.2912200]

10 **Simonyan K**, Zisserman A. Very deep convolutional networks for large-scale image recognition. 2014 Preprint. Available from: arXiv:14091556

11 **Szegedy C**, Liu W, Jia Y, Sermanet P, Reed S, Anguelov D, Erhan D, Vanhoucke V, Rabinovich A. Going deeper with convolutions. *IEEE CVPR* 2015; 1-9 [DOI: 10.1109/CVPR.2015.7298594]

12 **He K**, Zhang X, Ren S, Sun J. Deep Residual Learning for Image Recognition. 2015 Preprint. Available from: arXiv:1512.03385

13 **Devlin J**, Chang MW, Lee K, Toutanova K. Bert: Pre-training of deep bidirectional transformers for language understanding. 2018 Preprint. Available from: arXiv:1810.04805

14 **Long J**, Shelhamer E, Darrell T. Fully convolutional networks for semantic segmentation. 2015 Preprint. Available from: arXiv:1411.4038

15 **Ronneberger O**, Fischer P, Brox T. U-net: Convolutional networks for biomedical image segmentation. In: Navab N, Hornegger J, Wells W, Frangi A. Medical Image Computing and Computer-Assisted Intervention – MICCAI 2015. Lecture Notes in Computer Science: Springer, 2015: 234-241

16 **Pantanowitz L**, Sharma A, Carter AB, Kurc T, Sussman A, Saltz J. Twenty Years of Digital Pathology: An Overview of the Road Travelled, What is on the Horizon, and the Emergence of Vendor-Neutral Archives. *J Pathol Inform* 2018; **9**: 40 [PMID: 30607307 DOI: 10.4103/jpi.jpi_69_18]

17 **U.S. Food and Drug Administration.** 510(k) Substantial Equivalence Determination Summary for Phillips Intellisite Pathology Solution (PIPS). [cited 22 January 2021]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.accessdata.fda.gov/cdrh_docs/reviews/K172174.pdf.

18 **U.S. Food and Drug Administration.** 510(k) Substantial Equivalence Determination Summary for

Sectra Digital Pathology Module. [cited 22 January 2021]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.accessdata.fda.gov/cdrh_docs/reviews/K193054.pdf.

19 **Kothari S**, Phan JH, Stokes TH, Wang MD. Pathology imaging informatics for quantitative analysis of whole-slide images. *J Am Med Inform Assoc* 2013; **20**: 1099-1108 [PMID: 23959844 DOI: 10.1136/amiajnl-2012-001540]

20 **Al-Janabi S**, Huisman A, Vink A, Leguit RJ, Offerhaus GJ, ten Kate FJ, van Diest PJ. Whole slide images for primary diagnostics of gastrointestinal tract pathology: a feasibility study. *Hum Pathol* 2012; **43**: 702-707 [PMID: 21937077 DOI: 10.1016/j.humpath.2011.06.017]

21 **Litjens G**, Kooi T, Bejnordi BE, Setio AAA, Ciompi F, Ghafoorian M, van der Laak JAWM, van Ginneken B, Sánchez CI. A survey on deep learning in medical image analysis. *Med Image Anal* 2017; **42**: 60-88 [PMID: 28778026 DOI: 10.1016/j.media.2017.07.005]

22 **Gupta R**, Kurc T, Sharma A, Almeida JS, Saltz J. The emergence of pathomics. *Curr Pathobiol Rep* 2019; **7**: 73-84

23 **Thakur N**, Yoon H, Chong Y. Current Trends of Artificial Intelligence for Colorectal Cancer Pathology Image Analysis: A Systematic Review. *Cancers (Basel)* 2020; **12** [PMID: 32668721 DOI: 10.3390/cancers12071884]

24 **Yoon H**, Lee J, Oh JE, Kim HR, Lee S, Chang HJ, Sohn DK. Tumor Identification in Colorectal Histology Images Using a Convolutional Neural Network. *J Digit Imaging* 2019; **32**: 131-140 [PMID: 30066123 DOI: 10.1007/s10278-018-0112-9]

25 **Sena P**, Fioresi R, Faglioni F, Losi L, Faglioni G, Roncucci L. Deep learning techniques for detecting preneoplastic and neoplastic lesions in human colorectal histological images. *Oncol Lett* 2019; **18**: 6101-6107 [PMID: 31788084 DOI: 10.3892/ol.2019.10928]

26 **Iizuka O**, Kanavati F, Kato K, Rambeau M, Arihiro K, Tsuneki M. Deep Learning Models for Histopathological Classification of Gastric and Colonic Epithelial Tumours. *Sci Rep* 2020; **10**: 1504 [PMID: 32001752 DOI: 10.1038/s41598-020-58467-9]

27 **Russakovsky O**, Deng J, Su H, Krause J, Satheesh S, Ma S, Huang Z, Karpathy A, Khosla A, Bernstein M. Imagenet large scale visual recognition challenge. *Int J Comput Vis* 2015; **115**: 211-252

28 **Xu Y**, Jia Z, Wang LB, Ai Y, Zhang F, Lai M, Chang EI. Large scale tissue histopathology image classification, segmentation, and visualization *via* deep convolutional activation features. *BMC Bioinformatics* 2017; **18**: 281 [PMID: 28549410 DOI: 10.1186/s12859-017-1685-x]

29 **Sirinukunwattana K**, Ahmed Raza SE, Tsang YW, Snead DR, Cree IA, Rajpoot NM. Locality Sensitive Deep Learning for Detection and Classification of Nuclei in Routine Colon Cancer Histology Images. *IEEE Trans Med Imaging* 2016; **35**: 1196-1206 [PMID: 26863654 DOI: 10.1109/TMI.2016.2525803]

30 **Korbar B**, Olofson AM, Miraflor AP, Nicka CM, Suriawinata MA, Torresani L, Suriawinata AA, Hassanpour S. Looking under the hood: Deep neural network visualization to interpret whole-slide image analysis outcomes for colorectal polyps. *IEEE CVPRW* 2017; 821-827 [DOI: 10.1109/CVPRW.2017.114]

31 **Korbar B**, Olofson AM, Miraflor AP, Nicka CM, Suriawinata MA, Torresani L, Suriawinata AA, Hassanpour S. Deep Learning for Classification of Colorectal Polyps on Whole-slide Images. *J Pathol Inform* 2017; **8**: 30 [PMID: 28828201 DOI: 10.4103/jpi.jpi_34_17]

32 **Tomita N**, Abdollahi B, Wei J, Ren B, Suriawinata A, Hassanpour S. Attention-Based Deep Neural Networks for Detection of Cancerous and Precancerous Esophagus Tissue on Histopathological Slides. *JAMA Netw Open* 2019; **2**: e1914645 [PMID: 31693124 DOI: 10.1001/jamanetworkopen.2019.14645]

33 **Sali R**, Moradinasab N, Guleria S, Ehsan L, Fernandes P, Shah TU, Syed S, Brown DE. Deep Learning for Whole-Slide Tissue Histopathology Classification: A Comparative Study in the Identification of Dysplastic and Non-Dysplastic Barrett's Esophagus. *J Pers Med* 2020; **10** [PMID: 32977465 DOI: 10.3390/jpm10040141]

34 **Leon F**, Gelvez M, Jaimes Z, Gelvez T, Arguello H. Supervised classification of histopathological images using convolutional neuronal networks for gastric cancer detection. *IEEE STSIVA* 2019; 1-5 [DOI: 10.1109/STSIVA.2019.8730284]

35 **Kiani A**, Uyumazturk B, Rajpurkar P, Wang A, Gao R, Jones E, Yu Y, Langlotz CP, Ball RL, Montine TJ, Martin BA, Berry GJ, Ozawa MG, Hazard FK, Brown RA, Chen SB, Wood M, Allard LS, Ylagan L, Ng AY, Shen J. Impact of a deep learning assistant on the histopathologic classification of liver cancer. *NPJ Digit Med* 2020; **3**: 23 [PMID: 32140566 DOI: 10.1038/s41746-020-0232-8]

36 **Niazi MKK**, Tavolara TE, Arole V, Hartman DJ, Pantanowitz L, Gurcan MN. Identifying tumor in pancreatic neuroendocrine neoplasms from Ki67 images using transfer learning. *PLoS One* 2018; **13**: e0195621 [PMID: 29649302 DOI: 10.1371/journal.pone.0195621]

37 **Saltz J**, Gupta R, Hou L, Kurc T, Singh P, Nguyen V, Samaras D, Shroyer KR, Zhao T, Batiste R, Van Arnam J; Cancer Genome Atlas Research Network, Shmulevich I, Rao AUK, Lazar AJ, Sharma A, Thorsson V. Spatial Organization and Molecular Correlation of Tumor-Infiltrating Lymphocytes Using Deep Learning on Pathology Images. *Cell Rep* 2018; **23**: 181-193. e7 [PMID: 29617659 DOI: 10.1016/j.celrep.2018.03.086]

38 **Chang YH**, Thibault G, Madin O, Azimi V, Meyers C, Johnson B, Link J, Margolin A, Gray JW. Deep learning based Nucleus Classification in pancreas histological images. *Annu Int Conf IEEE Eng Med Biol Soc* 2017; **2017**: 672-675 [PMID: 29059962 DOI: 10.1109/EMBC.2017.8036914]

39 **Shapcott M**, Hewitt KJ, Rajpoot N. Deep Learning With Sampling in Colon Cancer Histology. *Front Bioeng Biotechnol* 2019; **7**: 52 [PMID: 30972333 DOI: 10.3389/fbioe.2019.00052]

40 **Wei JW**, Wei JW, Jackson CR, Ren B, Suriawinata AA, Hassanpour S. Automated Detection of Celiac Disease on Duodenal Biopsy Slides: A Deep Learning Approach. *J Pathol Inform* 2019; **10**: 7 [PMID: 30984467 DOI: 10.4103/jpi.jpi_87_18]

41 **Srivastava A**, Sengupta S, Kang SJ, Kant K, Khan M, Ali SA, Moore SR, Amadi BC, Kelly P, Syed S. Deep learning for detecting diseases in gastrointestinal biopsy images. *IEEE SIEDS* 2019; 1-4 [DOI: 10.1109/SIEDS.2019.8735619]

42 **Sali R**, Ehsan L, Kowsari K, Khan M, Moskaluk CA, Syed S, Brown DE. Celiacnet: Celiac disease severity diagnosis on duodenal histopathological images using deep residual networks. *IEEE BIBM* 2019; 962-967 [DOI: 10.1109/BIBM47256.2019.8983270]

43 **Sali R**, Adewole S, Ehsan L, Denson LA, Kelly P, Amadi BC, Holtz L, Ali SA, Moore SR, Syed S. Hierarchical Deep Convolutional Neural Networks for Multi-category Diagnosis of Gastrointestinal Disorders on Histopathological Images. 2020 Preprint. Available from: arXiv:2005.03868

44 **Martin DR**, Hanson JA, Gullapalli RR, Schultz FA, Sethi A, Clark DP. A Deep Learning Convolutional Neural Network Can Recognize Common Patterns of Injury in Gastric Pathology. *Arch Pathol Lab Med* 2020; **144**: 370-378 [PMID: 31246112 DOI: 10.5858/arpa.2019-0004-OA]

45 **Klein S**, Gildenblat J, Ihle MA, Merkelbach-Bruse S, Noh KW, Peifer M, Quaas A, Büttner R. Deep learning for sensitive detection of Helicobacter Pylori in gastric biopsies. *BMC Gastroenterol* 2020; **20**: 417 [PMID: 33308189 DOI: 10.1186/s12876-020-01494-7]

46 **Sirinukunwattana K**, Pluim JPW, Chen H, Qi X, Heng PA, Guo YB, Wang LY, Matuszewski BJ, Bruni E, Sanchez U, Böhm A, Ronneberger O, Cheikh BB, Racoceanu D, Kainz P, Pfeiffer M, Urschler M, Snead DRJ, Rajpoot NM. Gland segmentation in colon histology images: The glas challenge contest. *Med Image Anal* 2017; **35**: 489-502 [PMID: 27614792 DOI: 10.1016/j.media.2016.08.008]

47 **Xu Y**, Li Y, Liu M, Wang Y, Lai M, Eric I, Chang C. Gland instance segmentation by deep multichannel side supervision. Medical Image Computing and Computer-Assisted Intervention – MICCAI 2016. Lecture Notes in Computer Science: Springer, 2016: 496-504

48 **BenTaieb A**, Hamarneh G. Topology aware fully convolutional networks for histology gland segmentation. Medical Image Computing and Computer-Assisted Intervention – MICCAI 2016. Lecture Notes in Computer Science: Springer, 2016: 460-468

49 **Graham S**, Chen H, Gamper J, Dou Q, Heng PA, Snead D, Tsang YW, Rajpoot N. MILD-Net: Minimal information loss dilated network for gland instance segmentation in colon histology images. *Med Image Anal* 2019; **52**: 199-211 [PMID: 30594772 DOI: 10.1016/j.media.2018.12.001]

50 **Zhao P**, Zhang J, Fang W, Deng S. SCAU-Net: Spatial-Channel Attention U-Net for Gland Segmentation. *Front Bioeng Biotechnol* 2020; **8**: 670 [PMID: 32719781 DOI: 10.3389/fbioe.2020.00670]

51 **Xiao QE**, Chung PC, Tsai HW, Cheng KS, Chow NH, Juang YZ, Tsai HH, Wang CH, Hsieh TA. Hematoxylin and Eosin (H&E) Stained Liver Portal Area Segmentation Using Multi-Scale Receptive Field Convolutional Neural Network. *IEEE J Emerg Sel Top Circuits Syst* 2019; **9**: 623-634

52 **Xu J**, Luo X, Wang G, Gilmore H, Madabhushi A. A Deep Convolutional Neural Network for segmenting and classifying epithelial and stromal regions in histopathological images. *Neurocomputing* 2016; **191**: 214-223 [PMID: 28154470 DOI: 10.1016/j.neucom.2016.01.034]

53 **Wang X**, Fang Y, Yang S, Zhu D, Wang M, Zhang J, Tong KY, Han X. A hybrid network for automatic hepatocellular carcinoma segmentation in H&E-stained whole slide images. *Med Image Anal* 2021; **68**: 101914 [PMID: 33285479 DOI: 10.1016/j.media.2020.101914]

54 **Qaiser T**, Tsang YW, Taniyama D, Sakamoto N, Nakane K, Epstein D, Rajpoot N. Fast and accurate tumor segmentation of histology images using persistent homology and deep convolutional features. *Med Image Anal* 2019; **55**: 1-14 [PMID: 30991188 DOI: 10.1016/j.media.2019.03.014]

55 **Awan R**, Sirinukunwattana K, Epstein D, Jefferyes S, Qidwai U, Aftab Z, Mujeeb I, Snead D, Rajpoot N. Glandular Morphometrics for Objective Grading of Colorectal Adenocarcinoma Histology Images. *Sci Rep* 2017; **7**: 16852 [PMID: 29203775 DOI: 10.1038/s41598-017-16516-w]

56 **Abousamra S**, Fassler D, Hou L, Zhang Y, Gupta R, Kurc T, Escobar-Hoyos LF, Samaras D, Knudson B, Shroyer K. Weakly-Supervised Deep Stain Decomposition for Multiplex IHC Images. *IEEE ISBI* 2020; 481-485 [DOI: 10.1109/ISBI45749.2020.9098652]

57 **Fassler DJ**, Abousamra S, Gupta R, Chen C, Zhao M, Paredes D, Batool SA, Knudsen BS, Escobar-Hoyos L, Shroyer KR, Samaras D, Kurc T, Saltz J. Deep learning-based image analysis methods for brightfield-acquired multiplex immunohistochemistry images. *Diagn Pathol* 2020; **15**: 100 [PMID: 32723384 DOI: 10.1186/s13000-020-01003-0]

58 **Lee MJ**, Bagci P, Kong J, Vos MB, Sharma P, Kalb B, Saltz JH, Martin DR, Adsay NV, Farris AB. Liver steatosis assessment: correlations among pathology, radiology, clinical data and automated image analysis software. *Pathol Res Pract* 2013; **209**: 371-379 [PMID: 23707550 DOI: 10.1016/j.prp.2013.04.001]

59 **Forlano R**, Mullish BH, Giannakeas N, Maurice JB, Angkathunyakul N, Lloyd J, Tzallas AT, Tsipouras M, Yee M, Thursz MR, Goldin RD, Manousou P. High-Throughput, Machine Learning-Based Quantification of Steatosis, Inflammation, Ballooning, and Fibrosis in Biopsies From Patients With Nonalcoholic Fatty Liver Disease. *Clin Gastroenterol Hepatol* 2020; **18**: 2081-2090. e9 [PMID: 31887451 DOI: 10.1016/j.cgh.2019.12.025]

60 **Sun L**, Marsh JN, Matlock MK, Chen L, Gaut JP, Brunt EM, Swamidass SJ, Liu TC. Deep learning quantification of percent steatosis in donor liver biopsy frozen sections. *EBioMedicine* 2020; **60**: 103029 [PMID: 32980688 DOI: 10.1016/j.ebiom.2020.103029]

61 **Roy M**, Wang F, Vo H, Teng D, Teodoro G, Farris AB, Castillo-Leon E, Vos MB, Kong J. Deep-learning-based accurate hepatic steatosis quantification for histological assessment of liver biopsies. *Lab Invest* 2020; **100**: 1367-1383 [PMID: 32661341 DOI: 10.1038/s41374-020-0463-y]

62 **Salvi M**, Molinaro L, Metovic J, Patrono D, Romagnoli R, Papotti M, Molinari F. Fully automated quantitative assessment of hepatic steatosis in liver transplants. *Comput Biol Med* 2020; **123**: 103836 [PMID: 32658781 DOI: 10.1016/j.compbiomed.2020.103836]

63 **Bychkov D**, Linder N, Turkki R, Nordling S, Kovanen PE, Verrill C, Walliander M, Lundin M, Haglund C, Lundin J. Deep learning based tissue analysis predicts outcome in colorectal cancer. *Sci Rep* 2018; **8**: 3395 [PMID: 29467373 DOI: 10.1038/s41598-018-21758-3]

64 **Yue X**, Dimitriou N, Arandjelovic O. Colorectal cancer outcome prediction from H&E whole slide images using machine learning and automatically inferred phenotype profiles. 2019 Preprint. Available from: arXiv:1902.03582

65 **Kather JN**, Krisam J, Charoentong P, Luedde T, Herpel E, Weis CA, Gaiser T, Marx A, Valous NA, Ferber D, Jansen L, Reyes-Aldasoro CC, Zörnig I, Jäger D, Brenner H, Chang-Claude J, Hoffmeister M, Halama N. Predicting survival from colorectal cancer histology slides using deep learning: A retrospective multicenter study. *PLoS Med* 2019; **16**: e1002730 [PMID: 30677016 DOI: 10.1371/journal.pmed.1002730]

66 **Jiang D**, Liao J, Duan H, Wu Q, Owen G, Shu C, Chen L, He Y, Wu Z, He D, Zhang W, Wang Z. A machine learning-based prognostic predictor for stage III colon cancer. *Sci Rep* 2020; **10**: 10333 [PMID: 32587295 DOI: 10.1038/s41598-020-67178-0]

67 **Saillard C**, Schmauch B, Laifa O, Moarii M, Toldo S, Zaslavskiy M, Pronier E, Laurent A, Amaddeo G, Regnault H, Sommacale D, Ziol M, Pawlotsky JM, Mulé S, Luciani A, Wainrib G, Clozel T, Courtiol P, Calderaro J. Predicting Survival After Hepatocellular Carcinoma Resection Using Deep Learning on Histological Slides. *Hepatology* 2020; **72**: 2000-2013 [PMID: 32108950 DOI: 10.1002/hep.31207]

68 **Wulczyn E**, Steiner DF, Xu Z, Sadhwani A, Wang H, Flament-Auvigne I, Mermel CH, Chen PC, Liu Y, Stumpe MC. Deep learning-based survival prediction for multiple cancer types using histopathology images. *PLoS One* 2020; **15**: e0233678 [PMID: 32555646 DOI: 10.1371/journal.pone.0233678]

69 **Takamatsu M**, Yamamoto N, Kawachi H, Chino A, Saito S, Ueno M, Ishikawa Y, Takazawa Y, Takeuchi K. Prediction of early colorectal cancer metastasis by machine learning using digital slide images. *Comput Methods Programs Biomed* 2019; **178**: 155-161 [PMID: 31416544 DOI: 10.1016/j.cmpb.2019.06.022]

70 **Abrahao-Machado LF**, Scapulatempo-Neto C. HER2 testing in gastric cancer: An update. *World J Gastroenterol* 2016; **22**: 4619-4625 [PMID: 27217694 DOI: 10.3748/wjg.v22.i19.4619]

71 **Sharma H**, Zerbe N, Klempert I, Hellwich O, Hufnagl P. Deep convolutional neural networks for automatic classification of gastric carcinoma using whole slide images in digital histopathology. *Comput Med Imaging Graph* 2017; **61**: 2-13 [PMID: 28676295 DOI: 10.1016/j.compmedimag.2017.06.001]

72 **Redemann J**, Schultz FA, Martinez C, Harrell M, Clark DP, Martin DR, Hanson JA. Comparing Deep Learning and Immunohistochemistry in Determining the Site of Origin for Well-Differentiated Neuroendocrine Tumors. *J Pathol Inform* 2020; **11**: 32 [PMID: 33343993 DOI: 10.4103/jpi.jpi_37_20]

73 **Govind D**, Jen KY, Matsukuma K, Gao G, Olson KA, Gui D, Wilding GE, Border SP, Sarder P. Improving the accuracy of gastrointestinal neuroendocrine tumor grading with deep learning. *Sci Rep* 2020; **10**: 11064 [PMID: 32632119 DOI: 10.1038/s41598-020-67880-z]

74 **Burlingame EA**, McDonnell M, Schau GF, Thibault G, Lanciault C, Morgan T, Johnson BE, Corless C, Gray JW, Chang YH. SHIFT: speedy histological-to-immunofluorescent translation of a tumor signature enabled by deep learning. *Sci Rep* 2020; **10**: 17507 [PMID: 33060677 DOI: 10.1038/s41598-020-74500-3]

75 **Chen M**, Zhang B, Topatana W, Cao J, Zhu H, Juengpanich S, Mao Q, Yu H, Cai X. Classification and mutation prediction based on histopathology H&E images in liver cancer using deep learning. *NPJ Precis Oncol* 2020; **4**: 14 [PMID: 32550270 DOI: 10.1038/s41698-020-0120-3]

76 **Sirinukunwattana K**, Domingo E, Richman SD, Redmond KL, Blake A, Verrill C, Leedham SJ, Chatzipli A, Hardy C, Whalley CM, Wu CH, Beggs AD, McDermott U, Dunne PD, Meade A, Walker SM, Murray GI, Samuel L, Seymour M, Tomlinson I, Quirke P, Maughan T, Rittscher J, Koelzer VH; S:CORT consortium. Image-based consensus molecular subtype (imCMS) classification of colorectal cancer using deep learning. *Gut* 2021; **70**: 544-554 [PMID: 32690604 DOI: 10.1136/gutjnl-2019-319866]

77 **Kather JN**, Pearson AT, Halama N, Jäger D, Krause J, Loosen SH, Marx A, Boor P, Tacke F, Neumann UP, Grabsch HI, Yoshikawa T, Brenner H, Chang-Claude J, Hoffmeister M, Trautwein C, Luedde T. Deep learning can predict microsatellite instability directly from histology in gastrointestinal cancer. *Nat Med* 2019; **25**: 1054-1056 [PMID: 31160815 DOI: 10.1038/s41591-019-0462-y]

78 **Kather JN**, Heij LR, Grabsch HI, Loeffler C, Echle A, Muti HS, Krause J, Niehues JM, Sommer KAJ, Bankhead P, Kooreman LFS, Schulte JJ, Cipriani NA, Buelow RD, Boor P, Ortiz-Brüchle NN, Hanby AM, Speirs V, Kochanny S, Patnaik A, Srisuwananukorn A, Brenner H, Hoffmeister M, van den Brandt PA, Jäger D, Trautwein C, Pearson AT, Luedde T. Pan-cancer image-based detection of clinically actionable genetic alterations. *Nat Cancer* 2020; **1**: 789-799 [PMID: 33763651 DOI: 10.1038/s43018-020-0087-6]

79 **Echle A**, Grabsch HI, Quirke P, van den Brandt PA, West NP, Hutchins GGA, Heij LR, Tan X, Richman SD, Krause J, Alwers E, Jenniskens J, Offermans K, Gray R, Brenner H, Chang-Claude J, Trautwein C, Pearson AT, Boor P, Luedde T, Gaisa NT, Hoffmeister M, Kather JN. Clinical-Grade Detection of Microsatellite Instability in Colorectal Tumors by Deep Learning. *Gastroenterology* 2020; **159**: 1406-1416. e11 [PMID: 32562722 DOI: 10.1053/j.gastro.2020.06.021]

80 **Schmauch B**, Romagnoni A, Pronier E, Saillard C, Maillé P, Calderaro J, Kamoun A, Sefta M, Toldo S, Zaslavskiy M, Clozel T, Moarii M, Courtiol P, Wainrib G. A deep learning model to predict RNA-Seq expression of tumours from whole slide images. *Nat Commun* 2020; **11**: 3877 [PMID: 32747659 DOI: 10.1038/s41467-020-17678-4]

81 **Hosseini MS**, Chan L, Tse G, Tang M, Deng J, Norouzi S, Rowsell C, Plataniotis KN, Damaskinos S. Atlas of digital pathology: A generalized hierarchical histological tissue type-annotated database for deep learning. *IEEE CVPR* 2019; 11747-11756

82 **Chan L**, Hosseini MS, Rowsell C, Plataniotis KN, Damaskinos S. Histosegnet: Semantic segmentation of histological tissue type in whole slide images. *IEEE ICCV* 2019; 10661-10670 [DOI: 10.1109/ICCV.2019.01076]

83 **Binder T**, Tantaoui EM, Pati P, Catena R, Set-Aghayan A, Gabrani M. Multi-Organ Gland Segmentation Using Deep Learning. *Front Med (Lausanne)* 2019; **6**: 173 [PMID: 31428614 DOI: 10.3389/fmed.2019.00173]

84 **Louis DN**, Feldman M, Carter AB, Dighe AS, Pfeifer JD, Bry L, Almeida JS, Saltz J, Braun J, Tomaszewski JE, Gilbertson JR, Sinard JH, Gerber GK, Galli SJ, Golden JA, Becich MJ. Computational Pathology: A Path Ahead. *Arch Pathol Lab Med* 2016; **140**: 41-50 [PMID: 26098131 DOI: 10.5858/arpa.2015-0093-SA]

85 **Colen R**, Foster I, Gatenby R, Giger ME, Gillies R, Gutman D, Heller M, Jain R, Madabhushi A, Madhavan S, Napel S, Rao A, Saltz J, Tatum J, Verhaak R, Whitman G. NCI Workshop Report: Clinical and Computational Requirements for Correlating Imaging Phenotypes with Genomics Signatures. *Transl Oncol* 2014; **7**: 556-569 [PMID: 25389451 DOI: 10.1016/j.tranon.2014.07.007]

86 **U.S. Food and Drug Administration**. Proposed Regulatory Framework for Modifications to Artificial Intelligence/Machine Learning (AI/ML)-Based Software as a Medical Device (SaMD). [cited 22 January 2021]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/media/122535/download

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MINIREVIEWS

COVID-19 in normal, diseased and transplanted liver

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Abstract

Starting from December 2019 the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has extended in the entire world giving origin to a pandemic. Although the respiratory system is the main apparatus involved by the infection, several other organs may suffer coronavirus disease 2019 (COVID-19)-related injuries. The human tissues expressing angiotensin-converting enzyme 2 (ACE2) are all possible targets of viral damage. In fact myocarditis, meningo-encephalitis, acute kidney injury and other complications have been described with regard to SARS-CoV-2 infection. The liver has a central role in the body homeostasis contributing to detoxification, catabolism and also synthesis of important factor such as plasma proteins. ACE2 is significantly expressed just by cholangiocytes within the liver, however transaminases are increased in more than one third of COVID-19 patients, at hospital admission. The reasons for liver impairment in the course of this infection are not completely clear at present and multiple factors such as: Direct viral effect, release of cytokines, ischemic damage, use of hepatotoxic drugs, sepsis, and others, may contribute to damage. While COVID-19 seems to elicit just a transient alteration of liver function tests in subjects with normal hepatic function, of concern, more severe sequelae are frequently observed in patients with a reduced hepatic reserve. In this review we report data regarding SARS-CoV-2 infection in subjects with normal or diseased liver. In addition the risks of COVID-19 in immunosuppressed patients (either transplanted or suffering for autoimmune liver diseases) are also described.

Key Words: COVID-19; Liver; Non-alcoholic fatty-liver-disease; Cirrhosis; Liver transplant; Angiotensin-converting enzyme 2

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system is mainly involved in the infection other organs may be impaired by coronavirus disease 2019 (COVID-19). In this review we report the current finding regarding the liver during this infection. While mild liver effects occur in normal subjects with COVID-19, severe sequelae may be expected in individuals with a reduced hepatic reserve.

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INTRODUCTION

Between December 2019 and January 2020, first China and then the rest of the world observed the emergence of a new pathogen, responsible for the recent pandemic still plaguing our planet. On January 5, 2020, the WHO issued a global alert after 44 patients, hospitalized for pneumonia of unknown origin, were reported by Chinese national authorities[1]. On March 11, 2020, the WHO declared the new coronavirus, identified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and responsible for the so called coronavirus disease 2019 (COVID-19)[2], to be pandemic. To date (January 2021), the SARS-CoV-2 infection is approaching 100 million cases worldwide, while 2 million related deaths have been reported globally. SARS-CoV-2 is a virus belonging to the genus *Betacoronavirus*, family *Coronaviridae*, subgenus *Sarbecovirus*, discovered at the end of 2019. It is a single-stranded, positive-sense RNA virus with a 50-200 nm diameter. Since it has close genetic similarity with bat coronaviruses, a zoonotic origin has been suggested, based on the transmission from animal to humans. SARS-CoV-2 possesses four structural proteins called the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins; the N protein binds the virus genome while the S, E, and M proteins contribute in the assembly of the viral envelope[3].

Impairment of respiratory function is the most frequent clinical concern during infection by this new virus; however, other physiological organ systems can be affected. Among these, SARS-CoV-2-induced hepatic changes are gaining interest for their possible relationship with liver and patient outcome.

The liver plays a fundamental role in metabolism and the synthesis of plasma proteins, as well as in the manipulation and detoxification of diet xenobiotics[4]. Several conditions may lead to chronic hepatitis and cirrhosis or hepato-carcinoma, including viral agents, alcohol abuse, metabolic derangements, and others[5]. The patient affected by liver disease is fragile, and his clinical stability is always in perennial equilibrium. Among the direct causes of hepatic decompensation, the detrimental effect of infections, both viral and bacterial, is well known, and in this particular setting, COVID-19 does not seem less important[6]. This review summarizes the effects of SARS-CoV-2 infection in patients with liver disease, as well as in healthy and liver transplanted subjects. Data available on clinical complications and outcomes are also reported.

INTERACTION OF SARS-COV-2 WITH TISSUES

The SARS-CoV-2 molecular pathways employed by the virus to favor host interaction and invasion are of major interest. Several studies have been conducted on this issue, and the majority of evidence converges to identify the angiotensin-converting enzyme 2 (ACE2), a surface peptidase responsible for the regulation of blood pressure, as a key factor. In fact, Zhou *et al*[7] demonstrated that ACE2 is the main entry route of SARS-CoV-2 within the cell, as also previously observed for the SARS-CoV and HCoV-NL6 viruses[7]. During the previous SARS pandemic in 2003, it was demonstrated that the use of antibodies against ACE2 effectively counteracted viral replication. On the other hand, ACE1 antibodies did not have any effect[8]. The interaction between this new virus and ACE2 has also been investigated in a study conducted by Xu *et al*[9], where the strong link between the virus and this receptor was confirmed. ACE2 is present in

different organs and tissues, such as alveolar type 2 cells of the lung; epithelial cells of the nasal, nasopharyngeal, and oral mucosa; in the smooth muscles of the gastric and colonic mucosa; in enterocytes of the duodenum, jejunum, ileum, and colon; in myocardiocytes; in the cells of the proximal tubule of the kidney; and in cholangiocytes within the liver[10]. From all of the above, it is clear that any tissue expressing ACE2 might be a possible target for SARS-CoV-2, giving rise to different symptoms with a severity that may be a function of the baseline organ reserve. The main organs expressing ACE2 with the corresponding symptoms/complications COVID-19-related, are reported in Figure 1. Although the main clinical expression is represented by a flu-like mild syndrome, a severe bilateral interstitial pneumonia resulting in acute respiratory distress syndrome (ARDS) and requiring intensive care unit (ICU) management is not rare[11]. Other common clinical manifestations comprise: Nausea, vomiting, anorexia, diarrhea (2%-10% of patients), and altered liver function tests[12].

COVID-19 AND THE NORMAL LIVER

One large retrospective study, conducted in the city of New York, enrolled approximately 5700 COVID-19 patients. Liver transaminase levels were assessed at hospital admission. Increased aspartate transaminase (AST) or alanine transaminase (ALT) was found in 58% and 39% of patients, respectively[13]. Disease worsening during in-hospital stay was associated with a further increase of these enzymes. However, the pathogenetic mechanism leading to these alterations remained unclear. In fact, several factors may all have contributed to the liver changes in this heterogeneous group of patients, such as: (1) a direct viral cytopathic effect; (2) the activation of cytokine cascade; (3) the onset of multi-organ failure; (4) a state of disseminated intravascular coagulation; and (5) the use of potentially hepatotoxic drugs (such as remdesivir), among others[12]. As demonstrated by other research, in the course of COVID-19, the increase in liver enzymes is characterized by a preferential AST elevation, thus recalling the picture observed in alcoholic, metabolic, or ischemic liver conditions[14-16]. Abnormal levels of cholestasis markers are seldom observed[17], even if an increased gamma-glutamyltransferase (GGT) level was reported in 50% of patients in one study[18].

Even though SARS-CoV-2 viral particles were described in hepatocytes in two severe cases[19] a direct cytopathic effect does not seem to explain the liver changes since ACE2 is mainly expressed in the biliary tract, within the liver. On the other hand, despite: (1) the presence of the receptor on cholagiocytes; and (2) "*in vitro*" evidences demonstrating tight junction damage and bile acids transporter impairment in these cells[20]; cholestasis is seldom observed in COVID-19 clinical setting. With regard to liver damage, however, is to underscore that cardiomyopathy with the resulting myocardial dysfunction, is observed in nearly 33% of hospitalized COVID-19 patients. In this context, the onset of circulatory impairment could reasonably explain the biochemical pattern of the alteration of AST, ALT, and GGT, as in the more common picture of congestive liver disease[21-23]. Supporting this view, a postmortem analysis of liver tissue coming from COVID-19 patients denoted a necrotic injury associated with hypoperfusion and congestive changes[19]. Finally, even if liver involvement is not generally regarded as relevant in subjects without a pre-existing liver impairment, the evaluation of hepatic changes during COVID-19 remains difficult in the clinical setting, due to the lack of a clear understanding of this process[24]. In this perspective, bilirubin elevation, even if rarely observed, seems to expose the patient to an increased risk of mechanical ventilation or death in comparison with changes of other liver enzymes[25].

COVID-19 AND PATIENTS WITH NON-CIRRHOTIC, CHRONIC LIVER DISEASE

As stated in the previous paragraph, the alterations of liver function tests in the course of COVID-19 could be the result of different causal events that may act simultaneously. When damage occurs in a subject with an impaired hepatic functional reserve, a more difficult resolution of the clinical picture may be expected. Of more concern is the large United States study including more than 60 million electronic medical records, which also demonstrated that subjects affected by chronic liver diseases (CLD) were more exposed to acquiring COVID-19[26]. In this western research, the

Organ	Main site of ACE2 expression	Prossible injury or symptoms COVID-19 related
Heart 	Pericyte	Myocarditis Arrythmias Heart infarction
Kidney 	Proximal tubule cell	Tubular necrosis Acute kidney injury
Intestine 	Epithelial cell Enterocyte	Diarrea Nausea Vomiting Cell necrosis
Brain 	Brain ventricles Substantia nigra	Anosmia Dysgeusis Headache Meningo-encephalitis

Figure 1 Organs of human body (different from lung and liver) in which angiotensin-converting enzyme 2 was detected, the main site of expression and the possible coronavirus disease 2019 related symptoms/complications are reported in the corresponding columns.
COVID-19: Coronavirus disease 2019; ACE2: Angiotensin-converting enzyme 2.

prevalence of CLD in COVID-19 patients accounted for 5% of cases, while data coming from China demonstrated a baseline CLD in 2 to 11% of cases[27].

Several conditions may determine a CLD in humans, including those of a metabolic, toxic, viral, or autoimmune nature. Non-alcoholic- (or metabolic-associated-) fatty-liver-disease (NAFLD), a liver condition ranging from simple steatosis to non-alcoholic steatohepatitis and associated with metabolic syndrome, type II diabetes and obesity, has an estimated prevalence of 25% worldwide[28]. Because of its large burden, the role of NAFLD in determining the degree of liver injury in patients with COVID-19 has aroused much interest.

In China, 70 out of 324 COVID-19 patients (21.6%) were diagnosed with hepatic steatosis during radiological investigations by computed tomography. The severity of the clinical picture of COVID-19 was also increased in those with NAFLD[29]. Moreover, in a retrospective study on 76 patients with COVID-19, the presence of NAFLD was associated with the progression of lung impairment, with an odds ratio of 6.4[30]. In another study, NAFLD was again associated with a fourfold increased risk of a severe course of COVID-19[31]. The picture linking NAFLD to a severe outcome of this viral infection was later challenged by a study coming from Qatar[32]. This research, including 320 patients with NAFLD, showed that this metabolic condition was only associated with a worse outcome (increased ICU stay and requirement of mechanical ventilation) when univariate analysis was employed. On the other hand, the main predictor for mortality or a worst outcome at multivariate analysis were age > 50 years and diabetes, respectively. On the basis of the above studies, it is clear why the possible link between NAFLD and severity of COVID-19 remains controversial. Heterogeneity among studies, related to retrospective patient inclusion and classification, do not contribute to a clear picture. A further analysis possibly shed some light on this issue, linking the grade of liver fibrosis in NAFLD, rather than NAFLD by itself, to a worse COVID-19 outcome[33]. In this perspective, it seems that the extent of liver damage might have a more relevant role in comparison with the metabolic derangement observed in these patients.

Alcoholic liver disease (ALD) represents an important cause of hepatic morbidity and mortality worldwide[34]. Despite the fact that clinical data are not available on COVID-19 ALD patients, concern exists for the increased frailty of these subjects during the pandemic. On the other hand, psychological stress and social distancing seem to have increased individual alcoholic beverage consumption in general.

Alcohol abuse disorders frequently facilitate several comorbidities such as viral infections, diabetes or renal failure, all factors that may contribute to an increased risk of a severe course once SARS-CoV-2 infection occurs[35]. Moreover, increased consumption of alcoholic beverages is considered to be a risk factor for the development of respiratory tract infections that may be complicated by the onset of ARDS, one of the main features of severe COVID-19[17]. Also, in the recovery phase after COVID-19, previous alcohol abuse is considered a predisposing factor for the development of pulmonary fibrosis[36]. However, evidence-based indications in the management of ALD COVID-19-infected patients are lacking at present, and further studies are needed to improve our understanding on this issue. For the time being, expert opinion suggests, in general, to avoid the use of steroids in alcoholic hepatitis while concomitant uncontrolled infections are ongoing, because of the lowering effect on patient's immune defenses[37]. In clinical practice, on the other hand, the use of these drugs might be beneficial in the treatment of COVID-19 despite ALD, challenging our before-SARS-CoV-2 beliefs.

Another important challenge concerns the management of patients with autoimmune hepatitis on immunosuppressive therapy. The lack of data from scientific evidence has encouraged an empirical approach based on dose reduction of immunomodulatory therapy in order to prevent the most deleterious effects of COVID-19[38]. However, preliminary data from the city of Bergamo, in Italy, did not support this view since an increased risk was not observed in immunosuppressed patients during the SARS-CoV-2 epidemic. On the other hand, it must be considered that a possible reactivation of autoimmune hepatitis, after immunosuppressive therapy tapering, would probably require high-dose corticosteroids, greatly increasing the risk of infectious complications in these patients[39]. Therefore, current guidelines do not recommend the reduction of immunomodulatory therapy in the absence of SARS-CoV-2 infection; in the case of overt infection, dose adjustment may be pursued in order to increase the white blood cell count[40,41].

Few data are available on other forms of CLD. With regard to HBV, in a Chinese series represented by 105 hepatitis B surface antigen positive patients hospitalized for COVID-19 (1.9% with cirrhosis, 12.4% on antiviral therapy), 14 developed significant liver injury[12]. In these subjects, liver damage was associated with a severe course of infection in nearly 80% of cases. Since the possible prevention or attenuation of COVID-19 should be postulated with the use of antiviral drugs employed for hepatitis B virus (HBV) or hepatitis C virus (HCV), a Spanish study focused on subjects with chronic viral hepatitis following their specific antiviral regimen[42], with 1 out of 341 HCV patients and 8 out 1764 HBV patients developing COVID-19. Although the majority of them (nearly 80%) were hospitalized, no cases of fatal outcome were observed in this group. Finally, is to underscore that extensive use of intravenous steroid therapy to reduce the inflammatory state can lead to significant HBV reactivation. Routine testing for this virus would be wise in COVID-19 severe patients in order to adopt a timely prophylactic treatment[12].

No data are available at present on the impact of COVID-19 in patients with cholestatic CLD, such as primary biliary cholangitis or primary sclerosing cholangitis.

COVID-19 IN PATIENTS WITH CIRRHOSIS OF THE LIVER

Several factors may lead a cirrhotic patient from a compensated to a decompensated clinical condition. Among these, infections play a relevant role, also promoting the occurrence of acute-on-chronic-liver-failure (ACLF)[43,44]. In this perspective, SARS-CoV-2 infection, characterized by an important activation of the cytokine cascade, sepsis, and altered hepatic perfusion, may determine a significant impairment of hepatic reserve in cirrhosis[6]. Some studies evaluated the clinical outcome of COVID-19 in patients with liver cirrhosis. Preliminary data coming from two international registries and including 103 cirrhotic COVID-19 positive patients demonstrated an increased mortality, in comparison with the general population[45]. Moreover, increased fatality was strictly related to the degree of pre-existing liver impairment; in fact, more than 60% of Child-Pugh C patients died in this study. In the APCOLIS study, a worst COVID-19 outcome in cirrhotic patients was again confirmed[46]. In this research, 43 cirrhotic patients exhibited: (1) increased need of ICU care; (2) more liver–related complication; and (3) enhanced mortality in comparison with non-cirrhotic CLD patients. COVID-19 determined the onset of liver decompensation or ACLF in one out of five patients with cirrhosis. A Child Pugh score > 9 was a significant predictor of mortality.

A study conducted in Italy compared the 30-d in-hospital mortality in a group of patients admitted for COVID-19 infection with or without liver cirrhosis. The all-cause mortality rate was found to be significantly higher in cirrhotic patients with a model for end-stage liver disease score ≥ 15. In addition, this research also evidenced a deterioration of liver function in patients with a long-standing stable liver disease. In fact, a worsening of the Child-Pugh score was observed, from grade A at admission to status B/C during the hospital stay[47]. The mortality rate induced by COVID-19 in patients with decompensated cirrhosis was increased in comparison with other infective causes. In Figure 2, the main postulated mechanisms of liver injury, during COVID-19, are reported, together with the possible outcome as a function of previous liver condition. Finally, cirrhotic patients with hepatocarcinoma (HCC) deserve a separate consideration since neoplastic patients, in general, may have an additional immunologic impairment related to cancer and/or chemotherapy. A national study performed in China on 1590 patients in 575 hospitals showed that patients with an underlying malignancy had a higher risk of SARS-CoV-2 infection than the general population. Obviously, many patients with HCC had an underlying liver impairment that placed them in this higher risk group, with a decidedly more inauspicious clinical outcome[48]. Considering this increased risk, the America Association for Study on Liver Disease (AASLD) and the European Association for the Study of Liver (EASL) recommend a prolongation of the time of clinical and ultrasound surveillance of cirrhotic patients so as not to expose them to possible contagion in the places of treatment, deferring locoregional treatment for HCC where possible in patients with a relatively stable disease, and reserving interventions for patients in which the benefit outweighs the risk of acquiring SARS-CoV-2 infection[40,41]. However, the relationship between an impairment of the immune system (for cancer, immunological diseases, or transplant) and a worse COVID-19 outcome is not demonstrated as yet, as will be reported in details in the following paragraph.

COVID-19 IN LIVER TRANSPLANTED PATIENTS

As there is an assumption that the integrity of the immune-system is an important factor for preventing the most severe sequelae of COVID-19, since the pandemic began, concern has been raised about the possible outcome in liver transplant (LT) patients under continuous immunosuppressive regimen. However, current data do not confirm this worrisome view. An early pediatric analysis coming from the North of Italy (an area badly beaten by the pandemic) demonstrated just three children infected by COVID-19, and with an uneventful outcome, among those liver transplanted or under chemotherapy[39]. Data on adult LT recipients coming from the same geographic area gave a different picture[49]. Three patients having a LT more than ten years before, and with COVID-19, died from complications. All had metabolic impairment (diabetes, hypertension) and were > 65 years. Paradoxically, their immunosuppressive regimen was minimal in comparison with patients with a shorter transplant history, suggesting a possible protective rather detrimental effect of immunosuppression. A large series was published, gathering the data from two international registries (COVID-Hep and SECURE-Cirrhosis)[50]. In this study, data coming from 151 liver transplanted patients were compared with those of 627 controls. The main factors associated with death at multivariate analysis were: Age, creatinine levels, and non-liver malignancy. LT and immunosuppressive regimen were not associated to an increased risk of fatality. Finally, patients with LT required invasive ventilation more frequently (20% LT *vs* 5% control; $P < 0.001$), while the overall mortality was increased in the non LT control group (19% LT *vs* 27% control; $P = 0.05$). A Spanish prospective study reassessed this issue in 111 LT patients with COVID-19[51]. Although the fatality rate was slightly lower in LT patients, mycophenolate use (especially at > 1 g/daily dose) was associated with an increased risk of severe COVID-19 disease. From these findings, the authors suggested a possible benefit in mycophenolate tapering in COVID-19 LT patients, while a complete drug withdrawal was discouraged. Taken together, the available data do not suggest an increased fatality in LT patients with COVID-19; however, enhanced surveillance would be wise in these subjects because of the increased morbidity reported by some studies. While a possible detrimental effect has been suggested for mycophenolate, a clear picture of the relationship between immunosuppression and COVID-19 severity is still lacking.

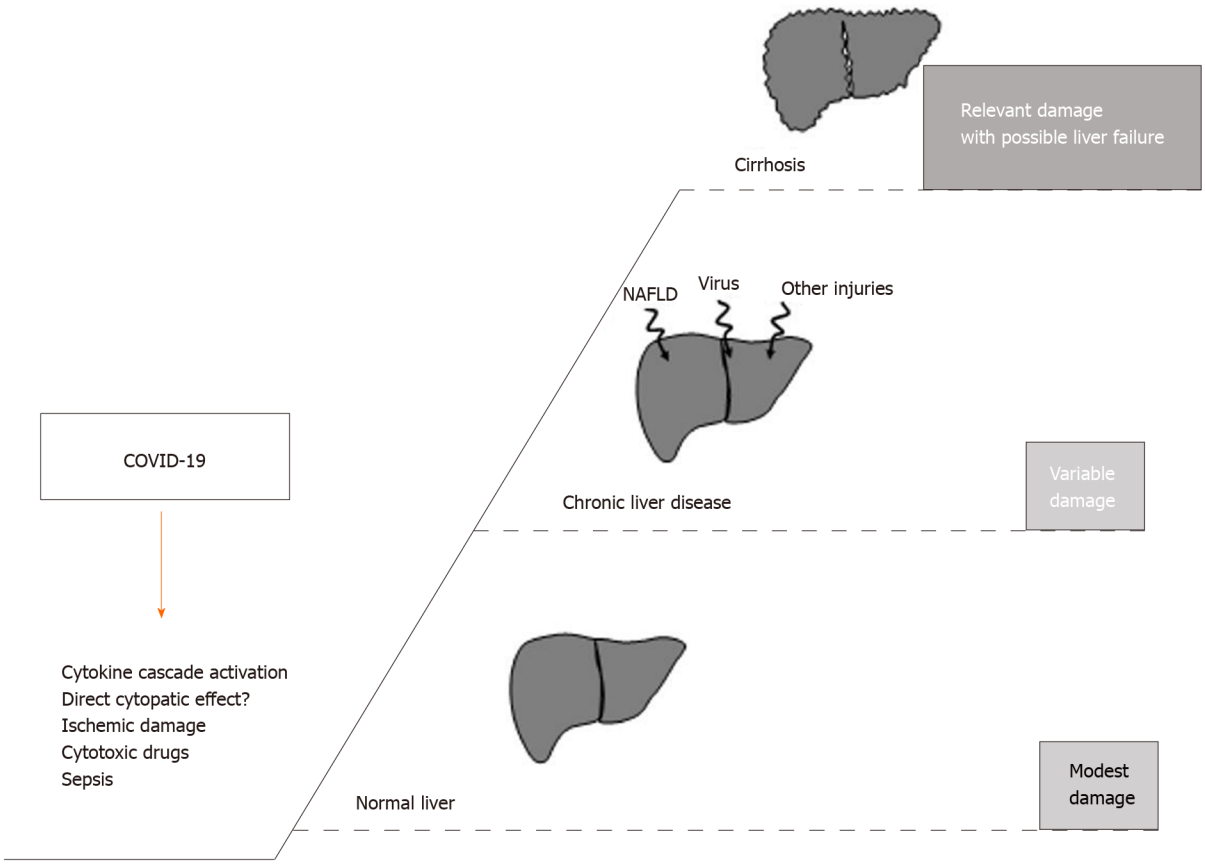


Figure 2 The putative mechanisms inducing liver injury in the course of coronavirus disease 2019 are reported in the left side of the figure (? = controversial data on the specific mechanism). The right part describes the possible outcome according to patient baseline liver condition. NAFLD: Non-alcoholic fatty-liver-disease; COVID-19: Coronavirus disease 2019.

CONCLUSION

The effects of COVID-19 on liver health are complex and largely dependent on the underling functional hepatic reserve. The reasons for the elevation of liver enzymes in a significant number of COVID-19 patients, is not clear at present. As a general rule, the effect of COVID-19 seems negligible on the normal liver, but concern exists for patients with impaired liver function. Expert societies, such as AASLD or EASL, are making a great effort to give indications on issues such as LT and the clinical management of liver disease patients during the SARS-CoV-2 pandemic, also in the lack of definitive evidence. While this scenario is quickly evolving, also for the possible emergence of some variant of concern[52] ,other issues are rising for patients with liver disease. Among the pandemic-associated and non-COVID-19-related adverse effects, the reduction of health resources for patients with liver disease may significantly impact their morbidity and mortality. A study in Italy evidenced that there was already a 25% reduction of transplant activity related to an increased ICUs saturation in the first 4 wk of the national pandemic[53]. Abrupt interruption of HCC surveillance in the majority of patients with liver cirrhosis will also have a cost in the future. Finally, a recent study modeling a one year delay in HCV cure since the pandemic estimated an increase of more than 100 thousand in liver related deaths and cancers in the population affected by this virus in the coming years[54]. In conclusion, while hepatologists are presently working in an emergency environment full of uncertainties and with limited resources, an "in search of lost time" attitude will probably be pursued by the same physicians in the future, when the pandemic ends.

REFERENCES

1 **Ciotti M**, Angeletti S, Minieri M, Giovannetti M, Benvenuto D, Pascarella S, Sagnelli C, Bianchi M, Bernardini S, Ciccozzi M. COVID-19 Outbreak: An Overview. *Chemotherapy* 2019; **64**: 215-223 [PMID: 32259829 DOI: 10.1159/000507423]

2 **Acuti Martellucci C**, Flacco ME, Cappadona R, Bravi F, Mantovani L, Manzoli L. SARS-CoV-2 pandemic: An overview. *Adv Biol Regul* 2020; **77**: 100736 [PMID: 32773099 DOI: 10.1016/j.jbior.2020.100736]

3 **Chen N**, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020; **395**: 507-513 [PMID: 32007143 DOI: 10.1016/S0140-6736(20)30211-7]

4 **Glaser T**, Baiocchi L, Zhou T, Francis H, Lenci I, Grassi G, Kennedy L, Liangpunsakul S, Glaser S, Alpini G, Meng F. Pro-inflammatory signalling and gut-liver axis in non-alcoholic and alcoholic steatohepatitis: Differences and similarities along the path. *J Cell Mol Med* 2020; **24**: 5955-5965 [PMID: 32314869 DOI: 10.1111/jcmm.15182]

5 **Wang FS**, Fan JG, Zhang Z, Gao B, Wang HY. The global burden of liver disease: the major impact of China. *Hepatology* 2014; **60**: 2099-2108 [PMID: 25164003 DOI: 10.1002/hep.27406]

6 **Bonnel AR**, Bunchorntavakul C, Reddy KR. Immune dysfunction and infections in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2011; **9**: 727-738 [PMID: 21397731 DOI: 10.1016/j.cgh.2011.02.031]

7 **Zhou P**, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, Guo H, Jiang RD, Liu MQ, Chen Y, Shen XR, Wang X, Zheng XS, Zhao K, Chen QJ, Deng F, Liu LL, Yan B, Zhan FX, Wang YY, Xiao GF, Shi ZL. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020; **579**: 270-273 [PMID: 32015507 DOI: 10.1038/s41586-020-2012-7]

8 **Li W**, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003; **426**: 450-454 [PMID: 14647384 DOI: 10.1038/nature02145]

9 **Xu X**, Chen P, Wang J, Feng J, Zhou H, Li X, Zhong W, Hao P. Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. *Sci China Life Sci* 2020; **63**: 457-460 [PMID: 32009228 DOI: 10.1007/s11427-020-1637-5]

10 **Zou X**, Chen K, Zou J, Han P, Hao J, Han Z. Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Front Med* 2020; **14**: 185-192 [PMID: 32170560 DOI: 10.1007/s11684-020-0754-0]

11 **Wu Z**, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA* 2020; **323**: 1239-1242 [PMID: 32091533 DOI: 10.1001/jama.2020.2648]

12 **Jothimani D**, Venugopal R, Abedin MF, Kaliamoorthy I, Rela M. COVID-19 and the liver. *J Hepatol* 2020; **73**: 1231-1240 [PMID: 32553666 DOI: 10.1016/j.jhep.2020.06.006]

13 **Richardson S**, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW; the Northwell COVID-19 Research Consortium, Barnaby DP, Becker LB, Chelico JD, Cohen SL, Cookingham J, Coppa K, Diefenbach MA, Dominello AJ, Duer-Hefele J, Falzon L, Gitlin J, Hajizadeh N, Harvin TG, Hirschwerk DA, Kim EJ, Kozel ZM, Marrast LM, Mogavero JN, Osorio GA, Qiu M, Zanos TP. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. *JAMA* 2020; **323**: 2052-2059 [PMID: 32320003 DOI: 10.1001/jama.2020.6775]

14 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

15 **Xu L**, Liu J, Lu M, Yang D, Zheng X. Liver injury during highly pathogenic human coronavirus infections. *Liver Int* 2020; **40**: 998-1004 [PMID: 32170806 DOI: 10.1111/liv.14435]

16 **Pratt DS**, Kaplan MM. Evaluation of abnormal liver-enzyme results in asymptomatic patients. *N Engl J Med* 2000; **342**: 1266-1271 [PMID: 10781624 DOI: 10.1056/NEJM200004273421707]

17 **Schaefer EAK**, Arvind A, Bloom PP, Chung RT. Interrelationship Between Coronavirus Infection and Liver Disease. *Clin Liver Dis (Hoboken)* 2020; **15**: 175-180 [PMID: 32489653 DOI: 10.1002/cld.967]

18 **Cai Q**, Huang D, Yu H, Zhu Z, Xia Z, Su Y, Li Z, Zhou G, Gou J, Qu J, Sun Y, Liu Y, He Q, Chen J, Liu L, Xu L. COVID-19: Abnormal liver function tests. *J Hepatol* 2020; **73**: 566-574 [PMID: 32298767 DOI: 10.1016/j.jhep.2020.04.006]

19 **Bradley BT**, Maioli H, Johnston R, Chaudhry I, Fink SL, Xu H, Najafian B, Deutsch G, Lacy JM, Williams T, Yarid N, Marshall DA. Histopathology and ultrastructural findings of fatal COVID-19 infections in Washington State: a case series. *Lancet* 2020; **396**: 320-332 [PMID: 32682491 DOI: 10.1016/S0140-6736(20)31305-2]

20 **Zhao B**, Ni C, Gao R, Wang Y, Yang L, Wei J, Lv T, Liang J, Zhang Q, Xu W, Xie Y, Wang X, Yuan Z, Zhang R, Lin X. Recapitulation of SARS-CoV-2 infection and cholangiocyte damage with human liver ductal organoids. *Protein Cell* 2020; **11**: 771-775 [PMID: 32303993 DOI: 10.1007/s13238-020-00718-6]

21 **Arentz M**, Yim E, Klaff L, Lokhandwala S, Riedo FX, Chong M, Lee M. Characteristics and Outcomes of 21 Critically Ill Patients With COVID-19 in Washington State. *JAMA* 2020; **323**: 1612-

1614 [PMID: 32191259 DOI: 10.1001/jama.2020.4326]
22 **van Deursen VM**, Damman K, Hillege HL, van Beek AP, van Veldhuisen DJ, Voors AA. Abnormal liver function in relation to hemodynamic profile in heart failure patients. *J Card Fail* 2010; **16**: 84-90 [PMID: 20123323 DOI: 10.1016/j.cardfail.2009.08.002]
23 **Weisberg IS**, Jacobson IM. Cardiovascular diseases and the liver. *Clin Liver Dis* 2011; **15**: 1-20 [PMID: 21111990 DOI: 10.1016/j.cld.2010.09.010]
24 **Moon AM**, Barritt AS 4th. Elevated Liver Enzymes in Patients with COVID-19: Look, but Not Too Hard. *Dig Dis Sci* 2020 [PMID: 32875529 DOI: 10.1007/s10620-020-06585-9]
25 **Hundt MA**, Deng Y, Ciarleglio MM, Nathanson MH, Lim JK. Abnormal Liver Tests in COVID-19: A Retrospective Observational Cohort Study of 1,827 Patients in a Major U.S. Hospital Network. *Hepatology* 2020; **72**: 1169-1176 [PMID: 32725890 DOI: 10.1002/hep.31487]
26 **Wang QQ**, Kaelber DC, Xu R, Volkow ND. COVID-19 risk and outcomes in patients with substance use disorders: analyses from electronic health records in the United States. *Mol Psychiatry* 2021; **26**: 30-39 [PMID: 32929211 DOI: 10.1038/s41380-020-00880-7]
27 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]
28 **Sheka AC**, Adeyi O, Thompson J, Hameed B, Crawford PA, Ikramuddin S. Nonalcoholic Steatohepatitis: A Review. *JAMA* 2020; **323**: 1175-1183 [PMID: 32207804 DOI: 10.1001/jama.2020.2298]
29 **Qian ZP**, Mei X, Zhang YY, Zou Y, Zhang ZG, Zhu H, Guo HY, Liu Y, Ling Y, Zhang XY, Wang JF, Lu HZ. [Analysis of baseline liver biochemical parameters in 324 cases with novel coronavirus pneumonia in Shanghai area]. *Zhonghua Gan Zang Bing Za Zhi* 2020; **28**: 229-233 [PMID: 32270660 DOI: 10.3760/cma.j.cn501113-20200229-00076]
30 **Ji D**, Qin E, Xu J, Zhang D, Cheng G, Wang Y, Lau G. Non-alcoholic fatty liver diseases in patients with COVID-19: A retrospective study. *J Hepatol* 2020; **73**: 451-453 [PMID: 32278005 DOI: 10.1016/j.jhep.2020.03.044]
31 **Zhou YJ**, Zheng KI, Wang XB, Sun QF, Pan KH, Wang TY, Ma HL, Chen YP, George J, Zheng MH. Metabolic-associated fatty liver disease is associated with severity of COVID-19. *Liver Int* 2020; **40**: 2160-2163 [PMID: 32573883 DOI: 10.1111/liv.14575]
32 **Mushtaq K**, Khan MU, Iqbal F, Alsoub DH, Chaudhry HS, Ata F, Iqbal P, Elfert K, Balaraju G, Almaslamani M, Al-Ejji K, AlKaabi S, Kamel YM. NAFLD is a predictor of liver injury in COVID-19 hospitalized patients but not of mortality, disease severity on the presentation or progression - The debate continues. *J Hepatol* 2021; **74**: 482-484 [PMID: 33223215 DOI: 10.1016/j.jhep.2020.09.006]
33 **Targher G**, Mantovani A, Byrne CD, Wang XB, Yan HD, Sun QF, Pan KH, Zheng KI, Chen YP, Eslam M, George J, Zheng MH. Risk of severe illness from COVID-19 in patients with metabolic dysfunction-associated fatty liver disease and increased fibrosis scores. *Gut* 2020; **69**: 1545-1547 [PMID: 32414813 DOI: 10.1136/gutjnl-2020-321611]
34 **Da BL**, Im GY, Schiano TD. Coronavirus Disease 2019 Hangover: A Rising Tide of Alcohol Use Disorder and Alcohol-Associated Liver Disease. *Hepatology* 2020; **72**: 1102-1108 [PMID: 32369624 DOI: 10.1002/hep.31307]
35 **Ahmed MZ**, Ahmed O, Aibao Z, Hanbin S, Siyu L, Ahmad A. Epidemic of COVID-19 in China and associated Psychological Problems. *Asian J Psychiatr* 2020; **51**: 102092 [PMID: 32315963 DOI: 10.1016/j.ajp.2020.102092]
36 **Ojo AS**, Balogun SA, Williams OT, Ojo OS. Pulmonary Fibrosis in COVID-19 Survivors: Predictive Factors and Risk Reduction Strategies. *Pulm Med* 2020; **2020**: 6175964 [PMID: 32850151 DOI: 10.1155/2020/6175964]
37 **Crabb DW**, Im GY, Szabo G, Mellinger JL, Lucey MR. Diagnosis and Treatment of Alcohol-Associated Liver Diseases: 2019 Practice Guidance From the American Association for the Study of Liver Diseases. *Hepatology* 2020; **71**: 306-333 [PMID: 31314133 DOI: 10.1002/hep.30866]
38 **Lleo A**, Invernizzi P, Lohse AW, Aghemo A, Carbone M. Management of patients with autoimmune liver disease during COVID-19 pandemic. *J Hepatol* 2020; **73**: 453-455 [PMID: 32283134 DOI: 10.1016/j.jhep.2020.04.002]
39 **D'Antiga L**. Coronaviruses and Immunosuppressed Patients: The Facts During the Third Epidemic. *Liver Transpl* 2020; **26**: 832-834 [PMID: 32196933 DOI: 10.1002/lt.25756]
40 **Fix OK**, Hameed B, Fontana RJ, Kwok RM, McGuire BM, Mulligan DC, Pratt DS, Russo MW, Schilsky ML, Verna EC, Loomba R, Cohen DE, Bezerra JA, Reddy KR, Chung RT. Clinical Best Practice Advice for Hepatology and Liver Transplant Providers During the COVID-19 Pandemic: AASLD Expert Panel Consensus Statement. *Hepatology* 2020; **72**: 287-304 [PMID: 32298473 DOI: 10.1002/hep.31281]
41 **Boettler T**, Newsome PN, Mondelli MU, Maticic M, Cordero E, Cornberg M, Berg T. Care of patients with liver disease during the COVID-19 pandemic: EASL-ESCMID position paper. *JHEP Rep* 2020; **2**: 100113 [PMID: 32289115 DOI: 10.1016/j.jhepr.2020.100113]
42 **Lens S**, Miquel M, Mateos-Muñoz B, García-Samaniego J, Forns X. SARS-CoV-2 in patients on antiviral HBV and HCV therapy in Spain. *J Hepatol* 2020; **73**: 1262-1263 [PMID: 32673740 DOI: 10.1016/j.jhep.2020.07.007]
43 **Jalan R**, Fernandez J, Wiest R, Schnabl B, Moreau R, Angeli P, Stadlbauer V, Gustot T, Bernardi M, Canton R, Albillos A, Lammert F, Wilmer A, Mookerjee R, Vila J, Garcia-Martinez R, Wendon J, Such J, Cordoba J, Sanyal A, Garcia-Tsao G, Arroyo V, Burroughs A, Ginès P. Bacterial infections in cirrhosis: a position statement based on the EASL Special Conference 2013. *J Hepatol* 2014; **60**:

1310-1324 [PMID: 24530646 DOI: 10.1016/j.jhep.2014.01.024]
44 **Arroyo V**, Moreau R, Jalan R. Acute-on-Chronic Liver Failure. *N Engl J Med* 2020; **382**: 2137-2145 [PMID: 32459924 DOI: 10.1056/NEJMra1914900]
45 **Moon AM**, Webb GJ, Aloman C, Armstrong MJ, Cargill T, Dhanasekaran R, Genescà J, Gill US, James TW, Jones PD, Marshall A, Mells G, Perumalswami PV, Qi X, Su F, Ufere NN, Barnes E, Barritt AS, Marjot T. High mortality rates for SARS-CoV-2 infection in patients with pre-existing chronic liver disease and cirrhosis: Preliminary results from an international registry. *J Hepatol* 2020; **73**: 705-708 [PMID: 32446714 DOI: 10.1016/j.jhep.2020.05.013]
46 **Sarin SK**, Choudhury A, Lau GK, Zheng MH, Ji D, Abd-Elsalam S, Hwang J, Qi X, Cua IH, Suh JI, Park JG, Putcharoen O, Kaewdech A, Piratvisuth T, Treeprasertsuk S, Park S, Wejnaruemarn S, Payawal DA, Baatarkhuu O, Ahn SH, Yeo CD, Alonzo UR, Chinbayar T, Loho IM, Yokosuka O, Jafri W, Tan S, Soo LI, Tanwandee T, Gani R, Anand L, Esmail ES, Khalaf M, Alam S, Lin CY, Chuang WL, Soin AS, Garg HK, Kalista K, Batsukh B, Purnomo HD, Dara VP, Rathi P, Al Mahtab M, Shukla A, Sharma MK, Omata M; APASL COVID Task Force, APASL COVID Liver Injury Spectrum Study (APCOLIS Study-NCT 04345640). Pre-existing liver disease is associated with poor outcome in patients with SARS CoV2 infection; The APCOLIS Study (APASL COVID-19 Liver Injury Spectrum Study). *Hepatol Int* 2020; **14**: 690-700 [PMID: 32623632 DOI: 10.1007/s12072-020-10072-8]
47 **Iavarone M**, D'Ambrosio R, Soria A, Triolo M, Pugliese N, Del Poggio P, Perricone G, Massironi S, Spinetti A, Buscarini E, Viganò M, Carriero C, Fagiuoli S, Aghemo A, Belli LS, Lucà M, Pedaci M, Rimondi A, Rumi MG, Invernizzi P, Bonfanti P, Lampertico P. High rates of 30-day mortality in patients with cirrhosis and COVID-19. *J Hepatol* 2020; **73**: 1063-1071 [PMID: 32526252 DOI: 10.1016/j.jhep.2020.06.001]
48 **Liang W**, Guan W, Chen R, Wang W, Li J, Xu K, Li C, Ai Q, Lu W, Liang H, Li S, He J. Cancer patients in SARS-CoV-2 infection: a nationwide analysis in China. *Lancet Oncol* 2020; **21**: 335-337 [PMID: 32066541 DOI: 10.1016/S1470-2045(20)30096-6]
49 **Bhoori S**, Rossi RE, Citterio D, Mazzaferro V. COVID-19 in long-term liver transplant patients: preliminary experience from an Italian transplant centre in Lombardy. *Lancet Gastroenterol Hepatol* 2020; **5**: 532-533 [PMID: 32278366 DOI: 10.1016/S2468-1253(20)30116-3]
50 **Webb GJ**, Marjot T, Cook JA, Aloman C, Armstrong MJ, Brenner EJ, Catana MA, Cargill T, Dhanasekaran R, García-Juárez I, Hagström H, Kennedy JM, Marshall A, Masson S, Mercer CJ, Perumalswami PV, Ruiz I, Thaker S, Ufere NN, Barnes E, Barritt AS 4th, Moon AM. Outcomes following SARS-CoV-2 infection in liver transplant recipients: an international registry study. *Lancet Gastroenterol Hepatol* 2020; **5**: 1008-1016 [PMID: 32866433 DOI: 10.1016/S2468-1253(20)30271-5]
51 **Colmenero J**, Rodríguez-Perálvarez M, Salcedo M, Arias-Milla A, Muñoz-Serrano A, Graus J, Nuño J, Gastaca M, Bustamante-Schneider J, Cachero A, Lladó L, Caballero A, Fernández-Yunquera A, Loinaz C, Fernández I, Fondevila C, Navasa M, Iñarrairaegui M, Castells L, Pascual S, Ramírez P, Vinaixa C, González-Dieguez ML, González-Grande R, Hierro L, Nogueras F, Otero A, Álamo JM, Blanco-Fernández G, Fábrega E, García-Pajares F, Montero JL, Tomé S, De la Rosa G, Pons JA. Epidemiological pattern, incidence, and outcomes of COVID-19 in liver transplant patients. *J Hepatol* 2021; **74**: 148-155 [PMID: 32750442 DOI: 10.1016/j.jhep.2020.07.040]
52 **Galloway SE**, Paul P, MacCannell DR, Johansson MA, Brooks JT, MacNeil A, Slayton RB, Tong S, Silk BJ, Armstrong GL, Biggerstaff M, Dugan VG. Emergence of SARS-CoV-2 B.1.1.7 Lineage - United States, December 29, 2020-January 12, 2021. *MMWR Morb Mortal Wkly Rep* 2021; **70**: 95-99 [PMID: 33476315 DOI: 10.15585/mmwr.mm7003e2]
53 **Angelico R**, Trapani S, Manzia TM, Lombardini L, Tisone G, Cardillo M. The COVID-19 outbreak in Italy: Initial implications for organ transplantation programs. *Am J Transplant* 2020; **20**: 1780-1784 [PMID: 32243677 DOI: 10.1111/ajt.15904]
54 **Blach S**, Kondili LA, Aghemo A, Cai Z, Dugan E, Estes C, Gamkrelidze I, Ma S, Pawlotsky JM, Razavi-Shearer D, Razavi H, Waked I, Zeuzem S, Craxi A. Impact of COVID-19 on global HCV elimination efforts. *J Hepatol* 2021; **74**: 31-36 [PMID: 32777322 DOI: 10.1016/j.jhep.2020.07.042]

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ORIGINAL ARTICLE

Basic Study

Upregulation of long noncoding RNA W42 promotes tumor development by binding with DBN1 in hepatocellular carcinoma

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) is a malignancy found globally. Accumulating studies have shown that long noncoding RNAs (lncRNAs) play critical roles in HCC. However, the function of lncRNA in HCC remains poorly understood.

AIM

To understand the effect of lncRNA W42 on HCC and dissect the underlying molecular mechanisms.

METHODS

We measured the expression of lncRNA W42 in HCC tissues and cells (Huh7 and SMMC-7721) by quantitative reverse transcriptase polymerase chain reaction. Receiver operating characteristic curves were used to assess the sensitivity and specificity of lncRNA W42 expression. HCC cells were transfected with pcDNA3.1-lncRNA W42 or shRNA-lncRNA W42. Cell functions were detected by cell counting Kit-8 (CCK-8), colony formation, flow cytometry and Transwell assays. The interaction of lncRNA W42 and DBN1 was confirmed by RNA immunoprecipitation and RNA pull down assays. An HCC xenograft model was used to assess the role of lncRNA W42 on tumor growth *in vivo*. The Kaplan-Meier curve was used to evaluate the overall survival and recurrence-free survival after surgery in patients with HCC.

RESULTS

In this study, we identified a novel lncRNA (lncRNA W42), and investigated its biological functions and clinical significance in HCC. LncRNA W42 expression

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was upregulated in HCC tissues and cells. Overexpression of lncRNA W42 notably promoted the proliferative and invasion of HCC, and inhibited cell apoptosis. LncRNA W42 directly bound to DBN1 and activated the downstream pathway. LncRNA W42 knockdown suppressed HCC xenograft tumor growth *in vivo*. The clinical investigation revealed that HCC patients with high lncRNA W42 expression exhibited shorter survival times.

CONCLUSION

In vitro and *in vivo* results suggested that the novel lncRNA W42, which is upregulated in HCC, may serve as a potential candidate prognostic biomarker and therapeutic target in HCC patients.

Key Words: Hepatocellular carcinoma; Long noncoding RNA; Long noncoding RNA W42; Tumor; DBN1; Cancer

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Core Tip: The results of this study demonstrated, for the first time, that long noncoding RNA (lncRNA) W42 expression was significantly higher in hepatocellular carcinoma (HCC) tissues than in normal tissues and that dysregulation of lncRNA W42 was positively correlated with tumor number, liver cirrhosis and tumor recurrence in patients with HCC. Moreover, increased lncRNA W42 expression was associated with a poor prognosis in patients with HCC. These findings suggest that lncRNA W42 is an important marker for indicting prognosis and may have potential as a diagnostic and therapeutic target for HCC.

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INTRODUCTION

Liver cancer is a major health problem[1] and is currently the third leading cause of cancer-related mortality in the world[2]. Among all primary liver cancers, hepatocellular carcinoma (HCC) accounts for approximately 90% of cases[3]. Several complex pathways with frequent mutations in HCC, including the telomere maintenance, cell cycle, WNT-β-catenin, epigenetic and chromatin remodeling pathways, have been well defined[4-8]. Although advances in the diagnosis and treatment of HCC have been made, its prognosis remains poorly understood. Even with radical treatment of HCC, the median 5-year survival rate remains lower than 50%[9-11].

Long noncoding RNAs (lncRNAs) are noncoding transcripts that exceed 200 nt in length; they have been defined as the largest subclass in the noncoding transcriptome in humans[12-14]. The majority of lncRNAs with limited protein coding potential are transcribed by RNA polymerase II and polyadenylated at their 5′ and 3′ ends, respectively[15]. Recent reports have shown that a growing number of cancer transcriptomes have indeed revealed that thousands of aberrant lncRNAs are closely related to a variety of cancer types[16-18], including HCC. In particular, previous studies have revealed that a number of lncRNAs are dysregulated and exert essential roles in HCC progression; these lncRNAs include BZRAP1-AS1[19], RAB5IF[20], SNHG15[21], MAGI2-AS3[22], ATB[23], *etc*. We also previously demonstrated that the decreased expression of long intergenic noncoding RNA P7 predicts unfavorable prognosis and promotes tumor proliferation *via* the modulation of the STAT1-MAPK pathway in HCC[24], and lncRNA W5 inhibits progression and predicts favorable prognosis in HCC[25].

In this report, for the first time, we identified a novel lncRNA, W42, and investigated its expression levels in HCC tumor tissues and its association with the clinical-

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pathological parameters of HCC patients. The expression, biological function and underlying molecular mechanisms of lncRNA W42 in HCC were also studied *in vivo* and *in vitro*.

MATERIALS AND METHODS

Clinical samples

Data were obtained from 92 patients with HCC (72 males and 20 females) who underwent surgery for HCC at the Department of Hepatobiliary Surgery of the Fifth Medical Center, Chinese PLA General Hospital between October 2013 and July 2019. After obtaining written informed consent from all patients, tumor tissues and paired adjacent noncancerous tissue samples were collected from patients with HCC in accordance with the institutional guidelines of the hospital's Ethics Committee. Resected tissues were immediately snap-frozen in liquid nitrogen within 30 min and preserved in an HCC Tissue Biobank until use. This study was approved by the Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital, and carried out according to the Declaration of Helsinki.

Cell lines

The human HCC cell lines HepG2 and Huh7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). The normal liver cell line LO2 and human HCC cell line SMMC-7721 were obtained from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China). SMMC-7721 cells were established from a 50-year-old Chinese male HCC patient with negative HBsAg and positive α-fetoprotein (AFP) by the scholars of Second Military Medical University (Shanghai, China) in 1977[26]. The use of the cell lines was approved by the Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital. All of the cell lines used in this study were maintained in DMEM (Thermo Fisher, Beijing, China) supplemented with 10% fetal bovine serum (Gibco, Beijing, China) in 5% CO_2 at 37°C.

RNA extraction and real-time polymerase chain reaction

Total RNA from frozen fresh HCC tissues and paired adjacent noncancerous tissues was extracted using TRIzol reagent (Thermo Fisher) according to the manufacturer's instructions. RNA concentration and purity were detected using a NanoDrop ND-1000 (Thermo Fisher, Beijing, China). LncRNA W42 expression levels were examined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) performed with Maxima SYBR Green on an ABI 7500 instrument (Applied Biosystems, CA, United States). All reactions were run at least three times using lncRNA W42-specific primers. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression was monitored as the endogenous control, and all samples were normalized to human GAPDH[24,27,28]. The primer sequences of lncRNA W42 were as follows: forward: 5′-TGTGAACCAGGTTTGCTGGA-3′, reverse: 5′-CTCAACCATGCCGACGAGAA-3′; GAPDH forward: 5′-CAGCCTCAAGATCATCAGCA-3′ reverse: 5′-TGTGGTCAT-GAGTCCTTCCA-3′. The amplification procedure was 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s, extension at 72°C for 30 s. The median of triplicate reactions was used to calculate relative lncRNA expression (ΔCt = Ct median lncRNA - Ct median GAPDH). Expression changes were calculated using the 2-ΔΔCt method.

Vector construction and Lentiviral Infection

The lncRNA W42 overexpression vector was constructed and cloned into the BamHI and EcorI sites of the pcDNA3.1 (+) vector, yielding pcDNA3.1-lncRNA W42. The primers used in this experiment were as follows: forward: 5′-AGCTGAGG-GAGCCGGCT-3′, reverse: 5′-ATTAATGAGAGAATTATGGTAT-3′. In addition, gene knockdown used RNA interference experiments. LncRNA W42 was knocked down by shRNA lentiviruses. shRNA-lncRNA W42-1 and shRNA-lncRNA W42-2 were designed and synthesized by RIBOBIO Co., Ltd. (Guangzhou, China). Their sequences were as follows: W42-1: 3′-dTdT GGCUCAGUCUUUGAUCAAA-5′; W42-2: 3′-dTdT CCUGUAGUAAACUACUCAA-5′; the shRNA negative control sequence number was siN05815122147 of RIBOBIO. SMMC7721 cells (1×10^4) were stably transfected with lentiviral vector containing lncRNA W42 with the help of Hanbio Biotechnology Co., Ltd. (Shanghai, China). After infection for 72 h, the cells were collected for RNA

quantitation of lncRNA W42 and targeted genes using qRT-PCR.

Nucleocytoplasmic separation experiment

A nucleocytoplasmic separation experiment was conducted with the PARIS™ Protein and RNA Isolation System (Ambion, Life Technologies Lithuania). The experiment followed the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was assessed by the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's protocols. Huh7 or SMMC7721 cells were plated in 12-well plates (5×10^5 cells/well) and then transfected with lncRNA W42 overexpression vector, and the numbers of cells per well were examined and counted by the absorbance (450 nm) of reduced WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-isulfophenyl)-2H-tetrazolium, monosodium salt] at the indicated time.

Colony formation assay

Huh7 or SMMC7721 cells were plated in 6-well plates and incubated in DMEM with 10% fetal bovine serum (FBS) with 5% CO_2 at 37°C. Six days later, the cells were fixed and stained with 0.1% crystal violet. The number of colonies, defined as > 50 cells/colony, was recorded and photographed[24].

Transwell assay

Cell migration and invasion were determined using Transwell chambers (Sigma). The upper chamber of the Transwell was treated with Matrigel (without Matrigel coating for the migration assay). Briefly, SMMC7721 or Huh7 cells (50000 cells/well) were resuspended in serum-free DMEM (Gibco). Then, the medium containing the cells was seeded into the upper chamber. In addition, the lower chamber was supplemented with medium containing 10% FBS (Gibco) as a chemoattractant. After incubation for 48 h, the cells that did not migrate or invade the membrane were scraped away, while the migrated and invaded cells in the lower chamber of the Transwell were fixed with methanol and stained with 0.1% crystal violet (Sigma). The number of migrated and invaded cells was counted using a microscope.

Cell apoptosis assay and cell cycle analysis

An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (BestBio, Shanghai, China) was used for the apoptosis assay and analyzed by flow cytometry. After treatment, SMMC7721 and Huh7 were washed three times with phosphate buffered saline (PBS) and stained with Annexin V-FITC/PI. After filtration, the suspension of each group was analyzed within 1 h using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, United States). The cells were transfected with lncRNA W42 overexpression vector or control and cultured for 48 h. Then the cells were washed twice with PBS and fixed in cold 70% ethanol for 1 h followed by incubation with 20 μg/mL PI (BestBio, Shanghai, China) and 200 μg/mL RNase A (Sigma) for 30 min. Finally, the cells were analyzed utilizing a flow cytometer (BD Biosciences, Franklin Lakes, NJ, United States).

RNA pull-down assays

RNA pull-down assays were performed as previously described[29,30]. Briefly, *in vitro* biotin-labeled RNAs (lncRNA W42 and its antisense RNA) were transcribed by biotin RNA labeling mix (Roche) and T7 RNA polymerase (Roche), treated with RNase-free DNase I (Promega) and purified using the RNeasy Mini Kit (QIAGEN). Biotinylated RNA was incubated with cell lysate, and precipitated proteins were separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to mass spectrometric (MS) analysis. RNA pull-down of AVAN-associated proteins was performed using biotinylated lncRNA W42 or antisense probes (immunoglobulin G, IgG). Isolated proteins were resolved by SDS-PAGE followed by silver staining. Silver staining was performed with the SilverQuest™ Silver Staining Kit (Invitrogen) following the manufacturer's recommendations.

RNA immunoprecipitation

The RNA immunoprecipitation (RIP) assay was performed as previously described[31,32]. With IgG antibodies as the isotype-matched control, nuclear extracts were immunoprecipitated with 2.5 mg hnRNP U (Abcam, clone 4D11, ab6106) overnight. RNA-protein-antibody complexes were captured using Dynabead Protein

A/G (Thermo Fisher Scientific). RNA was eluted by adding TRIzol directly to magnetic beads and isolated according to the manufacturer's instructions. cDNA was synthesized using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech) and analyzed by qPCR. The results were normalized to input RNA and are shown as fold-enrichment over control IgG RIP.

In vivo experiments

A total of 16 female 4-6-wk-old nude BALB/C mice were purchased from Charles River Laboratories (Beijing, China) and were maintained in a SPF (specific pathogen-free) facility under pathogen-free conditions at 22°C and 40%-50% humidity with a 12/12 h light/dark cycle, and had ad libitum access to food and water. Each mouse was randomly assigned to one of the following two groups: LncRNA W42 knockdown and control group (n = 8 per group). SMMC7721 cells (2×10^6) stably lncRNA W42 knockdown or empty vector were subcutaneously injected into the flanks of nude mice. Tumor growth was monitored by tumor volume and weight. After 4 d injection, tumor size was recorded every 3 d, and tumor volumes were calculated with the formula: Tumor volume (mm^3) = length × $width^2$ × 0.5. After 36 d, the mice were euthanized by i.p. injection of sodium pentobarbital (150 mg/kg), and tumor tissues were removed, weighed and fixed in polyformaldehyde after imaging of the tumors for further examination. All animal procedures were performed in strict accordance with the recommendations of the National Institutes of Health Laboratory Animal Care and Use Guidelines. This study was approved by the Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital (2020-002).

Statistical analysis

All data were analyzed using the SPSS 20.0 software package (Chicago, IL, United States). All statistical results are expressed as the mean ± SD. Kaplan-Meier analyses were used to represent the correlations between lncRNA W42 expression level and overall survival (OS) rate or recurrence-free survival (RFS). The data from the two groups were assessed using the Student t-test, the differences between various groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. All experiments were carried out at least three times. P values < 0.05 were considered significant.

RESULTS

LncRNA W42 was upregulated in human HCC tumor tissues and cell lines

Based on our previous bioinformatics analysis, we chose another 10 candidate lncRNAs and re-validated their expression in the normal liver cell line LO2 and the human HCC cell line Huh7 (Supplementary Figure 1A). Subsequently, we re-validated the expression of lncRNA W42 in 25 pairs of HCC tissues and paired adjacent noncancerous tissues. The results showed that lncRNA W42 was the most differentially expressed among these lncRNAs. Hence, we chose lncRNA W42 as a candidate lncRNA for HCC in the following study. To define the role of lncRNA W42 in human HCC, we first measured lncRNA W42 expression in 92 pairs of HCC tissues and adjacent noncancerous tissues by qRT-PCR. As shown in Figure 1, we observed that lncRNA W42 was significantly increased in HCC tissues compared with paired adjacent noncancerous tissues (P < 0.001, Figure 1A). Next, qRT-PCR analysis also showed significantly higher expression of lncRNA W42 in three human HCC cell lines (SMMC-7721, HepG2 and Huh7) than in the normal liver cell line LO2 (P < 0.05, Figure 1B). Moreover, we used receiver operating characteristic (ROC) curves to assess the sensitivity and specificity of lncRNA W42 expression to discriminate between tumor and noncancerous samples, the sensitivity was 74.76% and the specificity was 72.82%. It was noticeable that lncRNA W42 had predictive value, with an area under the curve (AUC, denotes discrimination accuracy) of 0.808 (Figure 1C), revealing that lncRNA W42 has adequate sensitivity and specificity to discriminate between HCC tissues and paired adjacent noncancerous tissues. In addition, the nucleocytoplasmic separation experiment showed that lncRNA W42 was mainly located in the nucleus (Figure 1D). In addition, we sequenced full-length lncRNA W42 and found that its length was 1598 nt and that it was located on chromosome 7q: 94423057-94429430 and shares a transcript with the COL1A2 gene (Supplementary Figure 1B and C). Thus, these results suggest that lncRNA W42 may provide imperative clinical guidance in HCC diagnosis and serve a critical role in HCC progression.

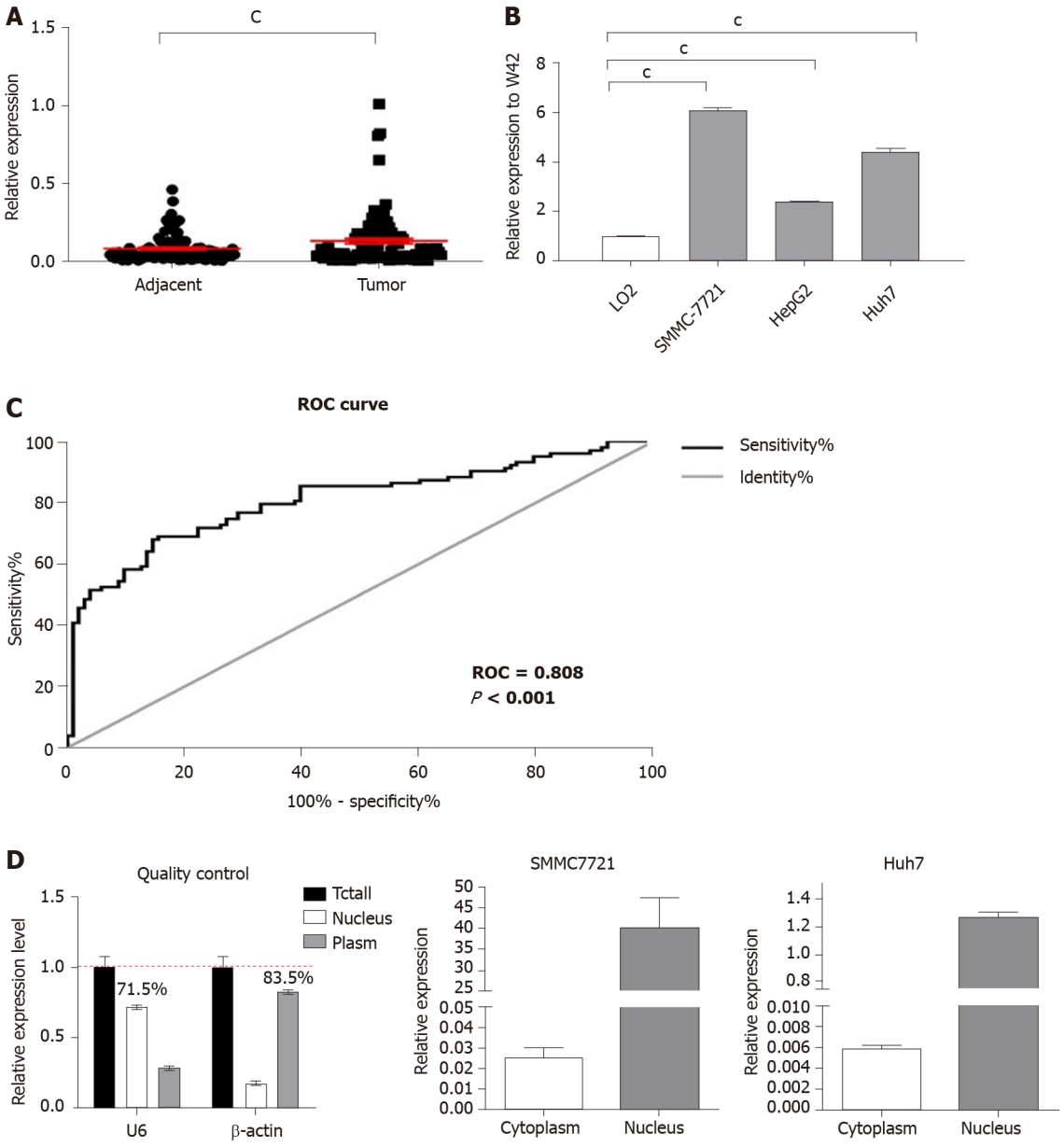
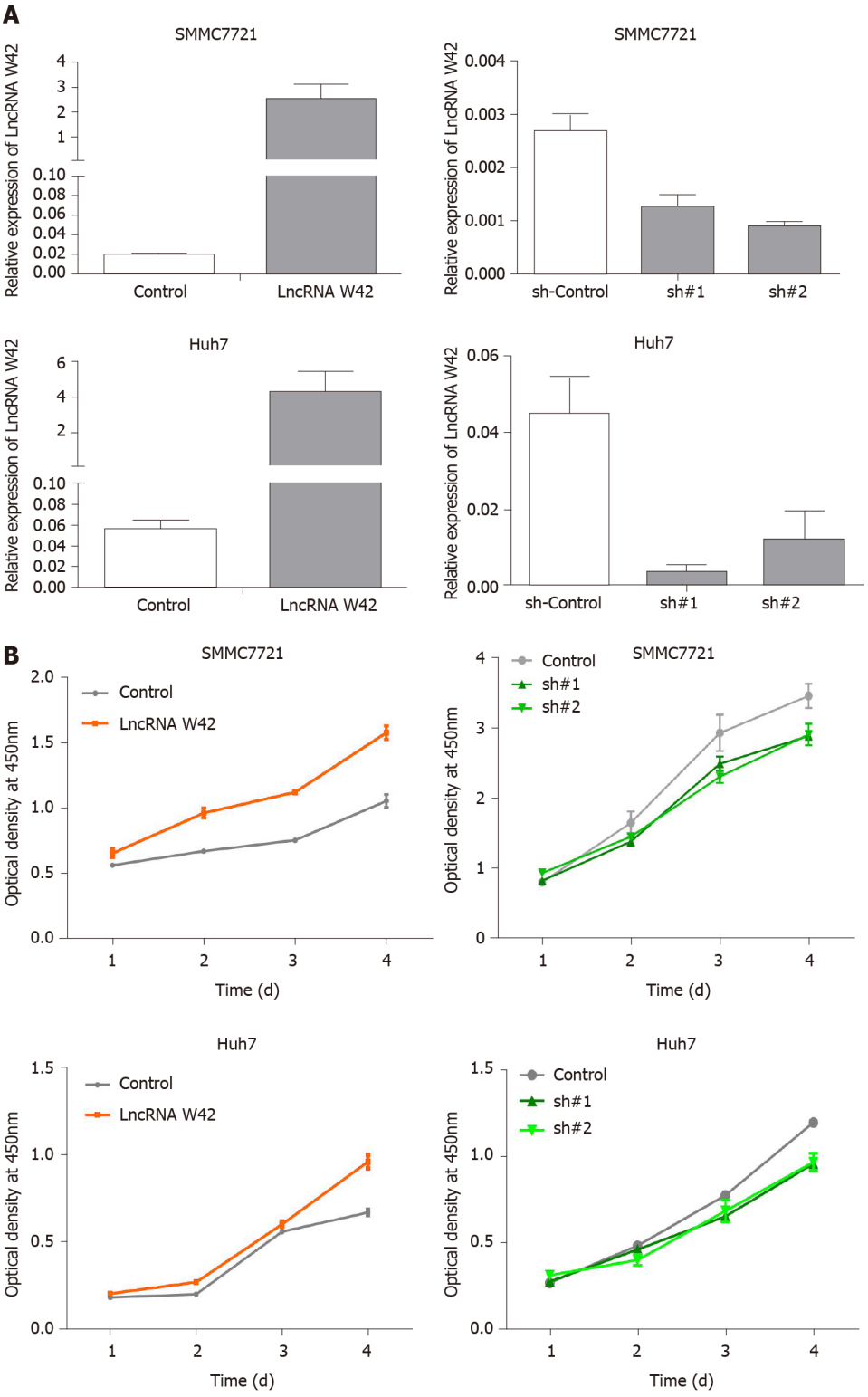
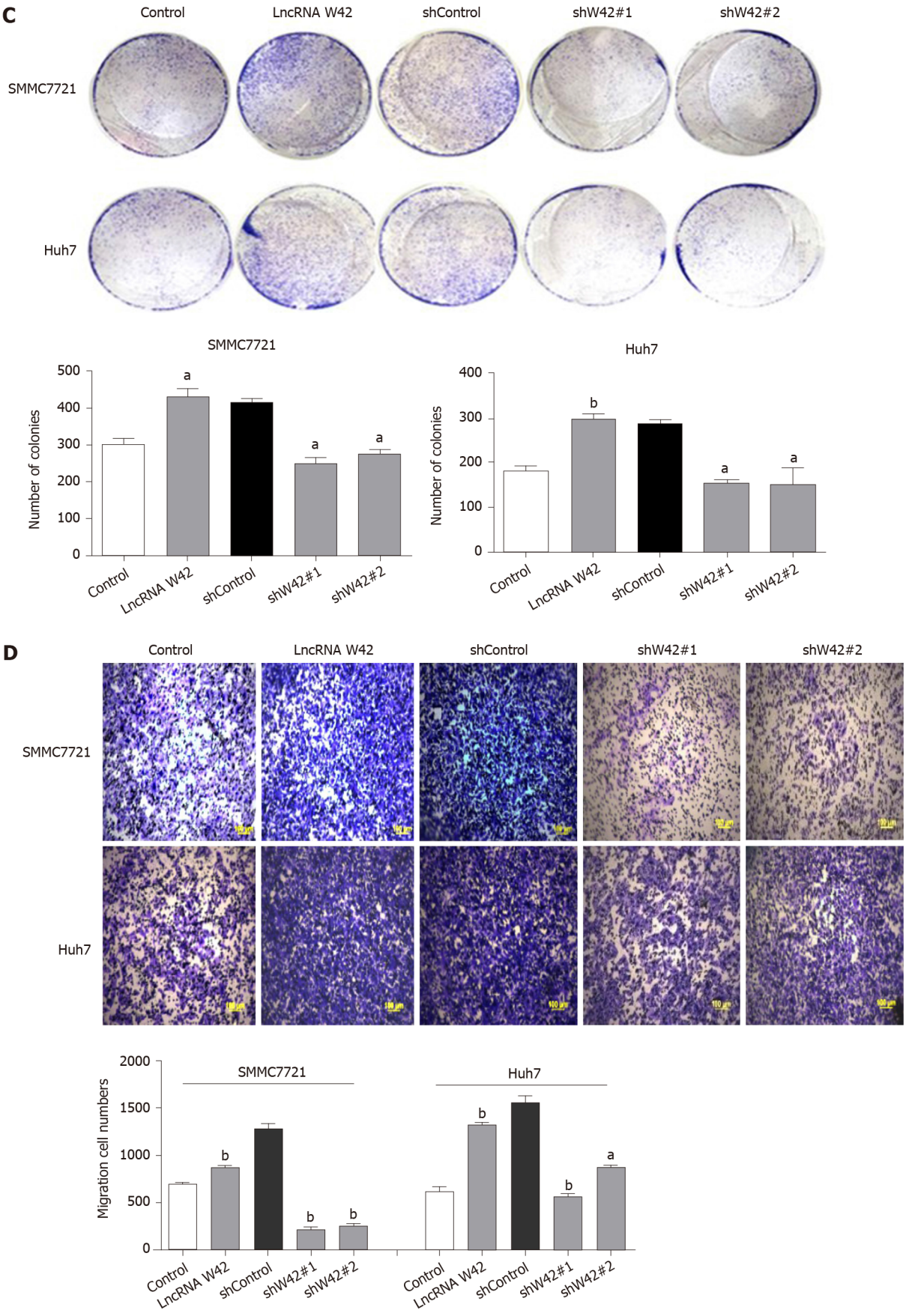


Figure 1 The levels of long noncoding RNA W42 expression in human hepatocellular carcinoma tissues and cell lines. A: Relative expression levels of long noncoding RNA (lncRNA) W42 in human hepatocellular carcinoma (HCC) tissue compared to paired adjacent noncancerous tissues (n = 92). LncRNA W42 expression was examined by quantitative reverse transcriptase polymerase chain reaction and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression ($^cP < 0.001$); B: The level of lncRNA W42 expression was higher in HCC cell lines than in the normal liver cell line LO2 ($^cP < 0.001$); C: The area under the receiver operating characteristic curve was used for prediction of HCC based on lncRNA W42 expression, using paired adjacent noncancerous tissues as a control; D: Quantitative reverse transcriptase polymerase chain reaction analysis of lncRNA W42 in subcellular fractions of SMMC 7721 and Huh7 cells. β-Actin and U6 acted as cytoplasmic and nuclear markers, respectively (n = 3). ROC: Receiver operating characteristic.

LncRNA W42 promoted proliferation and invasion and suppressed early apoptosis of HCC cells in vitro

To further investigate the biological functions of lncRNA W42 in HCC, we constructed a lentiviral vector for stably overexpressing lncRNA W42 in HCC cell lines, *i.e.*, SMMC7721 and Huh7, and designed two shRNAs for W42 (Figure 2A). CCK-8 assays revealed that overexpression of lncRNA W42 in these HCC cell lines significantly enhanced the cell proliferation ability (Figure 2B). Colony formation assays also indicated that the lncRNA W42-transfected HCC cells formed significantly more colonies than the control cells (Figure 2C). Conversely, we inhibited lncRNA W42 expression by transfecting lncRNA W42-specific shRNA into HCC cells. As indicated by CCK-8 assays, the repression of lncRNA W42 significantly decreased cell proliferation in HCC cells. Similarly, colony formation assays showed that compared to the control, knockdown of lncRNA W42 inhibited HCC cell colony formation. Moreover, overexpression of cellular lncRNA W42 not only increased HCC cell proliferation but


A
SMMC7721
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C
Control
LncRNA W42
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SMMC7721
Huh7
SMMC7721
Huh7
Number of colonies
500
400
300
200
100
0
400
300
200
100
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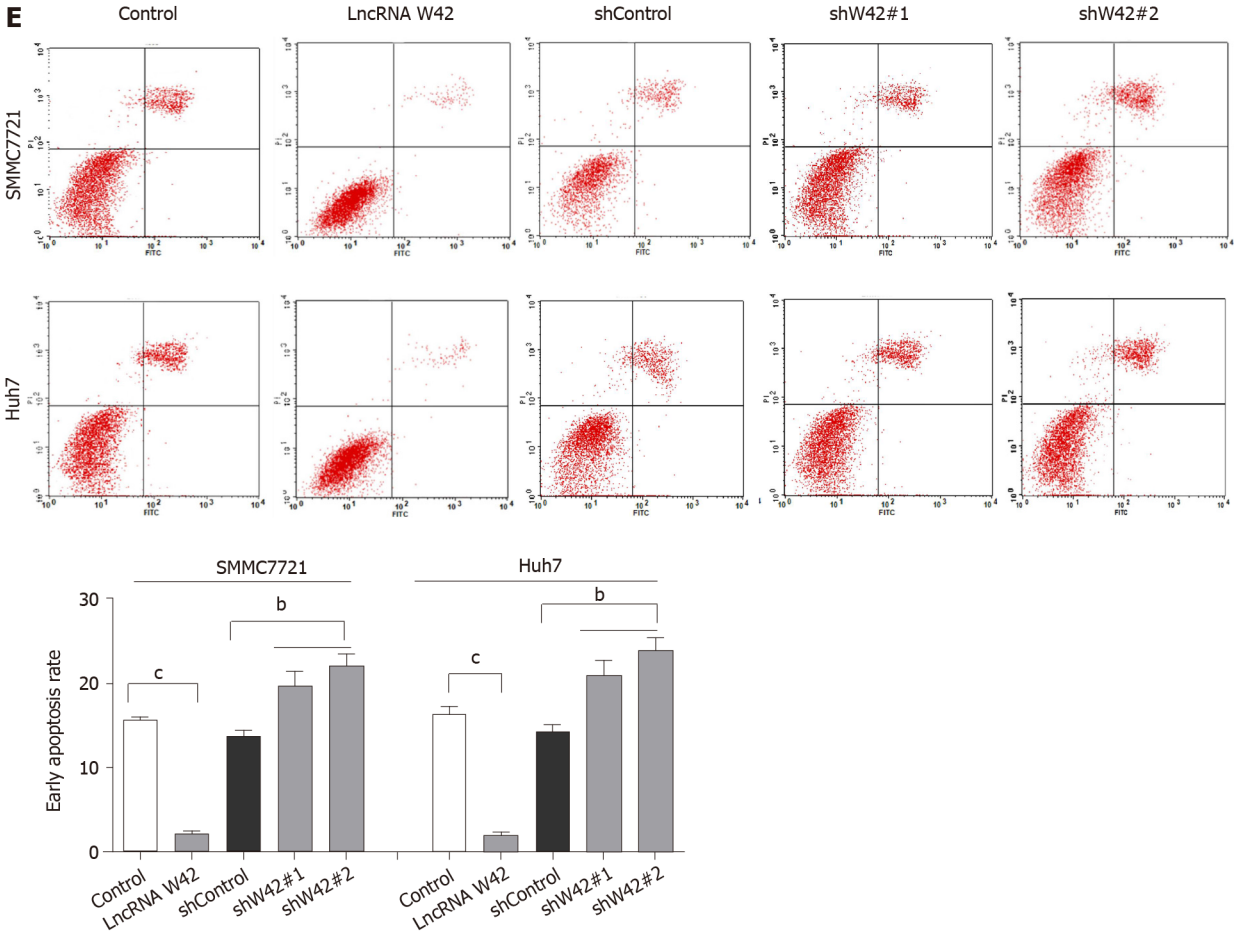


Figure 2 Long noncoding RNA W42 affects proliferation and invasion in hepatocellular carcinoma cells *in vitro*. A: Long noncoding RNA (lncRNA) W42 knockdown in SMMC 7721 and Huh7 cells. The expression level of lncRNA W42 was robustly enhanced or inhibited when cell lines were infected with lentivirus containing the lncRNA W42 coding sequence or two shRNAs targeting lncRNA W42, respectively. Empty vector and shRNA control were used as the negative controls; B: CCK-8 assays were used to determine the viability of SMMC7721 and Huh7 cells transfected with shRNA-lncRNA W42. The cell number was examined from 24 h to 96 h. The results are shown as the mean ± SE. $^{a}P < 0.05$, compared with the control by two-sided *t* test; C: Colony forming assays were performed to determine the proliferation of SMMC7721 and Huh7 cells transfected with shRNA-lncRNA W42; D: Transwell assays to measure the impact of lncRNA W42 knockdown on HCC migration ability; E: Annexin V-fluorescein isothiocyanate/propidium iodide assay for the effect of overexpression or knockdown of lncRNA W42 on early apoptosis of HCC cell lines. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. LncRNA: Long noncoding RNA.

also promoted the cell migration and invasion activity (Figure 2D) of HCC cells compared with that in the control group. In addition, we also examined apoptosis when over-expressing lncRNA W42 on HCC cells, the results showed that there was a significant decrease in the early apoptosis of HCC cells (Figure 2E). In addition, no significant difference was observed in the cell cycle analysis between the lncRNA W42 and control group (Supplementary Figure 2). Collectively, these data imply that lncRNA W42 exerts an important role in regulating HCC cell proliferation and invasion *in vitro*.

LncRNA W42 directly bound to DBN1 and enhanced DBN1-mediated tumor-promoting effects

Given that lncRNA W42 is mainly located in the nucleus, we further investigated the molecular mechanism of lncRNA W42. RNA pull-down assays and MS analysis were performed in SMMC7721 cells. The results showed that DBN1, which is a key player in the advancement of several cancers, bound lncRNA W42 in SMMC7721 cells more readily than in lncRNA W42 antisense control cells (Figure 3A). This result was further confirmed by a lncRNA W42 RNA pull-down western blot assay (Figure 3B). To validate the interaction between lncRNA W42 and DBN1, we immunoprecipitated DBN1 from SMMC7721 cells and quantified the protein-bound lncRNA W42. Significantly higher expression of lncRNA W42 was detected with endogenous (Figure 3C). Together, these results indicate that lncRNA W42 RNA physically interacts with DBN1.

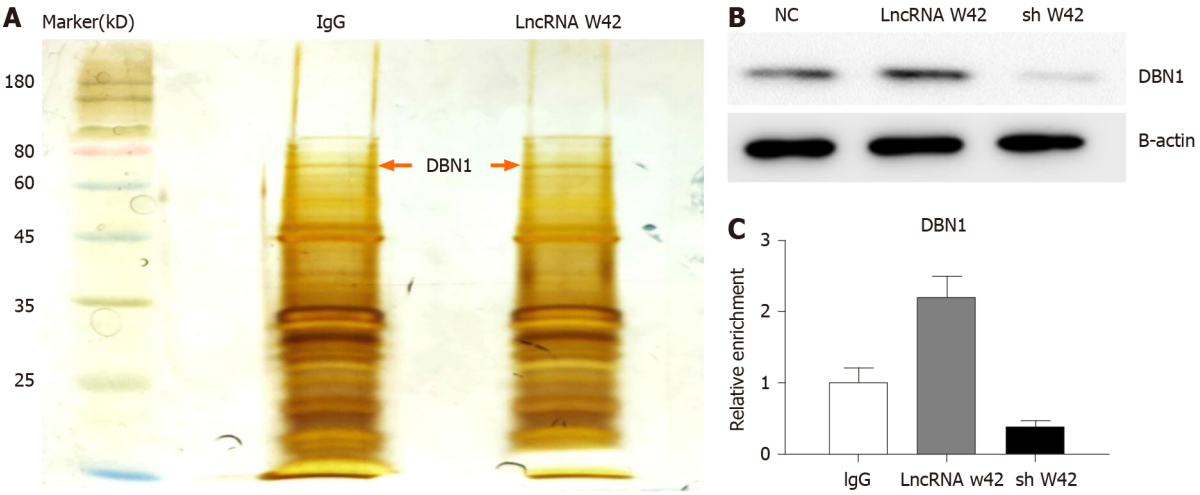


Figure 3 Long noncoding RNA W42 directly bound to DBN1 and enhanced DBN1-mediated tumor-promoting effects. A: RNA pull-down of AVAN-associated proteins using biotinylated long noncoding RNA (lncRNA) W42 or antisense probes (immunoglobulin G, IgG). Isolated proteins were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) followed by silver staining; B: Pull-down western blot showing that lncRNA W42 can bind directly to DBN1; C: RNA immunoprecipitation of DBN1 in SMMC7721 cells using anti-DBN1 or anti-IgG antibodies. The relative fold enrichment of lncRNA W42 was calculated by quantitative reverse transcriptase polymerase chain reaction. IgG: Immunoglobulin G; LncRNA: Long noncoding RNA; NC: Negative control.

Downregulation of lncRNA W42 inhibited HCC xenograft tumor growth in vivo

To elucidate the *in vivo* roles of lncRNA W42 in tumorigenesis, we determined whether decreased expression of lncRNA W42 could suppress tumor growth in the HCC xenograft tumor model. SMMC7721 cells with stable knockdown of lncRNA W42 or empty vector, generated by lentiviral vector transduction, were inoculated into nude mice. Thirty-six days after injection, the tumors were dissected from the mice (Supplementary Figure 3). Representative images of dissected tumors are shown in Figure 4A. As illustrated in Figure 4B, we observed that the growth rate of tumors in the lncRNA W42 knockdown group was lower than that in the control group. Six weeks after injection, the weight of tumors dissected from mice in the lncRNA W42 knockdown group was significantly lower than that in their control groups (Figure 4C). Consistent with the *in vitro* results, the tumor volumes and weights were markedly lower in the lncRNA W42 knockdown group than in the empty vector group, indicating that lncRNA W42 promotes HCC xenograft tumor growth *in vivo*.

Relationship between lncRNA W42 expression, clinicopathological characteristics and patient prognosis

We further determined whether lncRNA W42 expression was correlated with the clinicopathological characteristics and outcome of HCC patients. HCC patients (n = 92) were divided into a high lncRNA W42 expression group (above the average expression, n = 46) and a low lncRNA W42 expression group (below the average expression, n = 46). As illustrated in Table 1, no obvious differences were observed in age, tumor size, or AFP levels. However, lncRNA W42 expression was closely associated with tumor number, liver cirrhosis and tumor recurrence ($P < 0.05$).

Subsequently, we determined whether lncRNA W42 expression was associated with the prognosis of HCC patients. All 92 HCC patients included in this experiment were classified into two cohorts with high or low lncRNA W42 expression according to median lncRNA W42 expression values. Kaplan-Meier analysis in the 92 patients with HCC showed that the 3-year OS rate after surgery was 64.13% (P = 0.022; Figure 5A). Specifically, a higher lncRNA W42 expression level in HCC tissues was significantly associated with a reduction in OS (P = 0.005; Figure 5B) and RFS (P = 0.008; Figure 5C) during the 3 year follow up period, supporting the critical roles of lncRNA W42 in the pathogenesis of HCC.

DISCUSSION

With the advances of next-generation sequencing technology, lncRNAs have been reported to be aberrantly expressed in multiple types of cancers[33-35]. A number of lncRNAs have been validated to be critical regulators of biological processes in

Table 1 The relationship between long noncoding RNA W42 expression and clinicopathological features in hepatocellular carcinoma patients

Parameters	Group	Total	W42 expression		*P* value
			Low	High	
Gender	Male	72	35	37	0.810
	Female	20	11	9	
Age (yr)	< 60	27	15	12	0.681
	≥ 60	65	31	34	
Tumor size (cm)	< 5	52	25	27	0.846
	≥ 5	40	21	19	
AFP	< 20	27	11	16	0.411
	≥ 20	65	35	30	
Histological grade	Well/moderately	14	8	6	0.776
	Poorly	78	38	40	
Clinical stage	I and II	69	31	38	0.182
	III	23	15	8	
Tumor number	Solitary	69	49	20	0.009[b]
	Multiple	23	6	17	
Drinking status	Yes	34	19	23	0.999
	No	58	31	27	
Smoking status	Yes	48	22	26	0.563
	No	44	24	20	
HBV	Yes	66	30	36	0.419
	No	26	14	12	
Recurrence	Yes	29	9	20	0.016[a]
	No	79	49	30	
PVTT	Yes	42	22	20	0.847
	No	50	24	26	
Microvascular invasion	Yes	73	37	35	0.999
	No	19	9	10	
Liver cirrhosis	Absence	45	16	29	0.030[a]
	Presence	47	30	17	

[a]$P < 0.05$.

[b]$P < 0.01$. AFP: α-Fetoprotein; HBV: Hepatitis B virus; PVTT: Portal vein tumor thrombosis.

cancer[36]. In particular, several HCC hot lncRNAs, such as HULC[37], P53[38], MCM3AP-AS1[39], ATB[40], HOTAIR[14] and lnc00624[41], have been reported, and increasingly evidence showed that further investigation on the relationship between lncRNA and HCC might provide potential targets for targeted therapy of HCC. Herein, we revealed differential expression of lncRNA W42 using qRT-PCR analysis of HCC and paired adjacent noncancerous tissues. Our results showed that lncRNA W42 was upregulated in HCC tissues compared to their normal counterparts. The ROC AUC of lncRNA W42 was 0.808, suggesting its specificity and sensitivity in the diagnosis of HCC. We also identified the function of lncRNA W42 in HCC cells by applying gain-of-function and loss-of-function approaches. Overexpression of lncRNA W42 promoted the proliferation and invasion and suppressed early apoptosis of HCC cells. However, this phenomenon was reversed when lncRNA W42 was downregulated.

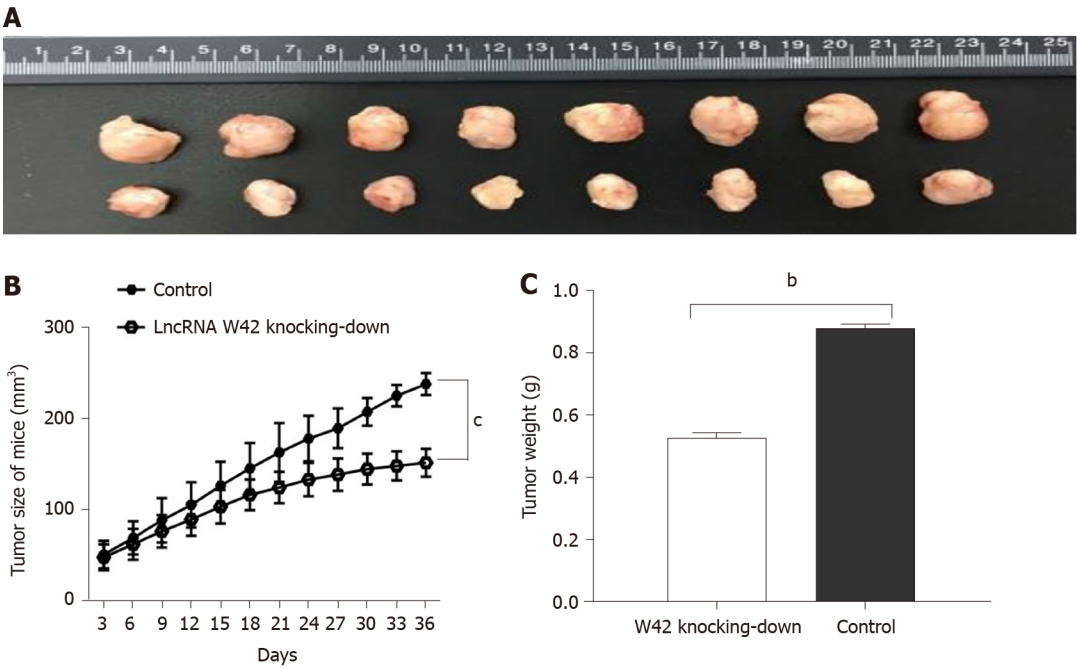


Figure 4 Effects of long noncoding RNA W42 expression on tumor growth in hepatocellular carcinoma nude mouse model. A: SMMC7721 cells (5×10^6) stably expressing long noncoding RNA (lncRNA) W42 were inoculated into hepatocellular carcinoma (HCC) nude mice, and the effect of lncRNA W42 on HCC tumor growth was examined every 3 d for 36 d ($n = 8$). Thirty-six days after injection, tumors were dissected from the mice in the lncRNA W42 knockdown and control groups. Representative images are shown; B: The growth rate of tumors in the lncRNA W42 knockdown group was lower than that in the control group. mean ± SE for eight mice in each group. $^{c}P < 0.001$ compared with the control group; C: The weights of tumors dissected from mice in the lncRNA W42 knockdown group were significantly lower than those of their control counterparts. The results are shown as the mean ± SE for eight mice in each group. $^{b}P < 0.01$ compared with control cells by two-sided *t* test. LncRNA: Long noncoding RNA.

DBN1 is an actin-binding protein that was initially found in embryonic chicken brains, and it was identified to be a neuron-specific protein[42]. However, several studies have shown the presence of DBN1 in various nonneuronal tissues, even in tumor tissues, such as recurrent non-small-cell lung cancer[43] and lymphoblastic leukemia[44] tissues. Herein, we showed that the overexpression of lncRNA W42 and DBN1 may be involved in the progression of HCC. Our results also showed that lncRNA W42 RNA physically interacts with DBN1 and that lncRNA W42 might directly bind to DBN1 and enhance the DBN1-mediated tumor-promoting effects. In addition, the mechanism by which lncRNA W42 regulates DBN1 warrants further investigation in subsequent studies.

Moreover, *in vivo* experiments revealed that lncRNA W42 knockdown inhibited tumor growth in nude mice, as indicated by tumor volume and weight. Subsequently, we will conduct *in vivo* experiments to analyze the effects of lncRNA W42 overexpression on liver tumors in nude mice. A clinical investigation showed that lncRNA W42 upregulation was closely correlated with tumor number, liver cirrhosis and tumor recurrence. More importantly, we observed that upregulated lncRNA W42 expression was closely associated with decreased OS rates and RFS rates. Based on these data, we propose that lncRNA W42 may play an important role in the development and progression of HCC.

Several previous studies have shown that liver cirrhosis is a predictor of and an independent risk factor for HCC recurrence and that patients with liver cirrhosis tend to express different levels lncRNAs[45-47]. Patients with liver cirrhosis usually have a higher probability of tumor development once a virus infection occurs because they are resistant to direct-acting antiviral agents. HCC develops frequently in the advanced fibrosis stage, and eradicating HBV or HCV infection has been a promising prophylactic therapy for preventing the occurrence of liver fibrosis and HCC. To date, several lncRNAs have been validated to be involved in HCC progression, but the role of lncRNAs in cirrhosis has not been well explained. On the other hand, lncRNA HULC is upregulated in the plasma samples of patients with HBV-related cirrhosis[48]. In line with these findings, we also noted that high expression of lncRNA W42 in patients was associated with cirrhosis, suggesting that the lncRNA profiles may be changed in HCC patients with cirrhosis. Furthermore, factors different from carcinogenesis or genetic and epigenetic factors are related to the flow from chronic hepatitis

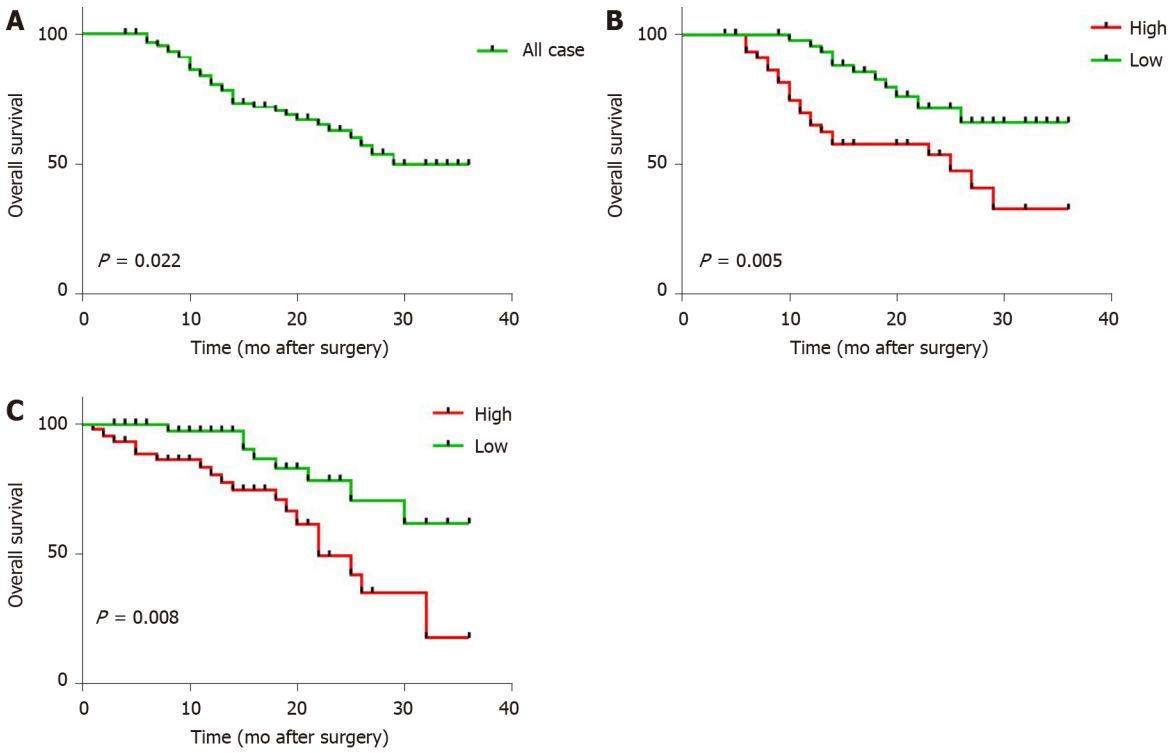


Figure 5 Association between long noncoding RNA W42 expression and hepatocellular carcinoma patient survival rates as determined by Kaplan-Meier analyses. A: The three-year overall survival rate of 92 hepatocellular carcinoma (HCC) patients was 64.13%; B: Patients with HCC with high long noncoding RNA (lncRNA) W42 expression levels showed significantly shorter overall survival than those with low lncRNA W42 expression levels (P = 0.005, log-rank test); C: Patients with HCC with high lncRNA W42 expression levels showed significantly shorter recurrence-free survival than those with low lncRNA W42 expression levels (P = 0.008, log-rank test).

to liver cirrhosis or from focal HCC carcinogenesis in the liver to multi-organ metastasis. As shown in Table 1, we observed that lncRNA W42 expression was closely associated with liver cirrhosis and tumor recurrence, but no obvious differences were observed in age, gender, tumor size, HBV and AFP levels. Of course, the relationship between lncRNA W42 and the etiology of HCC (HCV, HBV, NAFLD, alcohol, *etc.*) needs to be validated in a much larger cohort. Moreover, further study will explore the levels of lncRNA W42 in serum and the AUC values of AFP in tumor tissue to ascertain if lncRNA W42 provides a higher diagnostic capacity compared to AFP in HCC patients.

Several studies have shown that microRNA (miRNA) and circulating tumor DNA detected in blood and body fluids such as urine, are useful for screening the presence of various cancers[49,50]. Similarly, the abundant mRNA, circular RNA, and lncRNA in human blood could be utilized as potential biomarkers for the diagnosis of various cancers, which were revealed by extracellular vesicles long RNA sequencing[51]. However, whether lncRNA W42 could be detected in peripheral blood needs to be investigated in subsequent studies.

These results further support that lncRNA W42 might participate in the proliferation and apoptosis regulation of HCC cells. Of course, the proliferation results need to be confirmed by flow cytometry (*e.g.*, CFSE assay) in subsequent studies. In addition, the effect and underlying molecular mechanism of lncRNA W42 in HCC progression and potential lncRNA W42 targets, for example, the modulation of lncRNA W42 impacts on the response to chemo-or-targeted therapies, remain to be investigated in the future.

CONCLUSION

In conclusion, our results demonstrated, for the first time, that lncRNA W42 expression was significantly higher in HCC tissues than in normal tissues and that dysregulation of lncRNA W42 was positively correlated with tumor number, liver cirrhosis and tumor recurrence in patients with HCC. Moreover, increased lncRNA

W42 expression was associated with a poor prognosis in patients with HCC. These findings suggest that lncRNA W42 is an important marker for indicting prognosis and may have potential as a diagnostic and therapeutic target for HCC.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a malignancy found globally.

Research motivation

The function of long noncoding RNAs (lncRNAs) in HCC requires further investigation.

Research objectives

We aimed to understand the effect of lncRNA W42 on HCC and dissect the underlying molecular mechanisms.

Research methods

LncRNA W42 expression in HCC tissues and cells (Huh7 and SMMC-7721) was detected by quantitative reverse transcriptase polymerase chain reaction. After transfection with pcDNA3.1-lncRNA W42 or shRNA-lncRNA W42, cell functions were assessed by cell counting Kit-8 (CCK-8), colony formation, flow cytometry and Transwell assays. An HCC xenograft model was used to assess the role of lncRNA W42 on tumor growth *in vivo*. Receiver operating characteristic curves and Kaplan-Meier curves were used for clinical investigation.

Research results

LncRNA W42 expression was upregulated in HCC tissues and cells. LncRNA W42 directly bound to DBN1 and activated the downstream pathway to promote cell proliferation, and invasion of HCC. LncRNA W42 knockdown suppressed HCC xenograft tumor growth *in vivo*. HCC patients with high lncRNA W42 expression exhibited shorter survival times.

Research conclusions

Upregulation of lncRNA W42 promotes tumor development by binding with DBN1 in HCC.

Research perspectives

LncRNA W42, which is upregulated in HCC, may serve as a potential candidate prognostic biomarker and therapeutic target in HCC.

REFERENCES

1 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

2 **Wang H**, Huo X, Yang XR, He J, Cheng L, Wang N, Deng X, Jin H, Wang C, Zhao F, Fang J, Yao M, Fan J, Qin W. STAT3-mediated upregulation of lncRNA HOXD-AS1 as a ceRNA facilitates liver cancer metastasis by regulating SOX4. *Mol Cancer* 2017; **16**: 136 [PMID: 28810927 DOI: 10.1186/s12943-017-0680-1]

3 **Sartorius K**, Sartorius B, Aldous C, Govender PS, Madiba TE. Global and country underestimation of hepatocellular carcinoma (HCC) in 2012 and its implications. *Cancer Epidemiol* 2015; **39**: 284-290 [PMID: 25922178 DOI: 10.1016/j.canep.2015.04.006]

4 **Nault JC**, Datta S, Imbeaud S, Franconi A, Mallet M, Couchy G, Letouzé E, Pilati C, Verret B, Blanc JF, Balabaud C, Calderaro J, Laurent A, Letexier M, Bioulac-Sage P, Calvo F, Zucman-Rossi J. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat Genet* 2015; **47**: 1187-1193 [PMID: 26301494 DOI: 10.1038/ng.3389]

5 **Rogacki K**, Kasprzak A, Stępiński A. Alterations of Wnt/β-catenin signaling pathway in hepatocellular carcinomas associated with hepatitis C virus. *Pol J Pathol* 2015; **66**: 9-21 [PMID: 26017875 DOI: 10.5114/pjp.2015.51148]

6 **Zucman-Rossi J**, Villanueva A, Nault JC, Llovet JM. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. *Gastroenterology* 2015; **149**: 1226-1239. e4 [PMID: 26099527 DOI: 10.1053/j.gastro.2015.05.061]

7 **Ding SL**, Yang ZW, Wang J, Zhang XL, Chen XM, Lu FM. Integrative analysis of aberrant Wnt signaling in hepatitis B virus-related hepatocellular carcinoma. *World J Gastroenterol* 2015; **21**: 6317-6328 [PMID: 26034368 DOI: 10.3748/wjg.v21.i20.6317]

8 **Yao S**, Johnson C, Hu Q, Yan L, Liu B, Ambrosone CB, Wang J, Liu S. Differences in somatic mutation landscape of hepatocellular carcinoma in Asian American and European American populations. *Oncotarget* 2016; **7**: 40491-40499 [PMID: 27246981 DOI: 10.18632/oncotarget.9636]

9 **Li X**, Li C, Zhang L, Wu M, Cao K, Jiang F, Chen D, Li N, Li W. The significance of exosomes in the development and treatment of hepatocellular carcinoma. *Mol Cancer* 2020; **19**: 1 [PMID: 31901224 DOI: 10.1186/s12943-019-1085-0]

10 **Chen B**, Garmire L, Calvisi DF, Chua MS, Kelley RK, Chen X. Harnessing big 'omics' data and AI for drug discovery in hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2020; **17**: 238-251 [PMID: 31900465 DOI: 10.1038/s41575-019-0240-9]

11 **Vogel A**, Saborowski A. Current strategies for the treatment of intermediate and advanced hepatocellular carcinoma. *Cancer Treat Rev* 2020; **82**: 101946 [PMID: 31830641 DOI: 10.1016/j.ctrv.2019.101946]

12 **Wei L**, Wang X, Lv L, Liu J, Xing H, Song Y, Xie M, Lei T, Zhang N, Yang M. The emerging role of microRNAs and long noncoding RNAs in drug resistance of hepatocellular carcinoma. *Mol Cancer* 2019; **18**: 147 [PMID: 31651347 DOI: 10.1186/s12943-019-1086-z]

13 **Lim LJ**, Wong SYS, Huang F, Lim S, Chong SS, Ooi LL, Kon OL, Lee CG. Roles and Regulation of Long Noncoding RNAs in Hepatocellular Carcinoma. *Cancer Res* 2019; **79**: 5131-5139 [PMID: 31337653 DOI: 10.1158/0008-5472.CAN-19-0255]

14 **Wong CM**, Tsang FH, Ng IO. Non-coding RNAs in hepatocellular carcinoma: molecular functions and pathological implications. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 137-151 [PMID: 29317776 DOI: 10.1038/nrgastro.2017.169]

15 **Dhanoa JK**, Sethi RS, Verma R, Arora JS, Mukhopadhyay CS. Long non-coding RNA: its evolutionary relics and biological implications in mammals: a review. *J Anim Sci Technol* 2018; **60**: 25 [PMID: 30386629 DOI: 10.1186/s40781-018-0183-7]

16 **Chen X**, Wang S. LncRNA-ZFAS1 contributes to colon cancer progression through the miR-150-5p/VEGFA axis. *Ann Oncol* 2018; **29**: v52-v53 [DOI: 10.1093/annonc/mdy151.187]

17 **Kirchhoff T**, Simpson D, Hekal T, Ferguson R, Kazlow E, Moran U, Lee Y, Izsak A, Wilson MA, Shapiro R, Pavlick A, Osman I. Discovery of novel germline genetic biomarkers of melanoma recurrence impacting exonic and long non-coding RNA (lncRNA) transcripts. *Ann Oncol* 2018; **29** (suppl_8): viii442-viii466 [DOI: 10.1093/annonc/mdy289]

18 **Zhang J**, Han X, Jiang L, Han Z, Wang Z. LncRNA CERNA2 is an independent predictor for clinical prognosis and is related to tumor development in gastric cancer. *Int J Clin Exp Pathol* 2018; **11**: 5783-5791 [PMID: 31949664]

19 **Wang W**, Chen G, Wang B, Yuan Z, Liu G, Niu B, Chen Y, Zhou S, He J, Xue H. Long non-coding RNA BZRAP1-AS1 silencing suppresses tumor angiogenesis in hepatocellular carcinoma by mediating THBS1 methylation. *J Transl Med* 2019; **17**: 421 [PMID: 31847842 DOI: 10.1186/s12967-019-02145-6]

20 **Koo JI**, Lee HJ, Jung JH, Im E, Kim JH, Shin N, Sim DY, Hwang J, Kim SH. The Pivotal Role of Long Noncoding RNA RAB5IF in the Proliferation of Hepatocellular Carcinoma Via LGR5 Mediated β-Catenin and c-Myc Signaling. *Biomolecules* 2019; **9** [PMID: 31717443 DOI: 10.3390/biom9110718]

21 **Dai W**, Dai JL, Tang MH, Ye MS, Fang S. lncRNA-SNHG15 accelerates the development of hepatocellular carcinoma by targeting miR-490-3p/ histone deacetylase 2 axis. *World J Gastroenterol* 2019; **25**: 5789-5799 [PMID: 31636472 DOI: 10.3748/wjg.v25.i38.5789]

22 **Pu J**, Wang J, Wei H, Lu T, Wu X, Wu Y, Shao Z, Luo C, Lu Y. lncRNA MAGI2-AS3 Prevents the Development of HCC *via* Recruiting KDM1A and Promoting H3K4me2 Demethylation of the RACGAP1 Promoter. *Mol Ther Nucleic Acids* 2019; **18**: 351-362 [PMID: 31629962 DOI: 10.1016/j.omtn.2019.08.020]

23 **Wang CZ**, Yan GX, Dong DS, Xin H, Liu ZY. LncRNA-ATB promotes autophagy by activating Yes-associated protein and inducing autophagy-related protein 5 expression in hepatocellular carcinoma. *World J Gastroenterol* 2019; **25**: 5310-5322 [PMID: 31558875 DOI: 10.3748/wjg.v25.i35.5310]

24 **Cheng S**, Li T, Wang C, Wang K, Lai C, Yan J, Fan H, Sun F, Wang Z, Zhang P, Yu L, Hong Z, Lei G, Sun B, Gao Y, Xiao Z, Ji X, Wang R, Wu J, Wang X, Zhang S, Yang P. Decreased long intergenic noncoding RNA P7 predicts unfavorable prognosis and promotes tumor proliferation *via* the modulation of the STAT1-MAPK pathway in hepatocellular carcinoma. *Oncotarget* 2018; **9**: 36057-36066 [PMID: 30546827 DOI: 10.18632/oncotarget.23282]

25 **Lei GL**, Fan HX, Wang C, Niu Y, Li TL, Yu LX, Hong ZX, Yan J, Wang XL, Zhang SG, Ren MJ, Yang PH. Long non-coding ribonucleic acid W5 inhibits progression and predicts favorable prognosis in hepatocellular carcinoma. *World J Gastroenterol* 2021; **27**: 55-68 [PMID: 33505150 DOI: 10.3748/wjg.v27.i1.55]

26 **Dong RC**. Establishment of SMMC-7721 human liver cancer cell line and preliminary observation of its biological characteristics. *J Sec Milit Med Univ* 1980; **1**: 5-9

27 **Wang Y**, Fu J, Jiang M, Zhang X, Cheng L, Xu X, Fan Z, Zhang J, Ye Q, Song H. MiR-410 is overexpressed in liver and colorectal tumors and enhances tumor cell growth by silencing FHL1 *via* a direct/indirect mechanism. *PLoS One* 2014; **9**: e108708 [PMID: 25272045 DOI:

10.1371/journal.pone.0108708]
28 **Fu J**, Cheng L, Wang Y, Yuan P, Xu X, Ding L, Zhang H, Jiang K, Song H, Chen Z, Ye Q. The RNA-binding protein RBPMS1 represses AP-1 signaling and regulates breast cancer cell proliferation and migration. *Biochim Biophys Acta* 2015; **1853**: 1-13 [PMID: 25281386 DOI: 10.1016/j.bbamcr.2014.09.022]
29 **Xiu B**, Chi Y, Liu L, Chi W, Zhang Q, Chen J, Guo R, Si J, Li L, Xue J, Shao ZM, Wu ZH, Huang S, Wu J. LINC02273 drives breast cancer metastasis by epigenetically increasing AGR2 transcription. *Mol Cancer* 2019; **18**: 187 [PMID: 31856843 DOI: 10.1186/s12943-019-1115-y]
30 **Li D**, Liu X, Zhou J, Hu J, Zhang D, Liu J, Qiao Y, Zhan Q. Long noncoding RNA HULC modulates the phosphorylation of YB-1 through serving as a scaffold of extracellular signal-regulated kinase and YB-1 to enhance hepatocarcinogenesis. *Hepatology* 2017; **65**: 1612-1627 [PMID: 28027578 DOI: 10.1002/hep.29010]
31 **Yan X**, Zhang D, Wu W, Wu S, Qian J, Hao Y, Yan F, Zhu P, Wu J, Huang G, Huang Y, Luo J, Liu X, Liu B, Chen X, Du Y, Chen R, Fan Z. Mesenchymal Stem Cells Promote Hepatocarcinogenesis *via* lncRNA-MUF Interaction with ANXA2 and miR-34a. *Cancer Res* 2017; **77**: 6704-6716 [PMID: 28947421 DOI: 10.1158/0008-5472.CAN-17-1915]
32 **Li O**, Li Z, Tang Q, Li Y, Yuan S, Shen Y, Zhang Z, Li N, Chu K, Lei G. Long Stress Induced Non-Coding Transcripts 5 (LSINCT5) Promotes Hepatocellular Carcinoma Progression Through Interaction with High-Mobility Group AT-hook 2 and MiR-4516. *Med Sci Monit* 2018; **24**: 8510-8523 [PMID: 30472720 DOI: 10.12659/MSM.911179]
33 **An HX**, Xu B, Wang Q, Li YS, Shen LF, Li SG. Up-regulation of long non-coding RNA SNHG6 predicts poor prognosis in renal cell carcinoma. *Eur Rev Med Pharmacol Sci* 2018; **22**: 8624-8629 [PMID: 30575902 DOI: 10.26355/eurrev_201812_16626]
34 **Wang D**, Hu Y. Long Non-coding RNA PVT1 Competitively Binds MicroRNA-424-5p to Regulate CARM1 in Radiosensitivity of Non-Small-Cell Lung Cancer. *Mol Ther Nucleic Acids* 2019; **16**: 130-140 [PMID: 30861415 DOI: 10.1016/j.omtn.2018.12.006]
35 **Li L**, Peng M, Xue W, Fan Z, Wang T, Lian J, Zhai Y, Lian W, Qin D, Zhao J. Integrated analysis of dysregulated long non-coding RNAs/microRNAs/mRNAs in metastasis of lung adenocarcinoma. *J Transl Med* 2018; **16**: 372 [PMID: 30587197 DOI: 10.1186/s12967-018-1732-z]
36 **Liu L**, Zhou XY, Zhang JQ, Wang GG, He J, Chen YY, Huang C, Li L, Li SQ. LncRNA HULC promotes non-small cell lung cancer cell proliferation and inhibits the apoptosis by up-regulating sphingosine kinase 1 (SPHK1) and its downstream PI3K/Akt pathway. *Eur Rev Med Pharmacol Sci* 2018; **22**: 8722-8730 [PMID: 30575912 DOI: 10.26355/eurrev_201812_16637]
37 **Xin X**, Wu M, Meng Q, Wang C, Lu Y, Yang Y, Li X, Zheng Q, Pu H, Gui X, Li T, Li J, Jia S, Lu D. Long noncoding RNA HULC accelerates liver cancer by inhibiting PTEN *via* autophagy cooperation to miR15a. *Mol Cancer* 2018; **17**: 94 [PMID: 29895332 DOI: 10.1186/s12943-018-0843-8]
38 **Qin G**, Tu X, Li H, Cao P, Chen X, Song J, Han H, Li Y, Guo B, Yang L, Yan P, Li P, Gao C, Zhang J, Yang Y, Zheng J, Ju HQ, Lu L, Wang X, Yu C, Sun Y, Xing B, Ji H, Lin D, He F, Zhou G. Long Noncoding RNA p53-Stabilizing and Activating RNA Promotes p53 Signaling by Inhibiting Heterogeneous Nuclear Ribonucleoprotein K deSUMOylation and Suppresses Hepatocellular Carcinoma. *Hepatology* 2020; **71**: 112-129 [PMID: 31148184 DOI: 10.1002/hep.30793]
39 **Wang Y**, Yang L, Chen T, Liu X, Guo Y, Zhu Q, Tong X, Yang W, Xu Q, Huang D, Tu K. A novel lncRNA MCM3AP-AS1 promotes the growth of hepatocellular carcinoma by targeting miR-194-5p/FOXA1 axis. *Mol Cancer* 2019; **18**: 28 [PMID: 30782188 DOI: 10.1186/s12943-019-0957-7]
40 **Li W**, Kang Y. A new Lnc in metastasis: long noncoding RNA mediates the prometastatic functions of TGF-β. *Cancer Cell* 2014; **25**: 557-559 [PMID: 24823634 DOI: 10.1016/j.ccr.2014.04.014]
41 **Li Z**, Lu X, Liu Y, Zhao J, Ma S, Yin H, Huang S, Zhao Y, He X. Gain of LINC00624 Enhances Liver Cancer Progression by Disrupting the Histone Deacetylase 6/Tripartite Motif Containing 28/Zinc Finger Protein 354C Corepressor Complex. *Hepatology* 2020 [PMID: 32869873 DOI: 10.1002/hep.31530]
42 **Lin Q**, Tan HT, Lim TK, Khoo A, Lim KH, Chung MC. iTRAQ analysis of colorectal cancer cell lines suggests Drebrin (DBN1) is overexpressed during liver metastasis. *Proteomics* 2014; **14**: 1434-1443 [PMID: 24610677 DOI: 10.1002/pmic.201300462]
43 **Mitra R**, Lee J, Jo J, Milani M, McClintick JN, Edenberg HJ, Kesler KA, Rieger KM, Badve S, Cummings OW, Mohiuddin A, Thomas DG, Luo X, Juliar BE, Li L, Mesaros C, Blair IA, Srirangam A, Kratzke RA, McDonald CJ, Kim J, Potter DA. Prediction of postoperative recurrence-free survival in non-small cell lung cancer by using an internationally validated gene expression model. *Clin Cancer Res* 2011; **17**: 2934-2946 [PMID: 21242119 DOI: 10.1158/1078-0432.CCR-10-1803]
44 **Vaskova M**, Kovac M, Volna P, Angelisova P, Mejstrikova E, Zuna J, Brdicka T, Hrusak O. High expression of cytoskeletal protein drebrin in TEL/AML1pos B-cell precursor acute lymphoblastic leukemia identified by a novel monoclonal antibody. *Leuk Res* 2011; **35**: 1111-1113 [PMID: 21497902 DOI: 10.1016/j.leukres.2011.03.028]
45 **Zhang K**, Shi Z, Zhang M, Dong X, Zheng L, Li G, Han X, Yao Z, Han T, Hong W. Silencing lncRNA Lfar1 alleviates the classical activation and pyoptosis of macrophage in hepatic fibrosis. *Cell Death Dis* 2020; **11**: 132 [PMID: 32071306 DOI: 10.1038/s41419-020-2323-5]
46 **Li LJ**, Wu XY, Tan SW, Xie ZJ, Pan XM, Pan SW, Bai WR, Li HJ, Liu HL, Jiang J, Wu B. Lnc-TCL6 is a potential biomarker for early diagnosis and grade in liver-cirrhosis patients. *Gastroenterol Rep (Oxf)* 2019; **7**: 434-443 [PMID: 31857905 DOI: 10.1093/gastro/goz050]
47 **Zhang K**, Zhang M, Yao Q, Han X, Zhao Y, Zheng L, Li G, Liu Q, Chang Y, Zhang P, Cui H, Shi Z,

Chen T, Yao Z, Han T, Hong W. The hepatocyte-specifically expressed lnc-HSER alleviates hepatic fibrosis by inhibiting hepatocyte apoptosis and epithelial-mesenchymal transition. *Theranostics* 2019; **9**: 7566-7582 [PMID: 31695787 DOI: 10.7150/thno.36942]

48 **Zhang Y**, Li Z, Zhang Y, Zhong Q, Chen Q, Zhang L. Molecular mechanism of HEIH and HULC in the proliferation and invasion of hepatoma cells. *Int J Clin Exp Med* 2015; **8**: 12956-12962 [PMID: 26550214]

49 **He Q**, Fang Y, Lu F, Pan J, Wang L, Gong W, Fei F, Cui J, Zhong J, Hu R, Liang M, Fang L, Wang H, Yu M, Zhang ZF. Analysis of differential expression profile of miRNA in peripheral blood of patients with lung cancer. *J Clin Lab Anal* 2019; **33**: e23003 [PMID: 31541491 DOI: 10.1002/jcla.23003]

50 **Patel H**, Okamura R, Fanta P, Patel C, Lanman RB, Raymond VM, Kato S, Kurzrock R. Clinical correlates of blood-derived circulating tumor DNA in pancreatic cancer. *J Hematol Oncol* 2019; **12**: 130 [PMID: 31801585 DOI: 10.1186/s13045-019-0824-4]

51 **Li Y**, Zhao J, Yu S, Wang Z, He X, Su Y, Guo T, Sheng H, Chen J, Zheng Q, Li Y, Guo W, Cai X, Shi G, Wu J, Wang L, Wang P, Huang S. Extracellular Vesicles Long RNA Sequencing Reveals Abundant mRNA, circRNA, and lncRNA in Human Blood as Potential Biomarkers for Cancer Diagnosis. *Clin Chem* 2019; **65**: 798-808 [PMID: 30914410 DOI: 10.1373/clinchem.2018.301291]

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ORIGINAL ARTICLE

Retrospective Cohort Study

Understanding celiac disease monitoring patterns and outcomes after diagnosis: A multinational, retrospective chart review study

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Abstract

BACKGROUND

Long-term outcomes and monitoring patterns in real-world practice are largely unknown among patients with celiac disease.

AIM

To understand patterns of follow-up and management of patients with celiac disease, and to characterize symptoms and villous atrophy after diagnosis.

METHODS

A retrospective chart review study was performed using medical chart data of patients diagnosed with celiac disease. Three gastroenterology referral centers, with substantial expertise in celiac disease, participated in the United Kingdom, United States, and Norway. Demographic and clinical data were collected from

medical charts. Descriptive analyses were conducted on patients with biopsy-confirmed celiac disease, diagnosed between 2008 and 2012, with at least one follow-up visit before December 31, 2017. Patient demographic and clinical characteristics, biopsy/serology tests and results, symptoms, and comorbidities were captured at diagnosis and for each clinic visit occurring within the study period (*i.e.*, before the study end date of December 31, 2017).

RESULTS

A total of 300 patients were included in this study [72% female; mean age at diagnosis: 38.9 years, standard deviation (SD) 17.2]. Patients were followed-up for a mean of 29.9 mo (SD 22.1) and there were, on average, three follow-up visits *per* patient during the study period. Over two-thirds (68.4%) of patients were recorded as having ongoing gastrointestinal symptoms and 11.0% had ongoing symptoms and enteropathy during follow-up. Approximately 80% of patients were referred to a dietician at least once during the follow-up period. Half (50.0%) of the patients underwent at least one follow-up duodenal biopsy and 36.6% had continued villous atrophy. Patterns of monitoring varied between sites. Biopsies were conducted more frequently in Norway and patients in the United States had a longer follow-up duration.

CONCLUSION

This real-world study demonstrates variable follow-up of patients with celiac disease despite most patients continuing to have abnormal histology and symptoms after diagnosis.

Key Words: Celiac disease; Outcomes research; Endoscopy; Real-world; General practice; Villous atrophy

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Core Tip: Inadequately managed celiac disease can lead to health complications. However, there are limited published data on real-world monitoring and outcomes for patients with celiac disease. In this real-world study, country/site-specific differences in the routine monitoring of patients after diagnosis were evident, including differences in the frequency of follow-up biopsy. A large proportion of patients continued to have villous atrophy and symptoms after diagnosis and, therefore, there is a continued need for more routine follow-up assessments, including mucosal assessments of celiac disease activity.

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INTRODUCTION

Celiac disease is a chronic, immune-mediated disorder that affects genetically susceptible individuals. The only accepted current standard of care for celiac disease is a life-long gluten-free diet (GFD). Previous studies have reported that adherence rates to a GFD range between 42% and 91%[1,2]. Inadequately managed celiac disease can lead to health complications such as malnutrition, osteoporosis, neurologic complaints, and lymphoma[2]. It has been hypothesized that long-term management and regular follow-up of patients with celiac disease will improve adherence to a GFD, and improve disease outcomes including mucosal healing and symptom resolution. For this reason, long-term management and regular follow-up of patients with celiac disease are advocated by current practice guidelines[3,4], yet it is unclear how these are actually implemented in practice. It is understood, however, that practice patterns

09/11/2018; Health Research Authority 19/11/2018; and Regional Committees for Medical and Health Research Ethics 05/06/2018).

Informed consent statement: A waiver of consent was approved for the United States site; the other sites had collected patient consent for research with their patient database.

Conflict-of-interest statement: KEA Lundin has served as a speaker/consultant/advisory board member for Amyra Biotech AG, Bioniz Therapeutics, Chugai Pharmaceutical, Dr. Falk Pharma GMBH, Immusant Therapeutics, and Interexon Actobiotics. CP Kelly has served as a consultant/advisory board member for Aptalis, Cour Pharma, Glutenostics, ImmunogenX, Innovate, Janssen, Kanyos, Takeda Pharmaceuticals, Merck, and Theravance; CP Kelly has received a grant from Aptalis; S Kayaniyil, S Wang, RJ Wani, and R Sambrook are salaried employees of ICON, which received research funds from Takeda Pharmaceuticals for conducting the study and preparation of the manuscript for publication. K Chen was a salaried employee of Takeda Pharmaceuticals at the time of study. DA Leffler is a salaried employee of Takeda Pharmaceuticals; CP Kelly owns shares in Cour Pharma and Glutenostics; DS Sanders, C Barrett, S Yoosuf, and ES Pettersen have no conflicts of interest to declare.

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vary widely both between countries and between practices.

Given that celiac disease is a chronic disorder, it is important to understand real-world, long-term outcomes and routine monitoring practices; however, there are few published data in these areas. Therefore, the aims of this multinational study were twofold. First, to understand, in real-world clinical practice, patterns of patient follow-up and management and how these practices vary by country. The second aim was to characterize patient outcomes, specifically related to ongoing symptoms and ongoing villous atrophy after diagnosis. Together, these data may be helpful in informing clinical practice, studies, and interventions aimed at improving celiac disease outcomes, and for quality improvement initiatives.

MATERIALS AND METHODS

A retrospective chart review study was conducted using medical chart data of patients diagnosed with celiac disease. Three large gastroenterology centers with substantial expertise in celiac disease participated, capturing patients in the United Kingdom, the United States, and Norway. Each site contributed 100 patients. Ethics approval was obtained before data collection commenced.

Patients were eligible if they had biopsy-confirmed celiac disease[3,5,6], were diagnosed between 2008 and 2012, and had at least one follow-up visit before 31 December 2017. This study period was selected to allow for at least five years of follow-up after diagnosis. Patients were excluded if they had initiated a GFD before receiving a diagnosis of celiac disease.

Using the database of patients at each site, the assigned staff at each center identified eligible patients by first looking at the date of diagnosis. The data abstractor reviewed and identified eligible patients who were diagnosed in December 2012, and then continued review of eligibility for patients consecutively backwards from that date (back to a diagnosis date in 2008). After examining the date of diagnosis, other inclusion/exclusion criteria were assessed to verify patient eligibility for the study. All three sites were explicitly asked to follow the same approach regarding selection of consecutive patients to avoid selection bias. The assigned staff at each site responsible for data abstraction then entered de-identified data for eligible patients into a custom electronic case report form. All data collected were based on the patient's pre-existing medical record. No direct personal identifiers were attached to the abstracted data. Data describing patient demographic and clinical characteristics, biopsy/serology tests and results, symptoms, and comorbidities were captured at diagnosis and for each clinic visit occurring within the study period (*i.e.*, before the study end date of December 31, 2017).

In terms of diagnostic testing, available serology results were collected, including tissue transglutaminase-immunoglobulin (Ig) A, IgA endomysial antibody, total serum IgA, deamidated gliadin peptide (DGP) IgA, DGP IgG, and DGP IgA-IgG. As not all pathology reports across sites utilized Marsh-Oberhuber classification, a descriptive assessment of biopsy results was recorded as follows: normal, increased intraepithelial lymphocytes only, mild/partial villous atrophy, subtotal villous atrophy, total/complete atrophy, and other.

Analysis

Data are summarized by descriptive statistics [mean, standard deviation (SD), median, and interquartile range for continuous variables, and number and percentage for categorical variables]. Gastrointestinal symptoms and extraintestinal comorbidities/complications (termed extraintestinal manifestations) are described at diagnosis and during study follow-up.

The presence of symptoms during the follow-up period was characterized specifically for patients who had a symptom at diagnosis and a record of symptoms at least once during follow-up. For each patient, the duration of the follow-up period was calculated as the time from diagnosis to the last follow-up visit within the study period. The mean number of visits *per* patient and the number of follow-up visits *per* patient with biopsy data were summarized overall and by country.

Following the classification proposed by Kurien *et al*[2], subsets of study patients with available symptom (defined as diarrhea, abdominal pain, abdominal distention, poor appetite, weight loss, tiredness/lethargy, brain fog, malabsorption and/or bloating) and biopsy data were grouped into four main disease states at diagnosis and at each follow-up visit: class 1 (no symptoms and normal duodenal histology); class 2 (no symptoms and abnormal duodenal histology); class 3 (symptoms and normal

duodenal histology); class 4 (symptoms and abnormal duodenal histology). This classification provides an intuitive framework for assessing celiac disease outcomes and may help to identify patients with the highest risk of complications. In addition, biopsy results reported as mild/partial/subtotal/total/complete villous atrophy were considered as abnormal histology; all other findings were considered normal for this classification. Those with 'other' biopsy findings were excluded in the classification.

Analyses were based on available data. Descriptive statistics were restricted to the subset of patients for whom data were available, with relevant denominator information provided in the results. All analyses were conducted in SAS 9.4.

RESULTS

A total of 300 patients with celiac disease were included in this study, comprising 100 patients from each of the three participating gastroenterology referral centers in the United Kingdom, the United States, and Norway. Table 1 presents the demographic and clinical characteristics of included patients at diagnosis.

Patients were, on average, 39 years of age at diagnosis, with 24 patients (8%) less than 18 years of age; there were 216 females in the study (72.0%). The study populations across the three sites were quite similar with respect to age, gender, and ethnicity distributions (Table 1). Gastrointestinal symptoms were the most common reason leading to diagnosis. There was a significantly greater proportion of patients in the United Kingdom (57.0%, $n = 57$) who presented with extraintestinal manifestations at diagnosis compared with patients in the United States (17.0%) and Norway (17.0%) ($P < 0.0001$). Nutritional deficiency was the most commonly reported extraintestinal manifestation in the United States and Norway, whereas in the United Kingdom anemia was most frequently documented at diagnosis (Table 2). Almost all ($n = 299$, 99.7%) patients had an esophagogastroduodenoscopy (EGD) conducted at diagnosis, and two patients (0.7%) had an enteroscopy. Overall, 90.7% ($n = 272$) of patients had serologic testing concurrently with biopsy, and these findings were similar across patients at the three sites. Biopsy results are presented in Table 1. Serology results at diagnosis and during the follow-up period are presented in Supplemental Table 1.

The types of gastrointestinal and extraintestinal manifestations and associated conditions at diagnosis and during follow-up were similar across sites and are presented in Table 2. At diagnosis, 256 patients (85.3%) and 228 patients (76.0%) had at least one gastrointestinal or extraintestinal manifestation, respectively. The most common symptoms across all sites were diarrhea, abdominal pain and bloating and the most common laboratory findings included nutrient deficiencies, anemia and low bone mineral density. Interestingly, both weight loss and weight gain were more commonly reported in the United States compared to the United Kingdom and Norway. There were 147 patients (49.0%) who presented with diarrhea, 124 (41.3%) who presented with abdominal pain, and 90 (30.0%) who presented with bloating. In addition, 104 patients (34.7%) had documentation of a nutritional deficiency, and 34 patients (11.3%) presented with another autoimmune disease, in addition to celiac disease, at diagnosis. During follow-up, diarrhea [$n = 100$ (33.3%)], abdominal pain [$n = 93$ (31.0%)], and bloating [$n = 76$ (25.3%)] continued to be the most frequently reported gastrointestinal symptoms. Of the 256 patients who had gastrointestinal symptoms at diagnosis, 175 (68.4%) had at least one visit reporting gastrointestinal symptoms during the follow-up period.

The duration of follow-up and average number of follow-up visits for the overall study population and by country are presented in Table 3. Patients were followed up for a mean of 29.9 mo (SD: 22.1) and there were, on average, three follow-up visits *per* patient during the study period. Patients from the United States site had the longest follow-up duration during the study period (mean: 38.7 mo), compared with the United Kingdom and Norway sites (mean: 26.5 and 24.5 mo, respectively; $P < 0.0001$). Overall referral patterns to other specialists were captured, indicating that approximately 80% of patients were referred to a dietician at least once during the follow-up period. Details on the last-recorded follow-up with the patient indicated that almost half (48%) of all patients had a follow-up appointment scheduled. Some were discharged (approximately 10%) or were referred to another specialist (approximately 19%), otherwise, the last follow-up decision was recorded as 'unknown' or 'other'.

After EGD, bone densitometry was the next most frequently reported procedure during follow-up, performed in 89 patients (29.7%) from the overall study population. Bone densitometry was performed at least once in 45 United States patients (45.0%) during the follow-up period, compared with patients who received this procedure in

Table 1 Demographic and clinical characteristics of patients at diagnosis, by country

	All patients (*n* = 300)	United Kingdom patients (*n* = 100)	United States patients (*n* = 100)	Norway patients (*n* = 100)
	n (%)	*n* (%)	*n* (%)	*n* (%)
Age at diagnosis[1]				
mean (SD)	38.9 (17.2)	42.4 (16.9)	39.1 (14.6)	33.1 (18.6)
Median (IQR)	36 (25.0-50.0)	41 (29.0-55.5)	38.5 (26.5-49.0)	31 (21.0-46.0)
Sex				
Male	84 (28.0)	29 (29.0)	24 (24.0)	31 (31.0)
Race/Ethnicity				
White/Caucasian	287 (95.7)	94 (94.0)	96 (96.0)	97 (97.0)
Black/African-American/Caribbean	2 (0.7)	1 (1.0)	0 (0.0)	1 (1.0)
Hispanic/Latino	2 (0.7)	0 (0.0)	2 (2.0)	0 (0.0)
Asian	6 (2.0)	4 (4.0)	0 (0.0)	2 (2.0)
Other	1 (0.3)	1 (1.0)	0 (0.0)	0 (0.0)
Unknown	2 (0.7)	0 (0.0)	2 (2.0)	0 (0.0)
Confirmation of diagnosis				
Biopsy and serology	272 (90.7)	99 (99.0)	91 (91.0)	82 (82.0)
Biopsy only	28 (9.3)	1 (1.0)	9 (9.0)	18 (18.0)
Reason for diagnosis[2]				
Gastrointestinal symptoms	231 (77.0)	70 (70.0)	80 (80.0)	81 (81.0)
Extraintestinal manifestations[3]	91 (30.3)	57 (57.0)	17 (17.0)	17 (17.0)
Family history of celiac disease	56 (18.7)	15 (15.0)	18 (18.0)	23 (23.0)
Screening for associated disorders	26 (8.7)	5 (5.0)	6 (6.0)	15 (15.0)
Family history of autoimmune disorders	13 (4.3)	0 (0.0)	3 (3.0)	10 (10.0)
Procedures performed for celiac disease-related symptoms				
EGD	299 (99.7)	100 (100.0)	99 (99.0)	100 (100.0)
Bone densitometry	76 (25.3)	58 (58.0)	9 (9.0)	9 (9.0)
Colonoscopy	48 (16.0)	11 (11.0)	33 (33.0)	4 (4.0)
Abdominal imaging	9 (3.0)	2 (2.0)	4 (4.0)	3 (3.0)
Enteroscopy	2 (0.7)	1 (1.0)	1 (1.0)	0 (0.0)
Capsule endoscopy	1 (0.3)	1 (1.0)	0 (0.0)	0 (0.0)
Other	15 (5.0)	2 (2.0)	3 (3.0)	10 (10.0)
None	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Biopsy results				
Normal	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Increased intraepithelial lymphocytes	2 (0.7)	1 (1.0)	1 (1.0)	0 (0.0)
Mild/partial villous atrophy	77 (25.7)	22 (22.0)	36 (36.0)	19 (19.0)
Subtotal villous atrophy	115 (38.3)	31 (31.0)	40 (40.0)	44 (44.0)
Total/complete villous atrophy	103 (34.3)	46 (46.0)	22 (22.0)	35 (35.0)
Other	3 (1.0)	0 (0.0)	1 (1.0)	2 (2.0)

[1]One patient had date of birth missing and is not included in the calculation.
[2]Overall percentage may be greater than 100%, as more than one option could be indicated.

[3]Extraintestinal manifestations can be found in Table 2.
EGD: Esophagogastroduodenoscopy; IQR: Interquartile range; SD: Standard deviation.

the United Kingdom and Norway [*n* = 22 (22.0%) for both United Kingdom and Norway patients; $P < 0.001$]. As this procedure is not performed in the gastroenterology unit, the results of these tests were not routinely available.

A summary of endoscopies with duodenal biopsy performed during the follow-up period, overall and by country, is also presented in Table 3. Of the 300 patients included in this study, 150 (50.0%) had at least one endoscopy with duodenal biopsy during the follow-up period. Of these 150 patients, 116 (77.3%) had a single follow-up endoscopy with biopsy during the follow-up period and most (14.7%, *n* = 22/150) of the remaining 34 patients had two follow-up endoscopies. A significantly higher proportion of Norway patients received a follow-up biopsy (82.0%, *n* = 82) compared with patients in the United Kingdom (42.0%, *n* = 42) and United States (26.0%, *n* = 26) ($P < 0.0001$).

The proportion of patients in the four disease state classes at diagnosis and at last follow-up with available data within the study period are presented in Figure 1. Of patients in classes 2 or 4 at diagnosis (*n* = 295) and who had a follow-up biopsy (*n* = 150), 53 (36.6%) continued to have villous atrophy (classes 2 or 4) at their last follow-up visit with biopsy data. The proportions were similar for the United Kingdom, United States, and Norway sites, where 39.0% (*n* = 16), 40.0% (*n* = 10), and 34.6% (*n* = 27) of patients, respectively, remained in classes 2 or 4 based on the last available biopsy data within the study period.

Overall, there were 54 patients who were in class 1 (no symptoms and normal duodenal histology) by the last follow-up visit with biopsy data. Of the patients with data available for the classification at diagnosis and at the last follow-up, the proportion of patients in class 1 during the follow-up period was slightly higher in Norwegian patients [*n* = 34 (43.6%)] compared with patients from the United Kingdom [*n* = 12 (29.3%)] and the United States [*n* = 8 (32.0%)].

DISCUSSION

This real-world study characterizes patients with celiac disease over time, and provides insight into routine monitoring practices from three large referral centers in the United Kingdom, the United States, and Norway. The majority of patients were female, which is consistent with other reports on the demographics of the celiac disease patient population[7,8]. Patients were followed for a mean of 29.9 mo (median 25 mo) and there were, on average, three follow-up visits *per* patient. Over two thirds of patients had a documentation of gastrointestinal symptoms during the follow-up period, which may indicate inadequate control of celiac disease despite patients being on a GFD. In addition, the fact that a higher proportion of patients from the United Kingdom site presented with extraintestinal manifestations at diagnosis, compared with patients from the United States and Norway sites, indicates that differences may exist in diagnostic or referral practices between different countries. This is particularly true for the United Kingdom site, which was known to see a greater number of patients with neurological manifestations of celiac disease. It is therefore likely that the differences in extraintestinal manifestations at diagnosis between the countries are due to a combination of referral bias and ascertainment bias at the individual sites, such that some manifestations may be evaluated more frequently at some sites than others.

While the study did collect information on extraintestinal manifestations, including liver abnormalities, it did not specifically assess metabolic disorders of patients with celiac disease. Given that an increased risk of non-alcoholic fatty liver disease in patients with celiac disease on a GFD has been reported[9], it would be valuable for future long-term studies to examine such metabolic disorders in this patient population. Country/site-specific differences were also evident in the routine monitoring of patients after diagnosis. While the United States patients had the longest follow-up duration within the study period, compared with Norwegian and United Kingdom patients, a higher proportion of Norwegian patients received a follow-up biopsy, indicating differences in diagnostic or referral practices across the different sites/countries that may not necessarily be reflective of differences in national guidelines.

Table 2 Presentation of gastrointestinal and extraintestinal manifestations at diagnosis and at follow-up visits, by country

	All patients (*n* = 300)		United Kingdom patients (*n* = 100)		United States patients (*n* = 100)		Norway patients (*n* = 100)	
	At diagnosis, *n* (%)	At follow-up[1], *n* (%)	At diagnosis, *n* (%)	At follow-up[1], *n* (%)	At diagnosis, *n* (%)	At follow-up[1], *n* (%)	At diagnosis, *n* (%)	At follow-up[1], *n* (%)
Gastrointestinal manifestations[2]								
None[3]	44 (14.7)	191 (63.7)	26 (26.0)	69 (69.0)	10 (10.0)	56 (56.0)	8 (8.0)	66 (66.0)
Diarrhea	147 (49.0)	100 (33.3)	44 (44.0)	35 (35.0)	54 (54.0)	45 (45.0)	49 (49.0)	20 (20.0)
Abdominal pain	124 (41.3)	93 (31.0)	26 (26.0)	23 (23.0)	46 (46.0)	45 (45.0)	52 (52.0)	25 (25.0)
Abdominal distension	12 (4.0)	11 (3.7)	0 (0.0)	0 (0.0)	7 (7.0)	9 (9.0)	5 (5.0)	2 (2.0)
Poor appetite	5 (1.7)	6 (2.0)	0 (0.0)	1 (1.0)	2 (2.0)	2 (2.0)	3 (3.0)	3 (3.0)
Constipation	38 (12.7)	47 (15.7)	6 (6.0)	6 (6.0)	15 (15.0)	29 (29.0)	17 (17.0)	12 (12.0)
Weight loss	6 (2.0)	33 (11.0)	0 (0.0)	6 (6.0)	5 (5.0)	17 (17.0)	1 (1.0)	10 (10.0)
Weight gain	44 (14.7)	27 (9.0)	10 (10.0)	0 (0.0)	25 (25.0)	24 (24.0)	9 (9.0)	3 (3.0)
Malabsorption	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)
Bloating	90 (30.0)	76 (25.3)	26 (26.0)	26 (26.0)	31 (31.0)	31 (31.0)	33 (33.0)	19 (19.0)
Hard stools	4 (1.3)	6 (2.0)	0 (0.0)	0 (0.0)	4 (4.0)	4 (4.0)	0 (0.0)	2 (2.0)
Mouth ulcers	3 (1.0)	2 (0.7)	2 (2.0)	0 (0.0)	0 (0.0)	1 (1.0)	1 (1.0)	1 (1.0)
Extraintestinal manifestations[2]								
None[3]	72 (24.0)	153 (51.0)	37 (37.0)	71 (71.0)	24 (24.0)	38 (38.0)	11 (11.0)	44 (44.0)
Brain fog	11 (3.7)	10 (3.3)	0 (0.0)	0 (0.0)	10 (10.0)	10 (10.0)	1 (1.0)	0 (0.0)
Nutritional deficiency	104 (34.7)	108 (36.0)	27 (27.0)	27 (27.0)	34 (34.0)	59 (59.0)	43 (43.0)	22 (22.0)
Osteoporosis/osteopenia	21 (7.0)	54 (18.0)	5 (5.0)	16 (16.0)	11 (11.0)	26 (26.0)	5 (5.0)	12 (12.0)
Anemia	52 (17.3)	41 (13.7)	29 (29.0)	19 (19.0)	22 (22.0)	20 (20.0)	1 (1.0)	2 (2.0)
Malignancy	2 (0.7)	5 (1.7)	0 (0.0)	0 (0.0)	2 (2.0)	3 (3.0)	0 (0.0)	2 (2.0)
Cardiovascular disease	8 (2.7)	10 (3.3)	0 (0.0)	0 (0.0)	1 (1.0)	2 (2.0)	7 (7.0)	8 (8.0)
Infertility	4 (1.3)	5 (1.7)	0 (0.0)	2 (2.0)	4 (4.0)	3 (3.0)	0 (0.0)	0 (0.0)
Depression	11 (3.7)	12 (4.0)	1 (1.0)	2 (2.0)	8 (8.0)	8 (8.0)	2 (2.0)	2 (2.0)
Anxiety	8 (2.7)	9 (3.0)	1 (1.0)	0 (0.0)	4 (4.0)	8 (8.0)	3 (3.0)	1 (1.0)
Headaches	11 (3.7)	21 (7.0)	2 (2.0)	2 (2.0)	7 (7.0)	15 (15.0)	2 (2.0)	4 (4.0)
Neuropathy	3 (1.0)	4 (1.3)	0 (0.0)	2 (2.0)	3 (3.0)	2 (2.0)	0 (0.0)	0 (0.0)
Autoimmune disease	34 (11.3)	38 (12.7)	2 (2.0)	2 (2.0)	14 (14.0)	16 (16.0)	18 (18.0)	20 (20.0)
Skin and dental conditions	7 (2.3)	7 (2.3)	0 (0.0)	0 (0.0)	3 (3.0)	4 (4.0)	4 (4.0)	3 (3.0)
Hair loss	5 (1.7)	2 (0.7)	0 (0.0)	0 (0.0)	3 (3.0)	2 (2.0)	2 (2.0)	0 (0.0)
Liver abnormalities[4]	6 (2.0)	11 (3.7)	1 (1.0)	2 (2.0)	3 (3.0)	6 (6.0)	2 (2.0)	3 (3.0)
Musculoskeletal symptoms	28 (9.3)	38 (12.7)	5 (5.0)	7 (7.0)	9 (9.0)	11 (11.0)	14 (14.0)	20 (20.0)

[1]Denotes presence at any time point during follow-up.
[2]Total percentage may be greater than 100%.
[3]Denotes no presence for the entire follow-up period.
[4]Includes elevated alanine aminotransferase, aspartate aminotransferase, or alkaline phosphatase levels.

In this study, half of patients received at least one follow-up biopsy after diagnosis within the study period, with significant variability between sites. While there is currently no consistent recommendation to perform routine follow-up biopsy on all patients, recent European guidelines suggest a follow-up biopsy in adults one to two

Table 3 Descriptive characteristics of follow-up visits and endoscopies during the follow-up period, overall and by country

		Overall	United Kingdom	United States	Norway
Length of follow-up time[1] (mo)	Mean (SD)	29.9 (22.1)	26.5 (20.9)	38.7 (22.8)	24.5 (20.1)
	Median (IQR)	25 (12-47)	21 (9-42)	39.5 (17-60)	16 (12-32.5)
Number of follow-up visits (*per* patient)	Mean (SD)	3.0 (2.3)	2.7 (2.5)	4.0 (2.4)	2.3 (1.5)
	Median (IQR)	2 (1-4)	2 (1-3)	4 (2-6)	2 (1-3)
Number of follow-up visits with biopsy data (*per* patient)	Mean (SD)	0.7 (0.8)	0.5 (0.7)	0.3 (0.5)	1.2 (0.9)
	Median (IQR)	0.5 (0-1)	0 (0-1)	0 (0-1)	1 (1-1.5)
Endoscopy with duodenal biopsy during follow-up					
Yes	*n* (%)	150 (50.0)	42 (42.0)	26 (26.0)	82 (82.0)
No	*n* (%)	150 (50.0)	58 (58.0)	74 (74.0)	18 (18.0)
If 'Yes', number of endoscopies with duodenal biopsy during follow-up					
1	*n* (%)	116 (77.3)	37 (88.1)	22 (84.6)	57 (69.5)
2	*n* (%)	22 (14.7)	3 (7.1)	4 (15.4)	15 (18.3)
3	*n* (%)	9 (6.0)	1 (2.4)	0 (0.0)	8 (9.8)
> 3	*n* (%)	3 (2.0)	1 (2.4)	0 (0.0)	2 (2.4)
Timing of endoscopy with duodenal biopsy during follow-up[2]					
< 12 mo	*n* (%)	45 (30.0)	18 (42.9)	3 (11.5)	24 (29.3)
12-24 mo	*n* (%)	63 (42.0)	11 (26.2)	6 (23.1)	46 (56.1)
> 24 mo	*n* (%)	64 (42.7)	19 (45.2)	18 (69.2)	27 (32.9)

For unknown diagnosis days and months, the day was imputed to the 15th, and the month was imputed to June.
[1]Length of time from diagnosis to last follow-up available in charts, within the study period.
[2]Total percentage may be greater than 100%.
IQR: Interquartile range; SD: Standard deviation.

years after diagnosis and after starting a GFD to assess mucosal healing, as treatment of ongoing mucosal injury is less well defined and depends on likely etiology[3].

The grouping of patients into four disease state classes in this study allows for examination of the persistence of celiac disease symptoms as well as mucosal recovery/healing. Patients in this study with ongoing mucosal injury likely represent a combination of ongoing gluten exposure, slow recovery post diagnosis, and refractory celiac disease. Analysis of specific etiologies of ongoing villous atrophy, however, is outside the scope of this manuscript. Study results indicated that 36.6% of patients overall had presence of villous atrophy (classes 2 or 4) at the last follow-up visit with available biopsy data, with similar findings across sites. While it is unclear how many of these patients would progress to histologic remission given longer follow-up, these data suggest that a substantial proportion of patients may not be achieving therapeutic goals, even at specialized celiac disease centers. Furthermore, it is important to note that among those with at least one follow-up visit only half of patients had a follow-up biopsy to examine mucosal recovery. While the proportion of patients with persistent villous atrophy may be partially related to referral bias, the inclusion of patients diagnosed only at tertiary centers should have mitigated this. Conversely, patients who are not followed up or who receive care at less well-equipped centers may have even higher rates of inadequate disease control.

The reasons for the variability in follow-up, both within and between centers, are unclear. However, it seems that many of the patients in this study were either not continuing to see their gastroenterologist or not having a follow-up biopsy, which would limit the ability to assess continued presence of symptoms and villous atrophy. Yet, previous studies reported that having a follow-up biopsy did not impact long-term outcomes when compared with those who did not have a follow-up biopsy, possibly due to lack of effective interventions to address ongoing villous atrophy [10,11].

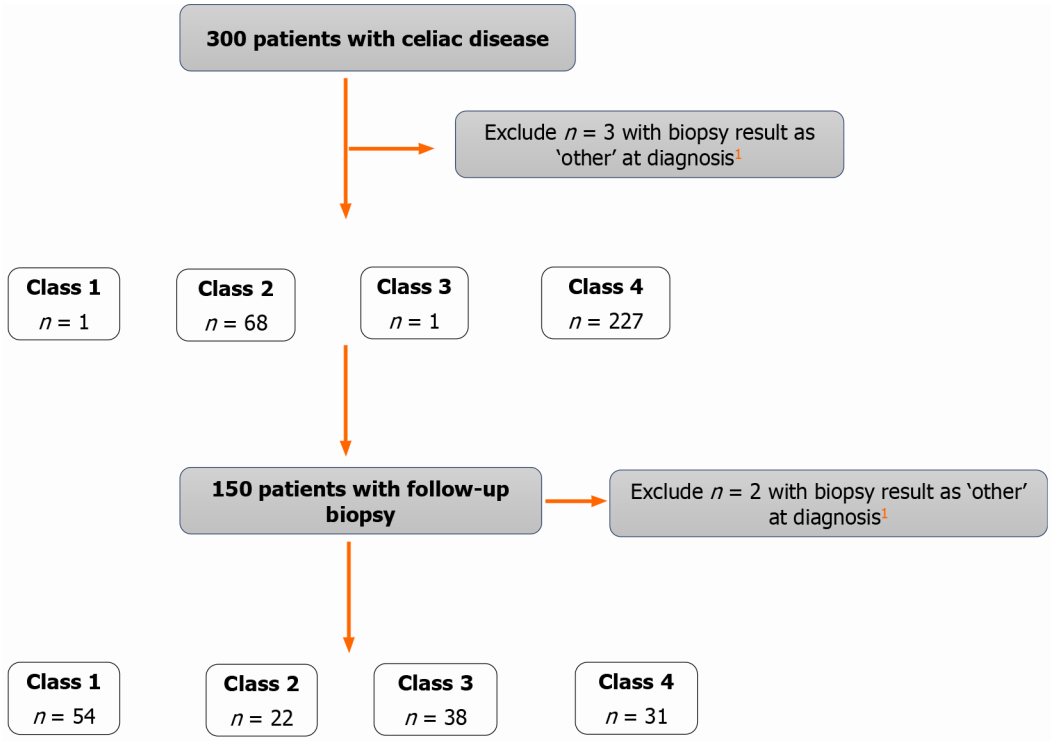


Figure 1 Number of patients grouped into the four disease state classes at diagnosis and at last follow-up with available biopsy data. Class 1: no symptoms and normal duodenal histology; Class 2: No symptoms and abnormal duodenal histology; Class 3: Symptoms and normal duodenal histology; Class 4: Symptoms and abnormal duodenal histology. [1]Patients with biopsy result indicated as 'other' in the data collection form were excluded from this classification.

Potential country differences in healthcare policies may also be at play here. Indeed one previous study conducted in Norway reported that only 6% of patients had prevalence of villous atrophy after a median follow-up of 8.1 years[10]. The authors of this Norwegian study indicated that this may be partially driven by the fact that, in Norway, patients diagnosed with celiac disease automatically qualify for a reimbursement to cover the extra costs associated with following a GFD. In another study, from Australia, rates of mucosal remission and response were 50% and 85% at one and five years, respectively[12]. In addition, Pekki *et al*[11] reported that 42% (n = 200) of the 476 patients examined in Finland, who had a repeat biopsy, continued to have atrophy after one year of follow-up[11]. In yet another study from Finland, the authors reported that 96% (n = 177) of patients had villous recovery after a mean of 11 years of follow-up while adhering to a GFD[13]. The present study, however, did not find a large difference by country for the proportion of patients with continued presence of villous atrophy during follow-up.

Strengths of this study are the inclusion of patients with biopsy-proven celiac disease, the multinational sample, and the use of consecutively diagnosed patients, which should have reduced selection bias. However, future research may be warranted to examine whether patterns of care are different in community-based compared with tertiary centers, and whether there are potential differences in outcomes for patients diagnosed by serology alone and followed up in general practice. Given that the sites in this study were large gastroenterology referral centers, it is anticipated that they should be reflective of practice patterns in similar centers within the countries studied, and where there were commonalities between the centers, these are likely generalizable. However, as this cannot be tested, it is also likely that the selected sites may not be truly representative of the country, and these findings would need to be confirmed by further research within each country. In addition, patterns of care are reflective of those in gastroenterology referral centers, and may be more rigorous than patterns of care in general practice.

Limitations of this study include the lack of information regarding adherence to a GFD, as this information is often not readily available in patient charts, although most patients (approximately 80%) were referred to a dietician at least once during the follow-up period. Future studies may be able to assess GFD adherence objectively through the presence of gluten immunogenic peptides in the urine[14]. There is also the possibility that variation in pathology assessment and reporting may influence

inter center results; although, good interobserver agreement for the detection of villous atrophy has been reported[15]. In addition, the majority of patients included in this study were diagnosed on the basis of symptoms, with approximately 12% diagnosed by screening alone. While asymptomatic patients may have different outcomes, related in part to GFD adherence, the current study was not designed to address this. However, it would be valuable for future studies to consider and compare outcomes based on whether diagnosis was based on asymptomatic *vs* symptomatic disease.

Further, it is unclear what proportion of patients in this study were diagnosed elsewhere and referred to one of the participating gastroenterology centers owing to lack of healing. This may have resulted in a higher proportion of patients with villous atrophy compared with a community setting. In addition, this study captured patient visits to the gastroenterologist only, and any continued management with another healthcare provider (*e.g.*, general practitioner, dietician) was not captured. Therefore, the results of this study are reflective of follow-up and outcomes for patients with celiac disease as *per* their management by the gastroenterologist. While it is expected that most patients will continue to be managed by a gastroenterologist, particularly if they continue to experience symptoms and have no evidence of mucosal healing, management by a general practitioner or other specialist (*e.g.*, dietician) may occur in parallel. In addition, given that the inclusion criteria required selection of patients with at least one follow-up visit within the study period, to report on follow-up patterns and outcomes, the study is unable to provide information on patients who did not return to the gastroenterologist for a follow-up visit during the study period. Further, comparisons made between sites/countries relied on standard parameters assessed across sites including celiac serologies (but heterogeneous in the frequency of retesting), symptoms assessment, GFD adherence and nutritional values. However, differences across the sites and the standard of practice would largely be the driver of follow-up endoscopy/biopsy, and the authors recognize this limitation in adequately comparing outcomes across patients.

There is a lack of clarity in guidelines on types of clinicians who are most appropriate to administer follow-up care and management for patients with celiac disease, and this may be especially important given increasing activity of non-traditional practitioners. Results from a patient survey indicated that 27% of patients had not visited a healthcare provider about celiac disease over the past five years, with almost half of these patients reporting that they felt that they were managing their celiac disease effectively on their own[16]. Therefore, despite the present study focusing specifically on management by gastroenterologists, it may be that some patients choose to manage celiac disease on their own and do not return for regularly scheduled visits.

This study provides valuable insight into the monitoring patterns and outcomes of patients with celiac disease managed at large referral centers in real-world practice. Overall, the monitoring of patients, including the rate of follow-up biopsy, varied across the participating sites, with a higher proportion of Norwegian patients receiving a follow-up biopsy compared with patients in the United Kingdom and United States. Differences were also observed in the presentation of extraintestinal manifestations at diagnosis across the sites. In addition, the study results indicate that a large proportion of patients continue to have villous atrophy and continue to experience symptoms after diagnosis; a finding that was consistent across sites. Pharmacological management may be required for patients who are adherent to a GFD but who still experience symptoms and mucosal injury.

CONCLUSION

In general, patients are not routinely monitored for the outcome of a GFD on symptoms, which may have an impact on intestinal health and can be a burden to patients. Overall, these data suggest that more routine follow-up assessment of celiac disease activity is needed. The inconsistent rates of mucosal assessment may be of concern, especially as many patients do not achieve histological remission. Novel, less invasive measures for assessment of ongoing villous atrophy, in combination with adjunctive pharmacologic therapy, may be needed to improve outcomes in patients with celiac disease.

ARTICLE HIGHLIGHTS

Research background

Long-term outcomes and monitoring patterns for patients with celiac disease in the real world are unknown.

Research motivation

Long-term management and regular follow-up of patients with celiac disease is thought to improve adherence to a gluten-free diet, improve mucosal healing, and symptom resolution. However, it is unclear how patients with celiac disease are managed in routine clinical practice. There is anecdotal evidence suggesting that practice patterns vary widely both between countries and between practices.

Research objectives

To understand real-world clinical practice, patterns of patient follow-up and management, and to characterize patient outcomes related to symptoms and villous atrophy after diagnosis.

Research methods

A retrospective observational study was performed using medical chart data of patients from three gastroenterology referral centers in the United Kingdom, United States, and Norway for patients diagnosed with celiac disease between 2008-2012.

Research results

300 patients were followed for a median of 25 mo. During follow-up, 68.4% of patients were recorded as having ongoing gastrointestinal symptoms and 11.0% had ongoing symptoms and enteropathy. 50.0% of patients underwent at least one follow-up duodenal biopsy and 36.6% had continued villous atrophy. Patterns of monitoring varied between sites.

Research conclusions

This real-world study demonstrates variable follow-up of patients with celiac disease even as most patients continue to have abnormal histology and symptoms after diagnosis.

Research perspectives

These data suggest that more routine follow-up assessment of celiac disease activity is needed. Novel and less invasive measures to assess ongoing villous atrophy, used in combination with adjunctive pharmacologic therapy, may be needed to improve outcomes in patients with celiac disease.

REFERENCES

1 **Hall NJ**, Rubin G, Charnock A. Systematic review: adherence to a gluten-free diet in adult patients with coeliac disease. *Aliment Pharmacol Ther* 2009; **30**: 315-330 [PMID: 19485977 DOI: 10.1111/j.1365-2036.2009.04053.x]

2 **Kurien M**, Trott N, Sanders DS. Long-term care for patients with coeliac disease in the UK: a review of the literature and future directions. *J Hum Nutr Diet* 2016; **29**: 617-623 [PMID: 27196331 DOI: 10.1111/jhn.12379]

3 **Al-Toma A**, Volta U, Auricchio R, Castillejo G, Sanders DS, Cellier C, Mulder CJ, Lundin KEA. European Society for the Study of Coeliac Disease (ESsCD) guideline for coeliac disease and other gluten-related disorders. *United European Gastroenterol J* 2019; **7**: 583-613 [PMID: 31210940 DOI: 10.1177/2050640619844125]

4 **Silvester JA**, Rashid M. Long-term follow-up of individuals with celiac disease: an evaluation of current practice guidelines. *Can J Gastroenterol* 2007; **21**: 557-564 [PMID: 17853949 DOI: 10.1155/2007/342685]

5 **Ludvigsson JF**, Bai JC, Biagi F, Card TR, Ciacci C, Ciclitira PJ, Green PH, Hadjivassiliou M, Holdoway A, van Heel DA, Kaukinen K, Leffler DA, Leonard JN, Lundin KE, McGough N, Davidson M, Murray JA, Swift GL, Walker MM, Zingone F, Sanders DS; BSG Coeliac Disease Guidelines Development Group; British Society of Gastroenterology. Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology. *Gut* 2014; **63**: 1210-1228 [PMID: 24917550 DOI: 10.1136/gutjnl-2013-306578]

6 **Rubio-Tapia A**, Hill ID, Kelly CP, Calderwood AH, Murray JA; American College of Gastroenterology. ACG clinical guidelines: diagnosis and management of celiac disease. *Am J*

Gastroenterol 2013; **108**: 656-76; quiz 677 [PMID: 23609613 DOI: 10.1038/ajg.2013.79]

7 **Megiorni F**, Mora B, Bonamico M, Barbato M, Montuori M, Viola F, Trabace S, Mazzilli MC. HLA-DQ and susceptibility to celiac disease: evidence for gender differences and parent-of-origin effects. *Am J Gastroenterol* 2008; **103**: 997-1003 [PMID: 18177450 DOI: 10.1111/j.1572-0241.2007.01716.x]

8 **Reilly NR**, Green PH. Epidemiology and clinical presentations of celiac disease. *Semin Immunopathol* 2012; **34**: 473-478 [PMID: 22526468 DOI: 10.1007/s00281-012-0311-2]

9 **Tovoli F**, Negrini G, Farì R, Guidetti E, Faggiano C, Napoli L, Bolondi L, Granito A. Increased risk of nonalcoholic fatty liver disease in patients with coeliac disease on a gluten-free diet: beyond traditional metabolic factors. *Aliment Pharmacol Ther* 2018; **48**: 538-546 [PMID: 29984415 DOI: 10.1111/apt.14910]

10 **Hære P**, Høie O, Schulz T, Schönhardt I, Raki M, Lundin KE. Long-term mucosal recovery and healing in celiac disease is the rule - not the exception. *Scand J Gastroenterol* 2016; **51**: 1439-1446 [PMID: 27534885 DOI: 10.1080/00365521.2016.1218540]

11 **Pekki H**, Kurppa K, Mäki M, Huhtala H, Laurila K, Ilus T, Kaukinen K. Performing routine follow-up biopsy 1 year after diagnosis does not affect long-term outcomes in coeliac disease. *Aliment Pharmacol Ther* 2017; **45**: 1459-1468 [PMID: 28326597 DOI: 10.1111/apt.14048]

12 **Newnham ED**, Shepherd SJ, Strauss BJ, Hosking P, Gibson PR. Adherence to the gluten-free diet can achieve the therapeutic goals in almost all patients with coeliac disease: A 5-year longitudinal study from diagnosis. *J Gastroenterol Hepatol* 2016; **31**: 342-349 [PMID: 26212198 DOI: 10.1111/jgh.13060]

13 **Tuire I**, Marja-Leena L, Teea S, Katri H, Jukka P, Päivi S, Heini H, Markku M, Pekka C, Katri K. Persistent duodenal intraepithelial lymphocytosis despite a long-term strict gluten-free diet in celiac disease. *Am J Gastroenterol* 2012; **107**: 1563-1569 [PMID: 22825364 DOI: 10.1038/ajg.2012.220]

14 **Moreno ML**, Cebolla Á, Muñoz-Suano A, Carrillo-Carrion C, Comino I, Pizarro Á, León F, Rodríguez-Herrera A, Sousa C. Detection of gluten immunogenic peptides in the urine of patients with coeliac disease reveals transgressions in the gluten-free diet and incomplete mucosal healing. *Gut* 2017; **66**: 250-257 [PMID: 26608460 DOI: 10.1136/gutjnl-2015-310148]

15 **Villanacci V**, Lorenzi L, Donato F, Auricchio R, Dziechciarz P, Gyimesi J, Koletzko S, Mišak Z, Laguna VM, Polanco I, Ramos D, Shamir R, Troncone R, Vriezinga SL, Mearin ML. Histopathological evaluation of duodenal biopsy in the PreventCD project. An observational interobserver agreement study. *APMIS* 2018; **126**: 208-214 [PMID: 29372596 DOI: 10.1111/apm.12812]

16 **Hughey JJ**, Ray BK, Lee AR, Voorhees KN, Kelly CP, Schuppan D. Self-reported dietary adherence, disease-specific symptoms, and quality of life are associated with healthcare provider follow-up in celiac disease. *BMC Gastroenterol* 2017; **17**: 156 [PMID: 29228908 DOI: 10.1186/s12876-017-0713-7]

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ORIGINAL ARTICLE

Retrospective Cohort Study

Development and validation of a prognostic model for patients with hepatorenal syndrome: A retrospective cohort study

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Abstract

BACKGROUND

Hepatorenal syndrome (HRS) is a severe complication of cirrhosis with high mortality, which necessitates accurate clinical decision. However, studies on prognostic factors and scoring systems to predict overall survival of HRS are not enough. Meanwhile, a multicenter cohort study with a long span of time could be more convincing.

AIM

To develop a novel and effective prognostic model for patients with HRS and clarify new prognostic factors.

METHODS

We retrospectively enrolled 1667 patients from four hospitals, and 371 eligible patients were finally analyzed to develop and validate a novel prognostic model for patients with HRS. Characteristics were compared between survivors and non-survivors, and potential prognostic factors were selected according to the impact on 28-d mortality. Accuracy in predicting 28-d mortality was compared between the novel and other scoring systems, including Model for End-Stage Liver Disease (MELD), Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA), and Chinese Group on the Study of Severe Hepatitis B-Acute-on-Chronic Liver Failure (COSSH-ACLF).

RESULTS

Five prognostic factors, comprised of gender, international normalized ratio, mean corpuscular hemoglobin concentration, neutrophil percentage, and stage,

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were integrated into a new score, GIMNS; stage is a binary variable defined by the number of failed organs. GIMNS was positively correlated with MELD, CLIF-SOFA, and COSSH-ACLF. Additionally, it had better accuracy [area under the receiver operating characteristic curve (AUROC): 0.830] than MELD (AUROC: 0.759), CLIF-SOFA (AUROC: 0.767), and COSSH-ACLF (AUROC: 0.759) in the derivation cohort ($P < 0.05$). It performed better than MELD and CLIF-SOFA in the validation cohort ($P < 0.050$) and had a higher AUROC than COSSH-ACLF ($P = 0.122$).

CONCLUSION

We have developed a new scoring system, GIMNS, to predict 28-d mortality of HRS patients. Mean corpuscular hemoglobin concentration and stage were first proposed and found to be related to the mortality of HRS. Additionally, the GIMNS score showed better accuracy than MELD and CLIF-SOFA, and the AUROC was higher than that of COSSH-ACLF.

Key Words: Hepatorenal syndrome; Prognostic factor; Mean corpuscular hemoglobin concentration; Mortality; Scoring system; Cohort study

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Core Tip: This multicenter retrospective cohort study investigated the prognostic factors for patients with hepatorenal syndrome and developed a novel prognostic model, named GIMNS. GIMNS contains five prognostic factors, comprised of gender, international normalized ratio, mean corpuscular hemoglobin concentration, neutrophil percentage, and stage, which had different expression levels between survivors and non-survivors. Stage, defined according to the number of organ failures and mean corpuscular hemoglobin concentration, was found to be an effective prognostic factor for the first time. The area under the operating characteristic curve reached 0.830 for 28-d mortality. The GIMNS score showed better accuracy than Model for End-Stage Liver Disease, Chronic Liver Failure-Sequential Organ Failure Assessment, and Chinese Group on the Study of Severe Hepatitis B-Acute-on-Chronic Liver Failure.

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INTRODUCTION

Hepatorenal syndrome (HRS) is a severe complication of cirrhosis characterized by increased splanchnic blood flow and rapid kidney dysfunction excluding other reasons[1]. It has high mortality within 2 wk, and the most effective treatment is liver transplantation, either liver only or simultaneous liver–kidney transplantation. Although pharmacological therapy, mainly vasoconstrictive (terlipressin or norepinephrine) plus albumin, can improve kidney function, it may make no difference to the percentage of patients who die or develop serious complications or the percentage of complications of any severity[2]. A retrospective study demonstrated that with present medical tools, there is still significantly high mortality in HRS despite guideline-based treatment[3]. According to another, single center study, occurrence of HRS is associated with a significantly worse outcome after living donor liver transplantation[4]. The focus of current HRS research is mainly on diagnosis and treatment, and there has been only a small number of studies on the prognosis of HRS. Thus, it is critical to develop a predictive tool to predict the mortality of HRS and to manage liver graft allocation on waiting lists.

A prospective study has demonstrated that age, serum bilirubin, and no reversal of creatinine after volume expansion independently predict mortality[5]. Another study has claimed that factors associated with poor prognosis are baseline bilirubin, no

reversibility of type-1 HRS, lack of resolution of the infection, and sepsis after diagnosis of type-1 HRS[6]. Acute-on-chronic liver failure (ACLF) grade is defined by the number of organ failures. A retrospective analysis from centers in Europe found that ACLF grade is the largest determinant of response and could affect the survival of HRS[7]. Among the predictors, the Model for End-stage Liver Disease (MELD) score has been widely applied for evaluating the severity of advanced liver diseases and liver graft allocation[8]. However, it has a limitation of failure to predict mortality after liver transplantation[9]. Therefore, a new effective model is urgently needed.

In this study, we aimed to construct a new prognostic model to predict mortality of HRS through a multicenter cohort study and retrospectively enrolled HRS patients from 2011 to 2019.

MATERIALS AND METHODS

Patient studies

Study population and data collection: In this retrospective cohort study we enrolled patients from four hospitals: First affiliated Hospital of Zhejiang University, Shulan Hospital, People's Hospital of Zhejiang Province, and People's Hospital of Shengzhou City. We studied a cohort with decompensated cirrhosis with acute renal injury from January 2011 to October 2019. Patients from the First Affiliated Hospital of Zhejiang University formed the derivation cohort and patients from the other three hospitals were the validation cohort. Demographic data (age and gender), history (vital signs and treatment), and laboratory parameters were available from medical records or the hospital database. The 28-d mortality was obtained from medical records or by directly contacting the patients or their families. All assays for serum biochemical parameters were routinely performed at the Central Clinical Laboratory of the four hospitals with the same type of testing equipment. The study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (No. 2019-1449-1), and complied with the ethical guidelines of the Declaration of Helsinki. The researchers only analyzed anonymous data, so informed consent was waived.

Inclusion and exclusion criteria: HRS was diagnosed based on criteria proposed by the International Club of Ascites in 2015. Acute kidney injury (AKI) was identified according to the standard Kidney Disease: Improving Global Outcomes criteria. The exclusion criteria were: (1) absence of ascites; (2) hepatocellular carcinoma; (3) other types of tumors; (4) chronic renal diseases; (5) liver transplantation; (6) age < 18 years; (7) thyroid diseases; (8) severe immunosuppression; (9) hospital stay < 1 wk; and (10) incomplete information. Finally, we excluded patients who were lost to follow-up (Figure 1).

Scoring models: The MELD score was calculated by the following formula: MELD = 3.78 ln [Total bilirubin (mg/dL)] + 11.2 ln (INR) + ln [serum creatinine (mg/dL)] + 6.43 (INR is international normalized ratio). The Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA) score summed the severity grades of organ failures. Liver failure was defined as bilirubin ≥ 12 mg/dL, coagulation failure as INR ≥ 2.5, brain failure as hepatic encephalopathy grade ≥ 3 (West Haven criteria), respiratory failure as a pulse oximetric saturation/fraction of inspired oxygen ratio ≤ 214 or arterial partial pressure of oxygen/fraction of inspired oxygen ratio ≤ 200 or the need for mechanical ventilation, and circulatory failure as the need for vasopressor therapy to achieve an adequate mean arterial pressure (MAP)[10]. The Chinese Group on the Study of Severe Hepatitis B-ACLF (COSSH-ACLF) was calculated as 0.741 INR + 0.523 hepatitis B virus (HBV)-SOFA + 0.026 age + 0.003 Total bilirubin. Stage was defined according to the number of organ failures, except renal failure. Stage 0 contained patients with zero or one organ failure, while stage 1 contained patients with more than two organ failures.

Statistical analysis: Clinicopathological features were summarized using medians and interquartile ranges or frequencies with percentages for normally distributed factors while using means with standard deviation for skewed distributed factors. Continuous variables were compared using *t* test or Mann–Whitney *U* test, and categorical variables were compared using the χ^2 test or Fisher's exact test. Patients were followed up from the day of diagnosis with HRS–AKI and ended at death or last follow–up at day 28. Univariate and multivariate Cox regression analyses were performed to establish the association between prognostic factors and overall survival of HRS. The

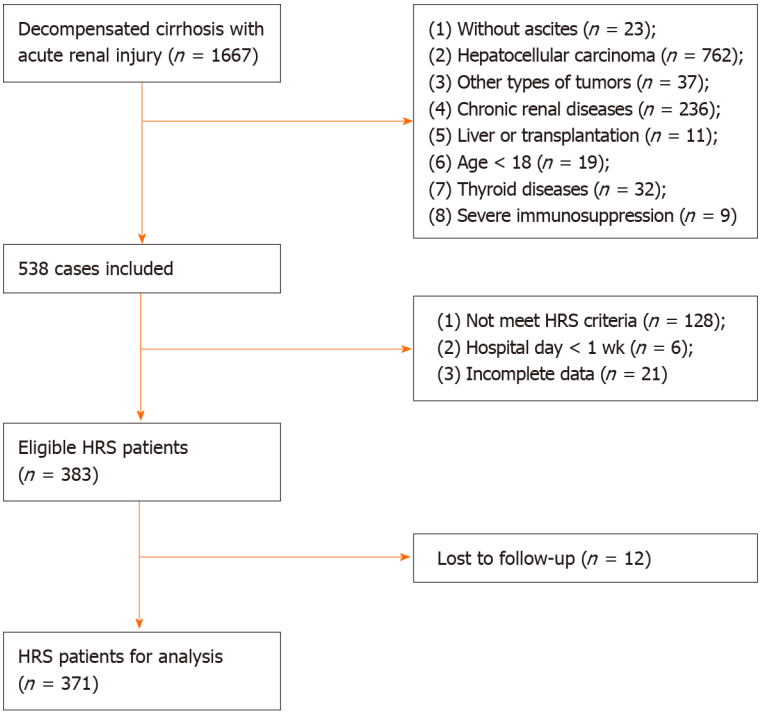


Figure 1 Workflow. The eligible cohort selection process after applying the inclusion and exclusion criteria from four hospitals. HRS: Hepatorenal syndrome.

Kaplan–Meier method was used to evaluate the survival probability of HRS, and a log–rank test was used to assess differences between groups. Hazard ratios were estimated using the Cox regression model. Statistical analyses were performed with R version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). All tests were two-sided, and $P < 0.05$ was considered statistically significant. The statistical methods of this study were reviewed by a member of the Biostatistics Service from the First Affiliated Hospital, College of Medicine, Zhejiang University.

RESULTS

Clinical characteristics

We retrospectively enrolled 1667 patients from four large general hospitals in Zhejiang province between January 2011 and October 2019. After inclusion and exclusion criteria, we had 383 eligible patients. Twelve patients were lost to follow-up due to a lack of contact information or survival information. Finally, we had 371 HRS patients for analysis: 248 in the derivation cohort and 123 in the validation cohort.

There were no significant differences in characteristics including demographic and clinical information between the derivation and validation cohorts ($P > 0.05$) (Table 1). Until the end of follow-up, 278 (74.9%) patients died, comprising 188 in the derivation cohort and 90 in the validation cohort. Male patients made up 76.8% (285) of the cohort, with 76.6% (190) and 77.2% (95) male in the derivation and validation cohorts, respectively. MAP, INR, alanine aminotransferase, serum bilirubin, number of failed organs, and the MELD, CLIF-SOFA, and COSSH-ACLF scores differed significantly between non-survivors and survivors in both cohorts (all $P < 0.05$). Age, gender, cause of cirrhosis, neutrophil percentage, hemoglobin, mean corpuscular hemoglobin (MCH) and MCH concentration (MCHC) differed between non-survivors and survivors only in the derivation cohort. Cirrhosis caused by HBV or HBV plus other reasons accounted for most of the patients (63.7% in the derivation cohort and 52.0% in the validation cohort). MAP was significantly higher in survivors than in non-survivors. Non-survivors had higher levels of INR and alanine aminotransferase compared with survivors. The MELD, CLIF-SOFA, and COSSH-ACLF scores were significantly higher in non-survivors in both cohorts ($P < 0.05$). Regarding treatment, all the eligible patients accepted rehydration treatment with albumin. In total, 21.3% (79) of patients received terlipressin plus albumin, and treatment was the same among the four hospitals ($P > 0.05$).

Table 1 Characteristics of patients with hepatorenal syndrome, *n* (%)

Variables	Derivation cohort (*n* = 248)			Validation cohort (*n* = 123)			[1]*P* value
	Non-survivor (*n* = 188)	Survivor (*n* = 60)	*P* value	Non-survivor (*n* = 90)	Survivor (*n* = 33)	*P* value	
Age (y)	59.11 ± 12.61	55.43 ± 12.04	0.048	56.94 ± 12.17	57.21 ± 11.81	0.913	0.379
Gender (male/female)	138/50	52/8	0.031	69/21	26/7	0.995	0.795
Causes of cirrhosis			0.046			0.741	0.096
HBV/HBV plus others	124 (66.0)	34 (56.7)		45 (50.0)	19 (57.6)		
Alcoholic	27 (14.4)	17 (28.3)		21 (23.3)	7 (21.2)		
Others	37 (19.7)	9 (15.0)		24 (26.7)	7 (21.2)		
MAP (mmHg)	95.16 ± 16.42	100.31 ± 18.44	0.041	93.1 ± 15.01	100.58 ± 18.33	0.023	0.483
INR	1.90 (1.70-2.60)	1.60 (1.30-1.90)	< 0.001	1.96 (1.54-2.40)	1.50 (1.31-1.90)	< 0.001	0.305
Neutrophil	75.68 ± 11.99	71.21 ± 13.03	0.015	76.03 ± 10.98	72.95 ± 10.97	0.170	0.647
RBC ($\times 10^9$/L)	3.15 ± 0.85	3.04 ± 0.85	0.404	3.16 ± 1.05	2.99 ± 0.78	0.388	0.950
Hemoglobin (g/L)	103.13 ± 25.48	95.14 ± 26.89	0.044	102.55 ± 29.63	95.41 ± 25.60	0.229	0.840
MCV (fl)	94.20 ± 9.16	92.21 ± 8.07	0.146	95.28 ± 11.72	94.00 ± 9.71	0.580	0.267
MCH (pg)	33.19 ± 3.78	31.45 ± 3.21	0.002	33.17 ± 3.93	32.21 ± 3.66	0.233	0.756
MCHC (g/L)	351.81 ± 18.15	341.77 ± 19.35	< 0.001	349.14 ± 19.13	342.83 ± 18.25	0.104	0.355
Platelet ($\times 10^9$/L)	66.5 (42.0-97.5)	69.0 (45.5-98.0)	0.419	64.0 (36.0-103.0)	75.0 (46.0-118.0)	0.250	0.737
Albumin (g/L)	29.12 ± 5.48	28.77 ± 5.37	0.668	29.22 ± 5.41	30.99 ± 5.61	0.115	0.274
A/G	1.00 (0.78-1.40)	0.92 (0.73-1.30)	0.267	1.13 ± 0.56	1.19 ± 0.51	0.620	0.400
ALT	60.00 (31.00-137.00)	32.50 (18.25-66.00)	0.001	59.00 (26.50-135.00)	36.00 (16.00-104.00)	0.035	0.673
Serum bilirubin	15.00 (5.32-27.50)	3.92 (1.10-12.80)	< 0.001	18.30 (6.75-25.30)	2.30 (1.17-23.30)	0.003	0.779
Cholinesterase (U/L)	1963.00 (1399.00-2559.00)	2264.00 (1410.50-3081.50)	0.197	1937.50 (1585.75-2637.20)	2376.00 (1716.00-3903.00)	0.152	0.198
Creatinine (mg/dL)	1.60 (0.92-2.70)	1.84 (1.14-2.80)	0.206	1.43 (0.85-2.20)	2.10 (1.10-2.90)	0.046	0.291
Sodium (mmol/L)	133.74 ± 6.79	135.66 ± 6.34	0.053	132.88 ± 5.89	136.02 ± 4.90	0.009	0.499
Potassium (mmol/L)	4.35 ± 1.01	4.18 ± 0.74	0.217	4.16 ± 0.80	4.31 ± 0.65	0.352	0.288
Number of failed organs	1 (0.75-2.00)	0 (0-1.00)	< 0.001	1 (0-2.00)	0 (0-1.00)	< 0.001	0.433
One organ failed	65 (34.6)	19 (31.7)	0.797	28 (31.1)	11 (33.3)	0.987	0.764
Two organs failed	50 (26.6)	1 (1.7)	< 0.001	26 (28.9)	3 (9.1)	0.040	0.596
≥ Three organs failed	26 (13.8)	1 (1.7)	0.017	12 (13.3)	1 (3.0)	0.188	0.990
MELD	29.50 ± 9.06	22.97 ± 8.00	< 0.001	28.04 ± 8.74	23.46 ± 9.47	0.013	0.276
CLIF-SOFA	10.79 ± 3.29	8.18 ± 3.08	< 0.001	10.86 ± 3.75	8.09 ± 3.37	< 0.001	0.912
COSSH-ACLF	7.57 ± 1.84	6.05 ± 1.27	< 0.001	7.57 ± 1.86	6.14 ± 1.49	< 0.001	0.958
Treatment			0.261			0.842	0.125
Terlipressin plus albumin	41 (21.8)	18 (30.0)		15 (16.7)	5 (15.2)		
Albumin only	147 (78.2)	42 (70.0)		75 (83.3)	28 (84.8)		

P non-survivor *vs* survivor.

[1]*P* derivation cohort *vs* validation cohort. A/G: Albumin/globulin; ALT: Alanine aminotransferase; CLIF-SOFA: Chronic Liver Failure-Sequential Organ Failure Assessment; COSSH-ACLF: Chinese Group on the Study of Severe Hepatitis B-Acute-on-Chronic Liver Failure; HBV: Hepatitis B virus; INR: International normalized ratio; MAP: Mean arterial pressure; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; MCV:

Mean cell volume; MELD: Model for End-Stage Liver Disease; RBC: Red blood cells.

Univariate and multivariate Cox regression

In the derivation cohort, gender, MAP, INR, neutrophil percentage, hemoglobin, MCH, MCHC, albumin/globulin, serum bilirubin, sodium, stage (defined above), and the MELD, CLIF-SOFA, and COSSH-ACLF scores were significantly associated with overall survival by univariate Cox regression (Table 2). We incorporated the above variables into multivariate Cox regression and finally five prognostic factors, including gender, INR, neutrophil percentage, MCHC and stage, were independently associated with overall survival.

The hazard ratio of stage was 2.724 (95% confidence interval, 1.877–3.952), and the hazard ratios of gender, INR, neutrophil percentage, and MCHC were 0.532, 1.408, 1.031, and 1.014, respectively. The risk of death in female patients was approximately 1.5 times that of male patients. Patients with ≥ two organ failures had up to 2.7 times greater mortality risk compared with those with ≤ one organ failure. Creatinine was not associated with overall survival of HRS patients, although AKI stage was defined by creatinine level.

GIMNS score development

According to the multivariate logistic regression based on 28-d mortality, the GIMNS score was developed. The formula is:

GIMNS = -1.412 gender + 0.053 neutrophil percentage + 0.014 MCHC + 2.073 ln (INR) + 1.231 stage - 9.217.

Mortality at 28 d in stratified HRS patients showed that patients with GIMNS > 2 were all dead, while those with GIMNS < 0 had the lowest mortality (30.34%) (Table 3). The mortality rates of patients with score 1–2 and 0–1 were 73.81% and 57.14%, respectively. Figure 2A showed the importance of each prognostic factor based on the logistic regression, and stage was the most important factor. Cumulative percentage also demonstrated this.

Characteristics in the GIMNS score

The GIMNS score contains five variables: gender, INR, MCHC, neutrophil percentage, and stage. The mosaic plot acquired from the derivation cohort could intuitively sense the distribution of the patients' survival statuses among different variables and establish the relationship between the variables (Figure 2B). From the mosaic plot, HBV-related cirrhosis accounted for the majority of the patients. For patients with HBV-related cirrhosis, male patients accounted for most, and 97.7% of the alcoholic HRS patients were male. As for patients with HRS of other causes, the proportion of female patients was larger than that of male patients. Although for every part of the whole mosaic plot, the number of patients with stage 1 disease was smaller than those with stage 0, the mortality was higher.

For the prognostic factors in the GIMNS score, we converted continuous variables into categorical variables by upper and lower quartiles (Figure 3A-C). INR was negatively correlated with survival probability. The survival probability of the upper quartile of INR was < 20%, which was lower compared with that of the others (P < 0.001). Similarly, patients with the lower quartile of neutrophil percentage had better overall survival compared with the others. Stratified MCHC also had different prognoses (P = 0.003).

Stage based on the number of organ failures (stage 0: 0–1, stage 1: ≥ 2) was the most important independent risk factor affecting overall survival of patients with HRS. The survival probability of stage 1 patients was < 10% while that of stage 0 patients was > 30%, which emphasized the essential role in predicting the prognosis of HRS (Figure 3D). Although female patients accounted for a small part of the whole cohort, they had a worse prognosis (P < 0.01) (Figure 3E).

GIMNS score and other scores

The GIMNS score was significantly higher in non-survivors in the derivation and validation cohorts (Figure 4A). The GIMNS score was divided into three parts according to the upper and lower quartiles. According to the Kaplan–Meier curves, mortality increased with GIMNS score (P < 0.001) (Figure 4B). The GIMNS score had a superior discrimination of risk of mortality of HRS patients. The GIMNS score was positively correlated with the MELD, CLIF-SOFA, and COSSH-ACLF scores (Figure 5A-C).

Table 2 Univariate and multivariate Cox regression.

Variables	Univariate Cox regression		Multivariate Cox regression	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	1.007 (0.995-1.019)	0.244		
Gender	0.586 (0.425-0.807)	0.001	0.532 (0.384-0.737)	< 0.001
Causes of cirrhosis	0.946 (0.785-1.140)	0.557		
MAP	0.990 (0.981-0.999)	0.024		
INR	1.842 (1.599-2.122)	< 0.001	1.408 (1.173-1.691)	< 0.001
Neutrophil	1.028 (1.015-1.041)	< 0.001	1.031 (1.018-1.044)	< 0.001
RBC	1.137 (0.959-1.348)	0.139		
Hemoglobin	1.007 (1.002-1.012)	0.012		
MCV	1.004 (0.988-1.021)	0.599		
MCH	1.066 (1.022-1.111)	0.003		
MCHC	1.019 (1.010-1.027)	< 0.001	1.014 (1.005-1.022)	0.002
Platelet	0.997 (0.994-1.000)	0.090		
Albumin	1.000 (0.973-1.028)	0.999		
A/G	1.257 (1.093-1.445)	0.001		
ALT	1.000 (1.000-1.001)	0.094		
Serum bilirubin	1.002 (1.001-1.003)	< 0.001		
Cholinesterase	1.000 (0.999-1.000)	0.160		
Creatinine	1.000 (0.999-1.001)	0.704		
Sodium	0.960 (0.939-0.982)	< 0.001		
Potassium	1.215 (1.032-1.430)	0.019		
Stage	3.805 (2.800-5.170)	< 0.001	2.724 (1.877-3.952)	< 0.001
MELD	1.077 (1.058-1.096)	< 0.001		
CLIF-SOFA	1.221 (1.166-1.278)	< 0.001		
COSSH-ACLF	1.467 (1.361-1.581)	< 0.001		

A/G: Albumin/globulin; ALT: Alanine aminotransferase; CI: Confidence interval; CLIF-SOFA: Chronic liver failure-sequential organ failure assessment; COSSH-ACLF: Chinese Group on the study of severe hepatitis B-acute-on-chronic liver failure; HR: Hazard ratio; INR: International normalized ratio; MAP: Mean arterial pressure; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; MCV: Mean cell volume; MELD: Model for end-stage liver disease; RBC: Red blood cells.

Table 3 Mortality at 28 d in patients with hepatorenal syndrome stratified according to the GIMNS score

GIMNS score	28-d mortality in HRS patients (%)
≥ 2	100.0
1-2	73.8
0-1	57.1
< 0	30.3

HRS: Hepatorenal syndrome.

Accuracy of the GIMNS score

The area under the receiver operating characteristic curve (AUROC) of the GIMNS score for 28-d mortality was 0.830, with a sensitivity of 0.735 and specificity of 0.787 at a cut-off value of 0.36 (Table 4). The mortality rate of the upper quartile GIMNS score

Table 4 Accuracy of different scoring systems in derivation and validation cohorts

Models	Cut-off	Sensitivity (%)	Specificity (%)	Youden	AUROC (95%CI)	*P* value	*P* value compared with GIMNS
Derivation cohort (*n* = 248)							
GIMNS	0.4	73.5	78.7	0.522	0.830 (0.778-0.882)	< 0.001	-
MELD	24.8	61.4	76.6	0.380	0.759 (0.670-0.821)	< 0.001	0.029
CLIF-SOFA	9.0	72.7	68.1	0.408	0.767 (0.706-0.827)	< 0.001	0.045
COSSH-ACLF	7.5	56.8	84.0	0.408	0.759 (0.696-0.821)	< 0.001	0.026
Validation cohort (*n* = 123)							
GIMNS	0.7	57.8	79.6	0.374	0.732 (0.642-0.821)	< 0.001	-
MELD	26.6	65.6	63.0	0.286	0.623 (0.520-0.726)	< 0.050	0.013
CLIF-SOFA	11.0	29.7	94.4	0.241	0.661 (0.563-0.758)	< 0.010	0.049
COSSH-ACLF	7.9	51.6	81.5	0.331	0.674 (0.577-0.770)	< 0.001	0.122

AUROC: Area under the receiver operating characteristic curve; CLIF-SOFA: Chronic liver failure-sequential organ failure assessment; COSSH-ACLF: Chinese Group on the study of severe hepatitis B-acute-on-chronic liver failure; MELD: Model for end-stage liver disease; CI: Confidence interval.

group was significantly higher than that of the lower quartile GIMNS score group. In the derivation cohort, the GIMNS score had significantly higher predictive power for the outcome of HRS patients than did the MELD, CLIF-SOFA, and COSSH-ACLF scores ($P < 0.05$) (Figure 5D). The AUROCs of GIMNS and other scoring systems are compared in Table 4.

Validation of performance of GIMNS score

The clinical and laboratory characteristics of the derivation and validation cohorts are shown in Table 1, which verified that there was no difference in the distribution of the characteristics between the cohorts. The mortality rate of the upper quartile GIMNS score group was significantly higher than that of the lower quartile GIMNS score group ($P < 0.001$). The AUROC of the GIMNS score model for 28-d mortality was 0.732, which was significantly better than that of the MELD ($P = 0.038$) and CLIF-SOFA ($P = 0.049$) scores and tended to be better than that of the COSSH-ACLF score ($P = 0.122$) (Figure 5E). The AUROCs of GIMNS and the other scoring systems were compared in Table 4.

DISCUSSION

In this study, we developed a novel model for prediction of prognosis of HRS called GIMNS, which had better accuracy than the traditional scoring systems MELD and CLIF-SOFA and a larger AUROC than COSSH-ACLF. GIMNS contains five prognostic factors: gender, INR, MCHC, neutrophil percentage, and stage.

INR is normally used in scoring systems to evaluate the severity of advanced liver diseases. In our study, different quartiles of INR indeed resulted in significantly different survival rates. Patients with higher INR have problems with blood coagulation and have a worse outcome. There was an obvious survival benefit in male patients, although the proportion of female patients was smaller, accounting for only 23.2% of the whole cohort. This suggests that female HRS patients should be given more attention than usual[11].

MCHC has traditionally been a subordinate process to diagnose the type of anemia combined with MCH and mean corpuscular volume. However, the association between MCHC and outcome of HRS, especially the role of MCHC in predicting survival, has been ignored. We found that when adjusted by other confounding factors in multivariate Cox analysis, the mortality risk of patients in the lower quartile of MCHC was nearly twice that of patients in the upper quartile (data not shown).

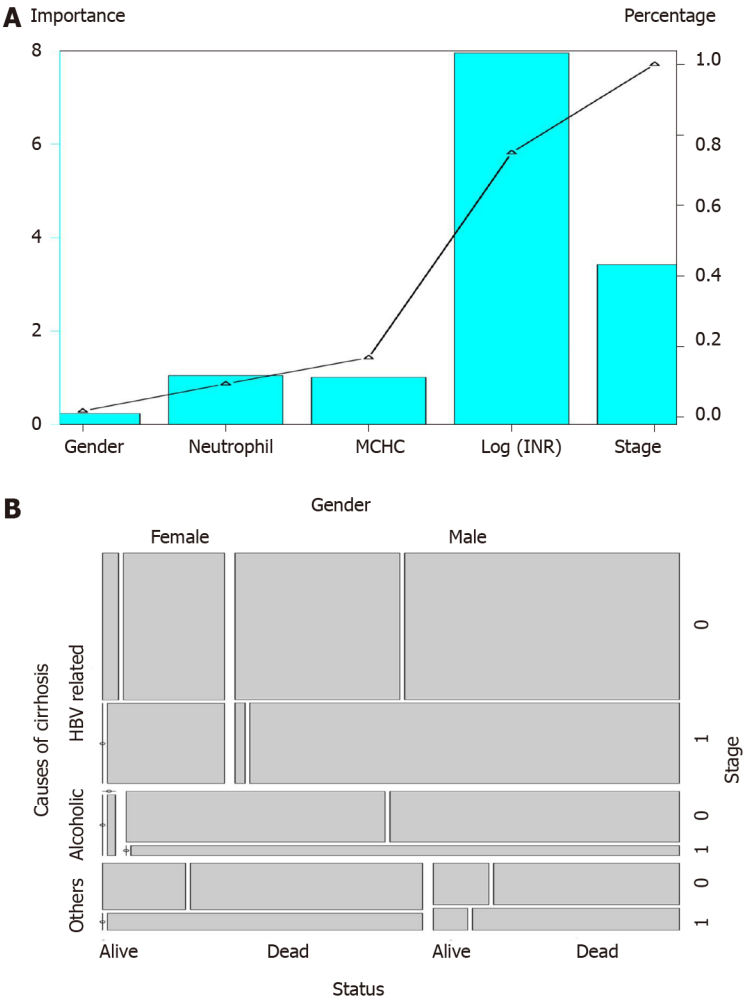


Figure 2 Characteristics associated with survival. A: Importance of each characteristic of GIMNS; B: Mosaic plot. Survival status of patients with different causes of cirrhosis, gender, and stage. HBV: Hepatitis B virus; INR: International normalized ratio; MCHC: Mean corpuscular hemoglobin concentration.

It has been shown that advanced cirrhosis is linked to increased serum levels of proinflammatory cytokines, which are common triggers of HRS[12,13]. These cytokines somehow cause kidney dysfunction. Neutrophil percentage reflects inflammatory state. Also, different neutrophil percentages divided by upper and lower quartiles resulted in different outcomes, meaning that patients with lower neutrophil percentage achieved a survival benefit.

For the first time, we incorporated the number of organ failures into the prognostic scoring system and divided the entire cohort into two, stage 0 with zero or one organ failure, except renal failure, and stage 1 with ≥ two organ failures. From the multivariate Cox analysis, we found that the 28-d mortality was significantly higher in stage 1 HRS, and the risk of stage 1 was almost three times that of stage 0 HRS. Organ failures have been used in the CLIF-SOFA score to evaluate the severity of advanced liver diseases[14]. Studies on the relationship between organ failure and prognosis of HRS are rare. It is essential to take this into consideration when developing a prognostic tool, either predicting the mortality or managing the liver transplantation waiting list.

The GIMNS score considered five prognostic factors and had better accuracy than the MELD and CLIF-SOFA scores in the derivation and validation cohorts. It also had a trend toward greater accuracy than the COSSH-ACLF had. The MELD score has been widely used in advanced liver diseases and plays an important role in liver graft allocation policy[15]. The MELD score is associated with 3-mo survival and can alter overall graft and outcome of HRS after transplantation. There is no doubt that the MELD score has been the first choice when evaluating prognosis of HRS and managing waiting lists for liver transplantation. However, with deeper understanding of HRS, more prognostic factors associated with HRS have been included, and a new score for predicting the mortality of HRS should be developed.

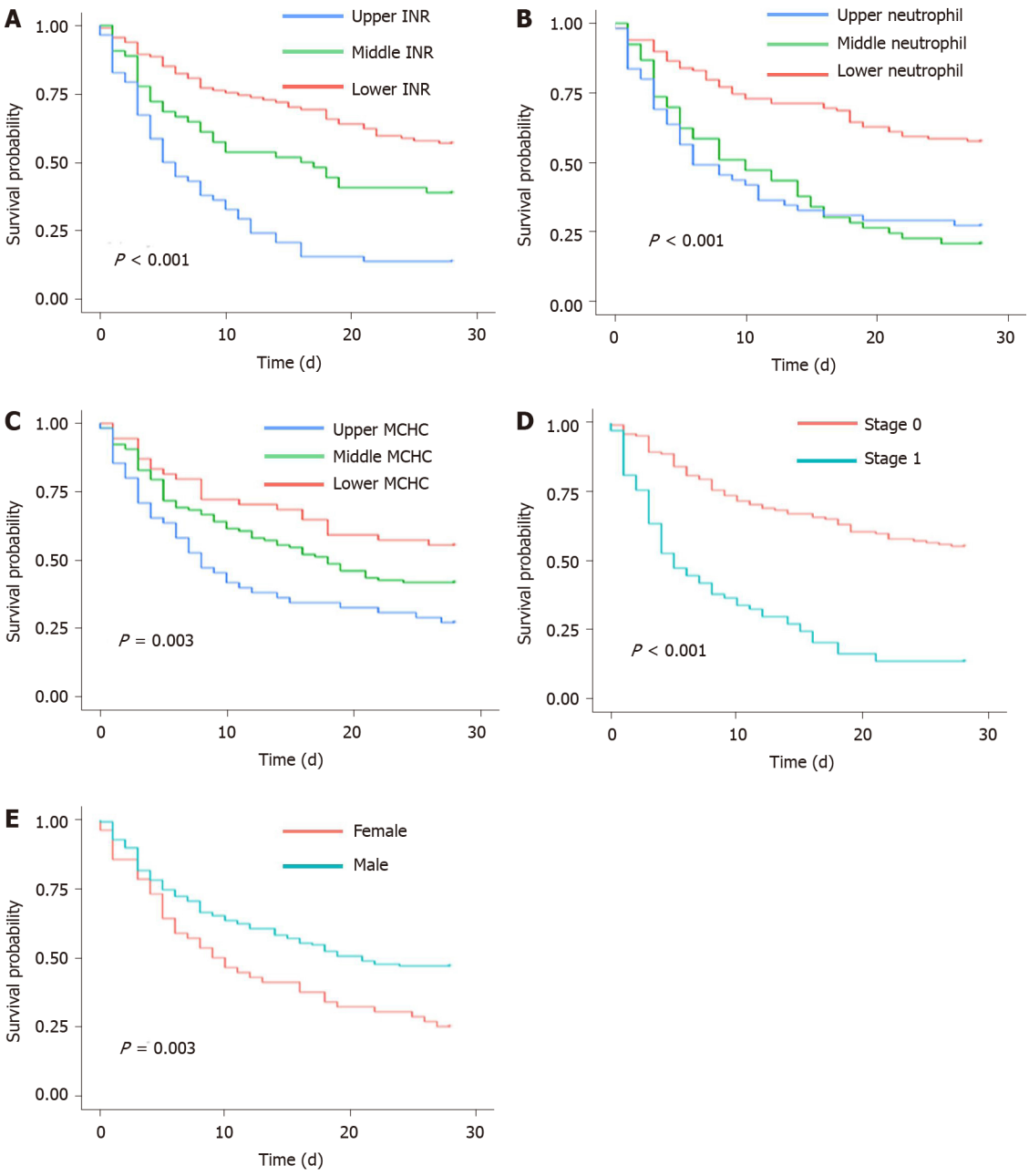


Figure 3 Kaplan–Meier curves of variables of GIMNS. A: Stratified international normalized ratio; B: Stratified neutrophil percentage; C: Stratified mean corpuscular hemoglobin concentration; D: Stage; E. Gender. INR: International normalized ratio; MCHC: Mean corpuscular hemoglobin concentration.

Preceding studies have found that gender is not associated with AKI, including HRS. However, we found that female patients were in more danger compared with male patients. Previous research has shown that predictors of AKI improvement are absence of alcoholic hepatitis, baseline creatinine, and male gender[11]. Male patients performed better in AKI, including HRS. In the derivation cohort, 72.2% of male patients and 86.8% of female patients died. The mortality risk in female patients was almost twice that in male patients. Thus, female patients have been ignored for a long time and more attention should be paid to this group.

INR has long been used as an indicator to evaluate advanced liver diseases, and it is widely used in different scoring systems, such as MELD, CLIF-SOFA, and COSSH-ACLF. INR can reflect coagulation function, and according to the Kaplan–Meier curves, patients with higher INR can have poor outcome. If the INR level is > 2.50, mortality is 95.0%, while if it is < 1.84, mortality can decrease to 64.0%. MCHC can also predict 28-d mortality of HRS partly because of kidney and liver dysfunction. Although the underlying pathological mechanism of MCHC remains unclear, it is associated with outcome of HRS and thus can act as a prognostic factor. Erythrocyte indices contain MCHC, MCH, and mean corpuscular volume. MCH and mean

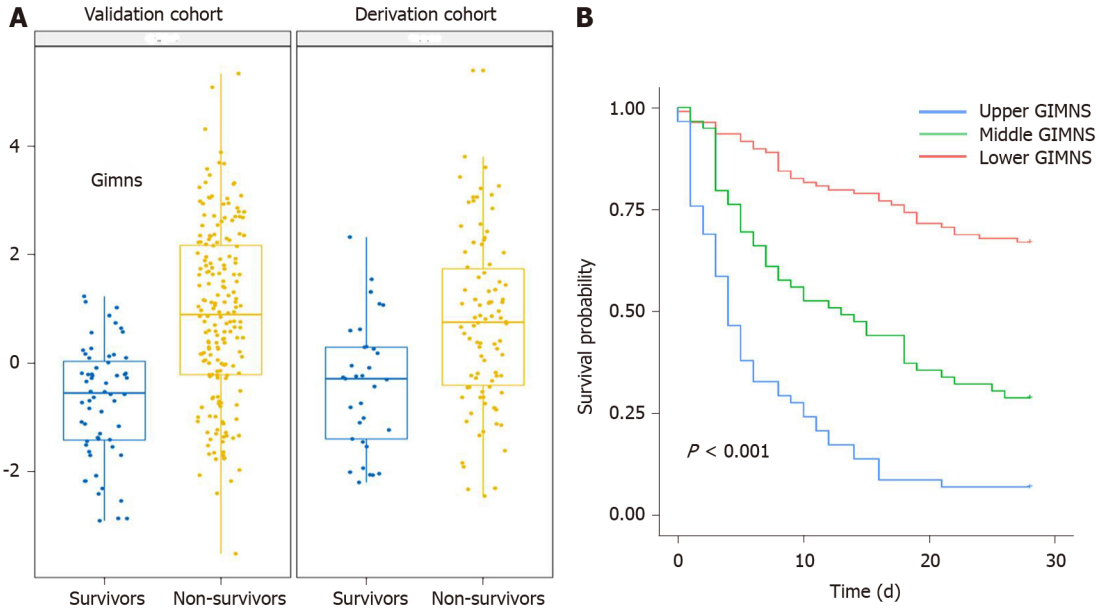


Figure 4 Distributions of the GIMNS score. A: The GIMNS score distribution between survivors and non-survivors in the derivation and validation cohorts; B: Kaplan–Meier curves of GIMNS.

corpuscular volume have no relationship with the prognosis of HRS. A previous retrospective study proposed that MCHC is independently associated with occurrence of HRS[16]. However, study on the association between MCHC and prognosis of HRS has not continued.

The GIMNS score is the first system to incorporate organ failure. It has been utilized in the ACLF score to stratify patients. It is classed as ACLF-1, ACLF-2, and ACLF-3 according to the number of organ failures, and this grade is significantly associated with outcome of ACLF. As HRS is a severe complication of end-stage cirrhosis, we previously proposed that stratification by the number of organ failures, like ACLF grade, could be useful for predicting prognosis of HRS. Finally, stage was found to be an important prognostic factor.

There have been some studies on factors associated with outcome of HRS, mainly mortality or reversal of HRS. One retrospective study has shown a death risk prediction score model included four independent risk factors: liver cancer, neutrophil > 70%, alanine aminotransferase > 40 U/L, and creatinine > 127 mmol/L[17]. It confirmed the essential role of neutrophil percentage in predicting mortality of HRS, although liver cancer should be excluded as it could be a competitive factor for mortality. We compared the creatinine levels between non-survivors and survivors in the derivation and validation cohorts, and there was no significant difference. Consistently, creatinine level was not associated with 28-d mortality. Another study showed that serum creatinine and urinary sodium at the time of diagnosis were associated with survival in univariate Cox analysis, but when adjusted by other confounding factors, serum creatinine had no relationship with outcome of HRS[18]. All the above results confirm that, although creatinine level defines the severity of AKI, it cannot help to predict the prognosis of HRS.

A retrospective study from a tertiary center created a new score that was an extension of the MELD score, including changes in serum bilirubin, creatinine, and albumin level during admission, to predict prognosis of type-1 HRS[19]. Although the concept of HRS-1 has been abandoned, it still helps to construct better prediction models. The MELD score has been widely used to evaluate advanced liver diseases, especially for liver graft allocation policy. However, it fails to predict outcomes of HRS after transplantation and could be a little outdated considering more prognostic factors have been found[20]. The new score derived from MELD proves this point.

Prognostic models for the short-term prognosis of HRS are not enough. Some commonly used prognostic models for end-stage liver disease, such as MELD, MELD-Na, COSSH-ACLF, and CLIF-SOFA, are developed for all end-stage liver diseases including severe hepatitis and cirrhosis. The novel model, GIMNS, takes HRS as the target disease. Moreover, our HRS patients were enrolled according to the latest diagnostic criteria of the International Club of Ascites in 2015, and the sample size is larger than other studies, which can increase the credibility of the results. We operated

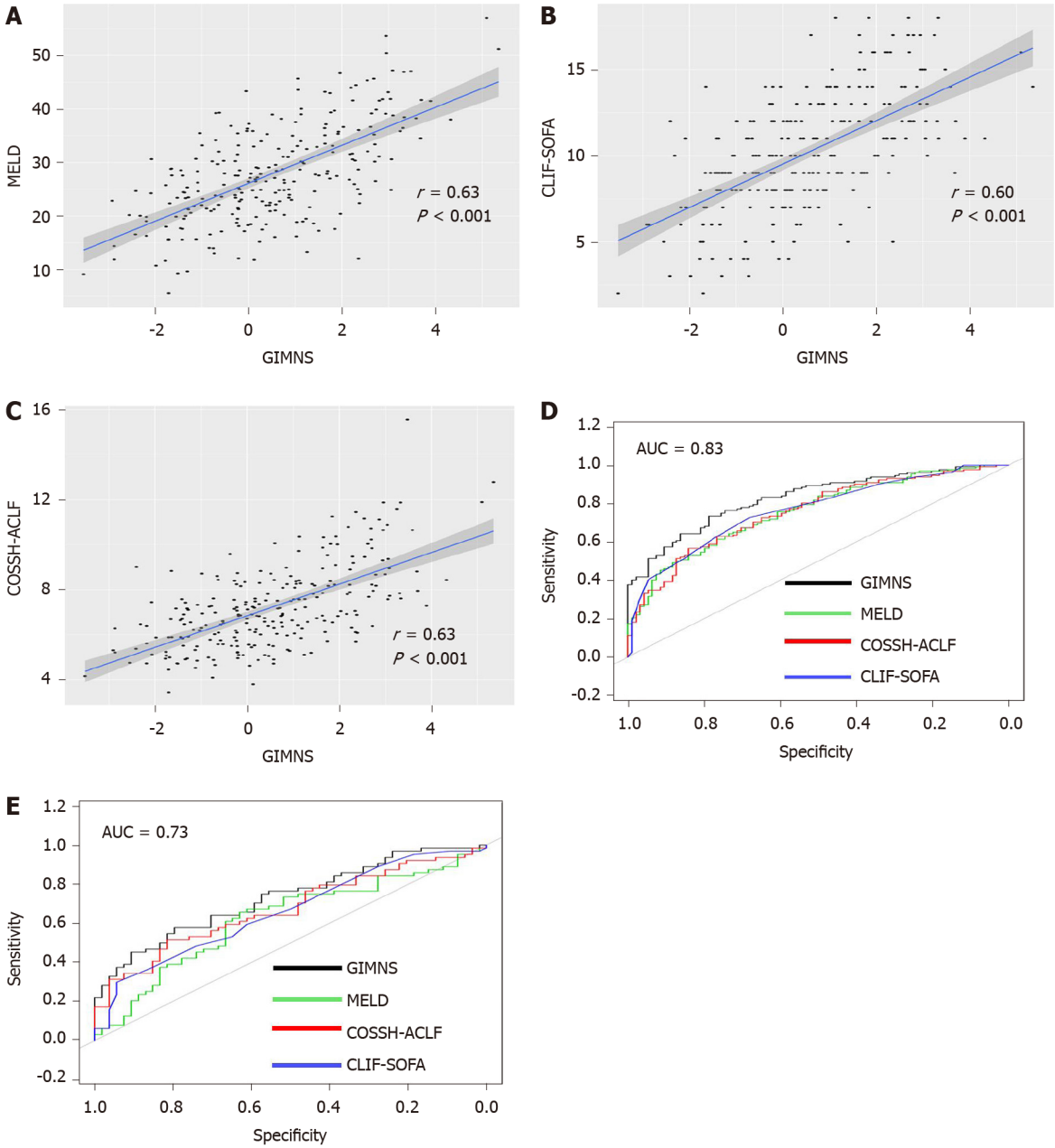


Figure 5 Relationship between GIMNS and other scoring systems and receiver operating characteristic curve curves. A: GIMNS and Model for End-stage Liver Disease; B: GIMNS and Chronic Liver Failure-Sequential Organ Failure Assessment; C: GIMNS and Chinese Group on the Study of Severe Hepatitis B-Acute-on-Chronic Liver Failure; D: Receiver operating characteristic curve curves in derivation cohort; E: Receiver operating characteristic curve in validation cohort. CLIF-SOFA: Chronic Liver Failure-Sequential Organ Failure Assessment; COSSH-ACLF: Chinese Group on the Study of Severe Hepatitis B-Acute-on-Chronic Liver Failure; MELD: Model for End-stage Liver Disease; AUC: Area under the curve.

external validation in three other hospitals to prevent overfitting. Finally, GIMNS performed better in both the derivation and validation cohorts. As its prognostic efficacy is better than MELD, COSSH-ACLF, and CLIF-SOFA, it has the value of clinical application.

Our new GIMNS score was based on all previous studies and showed better accuracy than MELD and the other commonly used scoring systems. Also, it is inexpensive and convenient to use, and MCHC and stage are given more attention. Our study had some limitations. First, this was a retrospective study, and therefore had a lower level of evidence compared with randomized controlled trials. We tried to balance the bias through external validation in three hospitals throughout Zhejiang Province, while developing the GIMNS score in one hospital. This could increase the credibility of our study. Second, it was hard to distinguish between HRS and other complications of AKI with cirrhosis, especially acute tubular necrosis. Thus, our cohort may have contained acute tubular necrosis patients, which could have interfered with our model development. Pathological sections are needed to separate HRS from acute

tubular necrosis. Finally, this study did not include urine output in the AKI diagnosis, which may have missed some eligible patients.

In summary, we developed a new scoring system, GIMNS, to predict 28-d mortality of HRS patients. The GIMNS score contains five elements: Gender, INR, MCHC, neutrophil percentage, and stage. Some of these elements have been widely used and others are recent additions. The GIMNS score has better accuracy than MELD and CLIF-SOFA, and the AUROC is larger than that of COSSH-ACLF. Prospective studies are needed for confirmation.

CONCLUSION

We developed a new scoring system, GIMNS, to predict 28-d mortality of HRS patients. Two of the five prognostic indicators, MCHC and stage, were proposed and found to be related to the mortality of HRS for the first time. Additionally, the GIMNS score showed better accuracy than MELD and CLIF-SOFA, and the AUROC was higher than that of COSSH-ACLF.

ARTICLE HIGHLIGHTS

Research background

Hepatorenal syndrome (HRS) is a severe complication of cirrhosis with high mortality. It has a unique *in vivo* pathological environment compared with other end-stage liver diseases. Meanwhile, prognostic factors associated with prognosis of HRS are not rich enough, and current prognostic scoring systems for end-stage liver diseases need to be updated to fit the application in HRS.

Research motivation

As the diagnostic criteria for HRS were confusing before 2015, a multicenter retrospective study, strictly following the criteria proposed by the International Club of Ascites in 2015, was designed to systematically evaluate the evolution of HRS over the past decade at our institution and to figure out what affects the prognosis of HRS.

Research objectives

Our goal was to develop a novel scoring system to predict 28-d mortality of HRS and evaluate the accuracy, sensitivity, and specificity compared with other scoring systems, including Model for End-stage Liver Disease, Chronic Liver Failure-Sequential Organ Failure Assessment, and Chinese Group on the Study of Severe Hepatitis B-Acute-on-Chronic Liver Failure.

Research methods

Demographic/clinical variables and medical records of HRS patients from January 2011 to October 2019 were collected from four tertiary medical centers, and strict eligibility criteria were applied. The final analysis included 371 patients with HRS. Univariate and multivariate Cox regression analyses were performed for prediction modeling of HRS.

Research results

We found that five indicators can be used as prognostic factors for HRS, including gender, international normalized ratio, mean corpuscular hemoglobin concentration, neutrophil percentage, and stage (defined as a binary variable by the number of failed organs). They formed a new score, GIMNS, based on the weight coefficient. As the GIMNS increases, the risk of mortality gets higher. The sensitivity and specificity of GIMNS were much higher than those of Model for End-stage Liver Disease, Chronic Liver Failure-Sequential Organ Failure Assessment, and Chinese Group on the Study of Severe Hepatitis B-Acute-on-Chronic Liver Failure in both the derivation and validation cohorts.

Research conclusions

We innovatively defined a new variable, stage (based on the number of failed organs), to predict the prognosis of HRS. Importantly, we developed a new scoring system, GIMNS, which was specifically for patients with HRS and performed better than

Model for End-stage Liver Disease, Chronic Liver Failure-Sequential Organ Failure Assessment, and Chinese Group on the Study of Severe Hepatitis B-Acute-on-Chronic Liver Failure in accuracy, sensitivity, and specificity. As the five prognostic factors in GIMNS are easily available, clinical application of GIMNS could be more convenient.

Research perspectives

The GIMNS scoring system should be validated in future prospective studies to get a more complete assessment.

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REFERENCES

1 **Yulyana Y**, Ho IA, Sia KC, Newman JP, Toh XY, Endaya BB, Chan JK, Gnecchi M, Huynh H, Chung AY, Lim KH, Leong HS, Iyer NG, Hui KM, Lam PY. Paracrine factors of human fetal MSCs inhibit liver cancer growth through reduced activation of IGF-1R/PI3K/Akt signaling. *Mol Ther* 2015; **23**: 746-756 [PMID: 25619723 DOI: 10.1038/mt.2015.13]

2 **Best LM**, Freeman SC, Sutton AJ, Cooper NJ, Tng EL, Csenar M, Hawkins N, Pavlov CS, Davidson BR, Thorburn D, Cowlin M, Milne EJ, Tsochatzis E, Gurusamy KS. Treatment for hepatorenal syndrome in people with decompensated liver cirrhosis: a network meta-analysis. *Cochrane Database Syst Rev* 2019; **9**: CD013103 [PMID: 31513287 DOI: 10.1002/14651858.CD013103.pub2]

3 **Bashir MH**, Iqbal S, Miller R, Singh J, Mubarak G, Likhtshteyn M, Bigajer E, Gallagher B, Hurairah A, Stefanov D, McFarlane SI, Ferstenberg R. Management and outcomes of hepatorenal syndrome at an urban academic medical center: a retrospective study. *Eur J Gastroenterol Hepatol* 2019; **31**: 1545-1549 [PMID: 31169567 DOI: 10.1097/MEG.0000000000001462]

4 **Okamura Y**, Hata K, Inamoto O, Kubota T, Hirao H, Tanaka H, Fujimoto Y, Ogawa K, Mori A, Okajima H, Kaido T, Uemoto S. Influence of hepatorenal syndrome on outcome of living donor liver transplantation: A single-center experience in 357 patients. *Hepatol Res* 2017; **47**: 425-434 [PMID: 27323334 DOI: 10.1111/hepr.12764]

5 **Salerno F**, Cazzaniga M, Merli M, Spinzi G, Saibeni S, Salmi A, Fagiuoli S, Spadaccini A, Trotta E, Laffi G, Koch M, Riggio O, Boccia S, Felder M, Balzani S, Bruno S, Angeli P; Italian Association of the Hospital Gastroenterologists (AIGO) investigators. Diagnosis, treatment and survival of patients with hepatorenal syndrome: a survey on daily medical practice. *J Hepatol* 2011; **55**: 1241-1248 [PMID: 21703199 DOI: 10.1016/j.jhep.2011.03.012]

6 **Barreto R**, Fagundes C, Guevara M, Solà E, Pereira G, Rodríguez E, Graupera I, Martín-Llahí M, Ariza X, Cárdenas A, Fernández J, Rodés J, Arroyo V, Ginès P. Type-1 hepatorenal syndrome associated with infections in cirrhosis: natural history, outcome of kidney function, and survival. *Hepatology* 2014; **59**: 1505-1513 [PMID: 24037970 DOI: 10.1002/hep.26687]

7 **Piano S**, Schmidt HH, Ariza X, Amoros A, Romano A, Hüsing-Kabar A, Solà E, Gerbes A, Bernardi M, Alessandria C, Scheiner B, Tonon M, Maschmeier M, Solè C, Trebicka J, Gustot T, Nevens F, Arroyo V, Gines P, Angeli P; EASL CLIF Consortium, European Foundation for the Study of Chronic Liver Failure (EF Clif). Association Between Grade of Acute on Chronic Liver Failure and Response to Terlipressin and Albumin in Patients With Hepatorenal Syndrome. *Clin Gastroenterol Hepatol* 2018; **16**: 1792-1800. e3 [PMID: 29391267 DOI: 10.1016/j.cgh.2018.01.035]

8 **Wu J**, Guo N, Zhang X, Xiong C, Liu J, Xu Y, Fan J, Yu J, Zhao X, Liu B, Wang W, Zhang J, Cao H, Li L. HEV-LF$_S$: A novel scoring model for patients with hepatitis E virus-related liver failure. *J Viral Hepat* 2019; **26**: 1334-1343 [PMID: 31294523 DOI: 10.1111/jvh.13174]

9 **Angeli P**, Gines P. Hepatorenal syndrome, MELD score and liver transplantation: an evolving issue with relevant implications for clinical practice. *J Hepatol* 2012; **57**: 1135-1140 [PMID: 22749942 DOI: 10.1016/j.jhep.2012.06.024]

10 **Organization Committee of 13th Asia-Pacific Congress of Clinical Microbiology and Infection**. 13th Asia-Pacific Congress of Clinical Microbiology and Infection Consensus Guidelines for diagnosis and treatment of liver failure. *Hepatobiliary Pancreat Dis Int* 2013; **12**: 346-354 [PMID: 23924491 DOI: 10.1016/s1499-3872(13)60055-7]

11 **Wong F**, Boyer TD, Sanyal AJ, Pappas SC, Escalante S, Jamil K. Reduction in acute kidney injury stage predicts survival in patients with type-1 hepatorenal syndrome. *Nephrol Dial Transplant* 2020; **35**: 1554-1561 [PMID: 30887050 DOI: 10.1093/ndt/gfz048]

12 **Solé C**, Solà E, Huelin P, Carol M, Moreira R, Cereijo U, Mas JM, Graupera I, Pose E, Napoleone L, dePrada G, Juanola A, Fabrellas N, Torres F, Morales-Ruiz M, Farrés J, Jiménez W, Ginès P. Characterization of inflammatory response in hepatorenal syndrome: Relationship with kidney

outcome and survival. *Liver Int* 2019; **39**: 1246-1255 [PMID: 30597709 DOI: 10.1111/Liv.14037]

13 **Ginès P**, Solà E, Angeli P, Wong F, Nadim MK, Kamath PS. Hepatorenal syndrome. *Nat Rev Dis Primers* 2018; **4**: 23 [PMID: 30213943 DOI: 10.1038/s41572-018-0022-7]

14 **Wu T**, Li J, Shao L, Xin J, Jiang L, Zhou Q, Shi D, Jiang J, Sun S, Jin L, Ye P, Yang L, Lu Y, Li T, Huang J, Xu X, Chen J, Hao S, Chen Y, Xin S, Gao Z, Duan Z, Han T, Wang Y, Gan J, Feng T, Pan C, Li H, Huang Y, Xie Q, Lin S, Li L; Chinese Group on the Study of Severe Hepatitis B (COSSH). Development of diagnostic criteria and a prognostic score for hepatitis B virus-related acute-on-chronic liver failure. *Gut* 2018; **67**: 2181-2191 [PMID: 28928275 DOI: 10.1136/gutjnl-2017-314641]

15 **Alessandria C**, Ozdogan O, Guevara M, Restuccia T, Jiménez W, Arroyo V, Rodés J, Ginès P. MELD score and clinical type predict prognosis in hepatorenal syndrome: relevance to liver transplantation. *Hepatology* 2005; **41**: 1282-1289 [PMID: 15834937 DOI: 10.1002/hep.20687]

16 **Koola JD**, Chen G, Malin BA, Fabbri D, Siew ED, Ho SB, Patterson OV, Matheny ME. A clinical risk prediction model to identify patients with hepatorenal syndrome at hospital admission. *Int J Clin Pract* 2019; **73**: e13393 [PMID: 31347754 DOI: 10.1111/ijcp.13393]

17 **Zhang S**, He LL, Wang XH, Dang ZB, Liu XL, Li MG, Wang XB, Yang ZY. A novel scoring model for predicting mortality risk in patients with cirrhosis and hepatorenal syndrome. *Eur J Gastroenterol Hepatol* 2018; **30**: 938-943 [PMID: 29634664 DOI: 10.1097/MEG.0000000000001127]

18 **Olivera-Martinez M**, Sayles H, Vivekanandan R, D' Souza S, Florescu MC. Hepatorenal syndrome: are we missing some prognostic factors? *Dig Dis Sci* 2012; **57**: 210-214 [PMID: 21850494 DOI: 10.1007/s10620-011-1861-1]

19 **Yang YW**, Wu CH, Hu RH, Ho MC, Tsai MK, Wu YM, Lee PH. Longitudinal assessment of prognostic factors for patients with hepatorenal syndrome in a tertiary center. *Hepatol Int* 2010; **4**: 507-510 [PMID: 20827408 DOI: 10.1007/s12072-010-9180-8]

20 **Sibulesky L**, Leca N, Blosser C, Rahnemai-Azar AA, Bhattacharya R, Reyes J. Is MELD score failing patients with liver disease and hepatorenal syndrome? *World J Hepatol* 2016; **8**: 1155-1156 [PMID: 27721921 DOI: 10.4254/wjh.v8.i27.1155]

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ORIGINAL ARTICLE

Observational Study

Inflammatory bowel disease in Tuzla Canton, Bosnia-Herzegovina: A prospective 10-year follow-up

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Institutional review board statement: The study was reviewed and approved by the Ethical Board of University Clinical Center Tuzla by the declaration 02-09/2-78/20, on the 01.12.2020.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Abstract

BACKGROUND

The incidence and prevalence of inflammatory bowel disease (IBD) vary between regions but have risen globally in recent decades. A lack of data from developing nations limits the understanding of IBD epidemiology.

AIM

To perform a follow-up review of IBD epidemiology in the Tuzla Canton of Bosnia-Herzegovina during a 10-year period (2009-2019).

METHODS

We prospectively evaluated the hospital records of both IBD inpatients and outpatients residing in Tuzla Canton for the specified period of time between January 1, 2009 and December 31, 2019. Since all our patients had undergone proximal and distal endoscopic evaluations at the hospital endoscopy unit, we used the hospital's database as a primary data source, alongside an additional cross-relational search of the database. Both adult and pediatric patients were included in the study. Patients were grouped by IBD type, phenotype, age, and gender. Incidence rates were calculated with age standardization using the European standard population. Trends in incidence and prevalence were evaluated as a 3-year moving average and average annual percentage change rates.

RESULTS

During the 10-year follow-up period, 651 patients diagnosed with IBD were monitored (of whom 334, or 51.3%, were males, and 317, or 48.7%, were females). Of all the patients, 346 (53.1%) had been diagnosed with ulcerative colitis (UC), 292 (44.9%) with Crohn's disease (CD), and 13 (2%) with indeterminate colitis (IC).

Clinical Center Tuzla. The data will be shared on reasonable request to the corresponding author with the permission of University Clinical Center Tuzla.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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We observed 440 newly diagnosed patients with IBD: 240 (54.5%) with UC, 190 (43.2%) with CD, and 10 (2.3%) with IC. The mean annual crude incidence rates were found to be 9.01/100000 population for IBD [95% confidence interval (CI): 8.17-9.85], with 4.91/100000 (95%CI: 4.29-5.54) for UC and 3.89/100000 (95%CI: 3.34-4.44) for CD. Calculated IBD prevalence in 2019 was 146.64/100000 (95%CI: 128.09-165.19), with 77.94/100000 (95%CI: 68.08-87.70) for UC and 65.77/100000 (95%CI: 54.45-74.1) for CD. The average annual IBD percentage change was 0.79% (95%CI: 0.60-0.88), with -2.82% (95%CI: -2.67 to -2.97) for UC and 6.92% (95%CI: 6.64-7.20) for CD. During the study period, 24,509 distal endoscopic procedures were performed. The incidence of IBD was 3.16/100 examinations (95%CI: 2.86-3.45) or 1.72/100 examinations (95%CI: 1.5-1.94) for UC and 1.36/100 examinations (95%CI: 1.17-1.56) for CD.

CONCLUSION

Trends in the incidence and prevalence of IBD in Tuzla Canton are similar to Eastern European averages, although there are significant epidemiological differences within geographically close and demographically similar areas.

Key Words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Indeterminate colitis; Epidemiology; Endoscopy

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Core Tip: The greatest gap in knowledge about the epidemiological picture of inflammatory bowel disease relates to developing countries, such as those in Eastern Europe. The heterogeneity between the results among the few available epidemiological studies concerning Eastern Europe is intriguing. Therefore, we aimed to perform a follow-up review of inflammatory bowel disease epidemiology in the Tuzla Canton of Bosnia-Herzegovina during a 10-year period (2009-2019).

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of diseases sharing characteristics of chronic and relapsing-remitting immune activation and inflammation within the gastrointestinal tract; this group shares several clinical and epidemiological characteristics[1].

Traditionally, a higher incidence of IBD is found in developed and industrialized countries. Recent reports indicate a stabilization of the incidence in most Western European countries, with the highest reported incidence in Netherlands [ulcerative colitis (UC) 17.2/100.000 inhabitants; Crohn's disease (CD) 10.5/100.000 inhabitants] and North American countries, with the highest reported incidence in Nova Scotia, Canada (UC 23.14/100.000 inhabitants; CD 23.82/100.000 inhabitants), and suggest an increase in northern European countries[2-4]. On the other hand, there is a trend of increasing incidence in areas with traditionally lower incidence rates, such as the countries of Eastern Europe[2].

The heterogeneity of results among the few available epidemiological studies concerning Eastern Europe is intriguing[5-11]. It is apparent that the epidemiological picture of the region needs to be investigated if we are to further our understanding of the nature of IBD. Building on the results of previous studies conducted in Tuzla Canton[10,11], this study aimed to contribute to understanding the epidemiological picture of IBD in the region of Southeastern Europe by evaluating the epidemiological characteristics of IBD in the Tuzla Canton of Bosnia-Herzegovina over a follow-up period of 10 years (2009-2019).

MATERIALS AND METHODS

Study population and data collection

We prospectively evaluated both IBD inpatients and outpatients residing in Tuzla Canton in northeast Bosnia-Herzegovina between January 1, 2009 and December 31, 2019 at the Department of Gastroenterology and Hepatology, University Clinical Center Tuzla. Both adult and pediatric patients were included in the study.

Tuzla Canton is one of ten cantons of the Federation of Bosnia-Herzegovina, the one of two entities in Bosnia-Herzegovina. As the most populous region with 12.5% of the nation's population, Tuzla Canton is also an industrial center. The population in the study area is homogenous in terms of ethnicity and genetic background.

Since all our patients had undergone proximal and distal endoscopic evaluations at the hospital endoscopy unit, we used the hospital's database as a primary data source, alongside an additional cross-relational search of the database. The study included only patients with a definitive diagnosis, based on widely accepted diagnostic criteria consisting of history data, clinical examinations, laboratory tests, radiology, endoscopy, and histology[12,13]; those with an uncertain diagnosis or without permanent residency in Tuzla Canton were excluded from the study.

Patients were grouped by IBD type, phenotype, age, and gender. As UC is categorized according to endoscopic findings relating to the disease localization, it was divided into proctitis, left-sided colitis, and extensive colitis[12]. CD was categorized according to the Montreal classification[13]. Indeterminate colitis (IC) was diagnosed in those patients for whom endoscopic, radiological, and pathohistological evaluations could not confirm either of the two main forms of IBD[1].

Statistical analysis

Statistical analyses were performed with SPSS software, version 26 (SPSS, IBM, United States). Calculations of disease incidence were performed based on the 2013 census and data from the Statistical Office of the Federation of Bosnia-Herzegovina.

Descriptive statistical parameters were used to determine the basic characteristics of the study population. The year of diagnosis was used to calculate incidence. Incidence and prevalence calculations were performed using census data from the Statistical Office of the Federation of Bosnia-Herzegovina. Ninety-five percent confidence intervals (95%CIs) for the incidence rate were calculated assuming a Poisson distribution of cases.

Crude annual incidence rates for both genders were calculated based on the number of diagnosed patients and the number of inhabitants, while the average incidence rate during the observed period was calculated based on the number of years of the study. Incidence rates were standardized using standard European age groups for each of the standardized age groups[14].

Morbidity trends from 2009 to 2019 were determined by moving 3-year averages with 95%CI and by calculating annual average percentage change. The annual incidence of new cases detected per 100 distal endoscopic procedures and colonoscopies was estimated. Trends in incidence and prevalence were estimated using a linear regression model, where applicable. Prevalence estimates during the observed period were produced based on the total number of detected cases and the number of inhabitants.

The statistical level of 95% ($P < 0.05$) was considered significant for all statistical tests. The statistic review of the study was performed by a biomedical statistician.

RESULTS

From January 1, 2009 to December 31, 2019, 651 patients diagnosed with IBD were monitored (of whom 334, or 51.3%, were males, and 317, or 48.7%, were females). Of these, 346 (53.1%) had been diagnosed with UC, 292 (44.9%) with CD, and the remaining 13 (2%) with IC.

In total, 440 new IBD patients were diagnosed: 240 (54.5%) with UC, 190 (43.2%) with CD, and 10 (2.3%) with IC. Among the newly diagnosed cases, 230 (52.3%) were males, and 210 (47.7%) were females.

Age distribution

The mean age (± SD) of all monitored patients was 46.2 ± 16.6 years, with a median of 47 years (25th and 75th percentiles: 33 and 58 years). The mean age (± SD) of newly diagnosed patients was 45.26 ± 17.38 years, with a median age of 47 years (25th and 75th

percentiles: 30 and 59 years). The mean age of newly diagnosed patients with UC was 48.16 ± 16.31 years, with a median age of 50 years (25th and 75th percentiles: 35 and 60 years), among whom the youngest patient was 15 and the oldest, 81 years old. The mean age of newly diagnosed patients with CD was 41.18 ± 18.01 years, with a median age of 40 years (25th and 75th percentiles: 26 and 55 years), among whom the youngest patient was 8 and the oldest, 80 years old. In total, 27 (6.1%) newly diagnosed patients younger than 19 years of age were registered. UC was diagnosed in five of these (18.5%), while CD was diagnosed in the remaining 22 (81.5%).

Distribution by gender

There were slightly more males among the newly diagnosed patients than women, with a ratio of 52.3% to 47.7%. Males were more likely to suffer from both major forms of IBD. Of the new cases of UC diagnosed, 124 were male (51.7%) and 116 (48.3%) were female (a ratio of 1.07:1). In total, 102 new cases of CD among the male population were registered (53.7%) and 88 (46.3%) new cases among the female population (a ratio of 1.16:1). Among the ten new cases of IC, the gender distribution was equal.

In general, no statistically significant difference in age was observed between genders at the time of diagnosis. The mean age (± SD) among male patients was 45.22 ± 18.01 (8-81) years, while the mean age of female patients was 45.3 ± 16.7 (11-80) years (t = 0.046, d.f. = 438, P = 0.964). A statistically significant difference in age between genders was observed among the patients diagnosed with UC. Males were older, with a mean age of 50.98 ± 18.12 (17-81) years, compared to females, with a mean age of 45.15 ± 16.05 (15-79) years (t = 2.805, d.f. = 238, P = 0.005). The same observation was true for IC.

Among the patients diagnosed with CD, a statistically significant difference between genders was observed in terms of age at the time of diagnosis, and females were older than males. Therefore, the average age of males was 37.53 ± 17.47 (8-76) years, while the average age of females was 45.41 ± 17.79 (11-80) years (t = 3, d.f. = 188, P = 0.002).

Incidence

The mean annual crude incidence of IBD during the study period was 9.01/100000 inhabitants (95%CI: 8.17-9.85), with an incidence of 9.64/100000 inhabitants (95%CI: 8.5-10.78) in the male population and 8.41/100000 inhabitants (95%CI: 7.16-9.65) in the female population. The age-standardized mean annual incidence was 8.9/100000 inhabitants (95%CI: 8.07-9.72). In general, the ratio between the incidence of individual forms (UC:CD:IC) of the disease during the study was 54.55%:43.18%:2.27%.

The mean crude annual incidence of UC was 4.91/100000 inhabitants per year (95%CI: 4.29-5.54), with an incidence of 5.16/100000 (95%CI: 4.25-6.07) among males, and 4.68/100000 (95%CI: 3.83-5.53) among females. The age-standardized incidence for the observed period was 4.9/100000 inhabitants per year (95%CI: 4.3-5.54). The average annual incidence during the last 5 years of the study (2015-2019) was 4.91/100000 inhabitants (95%CI: 4-5.83).

The mean crude annual incidence of CD was 3.89/100000 inhabitants (95%CI: 3.34-4.44), with a prevalence of 4.28/100000 (95%CI: 3.54-5.01) for males and 3.52/100000 (95%CI: 2.69-4.35) for females. The age-standardized incidence for the observed period was 3.76/100000 inhabitants (95%CI: 3.22-4.29). The average annual incidence for the last 5 years of the study was 4.05/100000 inhabitants (95%CI: 3.22-4.89).

The mean crude annual incidence of IC was 0.2/100000 inhabitants (95%CI: 0.08-0.33).

The mean crude annual incidence of IBD in the pediatric and adolescent population was 2.49/100000 inhabitants (95%CI: 1.55-3.43), with 0.46/100000 inhabitants (95%CI: 0.06-0.87) for UC and 2.03/100000 inhabitants (95%CI: 1.26-2.79) for CD.

Figure 1 presents the mean crude annual incidence of IBD clustered by age and gender. Overall, the highest incidence of IBD occurred in three age groups in the male population: Patients between 55 and 64 years (14/100000 inhabitants), between 25 and 34 years (10.8/100000 inhabitants), and between 45 and 54 years (10.6/100000 inhabitants).

In general, a higher incidence was recorded both in the younger female age groups and in the older male age groups among patients diagnosed with UC. The highest incidence was recorded among males aged between 55 and 64 (11.2/100000 inhabitants) and in the age group between 55 and 64 years among the female population, which was 6.9/100000 inhabitants.

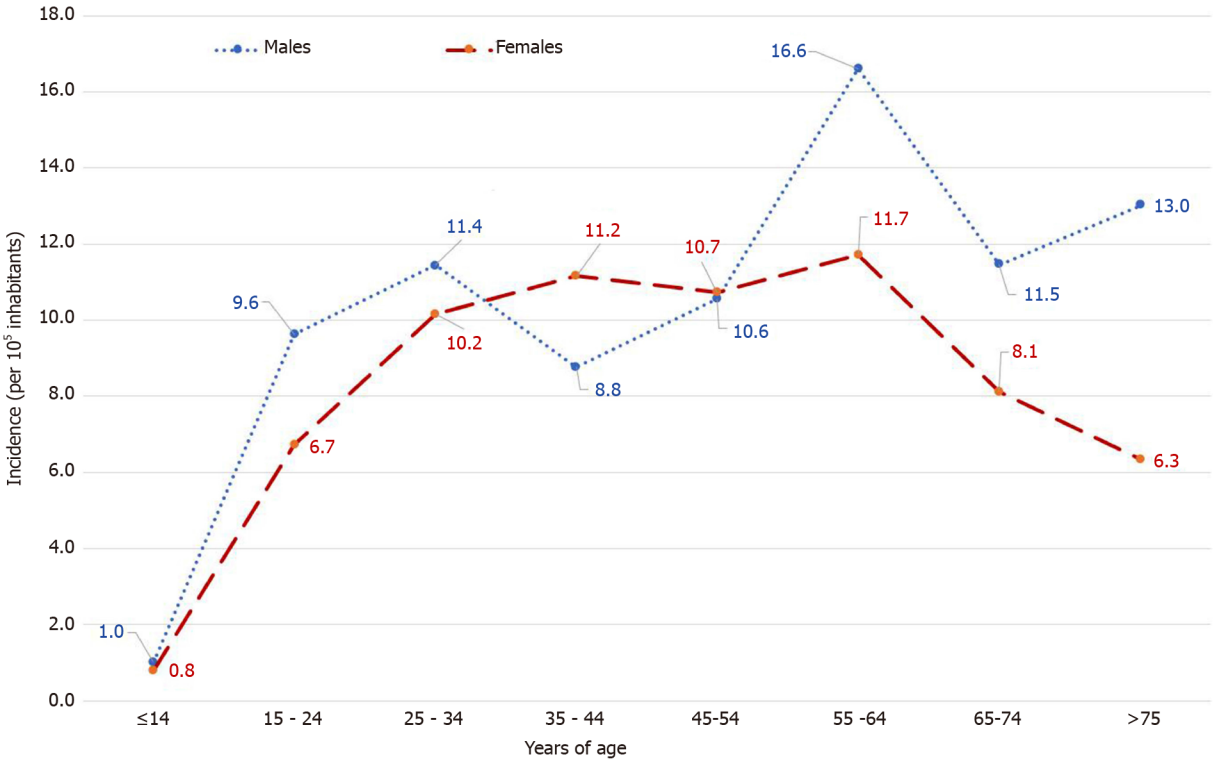


Figure 1 Inflammatory bowel diseases incidence clustered by age and gender.

The highest incidence of CD was recorded among age groups in the male population comprising patients between 15 and 34 years: 7/100000 inhabitants. Among the female population, the disease was most often diagnosed in patients between 45 and 54 years of age, which amounts to 5.4/100000 inhabitants.

Based on the 3-year average incidence rate, which is presented in Figure 2, the incidence of both major forms of IBD occurred fairly uniformly throughout the study. The average annual incidence percentage variation was 0.79% (95%CI: 0.60-0.88) or -2.82% (95%CI: -2.67 - -2.97) for UC and 6.92% (95%CI: 6.64-7.20) for CD. Summarizing the above, we concluded that the UC incidence trend is stable; however, the same cannot be concluded for CD, where the incidence trend is increasing (Figure 2).

During the study period, 24509 distal endoscopic procedures were performed (an average of 2228 per year). Figure 3 shows the trend of incidence per 100 colonoscopies. Any patient suspected of having IBD underwent a detailed endoscopic evaluation in the form of a gastroscopy and colonoscopy with—if the disease phenotype allowed—intubation of the terminal ileum. The incidence of IBD was 3.16/100 examinations (95%CI: 2.86-3.45) or 1.72/100 examinations (95%CI: 1.5-1.94) for UC and 1.36/100 examinations (95%CI: 1.17-1.56) for CD.

A summarized review of the 3-year average incidence rate reported in previous studies[10,11] and a recent study is presented in Figure 4. Data from the last year of the previous study (2006) and the first year of our study (2009) were used to fill in for missing years by averaging the values of these 2 years.

Prevalence

In the last year of the study, the prevalence of IBD was 147.44/100000 inhabitants (95%CI: 136.1-158.8): 154.48/100000 inhabitants (95%CI: 137.8-171.1) in the male population and 140.73/100000 inhabitants (95%CI: 125.2-156.2) in the female population. The age-standardized prevalence was 147.3/100000 inhabitants (95%CI: 136-158.7).

Clinical and phenotypical characteristics of IBD

The types of individual forms of IBD according to the Montreal classification at diagnosis are presented in Tables 1 and 2. In eight (0.12%) patients, a change in the previously made diagnosis was recorded.

Among 651 patients, 49 (7.53%) required surgical treatment. In total, 8 (2.31%) patients treated for UC and 41 (14%) treated for CD underwent surgical treatment due

Table 1 Clinical phenotypes of ulcerative colitis clustered by gender

	Males	Females	Total
Proctitis, *n* (%)	32 (13.3)	37 (15.4)	69 (28.7)
Left-sided colitis, *n* (%)	68 (28.3)	64 (26.7)	132 (55)
Extensive colitis, *n* (%)	24 (10)	15 (6.3)	39 (16.3)

Table 2 Clinical phenotypes of Crohn's disease according to Vienna classification

	n (%)
Age	
A1	13 (6.8)
A2	117 (61.6)
A3	60 (31.6)
Location	
L1	79 (41.5)
L2	52 (27.4)
L3	59 (31.1)
Behaviour	
B1	124 (65.3)
B2	49 (25.8)
B3	17 (8.9)
Perianal disease	
Present	12 (6.3)
Absent	178 (93.7)

to complications. The most common indication was lumen stenosis, which occurred in 23 (46.94%) patients. Multiple surgical treatments were performed in four (8.16%) patients. Comorbidities, primarily gastroenterological and autoimmune diseases, were also monitored during the study. Primary sclerosing cholangitis was diagnosed in four (1.37%) patients treated for CD and one (0.03%) treated for UC. Primary biliary cirrhosis, followed by liver transplantation, was noted in one (0.15%) patient. Autoimmune hepatitis was diagnosed in two (0.3%) patients, one of whom was treated for UC and the other for CD. Familial adenomatous polyposis or ankylosing spondylitis were diagnosed in two patients. Malignant colon disease occurred in five (0.76%) patients, four of whom were treated for UC (1.16%) and one for CD (0.03%). Fatal outcome occurred in six (0.9%) patients. Two patients died due to the consequences of malignant colon disease, two immediately after demanding surgical procedures, while one patient died due to the consequences of toxic megacolon accompanied by massive pulmonary embolism.

DISCUSSION

Until recently, scholars have considered IBD to be a group of diseases of developed Western societies. Recent research into the global epidemiological situation confirms a stabilization of the IBD incidence in these areas, with far higher prevalence than in less developed societies, such as in Eastern Europe[4]. The GBD (Global Burden of Disease) study observed the highest age-standardized prevalence in societies with the highest socio-demographic index (SDI)[15]. The study found the highest prevalence in the region of North America (422/100000 inhabitants) and Western Europe, especially the United Kingdom (449.6/100000 inhabitants). Bosnia-Herzegovina is currently classified as a country with a high middle SDI; according to the study, the values of

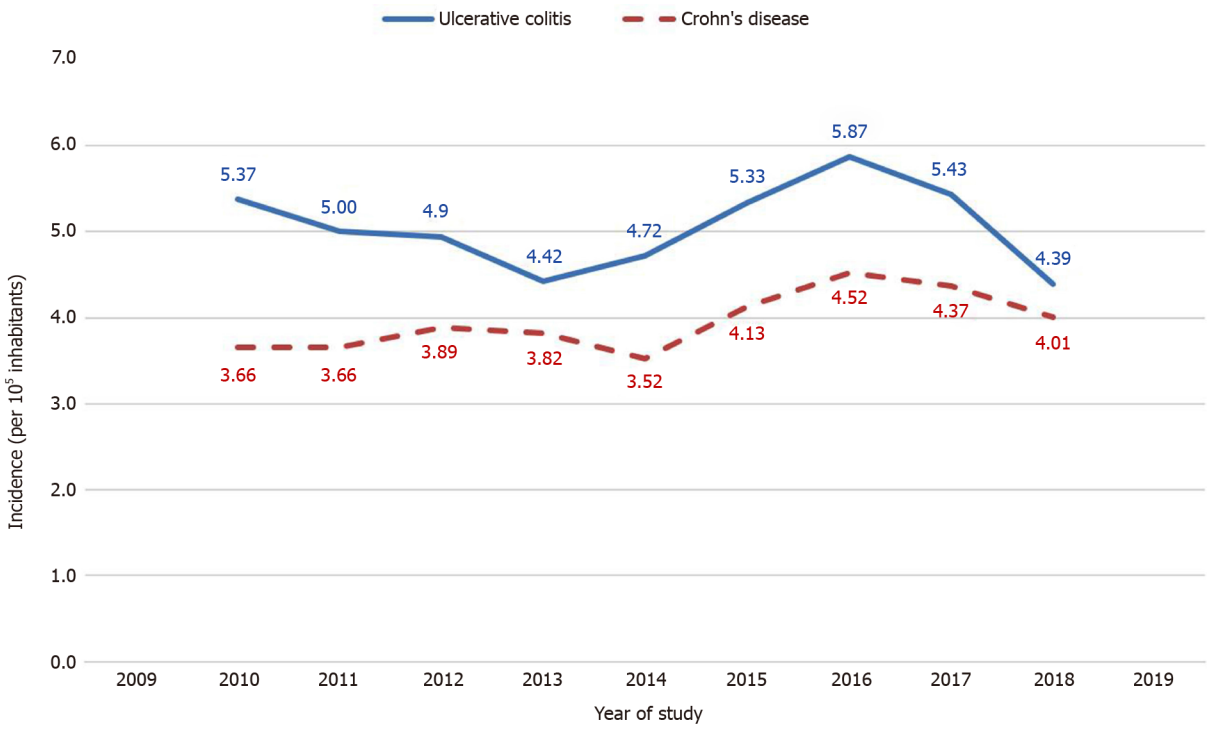


Figure 2 Annual incidence rates of two major forms of inflammatory bowel diseases presented as moving 3-yr average with a 95% confidence interval.

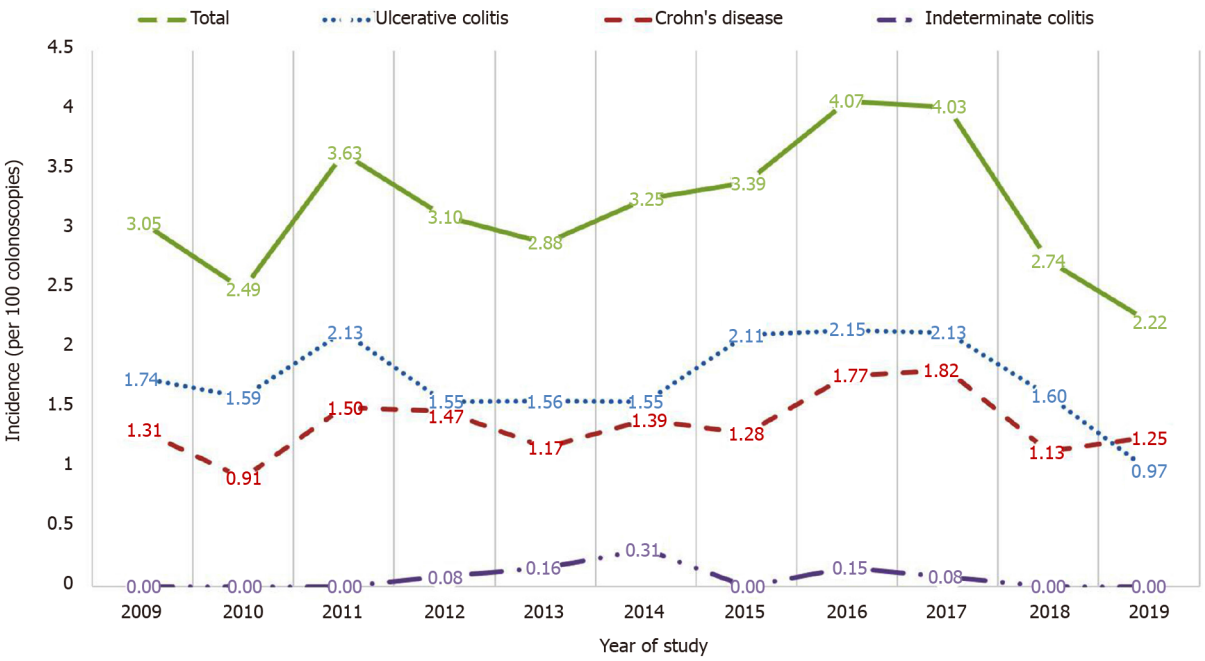


Figure 3 The trend of the annual incidence of inflammatory bowel diseases per 100 colonoscopies performed.

age-standardized prevalence do not differ significantly compared to regions and countries of a similar standard.

When it comes to neighbouring countries, the GBD study ranked Bosnia-Herzegovina among countries with an estimated age-standardized prevalence between 100 and 120/100000 inhabitants, which is still slightly lower than the results of our research. Among the former Yugoslavian republics, neighbouring Croatia and Slovenia lead, with an estimated prevalence of between 180 and 200/100000 inhabitants[15]. These amounts do not apply to Hungary; since the 1970s, several extensive studies have shown that Hungary hosts the highest incidence of IBD in

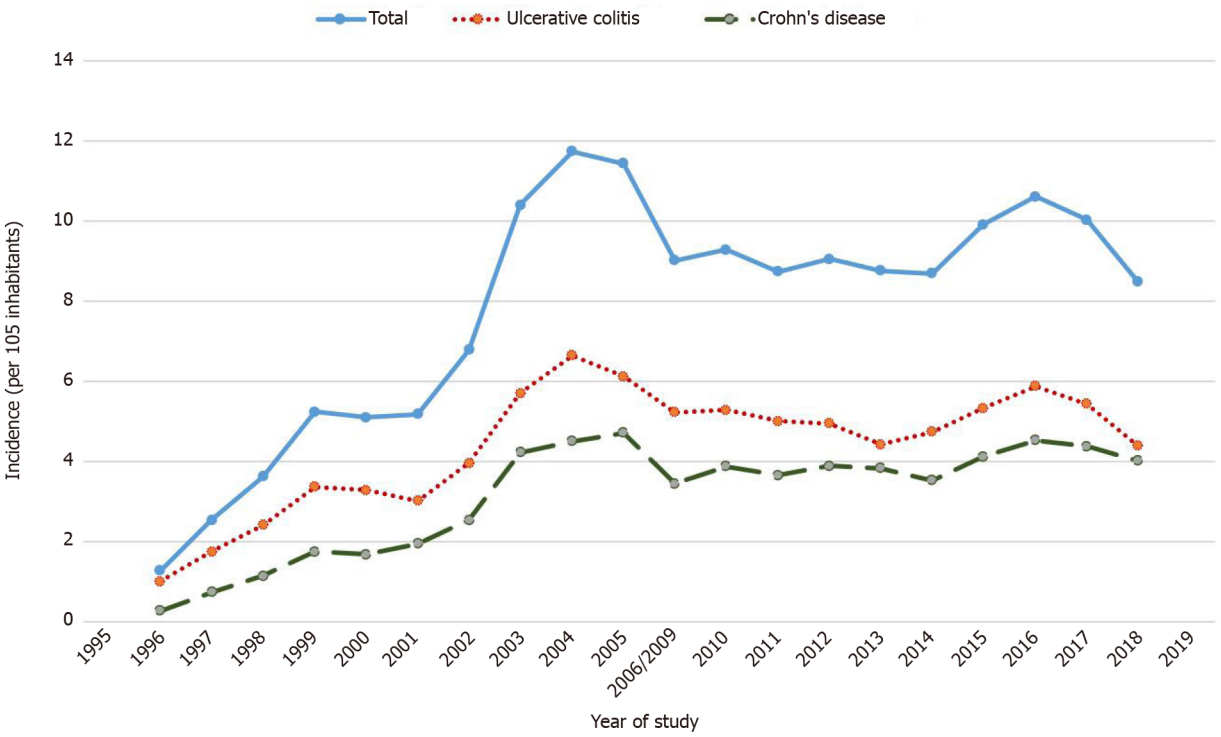


Figure 4 Summarized review of 3-yr average incidence rates on both previous and recent study. Since the time period 2006-2009 was not evaluated, we used the calculated average of 2006 and 2009.

Eastern Europe[16,17].

Our study recorded the highest prevalence among males over the age of 75, which is similar to the GBD study results. The highest prevalence among the female population in our study occurred among slightly younger age groups (44-54 years) than those globally[15].

Paralysis of the healthcare system caused by the war in our country at the end of the last century required a longer recovery, which is certainly reflected in the volume of diagnostic tests and the results of scientific research. When compared with incidence rates from 1995-2006[10,11], a stable trend can be seen in IBD incidence over the study's 10-year period. According to a study by Salkic *et al*[11] of the period between 1995 and 2006, an average of 397 colonoscopies per year were performed, which is 3.2 times fewer than the average number of diagnostic procedures performed annually during our study. The frequency of diagnosing UC per 100 colonoscopies performed during the previous study was 3.2 times higher than now; CD was diagnosed 2.2 times more often according to the given number of procedures performed.

The incidence rate among developing countries and regions such as Eastern Europe can be seen to have significantly increased when compared to previous reports[4]. Earlier studies from developing regions have shown an increase in the incidence of CD compared to UC[18], which was (based on the average percentile annual variation) also found to be the case in our study. Conducted in 2010 among 22 European countries, the ECCO-EpiCom cohort study[3] compared epidemiological differences between diseases in both Western and Eastern Europe, presenting the clearest and most concise epidemiological picture of IBD in Eastern Europe. It showed that the annual crude incidence of IBD in Eastern Europe was half that in Western Europe, with an average of 8.1/100000 inhabitants. The incidence of UC was 4.6/100000 inhabitants in 2010, while the incidence of CD was 3.3/100000 inhabitants, which are fairly uniform results, compared to the results presented in our study.

When it comes to the area of Southeastern Europe, the epidemiological situation is—except for Greece, Croatia, and Bosnia-Herzegovina[5-11] — poorly researched or unknown. Epidemiological studies among the pediatric population have been performed only in Slovenia[8]. The incidence calculated during our study period is somewhat lower compared to that in this region. A 10-year study (2000-2010) of both major forms of IBD in Zadar county has found a trend of increasing incidence, similar to previous studies from the Tuzla Canton[5,6,10,11]. The other recent Croatian study (conducted in Split-Dalmatia county), on the other hand, observed a decline in UC incidence and stabilization of CD incidence[7]. With the results of our study showing

stabilization of UC incidence alongside an increasing trend in CD incidence, we came to the conclusion that there are significant epidemiological differences within geographically close and demographically similar areas.

The population of Tuzla Canton is generally older than it was during the previous studies, which ultimately affected the results in terms of the epidemiological situation. Although the canton's population is still younger than the average European population, the patients involved in the study are generally older than the patients analysed in the ECCO-EpiCom study[3]. Despalatović *et al*[7] reported the highest incidence of UC within the 18-30 and 51-60 age groups. Their results are similar to ours and the results from a previous study from Tuzla Canton[11]. One can consider age structure to be a bimodal distribution of incidence. Similarities in the findings of these two studies can also be seen in relation to CD incidence, with the results showing a higher incidence of CD among the younger population[7,10].

When it comes to the pediatric and adolescent population, our study recorded a significantly lower incidence of IBD than research conducted in Croatia, Slovenia, and most of European countries[5,8,19], probably due to a relatively small number of colonoscopies performed in the pediatric and adolescent population in our center.

The main forms of IBD are characterized by a relapsing and remitting course, with a tendency to change localization and phenotype over time, although the results among studies are inconsistent[20-23]. The trend of localization change over time is more pronounced in UC than in CD. We registered a change in the localization of CD in 3.53% of patients during our study, which is significantly lower than those of the studies of Louis *et al*[21] and Lo *et al*[20]. Phenotype change was recorded in 16.47% of patients, which is close to the result of Lo *et al*[20] but drastically lower than that of Louis *et al*[21]'s study, which observed a change in phenotype in 45.9% of patients. Considering the new modalities of CD treatment—primarily in terms of the introduction of anti-TNF therapy—the reduction in phenotype change frequency is unsurprising. Modification of the early diagnosis was observed far less frequently than in the IBSEN study[24], in which the diagnosis of 9% of patients changed over time.

In comparison with an earlier study by Salkic *et al*[11], we observed a significantly higher incidence of proctitis at the expense of left-sided colitis. Given the significantly greater number of endoscopic procedures, our observation might have resulted from earlier detection of the disease. Interestingly, both studies detected the same incidence of extensive colitis, which was diagnosed in 16.3% of patients. The results of our study are close to the Eastern European average, with a higher incidence of left-sided colitis but a lower incidence of proctitis and extensive colitis. The localization and phenotype of CD do not differ significantly between Eastern and Western Europe. Except for a somewhat more frequent incidence of colic and stenotic types of CD, the results of our study do not differ significantly from the (Eastern) European average. The results of our study showed a higher incidence of the ileocolic form among the younger population, while the colic form was found to affect the older population more frequently (results which are analogous to previous studies)[25,26].

Despite the fact that patients with IBD develop a significantly higher risk of malignancy over time compared to the rest of the population[27], the results of our study show that a large number of newly diagnosed cases (128, or 37%, cases newly diagnosed with UC) undergo endoscopic examination less than two times. Similar results were obtained by Vienne *et al*[28] during a 7-year multicenter study in France: As many as 54% of patients with extensive colitis underwent endoscopic examination only once.

Etiological factors related to the onset or worsening of the course of the disease are numerous. IBD is mostly associated with industrialization, urbanization, and the Western way of life[2,4]. Tuzla Canton is the industrial and electric power center of Bosnia-Herzegovina, with coal as the primary raw material used for electricity production. Considering that the University Clinical Center Tuzla is the largest medical center in northeastern Bosnia-Herzegovina, it is not unrealistic to expect that the region suffers one of the highest IBD incidence rates in the country.

Population-based study in Bosnia-Herzegovina estimates that nearly half of all adults consume tobacco products on a daily basis[29]. Given the fact that tobacco consumption in public places in Bosnia-Herzegovina is not legally restricted, even a larger proportion of the population is exposed to tobacco smoke. The number of breastfeeding mothers in Bosnia-Herzegovina is far below the European average[30], which is alarming if the benefits of breastfeeding as a protective factor against the development of IBD are taken into account[31].

The design of our study was conceived according to a follow-up of patients over a 10-year period. Given the lack of an adequate information system from 2006 to 2009, 2009 was accepted as satisfactory for our study's beginning point. Our study is

retrospective-prospective in nature, based on respondents evaluated and treated at the University Clinical Center Tuzla, which is the only clinical center in the region and is a meeting place for all IBD-treated patients in the region and beyond. Given that the largest gap in the epidemiological picture of IBD relates to regions and developing countries[32] that include Bosnia-Herzegovina, we believe that our study (along with earlier reports[10,11]) can serve as a template for understanding the epidemiological picture in developing countries. In future, a multicenter study of the territory of Bosnia-Herzegovina and the countries of Southeastern Europe would greatly help us to understand this issue.

Conducted in a clinical center, this was a hospital study and, as such, has its limitations. The University Clinical Center Tuzla was the only institution capable of providing a comprehensive evaluation of patients with IBD in Tuzla Canton. Additionally, since all patients undergoing any form of IBD treatment must have a written recommendation for treatment in order to obtain reimbursement for the costs, any IBD patient from Tuzla Canton must eventually be evaluated in our center. We therefore feel safe in our assumption that despite it being single-hospital-based, our study is a confident representation of the epidemiological status of IBD in our region.

The need to conduct the study was made apparent by previous research conducted on this topic during the 11-year period from 1995 to 2006. Given the already mentioned technical limitations, the study did not follow the period from the end of 2006 to 2009. However, by monitoring prevalence, we can conclude that the incidence data throughout this period are close to data from the end of the previous study and from the beginning of our study.

CONCLUSION

The epidemiological image of IBD in Tuzla Canton is similar to the Eastern European average. Stagnation of the incidence of UC was registered, as well as an increase in the incidence of CD during the observed period. The prevalence of both major forms of IBD tends to increase. The incidence and prevalence of IC are similar to those of the Eastern European and the world average. The highest incidence of UC is observed in slightly older age groups, comparable to the average in Eastern Europe and the rest of the world. The highest incidence of CD is observed among younger age groups, between the second and fourth decades of life, as it follows Eastern European and world trends. Gender distribution among newly diagnosed patients during the study was approximately the same, with a slightly higher incidence among the males for all three most common forms of the disease. The results of the study did not show significant deviations according to the clinical and endoscopic characteristics of the disease compared to the European average.

ARTICLE HIGHLIGHTS

Research background

Recent epidemiological studies conducted in Southeastern Europe show increasing incidence of inflammatory bowel diseases (IBD) in areas previously characterized as low-incidence areas. However, the results are still heterogenous as studies were conducted in areas heterogenous in terms of ethnicity, genetics background and lifestyle.

Research motivation

The region of Eastern Europe, especially Balkan region, is poorly described in terms of epidemiology of inflammatory bowel disease. This obvious gap in our understanding of IBD epidemiology in this region of Europe was previously partially described, and this study represents a continuation of one of the longest continuous surveys in Europe at all with nearly 25 years of epidemiological data.

Research objectives

The authors sought to publish an epidemiological evaluation of IBD through a detailed 10-year follow-up of the clinical and epidemiological characteristics of the major forms of the disease.

Research methods

The study evaluated both IBD inpatients and outpatients residing in Tuzla Canton, and is single-hospital-based. Descriptive statistical parameters were used to determine basic characteristics of the study population. Incidence and prevalence calculations were performed using census data from the Statistical Office of the Federation of Bosnia-Herzegovina. The statistical level of 95% ($P < 0.05$) was considered significant for all statistical tests.

Research results

We observed 440 newly diagnosed patients with IBD: 240 (54.5%) with ulcerative colitis (UC), 190 (43.2%) with Crohn's disease (CD), and 10 (2.3%) with indeterminate colitis (IC). The mean annual crude incidence rates were found to be 9.01/100000 population for IBD [95% confidence interval (CI): 8.17-9.85], with 4.91/100000 (95%CI: 4.29-5.54) for UC and 3.89/100000 (95%CI: 3.34-4.44) for CD. Calculated IBD prevalence in 2019 was 146.64/100000 (95%CI: 128.09-165.19), with 77.94/100000 (95%CI: 68.08-87.70) for UC and 65.77/100000 (95%CI: 54.45-74.1) for CD. The average annual IBD percentage change was 0.79% (95%CI: 0.60-0.88), with -2.82% (95%CI: -2.67 - -2.97) for UC and 6.92% (95%CI: 6.64-7.20) for CD.

Research conclusions

We conclude that our region of Europe has a relatively stable incidence of UC (5/100000) and CD (4/100000), which is in line with previous predictions that IBD incidence in Eastern Europe is approximately half of the incidence in Western Europe. Whether this is the result of lower ascertainment or there are other factors included remains to be seen in future research.

Research perspectives

The most important factor for future research is why the incidence is lower in Eastern Europe. There are numerous factors to be considered, which include environmental, genetic, and sociological peculiarities of the region and its population. All of these factors should be taken in consideration during the design of future research.

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REFERENCES

1 **Feldman M**, Friedman LS, Brandt LJ. Sleisenger and Fordtran's gastrointestinal and liver disease: Pathophysiology/diagnosis/management. 10th ed. Philadelphia, PA: Saunders/Elsevier, 2016: 115-116

2 **Loftus EV Jr**. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 2004; **126**: 1504-1517 [PMID: 15168363 DOI: 10.1053/j.gastro.2004.01.063]

3 **Burisch J**, Pedersen N, Čuković-Čavka S, Brinar M, Kaimakliotis I, Duricova D, Shonová O, Vind I, Avnstrøm S, Thorsgaard N, Andersen V, Krabbe S, Dahlerup JF, Salupere R, Nielsen KR, Olsen J, Manninen P, Collin P, Tsianos EV, Katsanos KH, Ladefoged K, Lakatos L, Björnsson E, Ragnarsson G, Bailey Y, Odes S, Schwartz D, Martinato M, Lupinacci G, Milla M, De Padova A, D'Incà R, Beltrami M, Kupcinskas L, Kiudelis G, Turcan S, Tighineanu O, Mihu I, Magro F, Barros LF, Goldis A, Lazar D, Belousova E, Nikulina I, Hernandez V, Martinez-Ares D, Almer S, Zhulina Y, Halfvarson J, Arebi N, Sebastian S, Lakatos PL, Langholz E, Munkholm P; EpiCom-group. East-West gradient in the incidence of inflammatory bowel disease in Europe: the ECCO-EpiCom inception cohort. *Gut* 2014; **63**: 588-597 [PMID: 23604131 DOI: 10.1136/gutjnl-2013-304636]

4 **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 2017; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]

5 **Sincić BM**, Vucelić B, Persić M, Brncić N, Erzen DJ, Radaković B, Mićović V, Stimac D. Incidence of inflammatory bowel disease in Primorsko-goranska County, Croatia, 2000-2004: A prospective population-based study. *Scand J Gastroenterol* 2006; **41**: 437-444 [PMID: 16635912 DOI: 10.1080/00365520500320094]

6 **Klarin I**, Majnarić L, Jovanović Z, Nakić D, Vcev I, Vcev A. Epidemiology and clinical presentation

of inflammatory bowel disease in Zadar County, Croatia. *Coll Antropol* 2013; **37**: 1161-1170 [PMID: 24611329]

7 **Despalatović BR**, Bratanić A, Radić M, Jurišić Z, Tonkić A. Epidemiological trends of inflammatory bowel disease (IBD) in Split-Dalmatia County, Croatia from 2006 to 2014. *Eur J Intern Med* 2017; **46**: e17-e19 [PMID: 28882418 DOI: 10.1016/j.ejim.2017.08.026]

8 **Urlep D**, Blagus R, Orel R. Incidence Trends and Geographical Variability of Pediatric Inflammatory Bowel Disease in Slovenia: A Nationwide Study. *Biomed Res Int* 2015; **2015**: 921730 [PMID: 26688822 DOI: 10.1155/2015/921730]

9 **Malakos Z**, Saridi M, Latsou D, Katsanos K, Bakasis AD, Albani E, Christodoulou D. Increasing Incidence of Crohn's Disease in Epirus, Greece: A 30-Year Prospective Study from a Referral Center. [cited 5 May 2020]. In: Ecronicon [Internet]. Available from: https://www.ecronicon.com/ecgds/pdf/ECGDS-05-00211.pdf

10 **Pavlovic-Calic N**, Salkic NN, Gegic A, Smajic M, Alibegovic E. Crohn's disease in Tuzla region of Bosnia and Herzegovina: a 12-year study (1995-2006). *Int J Colorectal Dis* 2008; **23**: 957-964 [PMID: 18548259 DOI: 10.1007/s00384-008-0493-1]

11 **Salkic NN**, Pavlovic-Calic N, Gegic A, Jovanovic P, Basic M. Ulcerative colitis in the Tuzla region of Bosnia and Herzegovina between 1995 and 2006: epidemiological and clinical characteristics. *Eur J Gastroenterol Hepatol* 2010; **22**: 346-353 [PMID: 19474740 DOI: 10.1097/MEG.0b013e32832bfe05]

12 **Stange EF**, Travis SP, Vermeire S, Reinisch W, Geboes K, Barakauskiene A, Feakins R, Fléjou JF, Herfarth H, Hommes DW, Kupcinskas L, Lakatos PL, Mantzaris GJ, Schreiber S, Villanacci V, Warren BF; European Crohn's and Colitis Organisation (ECCO). European evidence-based Consensus on the diagnosis and management of ulcerative colitis: Definitions and diagnosis. *J Crohns Colitis* 2008; **2**: 1-23 [PMID: 21172194 DOI: 10.1016/j.crohns.2007.11.001]

13 **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus EV Jr, Peña AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5A-36A [PMID: 16151544 DOI: 10.1155/2005/269076]

14 **Pace M**, Lanzieri G, Glickman M, Županič T. Revision of the European Standard Population: report of Eurostat's task force. Publications O_ce of the European Union. 2013. [cited 21 June 2020]. In: ec.europa.eu [Internet]. Available from: https://ec.europa.eu/eurostat/documents/3859598/5926869/KS-RA-13-028-EN.PDF/e713fa79-1add-44e8-b23d-5e8fa09b3f8f

15 **GBD 2017 Inflammatory Bowel Disease Collaborators**. The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Gastroenterol Hepatol* 2020; **5**: 17-30 [PMID: 31648971 DOI: 10.1016/S2468-1253(19)30333-4]

16 **Lakatos L**, Kiss LS, David G, Pandur T, Erdelyi Z, Mester G, Balogh M, Szipocs I, Molnar C, Komaromi E, Lakatos PL. Incidence, disease phenotype at diagnosis, and early disease course in inflammatory bowel diseases in Western Hungary, 2002-2006. *Inflamm Bowel Dis* 2011; **17**: 2558-2565 [PMID: 22072315 DOI: 10.1002/ibd.21607]

17 **Kurti Z**, Vegh Z, Golovics PA, Fadgyas-Freyler P, Gecse KB, Gonczi L, Gimesi-Orszagh J, Lovasz BD, Lakatos PL. Nationwide prevalence and drug treatment practices of inflammatory bowel diseases in Hungary: A population-based study based on the National Health Insurance Fund database. *Dig Liver Dis* 2016; **48**: 1302-1307 [PMID: 27481587 DOI: 10.1016/j.dld.2016.07.012]

18 **Cosnes J**, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1785-1794 [PMID: 21530745 DOI: 10.1053/j.gastro.2011.01.055]

19 **Sýkora J**, Pomahačová R, Kreslová M, Cvalínová D, Štych P, Schwarz J. Current global trends in the incidence of pediatric-onset inflammatory bowel disease. *World J Gastroenterol* 2018; **24**: 2741-2763 [PMID: 29991879 DOI: 10.3748/wjg.v24.i25.2741]

20 **Lo B**, Vester-Andersen MK, Vind I, Prosberg M, Dubinsky M, Siegel CA, Bendtsen F, Burisch J. Changes in Disease Behaviour and Location in Patients With Crohn's Disease After Seven Years of Follow-Up: A Danish Population-based Inception Cohort. *J Crohns Colitis* 2018; **12**: 265-272 [PMID: 29506105 DOI: 10.1093/ecco-jcc/jjx138]

21 **Louis E**, Collard A, Oger AF, Degroote E, Aboul Nasr El Yafi FA, Belaiche J. Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. *Gut* 2001; **49**: 777-782 [PMID: 11709511 DOI: 10.1136/gut.49.6.777]

22 **Lovasz BD**, Lakatos L, Horvath A, Szita I, Pandur T, Mandel M, Vegh Z, Golovics PA, Mester G, Balogh M, Molnar C, Komaromi E, Kiss LS, Lakatos PL. Evolution of disease phenotype in adult and pediatric onset Crohn's disease in a population-based cohort. *World J Gastroenterol* 2013; **19**: 2217-2226 [PMID: 23599648 DOI: 10.3748/wjg.v19.i14.2217]

23 **Vester-Andersen MK**, Prosberg MV, Jess T, Andersson M, Bengtsson BG, Blixt T, Munkholm P, Bendtsen F, Vind I. Disease course and surgery rates in inflammatory bowel disease: a population-based, 7-year follow-up study in the era of immunomodulating therapy. *Am J Gastroenterol* 2014; **109**: 705-714 [PMID: 24642581 DOI: 10.1038/ajg.2014.45]

24 **Henriksen M**, Jahnsen J, Lygren I, Sauar J, Schulz T, Stray N, Vatn MH, Moum B; Ibsen Study

Group. Change of diagnosis during the first five years after onset of inflammatory bowel disease: results of a prospective follow-up study (the IBSEN Study). *Scand J Gastroenterol* 2006; **41**: 1037-1043 [PMID: 16938716 DOI: 10.1080/00365520600554527]

25 **Castro M**, Papadatou B, Baldassare M, Balli F, Barabino A, Barbera C, Barca S, Barera G, Bascietto F, Berni Canani R, Calacoci M, Campanozzi A, Castellucci G, Catassi C, Colombo M, Covoni MR, Cucchiara S, D'Altilia MR, De Angelis GL, De Virgilis S, Di Ciommo V, Fontana M, Guariso G, Knafelz D, Lambertini A, Licciardi S, Lionetti P, Liotta L, Lombardi G, Maestri L, Martelossi S, Mastella G, Oderda G, Perini R, Pesce F, Ravelli A, Roggero P, Romano C, Rotolo N, Rutigliano V, Scotta S, Sferlazzas C, Staiano A, Ventura A, Zaniboni MG. Inflammatory bowel disease in children and adolescents in Italy: data from the pediatric national IBD register (1996-2003). *Inflamm Bowel Dis* 2008; **14**: 1246-1252 [PMID: 18521916 DOI: 10.1002/ibd.20470]

26 **Charpentier C**, Salleron J, Savoye G, Fumery M, Merle V, Laberenne JE, Vasseur F, Dupas JL, Cortot A, Dauchet L, Peyrin-Biroulet L, Lerebours E, Colombel JF, Gower-Rousseau C. Natural history of elderly-onset inflammatory bowel disease: a population-based cohort study. *Gut* 2014; **63**: 423-432 [PMID: 23408350 DOI: 10.1136/gutjnl-2012-303864]

27 **Axelrad JE**, Lichtiger S, Yajnik V. Inflammatory bowel disease and cancer: The role of inflammation, immunosuppression, and cancer treatment. *World J Gastroenterol* 2016; **22**: 4794-4801 [PMID: 27239106 DOI: 10.3748/wjg.v22.i20.4794]

28 **Vienne A**, Simon T, Cosnes J, Baudry C, Bouhnik Y, Soulé JC, Chaussade S, Marteau P, Jian R, Delchier JC, Coffin B, Admane H, Carrat F, Drouet E, Beaugerie L. Low prevalence of colonoscopic surveillance of inflammatory bowel disease patients with longstanding extensive colitis: a clinical practice survey nested in the CESAME cohort. *Aliment Pharmacol Ther* 2011; **34**: 188-195 [PMID: 21615760 DOI: 10.1111/j.1365-2036.2011.04711.x]

29 **Gligorić D**, Petković S, Pepić A, Ateljević J, Vukojević B. The elasticity of tobacco products in BiH – macrodata analysis. *Tob Prev Cessation* 2019; **5**: A97 [DOI: 10.18332/tpc/105226]

30 **Šumanović-Glamuzina D**. Breastfeeding practice in Bosnia and Herzegovina. *J Pediatr Neonat Individual Med* 2013; **2**: 55-62 [DOI: 10.7363/020117]

31 **Xu L**, Lochhead P, Ko Y, Claggett B, Leong RW, Ananthakrishnan AN. Systematic review with meta-analysis: breastfeeding and the risk of Crohn's disease and ulcerative colitis. *Aliment Pharmacol Ther* 2017; **46**: 780-789 [PMID: 28892171 DOI: 10.1111/apt.14291]

32 **Kaplan GG**, Ng SC. Understanding and Preventing the Global Increase of Inflammatory Bowel Disease. *Gastroenterology* 2017; **152**: 313-321. e2 [PMID: 27793607 DOI: 10.1053/j.gastro.2016.10.020]

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META-ANALYSIS

Association between oral contraceptive use and pancreatic cancer risk: A systematic review and meta-analysis

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Abstract

BACKGROUND

Studies on the association of oral contraceptive (OC) use and pancreatic cancer showed inconsistent findings.

AIM

To evaluate the relationship between OC use and pancreatic cancer risk.

METHODS

A literature search for observational studies (case-control and cohort studies) was conducted up to December 2020. A meta-analysis was performed by calculating pooled relative risks (RRs) and 95% confidence intervals (CIs). Heterogeneity was assessed using Cochran's chi-square test and I^2 statistic. Subgroup analyses were performed by study design, source of controls in case-control studies, number of cases of pancreatic cancers, study quality according to Newcastle-Ottawa Scale score, geographical region and menopausal status. All analyses were performed using Review Manager 5.3 (RevMan 5.3).

RESULTS

A total of 21 studies (10 case-control studies and 11 cohort studies) were finally included in the present meta-analysis, comprising 7700 cases of pancreatic cancer in total. A significant association was observed between the ever use of OC and pancreatic cancer risk in the overall analysis (RR = 0.85; 95%CI = 0.73-0.98; P = 0.03). Duration of OC use (< 1 year, < 5 years, 5-10 years, > 10 years) was not significantly associated with the risk of pancreatic cancer. Subgroup analyses revealed a statistically significant subgroup difference for the geographic region in which the study was conducted (Europe *vs* Americas *vs* Asia; P = 0.07).

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Subgroup analyses showed a statistically significant decrease in pancreatic cancer risk and OC use in high-quality studies, studies conducted in Europe, and in postmenopausal women.

CONCLUSION

Despite the suggested protective effects of OC use in this meta-analysis, further epidemiological studies are warranted to fully elucidate the association between the use of OC and pancreatic cancer risk.

Key Words: Pancreatic cancer; Oral contraceptives; Risk factors; Risk assessment; Meta-analysis; Review

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Core Tip: Although the understanding of the etiology of pancreatic cancer has improved dramatically over the past decades, the link between pancreatic cancer risk and oral contraceptive (OC) use is still insufficiently known. This meta-analysis showed a significant association between OC use and pancreatic cancer risk (overall relative risk = 0.85; 95% confidence interval = 0.73-0.98). A better understanding of the risks of pancreatic cancer occurrence in women who use OC may be relevant for pancreatic cancer prevention strategy.

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INTRODUCTION

Pancreatic cancer is the seventh most common cause of death among malignant tumors in females, with about 220000 deaths worldwide in 2018[1,2]. Pancreatic cancer is one of the deadliest types of cancer, with an estimated overall 5-year survival rate less than 10%[1]. Pancreatic cancer mortality is characterized by a dramatic increase after the age of 30 years, reaching the highest burden in women at about 80-years-old[1,3].

An understanding of the etiology of pancreatic cancer has improved dramatically over the past decades and certain risk factors have been established including tobacco use, obesity, diabetes mellitus, chronic pancreatitis, positive family history and inherited genetic syndromes, high alcohol consumption, dietary factors, physical inactivity, workplace exposure to some chemicals, infections[4-7]. Although some risk factors have been identified, the causes of pancreatic cancer are still insufficiently known.

Regarding the link between pancreatic cancer risk and oral contraceptive (OC) use, epidemiological studies have shown conflicting results: Some findings have shown positive associations with risk of pancreatic cancer[8,9], whereas some studies have indicated inverse associations[10,11]. One previous meta-analysis of observational studies did not support the hypothesis that OC use is associated with pancreatic cancer risk (the pooled relative risk [RR] = 1.09, 95% confidence interval [CI] 0.96–1.23)[12].

A better understanding of risks of pancreatic cancer occurrence in women who use OC may be relevant for pancreatic cancer prevention strategy. The purpose of this study was to evaluate the relationship between the use of OC and pancreatic cancer risk by performing a meta-analysis of case-control and cohort studies.

MATERIALS AND METHODS

This meta-analysis was performed following the Preferred Reporting Items for

Systematic Reviews and Meta-Analyses guidelines[13].

Ethics statement

This study is a part of a research project approved by the Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac (No. 01-14321).

Literature search

We searched PubMed from inception through December 2020 using combinations of keywords: ("oral contraceptives" or "birth control pills") and ("pancreatic cancer" or "pancreatic tumor" or "pancreatic neoplasm") and ("risk" or "risk factors" or "risk assessment"). Additionally, references of retrieved studies and reviews were hand-searched to identify additional relevant studies (up to 31 December 2020). No language restrictions were applied in the search.

Inclusion and exclusion criteria

Two authors (II and MI) independently screened the titles and abstracts of studies retrieved by literature search. Subsequently, the full texts of articles that were identified as relevant were assessed. Any disagreements between the reviewers were resolved through consensus. Studies which reported on the association between the use of OC (the exposure of interest) and risk of pancreatic cancer (the outcome of interest) and that were designed as case-control studies and cohort studies were included. In cases of multiple publications reporting results from the same population, the most recent report and the one with the most data was used. Case reports, case-series, reviews, letters, animal studies and studies with incomplete data were excluded. Studies that did not report separate data for OC use, but instead reported "any hormone therapy" were excluded.

Data extraction and quality appraisal of included studies

Data extraction and quality appraisal were performed independently by two authors (II and MI). Details regarding the study's author and year of publication, study design, sample size, methods of exposure assessment, methods of outcome assessment, and main findings regarding the investigated outcome were extracted. Methodological quality of studies was assessed using the Newcastle-Ottawa Scale (NOS) for quality assessment of case-control studies and cohort-studies[14]. This tool rates three categories: Selection, comparability and exposure (in case-control studies) or outcome (in cohort studies) in observational studies using a star-system. We considered studies with 8 and 9 stars as high quality, 6 and 7 stars as medium quality, and ≤ 5 stars as low quality.

Statistical analysis

A meta-analysis of the comparison of nonusers *vs* users of OC was performed. Odds ratios (ORs), RRs, and hazard ratios (HRs) were extracted from included studies and transformed into RRs. It can be considered that OR and HR approximate RR because the absolute risk of pancreatic cancer is low[15]. For studies that did not report risk estimates for the comparison of ever *vs* never use of OC, we calculated ORs and RRs based on the available published data. We pooled risk estimates for pancreatic cancer and calculated overall RRs with 95%CIs. Risk effects were combined using the random-effects model[16]. The $P < 0.05$ was considered significant.

Heterogeneity was quantified using Cochran's chi-square test and I^2 statistic, with I^2 values of 30%-60%, 50%-90%, and 75%-100% indicating moderate, substantial and considerable heterogeneity, respectively[17]. To explore heterogeneity, we performed subgroup analyses by study design (case-control and cohort), source of controls in case-control studies (population and hospital), number of cases of pancreatic cancers (< 200 and ≥ 200), and assessed study quality (NOS score ≤ 7 and > 7), geographical region (Europe, Americas, Asia), and menopausal status (premenopausal and postmenopausal). Statistically significant subgroup differences were considered for $P < 0.1$[18].

The pooled RRs with corresponding 95%CIs were presented graphically with forest plots. Publication bias was assessed with a funnel plot. All statistical analyses were performed using Review Manager 5 software (RevMan version 5.3, The Nordic Cochrane Centre, The Cochrane Collaboration)[19].

RESULTS

Literature search results and characteristics of included studies

The results of the literature search are presented in Figure 1. We identified 21 studies that investigated the association between the use of OC and risk of pancreatic cancer and that fulfilled the inclusion criteria[8-11,20-36]. There were 10 case-control studies and 11 cohort studies included in the meta-analysis. Characteristics of the included studies are presented in Table 1. In total, the included studies comprised 7700 cases of pancreatic cancer. Among the case-control studies, six had population-based controls and four had hospital based-controls. The use of OC was assessed *via* interviews with trained interviewers in 11 studies by means of self-reported questionnaires in 9 studies, and one study used data from the National Register of Medicinal Products. Outcome assessment was verified through cancer and hospital registries, and in most of the studies, there was pathohistological verification of the diagnosis of pancreatic cancer. Seven studies were conducted in the European region, ten in the Americas (the United States and Canada), three in Asia, and one study was multicentric and conducted in all three regions. According to NOS scores, 12 studies were of high quality (3 case-control and 9 cohort), 8 of moderate quality (6 case-control and 2 cohort), and 1 case-control study was of low quality.

Ever use of OC and risk of pancreatic cancer

Pooled risk estimates of all included studies showed that the ever use of OC was statistically significantly associated with a decreased risk of pancreatic cancer (RR = 0.85, 95%CI: 0.73-0.98; $P = 0.03$) (Figure 2). There was substantial heterogeneity for this estimate ($I^2 = 78\%$; $P < 0.00001$). Pooled risk estimates from case-control studies were not statistically significant (RR = 0.85, 95%CI: 0.64-1.14), while the association between ever use of OC and decreased risk of pancreatic cancer was borderline significant in cohort studies (RR = 0.84, 95%CI: 0.70-1.00). Visual inspection of funnel plot (Figure 3) did not indicate any apparent presence of publication bias.

Duration of OC use and risk of pancreatic cancer

Ever use *vs* never use of OC was chosen as the primary assessment of exposure because some studies have reported that it was not possible to assess the years of oral contraceptive use in their sample[10] or simply did not inquire participants about the duration of use[8-11,23,29,34]. However, we pooled the results from the subset of studies that investigated the duration of use of OC and risk of pancreatic cancer and that reported comparable cut-off periods. Eight studies (2 case-control and 6 cohort) assessed the < 1 year duration of OC use and risk of pancreatic cancer and the pooled risk estimate was not statistically significant (RR = 1.08, 95%CI: 0.91-1.29; $P = 0.38$) (Figure 4A). Similarly, across one case-control and six cohort studies, the results were not significant for durations of use: < 5 years (RR = 1.07, 95%CI: 0.95-1.19; $P = 0.27$) (Figure 4B), 5-10 years (RR = 1.08, 95%CI: 0938-1.26; $P = 0.29$) (Figure 4C) and > 10 years (RR = 1.16, 95%CI: 0.92-1.45; $P = 0.20$) (Figure 4D).

Subgroup analyses

Results of the subgroup analyses by selected characteristics are presented in Table 2. Statistically significant results for subgroup differences were noted only for geographic region (Europe *vs* Americas *vs* Asia) where studies were conducted ($P = 0.07$), while there were no significant subgroup differences noted for study design (case-control *vs* cohort studies), source of controls in case-control studies (population *vs* hospital), number of pancreatic cancer cases (< 200 *vs* ≥ 200), study quality (NOS ≤ 7 *vs* NOS > 7), or menopausal status (premenopausal *vs* postmenopausal). Finally, subgroup analyses revealed a statistically significant association between the use of OC and decreased risk of pancreatic cancer in studies of high quality (RR = 0.80, 95%CI: 0.66-0.98; $P = 0.03$), studies conducted in Europe (OR = 0.67, 95%CI: 0.51-0.88; $P = 0.004$) and in postmenopausal women ($r = 0.88$, 95%CI: 0.79-0.98; $P = 0.02$).

DISCUSSION

In this systematic review and meta-analysis, we identified 10 case-control and 11 cohort studies that investigated the association between the use of OC and pancreatic cancer. Our pooled analysis of these 21 studies, which comprised 7700 cases of pancreatic cancer, showed that the ever use of OC was statistically significantly

Table 1 Characteristics of included studies

Study design	Ref.	Country	Sample size[1]	Exposure assessment	Outcome assessment	Risk estimate (95%CI)[2]	Adjustments	NOS score
Case-control								
	Bueno de Mesquita *et al*[20], 1992	Netherlands	82 cases and 252 controls	Interviewer administered questionnaire	Clinical diagnosis (histological verification, clinicians, laboratory records, registries)	OR = 0.83 (0.31-2.23)	Age, response status and life-time smoking of cigarettes	7
	Ji *et al*[21], 1996	China	184 cases and 680 controls	Interviewer administered questionnaire	Rapid reporting system within cancer registry	OR = 1.78 (0.91-3.47)	Age, income, education, smoking, BMI, green tea drinking, respondent status, age at first birth, intake of dietary vitamin C	7
	Kreiger *et al*[10], 2001	Canada	52 cases and 233 controls	Mailed questionnaire	Cancer registry	OR = 0.36 (0.13-0.96); *P* = 0.031	Age, smoking status, BMI, tofu, dietary fat, coffee consumption, age at menarche, age at menopause, parity, estrogen replacement therapy age at first full term pregnancy	8
	Duell *et al*[22], 2005	United States	241 cases and 818 controls	Interviewer administered questionnaire	Physician, SEER and histologic confirmation	OR = 0.95 (0.65-1.4)	Age, education, smoking	8
	Duell *et al*[23], 2009	Australia, Canada, The Netherlands, Poland	367 cases and 821 controls	Interviewer administered questionnaire	Clinicians, hospital records, pathology records, cancer registries	OR = 0.74 (0.43-1.26)	Smoking, schooling, age, center, type of interview	8
	Zhang *et al*[24], 2010	United States	284 cases and 1096 controls	Interviewer administered questionnaire	Pathology report confirmation	[3]	-	6
	Lucenteforte *et al* [8], 2011	Italy	285 cases and 713 controls	Interviewer administered questionnaire	Histologically confirmed	OR = 1.04 (0.55-1.98)	Study/center, age, education, area of residence, year of interview, history of diabetes, tobacco smoking	7
	Azeem *et al*[11], 2015	Czech Republic	129 cases and 97 controls	Interviewer administered questionnaire	Hospital diagnosis	OR = 0.21 (0.07-0.69)	Not specified (other monitored factors)	5
	Masoudi *et al*[9], 2017	Iran	153 cases and 202 controls	Interviewer administered questionnaire	Pathological reports	OR = 1.07 (0.62-1.84)	Smoking status, BMI, diabetes	6
	Archibugi *et al*[25], 2020	Italy	253 cases and 506 controls	Interviewer administered questionnaire	Histologically proven diagnosis	OR = 0.52 (0.31-0.89)	Age, BMI, first degree family history of pancreatic cancer, history of diabetes > 1 yr, history of chronic pancreatitis, heavy alcohol intake, smoking habit	7
Cohort								
	Skinner *et al*[26],	United States	243 cases and	Mailed questionnaire	Self-reported confirmed *via* medical	RR = 1.21	Age, time period, cigarette smoking, diabetes, BMI, height,	8

	2003		115474 non-cases		records, pathology reports, death certificates, physicians, next of kin interview	(0.91-1.61)	parity	
	Teras *et al*[27], 2005	United States	1959 cases among 387981 participants	Self-administered questionnaire	National Death Index and death certificates	[3]	-	8
	Navarro Silvera *et al*[28], 2005	Canada	187 cases and 89645 noncases	Self-administered questionnaire	Canadian Cancer Database and the National mortality database	HR = 1.10 (0.81-1.50)	Age, cigarette smoking intensity, cigarette smoking duration, BMI, height, study center, randomization group, parity	8
	Prizment *et al*[29], 2007	United States	228 cases and 37231 noncases	Self-administered questionnaire	National Death Index and State Health Registry of Iowa	HR = 0.90 (0.62-1.30)	Age	7
	Dorjgochoo *et al*[30], 2009	China	78 cases among 66661 participants	In-person interview and a self-reported questionnaire	Shanghai Cancer registry and Shanghai Vital Statistics Registry	HR = 0.83 (0.45-1.55)	Education, age at menarche, number of live births, cumulative breast feeding months, BMI, exercised regularly in past 5 yr, smoking, menopausal status, first-degree family history of cancer, other contraceptive methods	9
	Duell *et al*[31], 2013	Denmark, France, Germany, Greece, Italy, Netherlands, Norway, Spain, Sweden, United Kingdom	304 cases among 328610 participants	Self-administered questionnaire	Population cancer registries, health insurance records, hospital-based and pathology registries	[3]	-	8
	Lee *et al*[32], 2013	United States	323 cases among 118164 participants	Self-administered questionnaire	California Cancer Registry	[3]	-	8
	Kabat *et al*[33], 2017	United States	1003 cases and 157295 non-cases	Self-administered questionnaire	Self-reports verified by physician adjudicators through records of hospitalizations, surgeries, pathology reports and procedures	HR = 0.92 (0.80-1.06)	Age, smoking status, pack-years of smoking, BMI, educational level, ethnicity, allocation to study component, diabetes	7
	Andersson *et al*[34],2018	Sweden	110 cases and 16921 noncases	On-site questionnaires and examinations	Swedish Cancer register confirmed by pathology records, autopsy	HR = 0.68 (0.44-1.06)	Age, smoking, alcohol consumption, BMI	8
	Butt *et al*[35], 2018	Denmark	235 cases among 1.9 million women	National Register of Medicinal Product Statistics	Danish Cancer Register and Danish National Patient Register	RR = 0.90 (0.68-1.19)	Age, year, education, PCOS, endometriosis, parity	9
	Michels *et al*[36], 2018	United States	1000 cases and 195536 noncases	Mailed questionnaire	Cancer registries	HR = 1.11 (0.97-1.28)	Age, race, BMI, smoking status, alcohol use, number of cigarettes smoked per day	8

[1]For samples including both sexes only data for women were presented.

[2]Ever use of oral contraceptives *vs* never use.

[3]Not reported, derived from available published data.

CI: Confidence interval; NOS: Newcastle-Ottawa Scale; OR: Odds ratio; RR: Relative risk; HR: Hazard ratio; BMI: Body mass index; PCOS: Polycystic ovary syndrome.

associated with a decreased risk of pancreatic cancer. However, the association was not significant when the duration of OC use less than 1 year, less than 5 years, 5-10 years, and longer than 10 years was assessed in relation to pancreatic cancer risk. A

Table 2 Association between the use of oral contraceptives and risk of pancreatic cancer: Subgroup analysis

Characteristic	Subgroup	Number of studies	RR (95%CI)	I^2 within each subgroup	Test for subgroups differences
Study design	Case-control	10	0.85 (0.64-1.14)	60% (P = 0.008)	P = 0.92
	Cohort	11	0.84 (0.70-1.00)	85% (P < 0.00001)	
Source of controls in case-control studies	Population	6	0.75 (0.47-1.21)	63% (P = 0.02)	P = 0.47
	Hospital	4	0.94 (0.64-1.39)	61% (P = 0.06)	
Number of pancreatic cancer cases	< 200	8	0.80 (0.56-1.14)	66% (P = 0.004)	P = 0.77
	≥ 200	13	0.85 (0.71-1.00)	83% (P < 0.00001)	
Assessed study quality	NOS ≤ 7	9	0.93 (0.75-1.17)	56% (P = 0.02)	P = 0.33
	NOS > 7	12	0.80 (0.66-0.98)	84% (P < 0.00001)	
Geographic region[1]	Europe	7	0.67 (0.51-0.88)	55% (P = 0.04)	P = 0.07
	Americas	10	0.91 (0.74-1.11)	86% (P < 0.00001)	
	Asia	3	1.14 (0.76-1.73)	27% (P = 0.25)	
Menopausal status	Premenopausal	1	0.90 (0.68-1.19)	N/A	0.90
	Postmenopausal	4	0.88 (0.79-0.98)	17% (P = 0.31)	

[1]One study was multicentric and conducted in all three geographic regions so it was not included in the subgroup analysis by geographic region.
CI: Confidence interval; NOS: Newcastle-Ottawa Scale; RR: Relative risk.

significantly reduced risk of pancreatic cancer in women using OC was noted in higher quality studies, studies conducted in Europe, and in postmenopausal women.

Differences in pancreatic cancer incidence rates between sexes, namely a higher incidence of pancreatic cancer in men than in women[7], have led to investigations into possible reasons behind these differences. Apart from the possible influence of environmental factors, it was hypothesized that female sex hormones could be responsible for a lower incidence of pancreatic cancer in women. *In vitro* and *in vivo* studies have shown that the pancreas contains estrogen and androgen receptors and that estrogen inhibits and testosterone promotes the occurrence of some pancreatic cancers[37].

Numerous observational studies have investigated the role of the use of OC and risk of pancreatic cancer; however, the results were not consistent. While some authors have reported an inverse relationship between the use of OC and risk of pancreatic cancer[10,11,25,31,32], other studies have not confirmed these findings. However, none of the published studies found a significant positive relationship between the ever use of OC and pancreatic cancer. With regard to the duration of OC use, our pooled analyses did not identify a significant association with pancreatic cancer risk. Still, one cohort study revealed a significant increase in pancreatic cancer risk in women using OC < 1 year (HR = 1.65, 95%CI: 1.08-2.50)[28], but the number of cases of pancreatic cancer in the group of women who were taking OC less than 1 year was small. Also, one hospital-based case-control study (NOS score assessed as 6) found a borderline positive association for the duration of use of OC of 5-10 years and > 10 years and risk of pancreatic cancer[24], and, despite the small numbers of cases in these groups, the P for trend was significant (< 0.01). In contrast, Kreiger *et al*[10] found a significant decrease in pancreatic cancer risk in women using OC longer than 6 mo. These discrepancies in results across the studies might be explained by differences in study design, study population, assessment of exposure assessment, definitions of exposure,

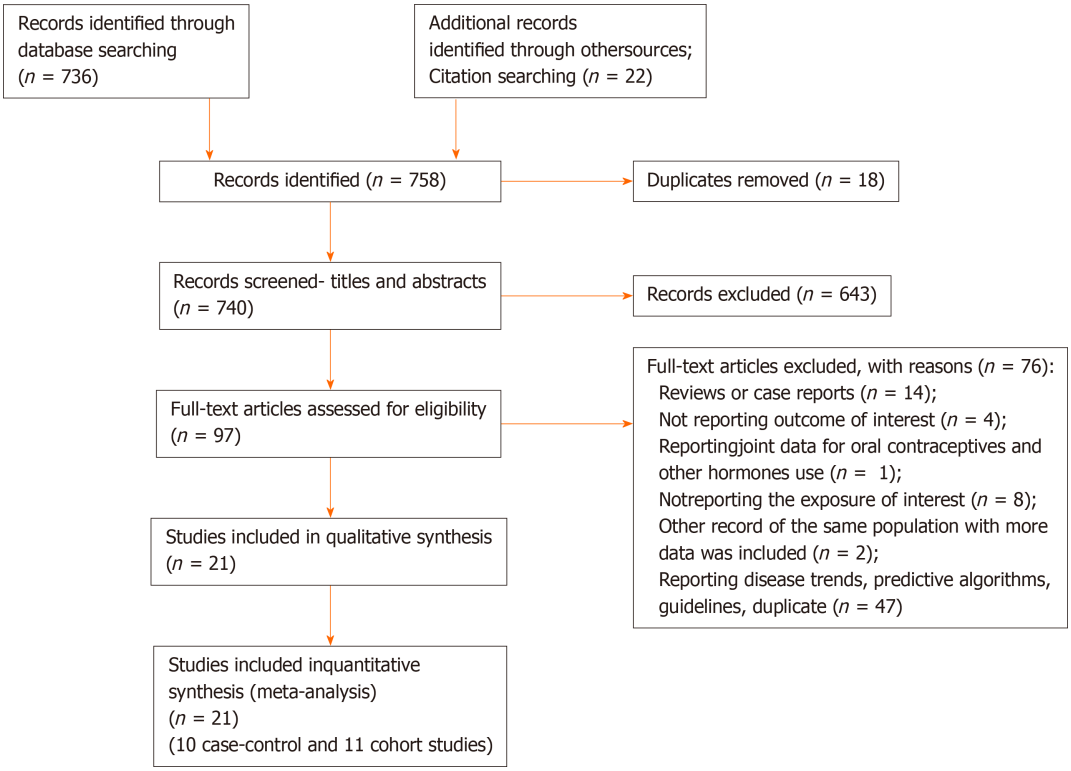


Figure 1 Flow-diagram of literature search.

and different cut-offs for duration of use of OC. Additionally, most of the studies have reported risk estimates adjusted for known and potential pancreatic cancer risk factors (age, diabetes, cigarette smoking, obesity), but with fewer studies also providing estimates adjusted for history of pancreatitis, positive family history of pancreatic cancer and high level of alcohol consumption[25,30,34,36] and one study reporting estimates adjusted only for age[29]. While some of the included studies have adjusted for factors which refer to diet, this most often involved body mass index (BMI)[9,10,21,25,26,28,30,33,34,36], with only two studies investigating nutrition variables such as green tea drinking and intake of dietary vitamin C[21], and coffee and tofu consumption and dietary fat intake[10]. Obesity is a risk factor for pancreatic cancer; however, the mechanisms are not fully known and may involve sex hormones[21]. High BMI might reflect high intake of dietary fat, although the findings regarding its association with pancreatic cancer risk are inconsistent[21,34]. Notably, adipose tissue produces estrogens and might have a protective role[34]. Therefore, dietary factors could confound the association between the risk for pancreatic cancer and the use of OC[10]. Similarly, studies investigating nutrition and pancreatic cancer risk should adjust for reproductive factors such as the use of OC.

Subgroup analyses identified a significantly lower risk of pancreatic cancer in women in European region who used OC (RR = 0.67, 95%CI: 0.51-0.88). Worldwide, highest incidence of pancreatic cancer in women was noted in Northern America, Western and Northern Europe[7]. Prevalence of OC use was the highest in Northern America, followed by Europe and Asia at 75%, 69% and 68%, respectively[38]. The differences in pancreatic cancer risk associated with the use of OC between different geographic regions could be explained by differences in exposure to environmental risk factors, genetic or cultural differences. Notably, most of the studies pooled in this meta-analysis have included relevant known or potential risk factors as covariates in the adjustments of risk estimates. It is also possible that regional differences in diagnosis and outcome assessment and reporting could contribute to the observed significant difference in subgroup analyses by geographic region. Possible explanations for the observed inverse relationship between the use of OC and pancreatic cancer in postmenopausal women could be related to the age of menopause, duration of menopause, duration of use of OC, and formulation of used OC.

Our literature search revealed one previously published meta-analysis that assessed the association between pancreatic cancer risk and female hormonal and menstrual factors[12], and one pooled analysis from the international pancreatic cancer case-control consortium[39]. In contrast to our results, a previous meta-analysis found no

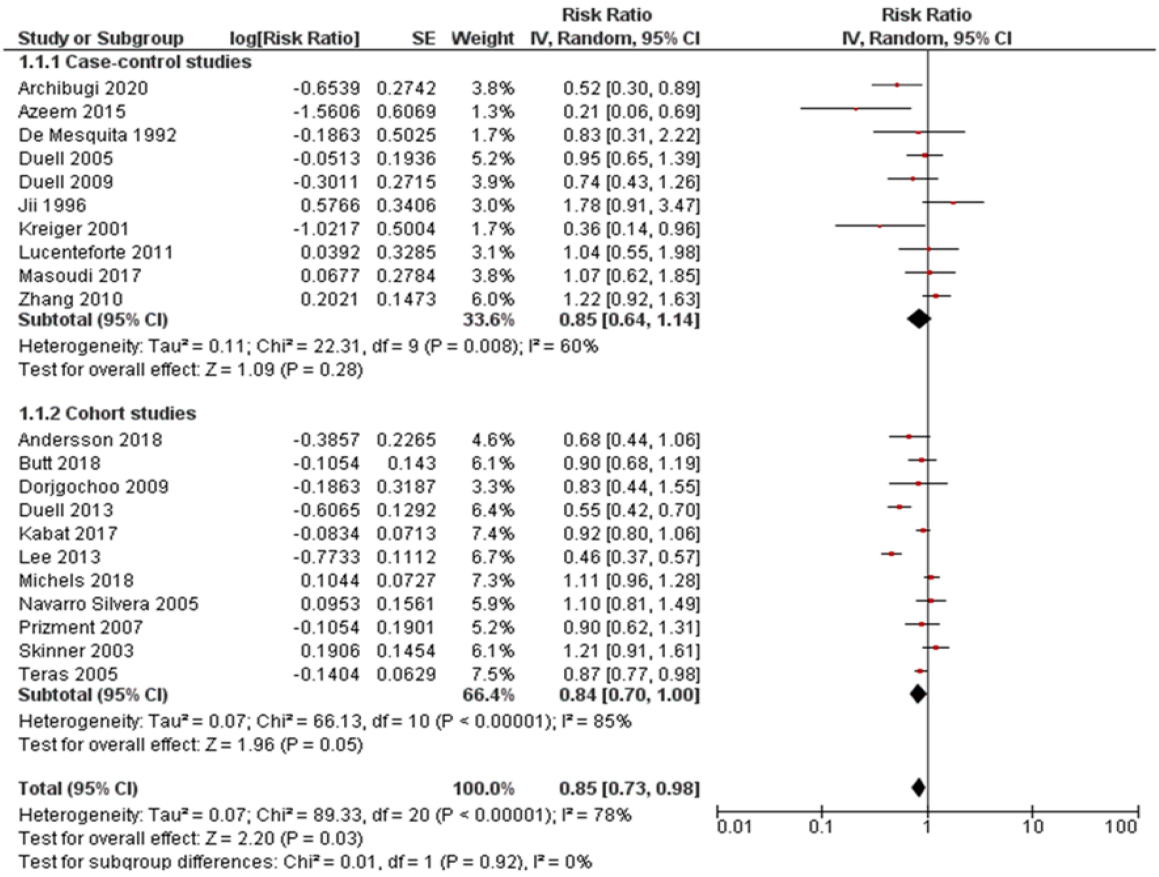


Figure 2 Forest plot of the association between the use of oral contraceptives and risk of pancreatic cancer. CI: Confidence interval.

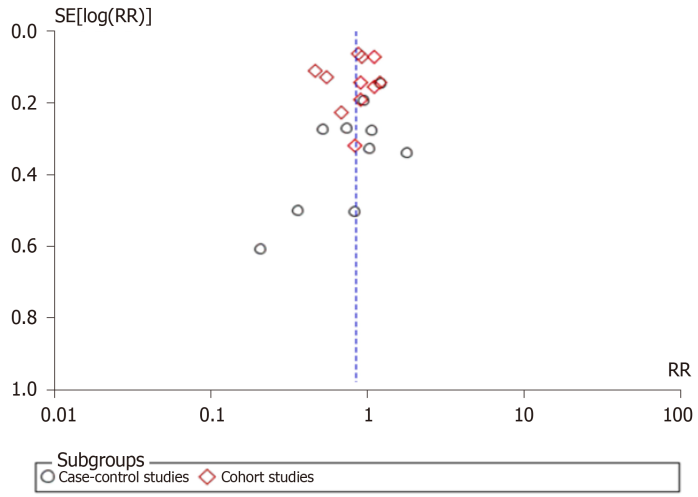
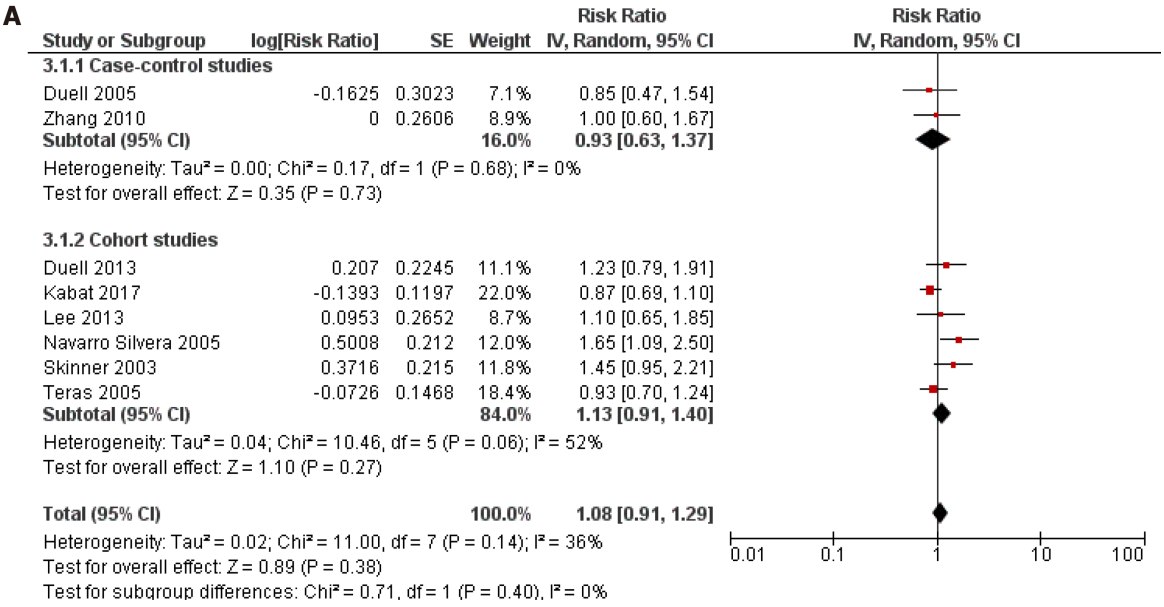


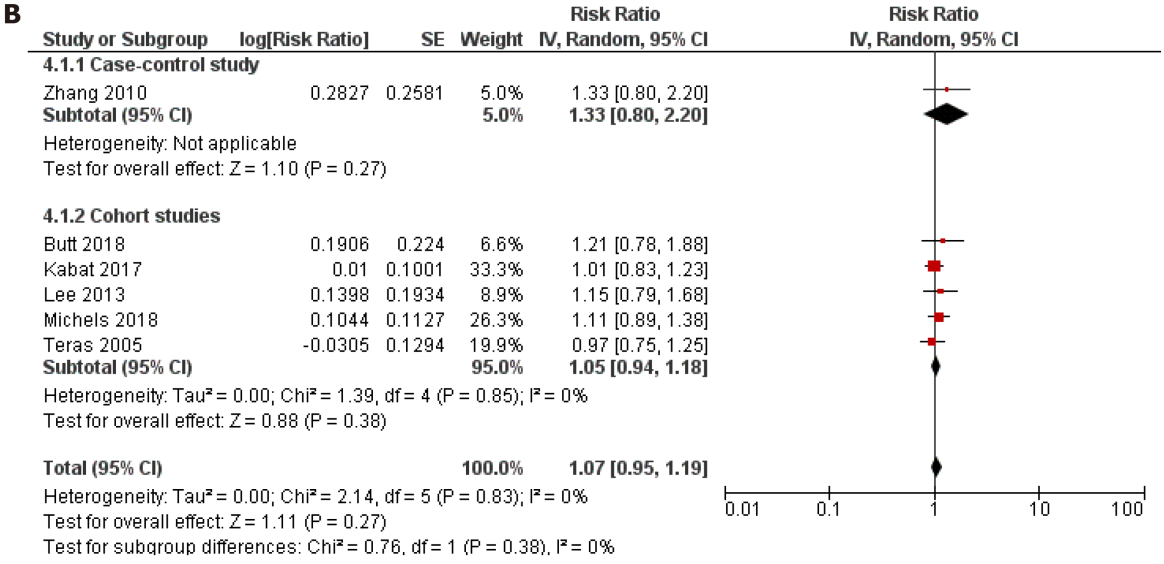
Figure 3 Funnel plot of studies investigating the use of oral contraceptives and risk of pancreatic cancer. RR: Relative risk.

significant associations between the risk of pancreatic cancer and OC use–pooled RR from six case-control studies and eight cohort studies was 1.09 (95%CI: 0.96-1.23)[12]. Subsequently, the authors noted that their subgroup analyses by study design showed a marginally significant increased risk of pancreatic cancer associated with OC use in cohort studies (RR = 1.09, 95%CI: 1.00-1.29). Our subgroup analyses by study design identified the opposite, namely, a borderline insignificant result for inverse association (RR = 0.84, 95%CI: 0.70-1.00). However, the authors identified publication bias for studies on exposure to OC, which could mask the true association. Our meta-analysis included an additional 4 case-control studies and 3 cohort studies, totaling 7700 cases of pancreatic cancer *vs* 5084 in the previous meta-analysis, and our analysis did not identify publication bias. Also, our study did not have language limitations in the

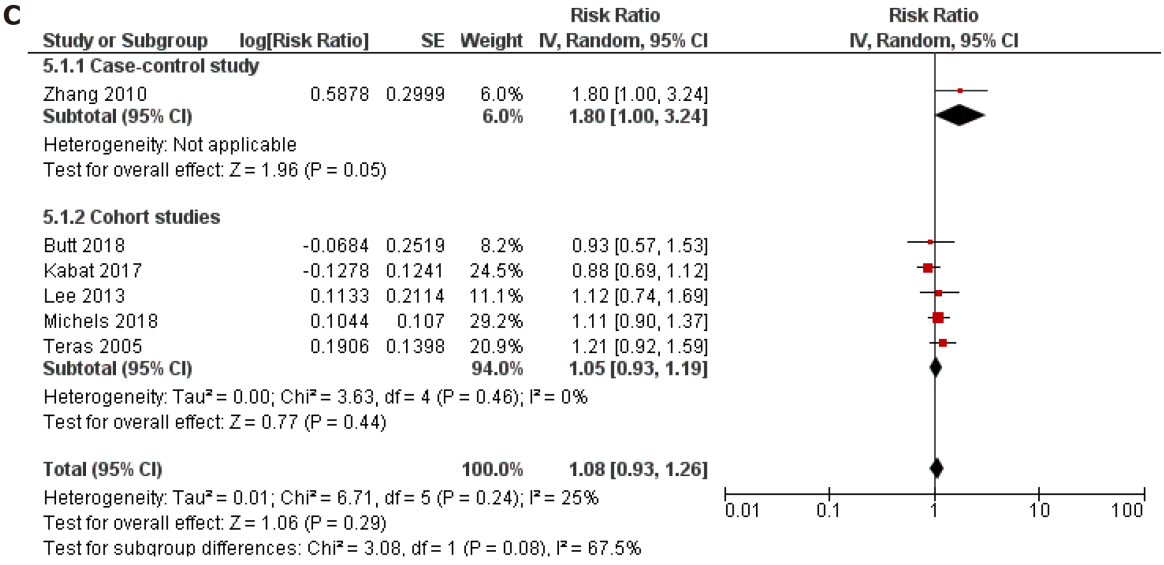
A


Study or Subgroup log[Risk Ratio] SE Weight Risk Ratio IV, Random, 95% CI
3.1.1 Case-control studies
Duell 2005 -0.1625 0.3023 7.1% 0.85 [0.47, 1.54]
Zhang 2010 0 0.2606 8.9% 1.00 [0.60, 1.67]
Subtotal (95% CI) 16.0% 0.93 [0.63, 1.37]
Heterogeneity: Tau² = 0.00; Chi² = 0.17, df = 1 (P = 0.68); I² = 0%
Test for overall effect: Z = 0.35 (P = 0.73)
3.1.2 Cohort studies
Duell 2013 0.207 0.2245 11.1% 1.23 [0.79, 1.91]
Kabat 2017 -0.1393 0.1197 22.0% 0.87 [0.69, 1.10]
Lee 2013 0.0953 0.2652 8.7% 1.10 [0.65, 1.85]
Navarro Silvera 2005 0.5008 0.212 12.0% 1.65 [1.09, 2.50]
Skinner 2003 0.3716 0.215 11.8% 1.45 [0.95, 2.21]
Teras 2005 -0.0726 0.1468 18.4% 0.93 [0.70, 1.24]
Subtotal (95% CI) 84.0% 1.13 [0.91, 1.40]
Heterogeneity: Tau² = 0.04; Chi² = 10.46, df = 5 (P = 0.06); I² = 52%
Test for overall effect: Z = 1.10 (P = 0.27)
Total (95% CI) 100.0% 1.08 [0.91, 1.29]
Heterogeneity: Tau² = 0.02; Chi² = 11.00, df = 7 (P = 0.14); I² = 36%
Test for overall effect: Z = 0.89 (P = 0.38)
Test for subgroup differences: Chi² = 0.71, df = 1 (P = 0.40), I² = 0%
Risk Ratio IV, Random, 95% CI
0.01 0.1 1 10 100

B


Study or Subgroup log[Risk Ratio] SE Weight Risk Ratio IV, Random, 95% CI
4.1.1 Case-control study
Zhang 2010 0.2827 0.2581 5.0% 1.33 [0.80, 2.20]
Subtotal (95% CI) 5.0% 1.33 [0.80, 2.20]
Heterogeneity: Not applicable
Test for overall effect: Z = 1.10 (P = 0.27)
4.1.2 Cohort studies
Butt 2018 0.1906 0.224 6.6% 1.21 [0.78, 1.88]
Kabat 2017 0.01 0.1001 33.3% 1.01 [0.83, 1.23]
Lee 2013 0.1398 0.1934 8.9% 1.15 [0.79, 1.68]
Michels 2018 0.1044 0.1127 26.3% 1.11 [0.89, 1.38]
Teras 2005 -0.0305 0.1294 19.9% 0.97 [0.75, 1.25]
Subtotal (95% CI) 95.0% 1.05 [0.94, 1.18]
Heterogeneity: Tau² = 0.00; Chi² = 1.39, df = 4 (P = 0.85); I² = 0%
Test for overall effect: Z = 0.88 (P = 0.38)
Total (95% CI) 100.0% 1.07 [0.95, 1.19]
Heterogeneity: Tau² = 0.00; Chi² = 2.14, df = 5 (P = 0.83); I² = 0%
Test for overall effect: Z = 1.11 (P = 0.27)
Test for subgroup differences: Chi² = 0.76, df = 1 (P = 0.38), I² = 0%
Risk Ratio IV, Random, 95% CI
0.01 0.1 1 10 100

C


Study or Subgroup log[Risk Ratio] SE Weight Risk Ratio IV, Random, 95% CI
5.1.1 Case-control study
Zhang 2010 0.5878 0.2999 6.0% 1.80 [1.00, 3.24]
Subtotal (95% CI) 6.0% 1.80 [1.00, 3.24]
Heterogeneity: Not applicable
Test for overall effect: Z = 1.96 (P = 0.05)
5.1.2 Cohort studies
Butt 2018 -0.0684 0.2519 8.2% 0.93 [0.57, 1.53]
Kabat 2017 -0.1278 0.1241 24.5% 0.88 [0.69, 1.12]
Lee 2013 0.1133 0.2114 11.1% 1.12 [0.74, 1.69]
Michels 2018 0.1044 0.107 29.2% 1.11 [0.90, 1.37]
Teras 2005 0.1906 0.1398 20.9% 1.21 [0.92, 1.59]
Subtotal (95% CI) 94.0% 1.05 [0.93, 1.19]
Heterogeneity: Tau² = 0.00; Chi² = 3.63, df = 4 (P = 0.46); I² = 0%
Test for overall effect: Z = 0.77 (P = 0.44)
Total (95% CI) 100.0% 1.08 [0.93, 1.26]
Heterogeneity: Tau² = 0.01; Chi² = 6.71, df = 5 (P = 0.24); I² = 25%
Test for overall effect: Z = 1.06 (P = 0.29)
Test for subgroup differences: Chi² = 3.08, df = 1 (P = 0.08), I² = 67.5%
Risk Ratio IV, Random, 95% CI
0.01 0.1 1 10 100

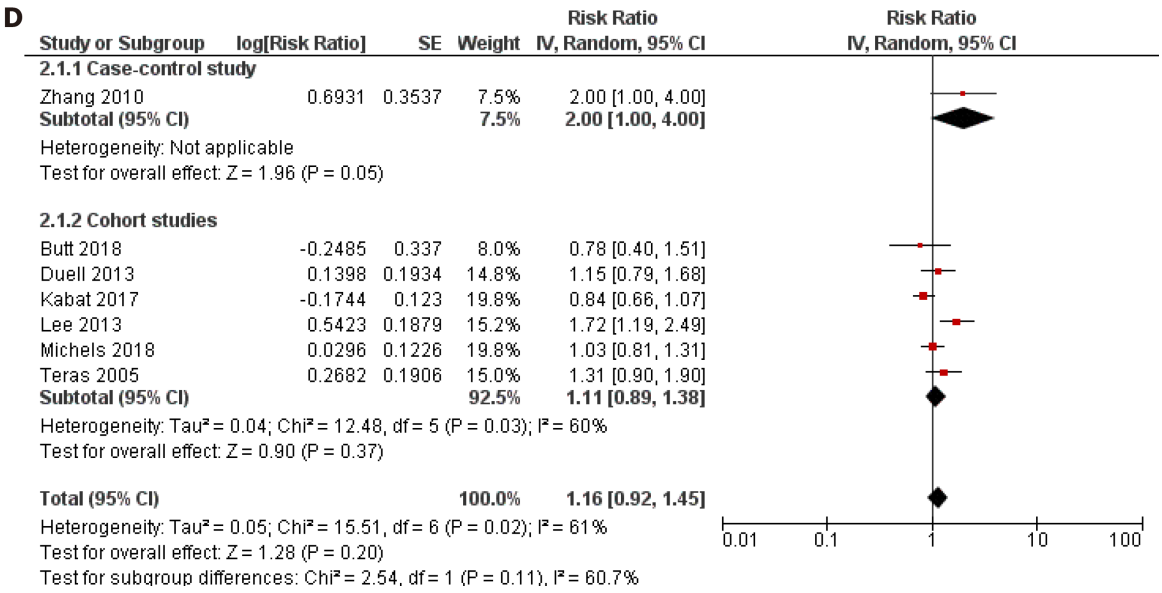


Figure 4 Forest plot of the association between pancreatic cancer risk and duration of oral contraceptives use. A: Less than 1 year; B: Less than 5 years; C: 5-10 years; D: Longer than 10 years. CI: Confidence interval.

literature search in contrast to previously conducted meta-analyses. A previous meta-analysis did not assess the quality of included studies, unlike our study that also explored subgroup differences in relation to study quality and found a statistically significant reduction in pancreatic cancer risk associated with the use of OC (RR = 0.67, 95%CI: 0.51-0.88) when pooling results from studies of high quality. The previous pooled analysis that included only case-control studies did not find a significant association between the ever use of OC and pancreatic cancer (OR = 0.83, 95%CI: 0.69-1.01).

Finally, studies included in this meta-analysis have been conducted in different time periods and the influence of different formulations of OC cannot be excluded. However, only one of the included studies inquired about the type of OC used by women[35], and did not find a significant association between pancreatic cancer and use of different types of hormonal contraceptives (RR = 0.92, 95%CI: 0.62-1.36 for oral combined 20-40 ug ethinyl estradiol, and RR = 1.16, 95%CI: 0.71-1.89 for progestin only). Further on, two studies made assumptions regarding the dose of OC by using the calendar year of use as a proxy for whether women were taking high-dose or low dose formulations[32,34]. Andersson *et al*[34] did not find a significant association between the risk of pancreatic cancer and use of OC in 1960-1970, after 1970, 1960-1980 or after 1980. However, Lee *et al*[32] found that a duration dose was present for high-dose use of OC (women who stopped using OC before 1974, *P* for trend 0.027), and in particular the risk for pancreatic cancer was increased in women using high-dose OC ≥ 10 years (RR = 2.08, 95%CI: 1.05-4.12). Due to the inconsistent results obtained by case-control and cohort studies, it is important to address this issue when planning future observational studies in order to provide further insight into the association between the use of OC and risk of pancreatic cancer.

Strengths and limitations

To the best of our knowledge, this represents the most comprehensive meta-analysis of observational studies which have investigated the association between the use of OC and risk of pancreatic cancer to date. Our analysis included 21 published studies with 7700 from various geographic regions which could add to the generalizability of the presented results. Also, the quality of included studies was relatively high and assessment of outcome involved pathohistological confirmation in majority of the cases.

However, our study had several limitations. First, this was a meta-analysis of observational studies and inherit limitations of study design of included studies cannot be excluded. Second, assessment of exposure was mostly based on self-report or obtained through interview, with only one study investigating the use of OC through a national medicinal registry. Further on, even though we applied a random-effects model in our meta-analyses, a high level of heterogeneity was found in some comparisons in our analyses, which we tried to explore by performing subgroup

analyses. We did not assess the association between pancreatic cancer and age at initiation of OC use, years since last use and intensity of use because only two studies reported this data and used different cut-offs. Our analyses pooled risk estimates adjusted for most potential cofactors as available in original studies, however it is not possible to exclude the influence of some confounding factors. Namely, the use of OC could be related with a higher socio-economic status which can in turn be related to a healthier diet[25]. Presence of bias due to the lack of confounding control cannot be excluded in included studies which have not considered dietary factors as possible confounding factors when investigating the association between the use of OC and risk for pancreatic cancer. Further on, for a few studies for which we needed to derive the necessary risk estimates there were no available adjusted risk estimates. Finally, an analysis of individual-patient data would have provided more precise results regarding the association between the use of OC and risk of pancreatic cancer.

CONCLUSION

Ever use of OC was associated with a decreased risk of pancreatic cancer in the present meta-analysis. However, more well-designed and detailed epidemiological studies are necessary in order to fully elucidate the association between the use of OC and pancreatic cancer.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cancer is the seventh most common cause of death among malignant tumors in women. It represents one of the deadliest types of cancer with overall 5-year survival rate < 10%.

Research motivation

Although the understanding of the etiology of pancreatic cancer has improved over the past decades and certain risk factors have been established, the causes of pancreatic cancer are still insufficiently known. Results of epidemiological studies show conflicting results regarding the association of the use of oral contraceptives (OC) and risk for pancreatic cancer.

Research objectives

The aim of this study was to evaluate the relationship between the use of OC and risk for pancreatic cancer.

Research methods

A comprehensive literature search was performed based on defined inclusion and exclusion criteria. Quality of included observational studies was assessed and data was extracted. A meta-analysis of ever-use *vs* never-use of OC and risk for pancreatic cancer was performed using Review Manager 5.3. In addition, the association between the duration of use of OC and pancreatic cancer risk was also assessed, and a subgroup analysis was performed.

Research results

A total of 7700 cases of pancreatic cancer from 21 studies (10 case-control and 11 cohort) were included in this meta-analysis. A significant association was observed between the ever-use of OC and pancreatic cancer risk (relative risk = 0.85; 95% confidence interval: 0.73-0.98), while the duration of use (< 1 year, < 5 years, 5-10 years, > 10 years) did not show a significant association. Subgroup analysis revealed a statistically significant decrease in pancreatic cancer and use of OC in high quality studies, studies conducted in Europe and in postmenopausal women.

Research conclusions

This meta-analysis suggests a protective effect of the use of OC and pancreatic cancer occurrence, however more epidemiological studies are necessary to fully elucidate this association.

Research perspectives

Further epidemiological studies are warranted to fully assess the association between the use of OC and risk for pancreatic cancer. These future studies investigating the risk for pancreatic cancer should be well-designed and include detailed questions regarding the use of OC.

REFERENCES

1 **International Agency for Research on Cancer**. Cancer Incidence in Five Continents. Vol. XI (electronic version). Lyon: International Agency for Research on Cancer. [cited 20 January 2021]. Available from: http://ci5.iarc.fr

2 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

3 **Ilić M**, Vlajinac H, Marinković J, Kocev N. Pancreatic cancer mortality in Serbia from 1991-2010 - a joinpoint analysis. *Croat Med J* 2013; **54**: 369-375 [PMID: 23986278 DOI: 10.3325/cmj.2013.54.369]

4 **GBD 2017 Pancreatic Cancer Collaborators**. The global, regional, and national burden of pancreatic cancer and its attributable risk factors in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Gastroenterol Hepatol* 2019; **4**: 934-947 [PMID: 31648972 DOI: 10.1016/S2468-1253(19)30347-4]

5 **Zanini S**, Renzi S, Limongi AR, Bellavite P, Giovinazzo F, Bermano G. A review of lifestyle and environment risk factors for pancreatic cancer. *Eur J Cancer* 2021; **145**: 53-70 [PMID: 33423007 DOI: 10.1016/j.ejca.2020.11.040]

6 **Lin QJ**, Yang F, Jin C, Fu DL. Current status and progress of pancreatic cancer in China. *World J Gastroenterol* 2015; **21**: 7988-8003 [PMID: 26185370 DOI: 10.3748/wjg.v21.i26.7988]

7 **Ilic M**, Ilic I. Epidemiology of pancreatic cancer. *World J Gastroenterol* 2016; **22**: 9694-9705 [PMID: 27956793 DOI: 10.3748/wjg.v22.i44.9694]

8 **Lucenteforte E**, Zucchetto A, Bosetti C, Talamini R, Negri E, Serraino D, Franceschi S, Lipworth L, La Vecchia C. Reproductive and hormonal factors and pancreatic cancer risk in women. *Pancreas* 2011; **40**: 460-463 [PMID: 21343831 DOI: 10.1097/MPA.0b013e31820bf986]

9 **Masoudi S**, Momayez Sanat Z, Mahmud Saleh A, Nozari N, Ghamarzad N, Pourshams A. Menstrual and Reproductive Factors and Risk of Pancreatic Cancer in Women. *Middle East J Dig Dis* 2017; **9**: 146-149 [PMID: 28894516 DOI: 10.15171/mejdd.2017.65]

10 **Kreiger N**, Lacroix J, Sloan M. Hormonal factors and pancreatic cancer in women. *Ann Epidemiol* 2001; **11**: 563-567 [PMID: 11709276 DOI: 10.1016/s1047-2797(01)00219-8]

11 **Azeem K**, Horáková D, Tomášková H, Ševčíková J, Vlčková J, Pastucha D, Procházka V, Shonová O, Martínek A, Janout V, Žídková V, Kollárová H. [A multifactor epidemiological analysis of risk factors for pancreatic cancer in women]. *Epidemiol Mikrobiol Imunol* 2015; **64**: 34-40 [PMID: 25872994]

12 **Tang B**, Lv J, Li Y, Yuan S, Wang Z, He S. Relationship between female hormonal and menstrual factors and pancreatic cancer: a meta-analysis of observational studies. *Medicine (Baltimore)* 2015; **94**: e177 [PMID: 25700305 DOI: 10.1097/MD.0000000000000177]

13 **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009; **6**: e1000097 [PMID: 19621072 DOI: 10.1371/journal.pmed.1000097]

14 **Wells GA**, Shea B, O'Connell D, Peterson J, Welch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. Ottawa, ON: Ottawa Hospital Research Institute, 2014. [cited 20 January 2021]. Available from: https://www.ohri.ca/programs/clinical_epidemiology/oxford.asp

15 **Greenland S**. Quantitative methods in the review of epidemiologic literature. *Epidemiol Rev* 1987; **9**: 1-30 [PMID: 3678409 DOI: 10.1093/oxfordjournals.epirev.a036298]

16 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188 [PMID: 3802833 DOI: 10.1016/0197-2456(86)90046-2]

17 **Higgins JPT**, Green S (editors). Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0. The Cochrane Collaboration, 2011. [cited 20 January 2021]. Available from: https://www.handbook.cochrane.org

18 **Richardson M**, Garner P, Donegan S. Interpretation of subgroup analyses in systematic reviews: a tutorial. *Clin Epidemiol Glob Health* 2019; **7**: 192-198 [DOI: 10.1016/j.cegh.2018.05.005]

19 **RevMan**. 2014 [Computer program] Nordic Cochrane Centre, The Cochrane Collaboration. Review Manager (RevMan). Version 5.3. Copenhagen: Nordic Cochrane Centre, The Cochrane Collaboration, 2014

20 **Bueno de Mesquita HB**, Maisonneuve P, Moerman CJ, Walker AM. Anthropometric and reproductive variables and exocrine carcinoma of the pancreas: a population-based case-control study in The Netherlands. *Int J Cancer* 1992; **52**: 24-29 [PMID: 1500223 DOI: 10.1002/ijc.2910520106]

21 **Ji BT**, Hatch MC, Chow WH, McLaughlin JK, Dai Q, Howe GR, Gao YT, Fraumeni JF Jr.

Anthropometric and reproductive factors and the risk of pancreatic cancer: a case-control study in Shanghai, China. *Int J Cancer* 1996; **66**: 432-437 [PMID: 8635856 DOI: 10.1002/(SICI)1097-0215(19960516)66:4<432::AID-IJC4>3.0.CO;2-X]

22 **Duell EJ**, Holly EA. Reproductive and menstrual risk factors for pancreatic cancer: a population-based study of San Francisco Bay Area women. *Am J Epidemiol* 2005; **161**: 741-747 [PMID: 15800266 DOI: 10.1093/aje/kwi104]

23 **Duell EJ**, Maisonneuve P, Baghurst PA, Bueno-de-Mesquita HB, Ghadirian P, Miller AB, Zatonski W, Vrieling A, Boffetta P, Boyle P. Menstrual and reproductive factors and pancreatic cancer in the SEARCH program of the IARC. *Cancer Causes Control* 2009; **20**: 1757-1762 [PMID: 19653108 DOI: 10.1007/s10552-009-9408-x]

24 **Zhang Y**, Coogan PF, Palmer JR, Strom BL, Rosenberg L. A case-control study of reproductive factors, female hormone use, and risk of pancreatic cancer. *Cancer Causes Control* 2010; **21**: 473-478 [PMID: 19941157 DOI: 10.1007/s10552-009-9478-9]

25 **Archibugi L**, Graglia B, Valente R, Stigliano S, Roberto M, Capalbo C, Marchetti P, Nigri G, Capurso G. Gynecological and reproductive factors and the risk of pancreatic cancer: A case-control study. *Pancreatology* 2020; **20**: 1149-1154 [PMID: 32800645 DOI: 10.1016/j.pan.2020.07.398]

26 **Skinner HG**, Michaud DS, Colditz GA, Giovannucci EL, Stampfer MJ, Willett WC, Fuchs CS. Parity, reproductive factors, and the risk of pancreatic cancer in women. *Cancer Epidemiol Biomarkers Prev* 2003; **12**: 433-438 [PMID: 12750238]

27 **Teras LR**, Patel AV, Rodriguez C, Thun MJ, Calle EE. Parity, other reproductive factors, and risk of pancreatic cancer mortality in a large cohort of U.S. women (United States). *Cancer Causes Control* 2005; **16**: 1035-1040 [PMID: 16184468 DOI: 10.1007/s10552-005-0332-4]

28 **Navarro Silvera SA**, Miller AB, Rohan TE. Hormonal and reproductive factors and pancreatic cancer risk: a prospective cohort study. *Pancreas* 2005; **30**: 369-374 [PMID: 15841050 DOI: 10.1097/01.mpa.0000160301.59319.ba]

29 **Prizment AE**, Anderson KE, Hong CP, Folsom AR. Pancreatic cancer incidence in relation to female reproductive factors: Iowa Women's Health Study. *JOP* 2007; **8**: 16-27 [PMID: 17228129]

30 **Dorjgochoo T**, Shu XO, Li HL, Qian HZ, Yang G, Cai H, Gao YT, Zheng W. Use of oral contraceptives, intrauterine devices and tubal sterilization and cancer risk in a large prospective study, from 1996 to 2006. *Int J Cancer* 2009; **124**: 2442-2449 [PMID: 19170208 DOI: 10.1002/ijc.24232]

31 **Duell EJ**, Travier N, Lujan-Barroso L, Dossus L, Boutron-Ruault MC, Clavel-Chapelon F, Tumino R, Masala G, Krogh V, Panico S, Ricceri F, Redondo ML, Dorronsoro M, Molina-Montes E, Huerta JM, Barricarte A, Khaw KT, Wareham NJ, Allen NE, Travis R, Siersema PD, Peeters PH, Trichopoulou A, Fragogeorgi E, Oikonomou E, Boeing H, Schuetze M, Canzian F, Lukanova A, Tjønneland A, Roswall N, Overvad K, Weiderpass E, Gram IT, Lund E, Lindkvist B, Johansen D, Ye W, Sund M, Fedirko V, Jenab M, Michaud DS, Riboli E, Bueno-de-Mesquita HB. Menstrual and reproductive factors in women, genetic variation in CYP17A1, and pancreatic cancer risk in the European prospective investigation into cancer and nutrition (EPIC) cohort. *Int J Cancer* 2013; **132**: 2164-2175 [PMID: 23015357 DOI: 10.1002/ijc.27875]

32 **Lee E**, Horn-Ross PL, Rull RP, Neuhausen SL, Anton-Culver H, Ursin G, Henderson KD, Bernstein L. Reproductive factors, exogenous hormones, and pancreatic cancer risk in the CTS. *Am J Epidemiol* 2013; **178**: 1403-1413 [PMID: 24008905 DOI: 10.1093/aje/kwt154]

33 **Kabat GC**, Kamensky V, Rohan TE. Reproductive factors, exogenous hormone use, and risk of pancreatic cancer in postmenopausal women. *Cancer Epidemiol* 2017; **49**: 1-7 [PMID: 28521283 DOI: 10.1016/j.canep.2017.05.002]

34 **Andersson G**, Borgquist S, Jirström K. Hormonal factors and pancreatic cancer risk in women: The Malmö Diet and Cancer Study. *Int J Cancer* 2018; **143**: 52-62 [PMID: 29424426 DOI: 10.1002/ijc.31302]

35 **Butt SA**, Lidegaardi Ø, Skovlund C, Hannaford PC, Iversen L, Fielding S, Mørch LS. Hormonal contraceptive use and risk of pancreatic cancer-A cohort study among premenopausal women. *PLoS One* 2018; **13**: e0206358 [PMID: 30376560 DOI: 10.1371/journal.pone.0206358]

36 **Michels KA**, Brinton LA, Pfeiffer RM, Trabert B. Oral Contraceptive Use and Risks of Cancer in the NIH-AARP Diet and Health Study. *Am J Epidemiol* 2018; **187**: 1630-1641 [PMID: 29394309 DOI: 10.1093/aje/kwx388]

37 **Wahi MM**, Shah N, Schrock CE, Rosemurgy AS 2nd, Goldin SB. Reproductive factors and risk of pancreatic cancer in women: a review of the literature. *Ann Epidemiol* 2009; **19**: 103-111 [PMID: 19185803 DOI: 10.1016/j.annepidem.2008.11.003]

38 **United Nations**. United Nations, Department of Economic and Social Affairs, Population Division (2015). Trends in Contraceptive Use Worldwide 2015 (ST/ESA/SER.A/349). [cited 20 January 2021]. Available from: https://www.un.org/development/desa/pd/sites/www.un.org.development.desa.pd/files/undesa_pd_report_2015_trends_contraceptive_use.pdf

39 **Lujan-Barroso L**, Zhang W, Olson SH, Gao YT, Yu H, Baghurst PA, Bracci PM, Bueno-de-Mesquita HB, Foretová L, Gallinger S, Holcatova I, Janout V, Ji BT, Kurtz RC, La Vecchia C, Lagiou P, Li D, Miller AB, Serraino D, Zatonski W, Risch HA, Duell EJ. Menstrual and Reproductive Factors, Hormone Use, and Risk of Pancreatic Cancer: Analysis From the International Pancreatic Cancer Case-Control Consortium (PanC4). *Pancreas* 2016; **45**: 1401-1410 [PMID: 27088489 DOI: 10.1097/MPA.0000000000000635]

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CASE REPORT

Cyclophosphamide-associated enteritis presenting with severe protein-losing enteropathy in granulomatosis with polyangiitis: A case report

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Abstract

BACKGROUND

Although cyclophosphamide (CPA) is the key drug for the treatment of autoimmune diseases including vasculitides, it has some well-known adverse effects, such as myelosuppression, hemorrhagic cystitis, infertility, and infection. However, CPA-associated severe enteritis is a rare adverse effect, and only one case with a lethal clinical course has been reported. Therefore, the appropriate management of patients with CPA-associated severe enteritis is unclear.

CASE SUMMARY

We present the case of a 61-year-old woman diagnosed with granulomatosis with polyangiitis based on the presence of symptoms in ear, lung, and, kidney with positive myeloperoxidase-antineutrophil cytoplasmic antibody. She received pulsed methylprednisolone followed by prednisolone 55 mg/d and intravenous CPA at a dose of 500 mg/mo. Ten days after the second course of intravenous CPA, she developed nausea, vomiting, and diarrhea, and was admitted to the hospital. Laboratory testing revealed hypoalbuminemia, suggesting protein-losing enteropathy. Computed tomography revealed wall thickening of the stomach, small intestine, and colon with contrast enhancement on the lumen side. Antibiotics and immunosuppressive therapy were not effective, and the patient's enteritis did not improve for > 4 mo. Because her condition became seriously exhausted, corticosteroids were tapered and supportive therapies including intravenous hyperalimentation, replenishment of albumin and gamma globulin, plasma exchange, and infection control were continued. These supportive therapies improved her condition, and her enteritis gradually regressed. She was finally discharged 7 mo later.

CONCLUSION

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Immediate discontinuation of CPA and intensive supportive therapy are crucial for the survival of patients with CPA-associated severe enteritis.

Key Words: Antineutrophil cytoplasmic antibody; Cyclophosphamide; Enteritis; Granulomatosis with polyangiitis; Plasma exchange; Vasculitis; Case report

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Core Tip: Cyclophosphamide-associated enteritis is rare, but a fatal complication of the treatment for the vasculitides. This is the first successful report of the treatment for the severe cyclophosphamide-associated enteritis, and indicates the importance of immediate discontinuation of cyclophosphamide and intensive supportive therapy.

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INTRODUCTION

Cyclophosphamide (CPA) is an alkylating agent that is frequently used in vasculitides as an induction therapy. Particularly in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, the addition of immunosuppressants is recommended for patients with a higher five-factor score[1]. CPA is metabolized into the active form in the liver and covalently binds to the nucleus[2]. Thereafter, it manifests antiproliferative effects by inhibiting deoxyribonucleic acid replication[2]. Although CPA has an established therapeutic effect in vasculitides, it sometimes results in adverse effects including myelosuppression, hemorrhagic cystitis, increased susceptibility to infection, and infertility[3]. However, the development of enteritis due to CPA use in patients with autoimmune diseases is rare, with only one case report in which Yang *et al*[4] presented a lethal case of CPA-associated enteritis in a patient with microscopic polyangiitis. Here, we report a severe case of CPA-associated enteritis in a patient who presented with prominent protein-losing enteropathy, who was successfully treated with supportive therapy including plasma exchange (PE).

CASE PRESENTATION

Chief complaints

A 61-year-old Japanese woman developed nausea, vomiting, and diarrhea.

History of present illness

Three months before the first admission, she experienced nasal congestion, aural fullness, and auditory disturbance, and was diagnosed with otitis media. Also, two months before presentation, she experienced cough worsening, and chest radiography revealed a pulmonary infiltrative shadow. Two months before presentation, she was referred to our hospital for the evaluation of pulmonary consolidation with positive myeloperoxidase-ANCA (MPO-ANCA). She was admitted to the previous hospital. She had a body temperature of 38 °C and elevated C-reactive protein level (7.7 mg/dL). Although she was treated with antibiotics, her symptoms, inflammatory markers, and chest infiltrates did not improve. Laboratory examination showed a high titer of MPO-ANCA (Table 1), and she was referred to our hospital. After admission, her creatinine level increased with proteinuria, glomerular hematuria, and granular casts, indicating the presence of rapidly progressive glomerulonephritis (RPGN). Granulomatosis with polyangiitis (GPA) was diagnosed on the basis of the presence of otitis media, sinusitis, pulmonary nodule, RPGN, and positive MPO-ANCA. She received pulsed methylprednisolone followed by prednisolone (PSL) 55 mg/d in

Table 1 Laboratory findings

	First admission	Second admission
Urinalysis		
Protein	1+	2+
Occult blood	1+	-
Red blood cell	10-29/HPF[1]	< 4/HPF
Casts	+[2]	+[3]
Spot protein-creatinine ratio (g/g Cr)	0.38	0.20
WBC (/μL)	14500	6100
Hb (g/dL)	10.9	14.9
Plt (10^4/μL)	43.4	35.1
T-Bil (mg/dL)	0.3	0.8
AST (U/L)	25	20
ALT (U/L)	30	18
γ-GTP (U/L)	24	18
ALP (U/L)	239	102
LDH (U/L)	207	200
CK (U/L)	58	37
TP (g/dL)	7.1	4.3
Alb (g/dL)	2.6	2.5
BUN (mg/dL)	11	35
Cr (mg/dL)	0.5	0.7
CRP (mg/dL)	14.2	0.2
MPO-ANCA (U/mL)	194.0	1.0

[1]Deformed erythrocytes.
[2]Casts: waxy casts, leukocyte casts.
[3]Casts: granular casts.
Alb: Albumin; ANCA: Antineutrophil cytoplasmic antibody; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; CK: Creatine kinase; Cr: Creatinine; CRP: C-reactive protein; Hb: Hemoglobin; HPF: High-power field; LDH: Lactate dehydrogenase; MPO: Myeloperoxidase; Plt: Platelets; T-Bil: Total bilirubin; TP: Total protein; WBC: White blood cells.

combination with intravenous CPA at a dose of 500 mg/mo. Her condition significantly improved, and she was discharged 9 d after the second course of intravenous CPA when the PSL dose was 45 mg/d. She developed nausea, vomiting, and diarrhea the day after discharge, and was admitted to our hospital.

History of past illness
She had been diagnosed with Grave's disease since the age of 40.

Personal and family history
The patient had no family history.

Physical examination
On admission, her consciousness was clear, body temperature was 36.3 °C, and body pressure was 106/75 mmHg. Her abdomen was soft and flat, with pain in the left lower quadrant without defense or rebound tenderness.

Laboratory examinations
Laboratory testing indicated hypoalbuminemia and hypogammaglobulinemia, suggesting protein-losing enteropathy that resulted in the leakage of proteins from the gastrointestinal tract (Table 1).

Imaging examinations

Abdominal radiography did not show niveau or free air. Computed tomography (CT) revealed wall thickening of the stomach, small intestine, and colon with contrast enhancement on the lumen side (Figure 1A). The contrast enhancement of the outer layer of the intestine was poor, indicating edematous change. Upper gastrointestinal endoscopy showed edematous thickening of the stomach and diffusely distributed erosion throughout the descending duodenum (Figure 1B). Colonoscopy showed generalized edema and depression with erythema, mainly at the end of the ileum. The depression had no ulcerated surface.

Further diagnostic work-up

Biopsy showed the absence of malignancy and vasculitis, as well as neoplastic changes including hyperplasia. Mild inflammatory cellular infiltration and some granulation tissues and ulcers were present. Cytomegalovirus staining was negative. Because the differential diagnoses included infectious enteritis, GPA-associated gastroenteritis, and drug-induced enteritis, the patient was initially treated with antibiotics including meropenem. However, hypoalbuminemia progressed. Therefore, we replenished albumin and gamma globulin and performed PE. Her condition did not improve, and her serum albumin levels remained approximately 1.5 mg/dL for > 1 mo. This clinical course ruled out the presence of an infectious etiology. Therefore, we decided to augment immunosuppression, and increased the PSL dose to 60 mg/d and added tacrolimus. Nevertheless, the enteritis did not respond to these treatments, which was contradictory to the autoimmune phenomenon, including GPA-associated enteritis. At this point, infectious and vasculitic etiologies were unlikely.

FINAL DIAGNOSIS

The CPA-associated enteropathy was considered, as previously reported by Yang *et al*[4].

TREATMENT

Because her condition became seriously exhausted, we tapered the steroid dose and continued supportive therapy including intravenous hyperalimentation, albumin supplementation, PE, and infection control (Figure 2).

OUTCOME AND FOLLOW-UP

The supportive therapies improved the patient's condition, and her enteritis gradually regressed (Figure 3). She was finally discharged 7 mo after the second admission and underwent rehabilitation. At 2 years from discharge, she is now in complete remission and has returned to her previous work.

DISCUSSION

The present case was complicated with severe enteritis, which was considered to be an adverse effect of CPA. Although the patient had severe protein-losing enteropathy, her condition was improved by aggressive supportive therapies with repeated administrations of albumin and globulin for several months.

CPA is one of the frequently used immunosuppressant drugs for vasculitidis. Gastrointestinal involvement of GPA is one of the conditions requiring CPA[5]. However, CPA-associated enteritis is very rare, and there exists only one case describing a lethal course in a patient with GPA[4].

When the present patient manifested abdominal symptoms, her vasculitis was in remission, which was supported by the absence of ear symptoms, normal urinalysis, normal inflammatory marker levels, and negative MPO-ANCA. In addition, there were no histologic signs of vasculitis, granulomatous colitis, or gastritis in biopsy tissues. Further, treatment with immunosuppressants did not improve her condition, which was similar to the previously described case[4,6-8]. In addition, the blood

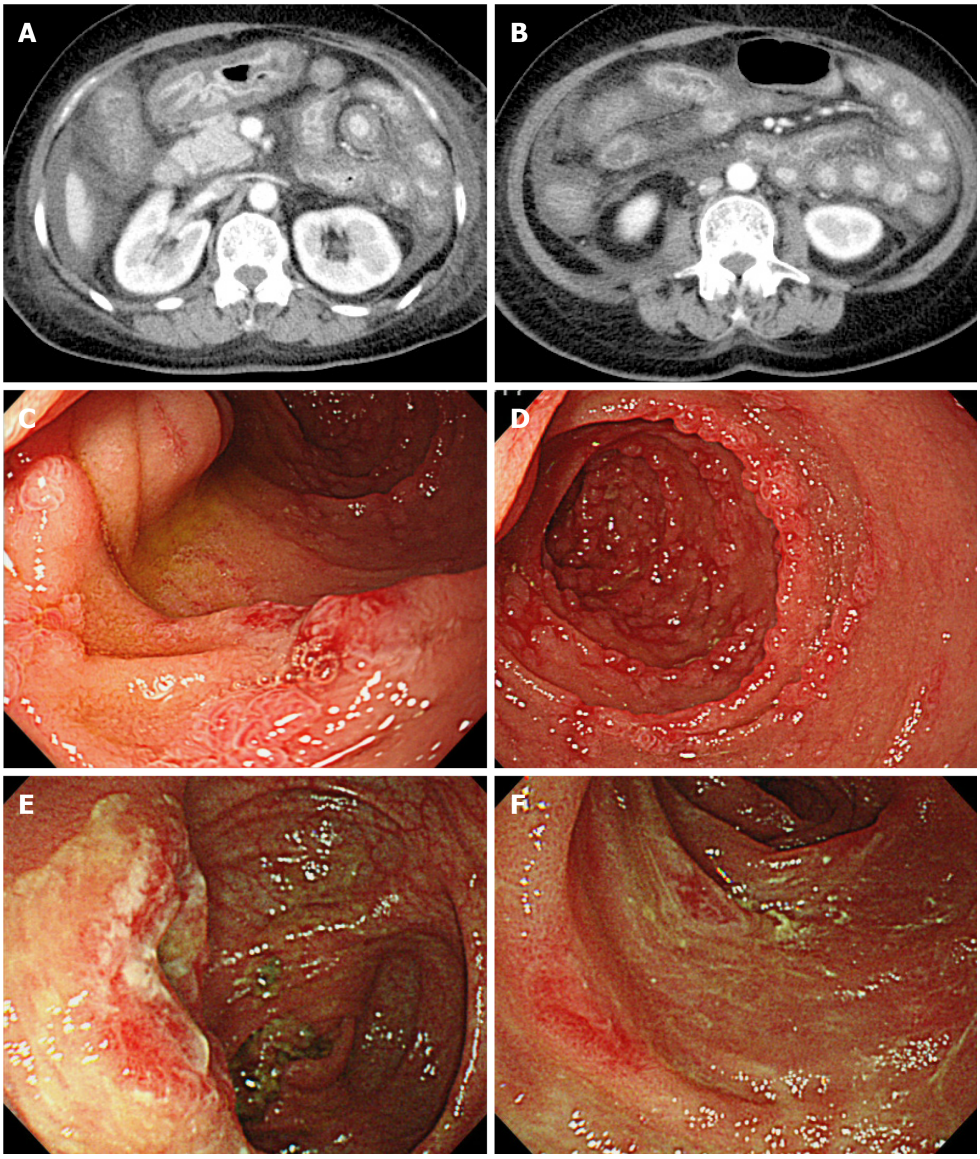


Figure 1 Clinical images on admission. A and B: Computed tomography scans of the abdomen showing diffuse wall thickening of the stomach, small intestine, and colon; C and D: Upper gastrointestinal endoscopy images showing diffuse erosion throughout the descending duodenum; E and F: Colonoscopy images showing generalized edema and depression with erythema mainly at the end of the ileum.

culture, fecal culture, *Clostridium difficile* toxin, and cytomegalovirus tests were all negative. Because it was possible that these test results were false negative, antibiotics were administered, which did not improve the enteritis.

The mechanisms by which CPA causes enteritis are not well defined. In the field of oncology, CPA is likely to be associated with gastrointestinal involvement and mucositis, which may be related to the gut microbiota[9]. In a mouse model, Viaud *et al*[10] reported that CPA altered the microbiota composition in the small intestine and induced the translocation of selected species of gram-positive bacteria into secondary lymphoid organs.

We compared the clinical characteristics between the present case and the previous case. In the present case, symptoms appeared 38 d after the first intravenous CPA (500 mg/mo), whereas the previous case showed symptoms 2 wk after the start of oral CPA (100 mg/d). Therefore, the cumulative dose of CPA at the onset of symptoms was 1 g in the present case and 1.4 g in the previous case. Gastrointestinal lesions were distributed in the stomach, duodenum, small bowel, and colon in the present case, whereas they were limited to the small bowel and colon in the previous case. In the present case, CT showed massive wall thickening with prominent contrast enhancement in the mucosa. The outer layer showed edematous thickening. Upper gastrointestinal endoscopy showed edematous thickening of the stomach and diffusely distributed erosion throughout the descending duodenum. Colonoscopy showed generalized edema from the transverse colon to the rectum. The clinical

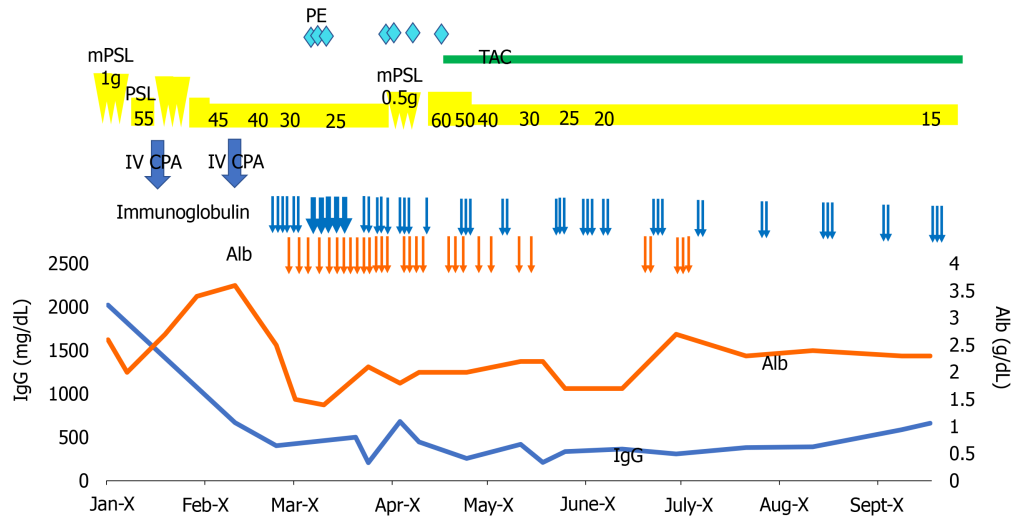


Figure 2 Clinical course. Alb: Albumin; IV CPA: Intravenous cyclophosphamide; mPSL: Methylprednisolone; PE: Plasma exchange; PSL: Prednisolone; TAC: Tacrolimus.

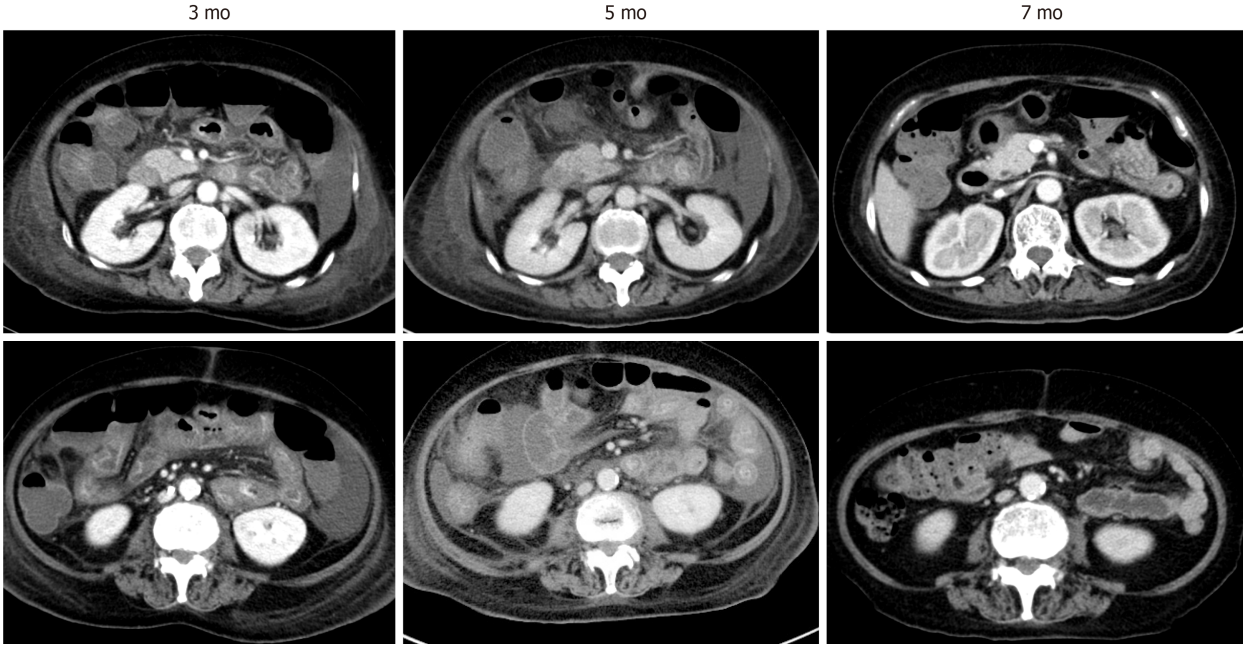


Figure 3 Improvement of computed tomography findings. The computed tomography images were taken at different time points after the second hospitalization, as indicated.

imaging findings in the previous case were similar to the diffuse mural thickening on CT and denuded and erythematous mucosa on endoscopy in the present case.

The difference from the previous report was that the present patient survived with intensive supportive therapy. In the previous case, CPA was continued for 1 mo with reduction to 50 mg/d after the complication of enteritis appeared, and then stopped. Therefore, the patient received 2.1 g CPA in total. In the present case, intravenous CPA was immediately stopped after the development of abdominal symptoms and the patient received 1 g CPA in total. Whether the cumulative dose of CPA influences the length of enteritis is uncertain, but the lower total CPA dose may be one of the reasons for the survival of the present patient. Nonetheless, this case confirmed that enteritis could develop not only with oral administration but also with intravenous injection of CPA.

With respect to treatment, no specific therapy exists for CPA-induced enteritis. However, the associated tissue injury is severe and can last for months, resulting in the loss of serum proteins including albumin and gamma globulin. Therefore, aggressive

supportive therapies including hyperalimentation, replenishment of albumin and gamma globulin, and PE in severe cases are important. Because continuation of CPA is difficult in patients who experienced CPA-induced enteritis, rituximab or azathioprine can be the alternative treatment for GPA.

CONCLUSION

In conclusion, severe enteritis is a rare but life-threatening adverse effect of CPA. Immediate discontinuation of CPA and persistent supportive treatment are crucial for survival.

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REFERENCES

1 **Guillevin L**, Pagnoux C, Seror R, Mahr A, Mouthon L, Toumelin PL; French Vasculitis Study Group (FVSG). The Five-Factor Score revisited: assessment of prognoses of systemic necrotizing vasculitides based on the French Vasculitis Study Group (FVSG) cohort. *Medicine (Baltimore)* 2011; **90**: 19-27 [PMID: 21200183 DOI: 10.1097/MD.0b013e318205a4c6]

2 **de Jonge ME**, Huitema AD, Rodenhuis S, Beijnen JH. Clinical pharmacokinetics of cyclophosphamide. *Clin Pharmacokinet* 2005; **44**: 1135-1164 [PMID: 16231966 DOI: 10.2165/00003088-200544110-00003]

3 **Ahmed AR**, Hombal SM. Cyclophosphamide (Cytoxan). A review on relevant pharmacology and clinical uses. *J Am Acad Dermatol* 1984; **11**: 1115-1126 [PMID: 6392368 DOI: 10.1016/s0190-9622(84)80193-0]

4 **Yang LS**, Cameron K, Papaluca T, Basnayake C, Jackett L, McKelvie P, Goodman D, Demediuk B, Bell SJ, Thompson AJ. Cyclophosphamide-associated enteritis: A rare association with severe enteritis. *World J Gastroenterol* 2016; **22**: 8844-8848 [PMID: 27818600 DOI: 10.3748/wjg.v22.i39.8844]

5 **Haworth SJ**, Pusey CD. Severe intestinal involvement in Wegener's granulomatosis. *Gut* 1984; **25**: 1296-1300 [PMID: 6500368 DOI: 10.1136/gut.25.11.1296]

6 **Eriksson P**, Segelmark M, Hallböök O. Frequency, Diagnosis, Treatment, and Outcome of Gastrointestinal Disease in Granulomatosis with Polyangiitis and Microscopic Polyangiitis. *J Rheumatol* 2018; **45**: 529-537 [PMID: 29419474 DOI: 10.3899/jrheum.170249]

7 **Chow FY**, Hooke D, Kerr PG. Severe intestinal involvement in Wegener's granulomatosis. *J Gastroenterol Hepatol* 2003; **18**: 749-750 [PMID: 12753164 DOI: 10.1046/j.1440-1746.2003.30472.x]

8 **Müller-Ladner U**. Vasculitides of the gastrointestinal tract. *Best Pract Res Clin Gastroenterol* 2001; **15**: 59-82 [PMID: 11355901 DOI: 10.1053/bega.2000.0156]

9 **Liu T**, Wu Y, Wang L, Pang X, Zhao L, Yuan H, Zhang C. A More Robust Gut Microbiota in Calorie-Restricted Mice Is Associated with Attenuated Intestinal Injury Caused by the Chemotherapy Drug Cyclophosphamide. *mBio* 2019; **10**: e02903-18 [PMID: 30862756 DOI: 10.1128/mBio.02903-18]

10 **Viaud S**, Saccheri F, Mignot G, Yamazaki T, Daillère R, Hannani D, Enot DP, Pfirschke C, Engblom C, Pittet MJ, Schlitzer A, Ginhoux F, Apetoh L, Chachaty E, Woerther PL, Eberl G, Bérard M, Ecobichon C, Clermont D, Bizet C, Gaboriau-Routhiau V, Cerf-Bensussan N, Opolon P, Yessaad N, Vivier E, Ryffel B, Elson CO, Doré J, Kroemer G, Lepage P, Boneca IG, Ghiringhelli F, Zitvogel L. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science* 2013; **342**: 971-976 [PMID: 24264990 DOI: 10.1126/science.1240537]

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FRONTIER

Cystic pancreatic lesions, the endless dilemma

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Abstract

Cystic pancreatic lesions involve a wide variety of pathological entities that include neoplastic and non-neoplastic lesions. The proper diagnosis, differentiation, and staging of these cystic lesions are considered a crucial issue in planning further management. There are great challenges for their diagnostic models. In our time, new emerging methods for this diagnosis have been discovered. Endoscopic ultrasonography-guided fine-needle aspiration cytology with chemical and molecular analysis of cyst fluid and EUS-guided fine needle-based confocal laser endomicroscopy, through the needle microforceps biopsy, and single-operator cho-langioscopy/pancreatoscopy are promising methods that have been used in the diagnosis of cystic pancreatic lesions. Hereby we discuss the diagnosis of cystic pancreatic lesions and the benefits of various diagnostic models.

Key Words: Pancreatic cystic lesion; Endoscopic diagnosis; Endoscopic ultrasonography; Cyst fluid markers; Endoscopic ultrasonography-guided fine needle-based confocal laser endomicroscopy; Through the needle microforceps biopsy; Single operator cholangioscopy/pancreatoscopy

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Core Tip: Pancreatic cystic lesions are commonly recognized with increasing frequency and have become a more common finding in clinical practice. Today, there has been an increased incidence in cystic pancreatic lesions that have in turn caused a lot of diagnostic modalities to emerge. We discuss the various methods of the diagnosis of cystic pancreatic lesions and compare these modalities.

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INTRODUCTION

Currently, pancreatic cystic lesions (PCLs) are commonly recognized with increasing frequency and have become a more common finding in clinical practice; their detection rate is rising with the advances in imaging technology, and there is an increased incidence of detection of unsuspected small PCLs[1]. The frequency of detection of pancreatic cysts reported in the literature ranges from 0.7% to 36.7%[2,3].

They comprise a broad spectrum of entities ranging from benign cysts to potentially malignant or malignant cystic lesions[4,5]; they differ greatly in the clinical behavior and malignant potential that give these lesions a diagnostic challenge[1].

The proper diagnosis, differentiation between neoplastic and non-neoplastic PCLs, and staging of these cystic lesions are considered a crucial issue for planning further management[6]. Endoscopic ultrasonography (EUS) and transpapillary diagnosis are the two major endoscopic approaches used. EUS-guided fine-needle aspiration (FNA) cytology, EUS-guided fine needle-based confocal laser endomicroscopy (nCLE), and needle microforceps biopsy are promising methods that have been used in the differential diagnosis of mucinous and non-mucinous pancreatic cysts[7].

The great benefits of EUS in detecting small lesions, making a differential diagnosis, and tumor staging provide a great challenge as many benign and malignant cystic lesions and also inflammatory cysts have a similar endosonographic appearance. Moreover, the permission of the evaluation of cyst fluid obtained by EUS-FNA for the early diagnosis or prediction of prognosis is a major concern to increase the diagnostic accuracy for more proper management[7].

EUS-FNA enables the use of aspirated samples for cytopathology examination and biochemical analyses, which provide an opportunity to further enhance diagnosis and medical decision-making[8] (Figure 1); therefore, the addition of FNA to various imaging modalities has been associated with increasing the diagnostic accuracy of PCLs[9].

The combination of both EUS-FNA findings with cystic fluid tumor markers analysis, along with clinical, radiologic, histologic, genetic, and molecular characteristics, enhances the diagnostic accuracy of PCLs and helps to construct a novel model in the era of cystic pancreatic lesion diagnosis[10].

EUS-guided radiofrequency ablation (EUS-RFA) is now considered an interesting alternative to doing major surgery with high morbidity and mortality for small, mostly benign, pancreatic lesions[11,12].

Owing to the close proximity of the transducer of EUS to the liver, from the transgastric and transduodenal routes, EUS allows good visualization of the liver anatomy and its vasculature by providing detailed images, and this plays a significant role in detecting small-sized liver metastasis from malignant pancreatic lesions, as well as potentially obtaining EUS-FNA with higher diagnostic accuracy[13]. This can be achieved in most liver segments except segment VII and VI which are very difficult to be visualized during EUS examination.

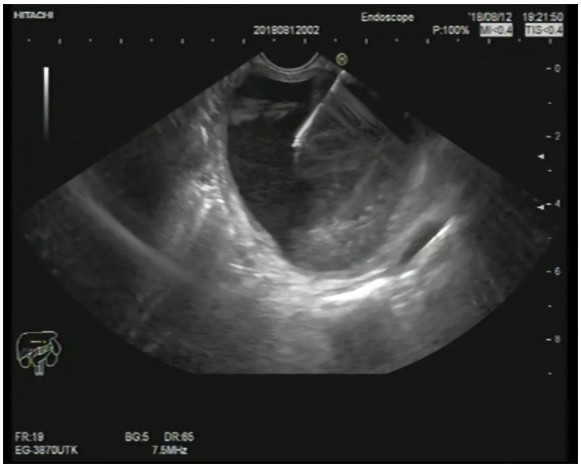


Figure 1 Endoscopic ultrasonography-guided fine-needle aspiration of a pancreatic mucinous cystic neoplasm.

CYSTIC LESIONS OF THE PANCREAS: TYPES, DIAGNOSIS

Pancreatic cysts are classified into inflammatory fluid collections and neoplastic and non-neoplastic pancreatic cysts. They may also be related to other diseases such as polycystic kidney disease or Von Hippel Lindau disease[14].

According to the revised Atlanta classification, inflammatory fluid collections are further categorized into acute peripancreatic fluid collections, pseudocysts, acute necrotic collections, and walled-off pancreatic necrosis[15].

Pancreatic cysts can range from simple cysts to neoplastic ones, so proper diagnosis and management are important. Mucinous cysts (Figure 2), for example, carry unpredictable potential malignant transformation[16]. Various methods are used to diagnose the type of the cystic lesion such as sonographic appearance, cytopathological examination of the cystic fluid, and tumor markers analysis from the cystic fluid.

Some sonographic findings are indicative of malignancy, including a thick wall, septations, and the presence of mural nodules[17]. Still, sonographic appearance or cytopathological examination has a low predictive value for diagnosis[18,19]. Mucin staining, carcinoembryonic antigen (CEA), CA19-9, and amylase levels in the cystic fluid improve the diagnosis of pancreatic cysts[20-22]. A study carried out by Okasha *et al*[10] shows that the highest AUC was that of cystic CEA, with a cutoff value of 160 ng/mL, and that it had a sensitivity of 60.4% and a specificity of 85%. It also mentioned that the best cutoff value for cystic CA19-9 was 1318 U/mL with a sensitivity of 64.1% and a specificity of 68.1%. The cutoff value of the cyst amylase level was 5500 U/L, with 84.2% sensitivity and 37.1% specificity. The sensitivity of mucin staining in detecting mucinous cystic neoplasm (MCN) was 85.45%, and specificity was 86.05% with an accuracy of 85.87%.

Low CEA with cuboidal epithelial cells, clear cytoplasm, and excess glycogen can diagnose serous cystic lesions (Figure 3)[23]. The presence of mucin, ovarian-like stroma with a degree of cell atypia is mainly found with mucinous pancreatic cysts[24]. Intraductal papillary mucinous neoplasm (IPMN) also produces mucin but is connected to the pancreatic duct with various degrees of dysplasia[23]. Malignant transformation in IPMN can be diagnosed by measuring cyst fluid CA19-9[25,26]. CEA > 192 ng/mL can differentiate between mucinous and non-mucinous cysts[27]. Cystic fluid amylase is helpful in diagnosing pseudocysts, especially if above > 250 IU/L[28], but cannot distinguish mucinous from non-mucinous cysts[29]. Table 1 shows different types of pancreatic cysts.

IMAGING DIAGNOSIS OF CYSTIC LESIONS OF THE PANCREAS

Ultrasound, computed tomography (CT) scan, and EUS are the most commonly used imaging modalities in diagnosing cystic pancreatic lesions. Still, magnetic resonance imaging (MRI), particularly T2-weighted images[30], and magnetic resonance cholangiopancreatography have better results in detecting the communication between pancreatic cysts and the pancreatic duct system, in addition to better detection for the

Table 1 Different parameters in different types of pancreatic cysts

Type of cyst	CEA	CA-19-9	Amylase	Mucin stain
Inflammatory pseudocyst	Normal	↑	↑	Negative
Lymphoepithelial cysts	Normal	Normal	Normal	Negative
Serous cystic neoplasms	Normal	Normal	Normal	Negative
Mucinous cystic neoplasm	↑	↑	Normal	Positive
Intraductal papillary mucinous neoplasm	↑/Normal	↑/Normal	↑/Normal	Positive
Solid papillary neoplasm	-	-	-	Negative

CEA: Carcinoembryonic antigen.



Figure 2 Mucinous cystic neoplasm of pancreatic tail with a mural nodule showing a vessel inside.

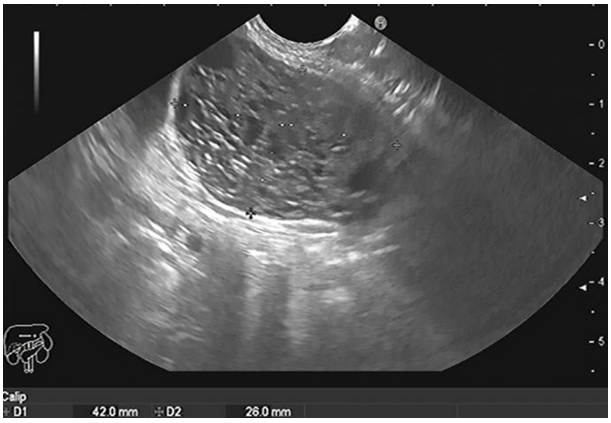


Figure 3 Microcystic serous cystadenoma of the body of the pancreas.

mural nodules[31-35]. Variable data are available for comparing the accuracy of CT and MRI in diagnosing pancreatic cysts. (Figure 4). A study carried out by Visser *et al*[36] concluded similar accuracy for both modalities. Procacci *et al*[37] described successful CT diagnosis for pancreatic cysts in 60% of examined cases. Another study done by Abraham *et al*[38] showed that both modalities carried equal performance accuracy.

Contrast-enhanced ultrasound is one of the most used imaging techniques. According to it, pancreatic lesions are divided into type I, with unilocular cysts; type II, presented as microcystic lesions; type III, with macrocystic lesions; and, lastly, type IV, containing cystic lesions with solid components or an irregularly thickened cystic wall[39]. According to this classification, type I is mostly a pancreatic pseudocyst.



Figure 4 Computed tomography shows inflammatory pancreatic pseudocyst.

Type II represents serous cystadenoma mostly. Type III includes mainly mucinous cystadenoma. Lastly, type IV is for solid pseudopapillary tumors and pancreatic masses with cystic degeneration[40].

Serous cystadenoma usually shows a honeycomb appearance in CT scan and EUS. The presence of central calcification is pathognomonic[41]. It is presented either as oligocystic or microcystic lesion[42] with low malignant potential transformation[43] and can be in any part of the pancreas.

Mucinous pancreatic cysts usually appear as unilocular lesions in a CT scan or EUS with no communication with the main pancreatic duct (MPD)[24]. They mainly appear in the body and tail of the pancreas. They have a high risk of malignant transformation [24] and generally appear in females in the fourth decade with mucin and ovarian-like stroma inside the cystic fluid. The presence of mural nodules during EUS examination favors malignant diagnosis. According to a study by Okasha *et al*[9], during the examination of 77 patients with PCLs, 11 out of 28 had mural nodules during EUS examination, 9 of which were proved to be mucinous cystadenocarcinoma.

IPMNs are divided into main duct, branched duct, or mixed according to the communication with the main or side branch pancreatic ducts[44]. The lesions are mostly solitary and located in the pancreatic head, but multifocal lesions can be detected[45].

ENDOSCOPIC DIAGNOSIS OF CYSTIC LESIONS OF THE PANCREAS

Some pancreatic tumors may exhibit cystic degeneration such as solid pseudopapillary neoplasms, cystic neuroendocrine tumor, and ductal adenocarcinoma[46]. However; the most common are MCNs, IPMNs, serous neoplasms (SCNs), and pseudocysts. In most cases, IPMN is multilocular connected to the pancreatic duct and usually located at the pancreatic head, whereas MCNs are rounded with a thick wall and located at the pancreatic tail. Mural nodules can sometimes be detected in both types of cysts, such as localized epithelial protrusions, and represent high-risk stigmata for both MCNs and IPMNs, but they are not typically found in SCNs. Hence, both IPMNs and MCNs have malignant potential. By contrast, SCNs do not have a predilection toward malignant transformation. Therefore, it is important to determine cyst type and its malignant potential so as to assign a treatment strategy and follow-up plan.

For the diagnosis of cystic lesions of the pancreas, 3 approaches have been described: (1) Diagnosis by EUS that involves morphology of the cyst during EUS, EUS-FNA, EUS-guided fine nCLE, through the needle biopsy (TTNB), and contrast-enhanced harmonic EUS; (2) Endoscopic retrograde cholangiopancreatography (ERCP); and (3) Single-operator cholangioscopy/pancreatoscopy.

DIAGNOSIS BY EUS

EUS became an indispensable tool for the diagnosis of cystic pancreatic lesions and the treatment of various nonmalignant pancreatic cysts. Nevertheless, EUS has significant importance in the early detection of small lesions, and the diagnostic accuracy of EUS imaging ranges from 40% to 96% when compared to histopathological examination of the surgical specimen[16].

In a single prospective study, the sensitivity and specificity were 56% and 45%, respectively. For EUS morphological diagnosis, by differentiating mucinous from non-mucinous cysts, the overall accuracy therefore lowered to 51%[22]. In contrast, EUS was superior to CT for detecting lesions, especially those less than 2 cm in size (83% *vs* 33%)[47].

In addition, EUS was equivalent to MRI in detecting communications of the PCLs to the MPD, thus differentiating IPMNs from MCNs and SCNs[48]. Furthermore, Binmoeller *et al*[47], in their consensus guidelines, recommended initial evaluation of IPMNs by EUS regardless of the presence or absence of high-risk stigmata or worrisome features in CT or MRI. In that study, the diagnostic accuracy was 100% when compared to ultrasound, CT, or MRI, which showed a rate of detection of malignant IPMNs at 47%, 53%, and 53%, respectively. Additional reports depicted that EUS is the modality of highest sensitivity for detecting mural nodules in malignant IPMNs[49-51].

EUS-FNA and cyst fluid analysis

Typically, the morphological features of cystic pancreatic lesions are not an independent factor in differentiating malignant from nonmalignant lesions. In addition, recent reports concluded that the appearance of PCLs during EUS evaluation was not sufficient in predicting their malignant potential[52,53].

In their study that included 77 patients with PCLs, Okasha *et al*[9] conducted FNA guided by either EUS or abdominal ultrasound for over 2 years. As a result, 28 (39.3%) had MCN, and 11 had mural nodules on EUS examination. Nine were proved to be mucinous cystadenocarcinoma, and 2 were diagnosed as IPMN, representing 81.8% of all cases of mucinous adenocarcinoma and 28.6% of all cases of IPMN, respectively. CEA was analyzed in 62 patients (80.5%). The cutoff value of 105 ng/dL showed a statistical difference between mucinous and non-mucinous lesions with a sensitivity and specificity of 80% and 77%, respectively. The reported accuracy was 78% for mucinous lesions. In addition, CA19-9 did not add much in differentiating neoplastic from non-neoplastic lesions. By contrast, the accuracy of positive mucin staining in differentiating between mucinous and non-mucinous, as well as neoplastic and non-neoplastic lesions was 91% and 80%, respectively. On the whole, the afore mentioned study concluded that cyst fluid analysis increases the yield of diagnostic accuracy of the PCL when there is insufficient clinical and poor morphological data using different imaging modalities[9].

In their study, Hawes *et al*[54] had depicted the essential role of EUS-FNA of the cyst fluid and its analysis regarding CEA, amylase, and cytology for the diagnosis of indeterminate cysts whose potential nature could not be predicted by imaging alone. On the other hand, cyst wall sampling using EUS-FNA may increase the diagnostic yield by 37%[55].

In the meantime, EUS-FNA shows the highest utility for high-risk stigmata for the malignant potential of PCLs, namely mural nodule or mass lesion, cyst size > 3 cm, or MPD dilation during EUS evaluation[52,56-62].

Molecular analysis of cyst fluid has been proposed to increase the diagnostic yield of PCLs. Typically, KRAS mutation analysis has increased the diagnostic accuracy of IPMNs to 81%[63]; when combined with CEA, it increased its sensitivity to 84% for mucinous cysts[64].

An international, multi-institution survey of the use of EUS in the diagnosis of PCLs

A committee member of the International Society of EUS Task Force conducted and drafted a multicenter survey-based study. This survey was distributed among 60 EUS experts in their centers around the world, this survey discussed the role of FNA in cystic pancreatic lesions, the analysis of cyst fluid and its role in differentiating specific types of various PCLs, and the bleeding risk that may complicate this procedure. It has been concluded that the utility of EUS diagnosis of PCLs based on the morphology is limited, but when combined with other imaging modalities such as CT or MRI, as well as FNA, it will lead to augmentation of the diagnostic yield and increased sensitivity and specificity of EUS as a diagnostic tool[7].

EUS-guided fine nCLE

Confocal laser endomicroscopy (nCLE) technology has recently been added as a diagnostic tool for PCLs evaluation. It permits visualization of the epithelial lining inside the cyst. In addition, IV fluorescein can be used during EUS-nCLE to further display the blood vessels supplying the cyst as well as other structures such as mural nodules[65].

Furthermore, each cyst type has a characteristic pattern when being visualized during EUS-nCLE. For instance, IPMN has finger-like projections and branched vascularity, whereas MCNs are lined by multiple epithelial layers. Typically, SCNs appear as a densely arranged superficial network of capillary vessels, whereas pseudocysts and SPENs appear as lightly colored inflammatory cells against a dark background and nets of dark cells separated by blood vessels[66].

INSPECT, DETECT, CONTACT-1 and -2, and INDEX trials are recent studies that validated the safety and feasibility of EUS-nCLE and its ability to augment the yield diagnosis of PCLs. In the most recent CONTACT-2 study, which included 78 patients, the sensitivity for EUS-nCLE to diagnose premalignant PCLs from benign lesions was 96%, and specificity was 95%, whereas for positive predictive value, the negative predictive values were 98% and 91%, respectively[67].

By contrast, in their study that evaluated the inter-observer agreement of EUS-nCLE, Karia *et al*[68] concluded that the inter-observer agreement and accuracy were low and that further studies are needed to validate EUS-nCLE as an accurate diagnostic tool.

Contrast-enhanced EUS

Contrast-enhanced EUS (CE EUS) can be a useful and substantial tool in differentiating between different types of pancreatic cystic neoplasms (PCNs). Recently, it has been widely applied. The CE EUS agent is a type of contrast that does not interfere with the interstitial space. As a result, it has the ability to reflect the microcirculation of tissues required for clinical correlation and diagnosis of various PCNs. Recent studies proved that CE EUS can differentiate solid pancreatic lesions of different origins. In their study, Zhong *et al*[69] revealed that CE EUS had an added accuracy in comparison to CT/MRI and fundamental B-mode EUS (CEEUS *vs* CT: 92.3% *vs* 76.9%; CE EUS *vs* MRI: 93.0% *vs* 78.9%; CE EUS *vs* fundamental B-mode EUS: 92.7% *vs* 84.1%) and that was consistent with previous reports.

TTNB

TTNB is a method used for better tissue acquisition in pancreatic cysts, which increases the diagnostic yield. Taking a biopsy from the wall of the cystic lesion ensures a better cytological gain. TTNB was first used in 2010 by Aparicio *et al*[70], associated with a spyglass fiber optic probe. Then, Moray microforceps were introduced in the field and proved better histological diagnosis[71,72].

The cyst is firstly punctured by a 19-gauge EUS-FNA needle. The forceps are then introduced through the fine needle, trans-gastric or trans-abdominal, to reach the wall of the cyst. The jaws of the forceps are pushed openly against the cystic wall and then closed to be pulled back, causing tenting. Crinò *et al*[73] recommended 3 passes per patient for adequate tissue acquisition and one bite with a "tent sign" for effective grip by the forceps. Yang *et al*[74] suggested 3 bites per pass, with a total of 3 passes for each case.

Technical failure is attributed to loss of echoendoscope flexibility when both the needle and forceps are introduced or when the site of puncture is difficult. Basar *et al*[75] recorded failed biopsy in 9.6% of the cases. Aparicio *et al*[70] attributed inadequate material to clots in 10 cases and amorphous materials in another 5 investigated cases. Kovacevic *et al*[76] linked failure to difficult transgastric puncture and difficult manipulation with a maximally flexed echoendoscope.

DIAGNOSIS BY ERCP

ERCP is not routinely required for the evaluation of PCLs. Nevertheless, duodenoscopy may reveal some specific findings in the main-duct IPMN, such as mucinous material flowing out of the patulous pancreatic duct (Fish Mouth appearance), which is seen in 20% to 55% of patients with main-duct IPMNs and is considered a pathognomonic finding for this type of IPMN[77-80].

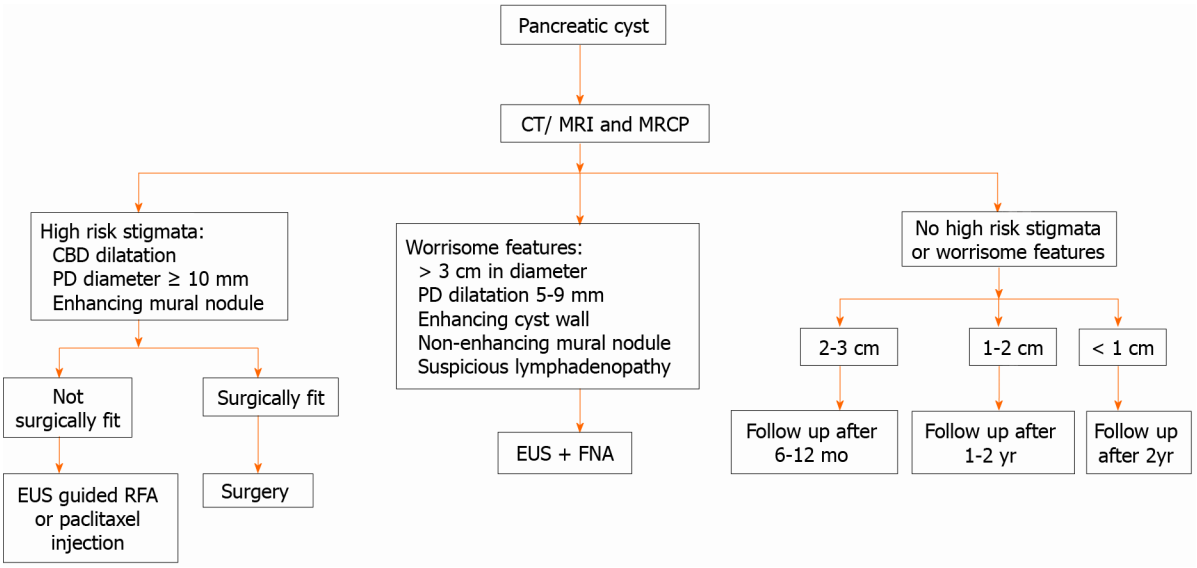


Figure 5 Approach to a patient with a pancreatic cyst. CT: Computed tomography; MRI: Magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography; CBD: Common bile duct; PD: Pancreatic duct; EUS: Endoscopic ultrasonography; RFA: Radiofrequency ablation; FNA: Fine-needle aspiration.

Pancreatoscopy is an emerging alternative for direct visualization of stones, tumors, and mucus, and when combined with an intraductal ultrasound, its accuracy in differentiating between benign and malignant IPMN reached 88% in one study[77].

ERCP-guided tissue acquisition, whether by brush cytology, biopsy specimens of fixed filling defects or strictures, or random biopsy specimens of dilated duct walls, is used for the evaluation of PCLs. Although ERCP tissue sampling has a relatively low diagnostic yield, transpapillary biopsy with standard or pediatric-sized forceps resulted in a diagnosis of 11 out of 13 patients with IPMN in one study[78].

Pancreatoscopy using a video scope with narrow-band imaging or *via* a fiberoptic probe has been described in several reports to acquire issue or cytology[81-84].

SINGLE-OPERATOR CHOLANGIOSCOPY/PANCREATOSCOPY

A mother-baby endoscope was used for a while during pancreatobiliary examination but had many problems. Single-operator cholangioscopy/pancreatoscopy has succeeded in overcoming the problems faced by the mother-baby endoscope: high cost, fragile baby scope, and absence of an irrigation channel. In addition to that, single-operator cholangioscopy/pancreatoscopy has a disposable access catheter for reusable optic probe[85]. A microforcep can be introduced to gain a specimen for histopathological examination[86,87]. A study was done to evaluate the benefit of using single-operator cholangioscopy/pancreatoscopy in diagnosing IPMN with 100% sensitivity and specificity for detecting the malignancy, as well as determining the operative excision line in 3 patients[84]. Another case report for a 74-year-old man with chronic pancreatitis and marked weight loss was diagnosed by a single-operator cholangioscopy/pancreatoscopy with IPMN[88].

We demonstrated a simplified flow chart for the diagnosing of PCLs as shown in Figure 5.

EUS-RFA

RFA through a 19G needle using a monopolar probe has been recently described for the management of PCLs. It has been considered a safe and effective procedure that depends on thermal ablation and induces coagulative necrosis of thermosensitive pancreatic tissue[89-91].

In their study, Pai *et al*[91] conducted RFA ablation on 8patients, where 6 had a PCN and 2 had a cystic neuroendocrine tumor in the head region. It was concluded that EUS-RFA is a safe procedure and that the response rate demonstrated a 100%

resolution of PCNs in most cases[92].

By contrast, potential adverse events may arise as a result of the effect of thermal injury, which can lead to biliary leakage and pancreatic and vascular injury[78]. Nevertheless, the data of PCNs' EUS-RFA are limited to a few human case series and animal data, and an ongoing Phase II multicenter trial of EUS-RFA has been conducted to evaluate the outcomes of the pancreatic cyst at 12 mo following cyst RFA[93].

THE FUTURE RESEARCH DIRECTIONS IN THE DIAGNOSIS OF CYSTIC PANCREATIC LESIONS AND THE ROLE OF MOLECULAR ANALYSIS OF PANCREATIC CYSTIC FLUID

Testing for mutations related to pancreatic malignancies increases the diagnostic accuracy of cystic pancreatic lesions[62,94-100]. The routine molecular analysis of *KRAS*/*GNAS* mutations gives high sensitivity and specificity, reaching 90% in detecting mucinous lesions[101-104] *KRAS* mutations still do not grade the level of malignancy. Springer *et al*[105] concluded in their study that mutations in the von Hippel-Lindau (*VHL*) gene (3p35), with a loss of heterozygosity at gene locus on chromosome 3 and chromosome 3p aneuploidy, were detected in approximately 67% of serous cystic adenomas.

A study carried out by Arner *et al*[106] mentioned that "The addition of DNA molecular analysis alters the clinical management of PCLs most often when CEA levels are intermediate (45-800 ng/mL) or when no CEA concentration is available".

GNAS mutations alone can be detected in up to 66% of mucinous neoplastic lesions, and their detection may reach 96% of cases when combined with *KRAS* mutations [107]. They were also found in 100% of IPMN cases with sensitivity and specificity of 89% and 100%, respectively, for the detection of a mucinous cyst[108].

Other investigated mutations include *TP53* mutations, deletions in p16/*CDKN2A*, *SMAD4*, mutations in *TP53*, *PIK3CA*, and/or *PTEN*. They are highly sensitive for IPMN[108]. According to a systematic review by Zhang and Pitman[109], although molecular testing is not an alternative to cytological and chemical testing, it can still add value to the diagnostic yielding.

Studies using DNA analysis of cyst fluid seem to be promising but require further attention.

The last randomized clinical studies discussing the radiological and imaging modalities of PCLs are shown in Table 2[108,110-120]; however further studies are warranted not only to improve diagnostic ability but also determine a clear strategy for the use of novel endoscopic techniques for the diagnosis of PCLs.

CONCLUSION

The real clinical challenge in the management of cystic pancreatic lesions is to identify those patients who should undergo pancreatic resection for early non-metastatic cancer and high-risk precancerous cystic lesions while appropriately observing those patients with limited or no potential for malignant transformation. In the context of many aspects of the EUS management of PCLs, available data are currently insufficient to make evidenced-based decisions.

In conclusion, by combining the use of EUS modalities with cystic fluid tumor markers, analysis constructs a novel diagnostic model for PCLs. Also, it indeed highlights that the accurate diagnosis of PCLs requires a well-experienced multidisciplinary and multimodal team approach, side by side with the integration of clinical findings, imaging, cytology, cyst fluid analysis, and molecular testing.

Table 2 The last randomized clinical studies in the radiological and imaging modalities of pancreatic cystic lesions

Ref.	Country	Sample size	Study period/design	Aim of study	Conclusion
Singhi *et al*[108], 2018	Pittsburgh	595	2014-2017/prospective	Evaluation of preoperative pancreatic cyst fluid (PCF) DNA testing	Preoperative next-generation sequencing of PCF for *KRAS/GNAS* mutations is highly sensitive for IPMNs and specific for mucinous PCLs. In addition, the combination of *TP53/PIK3CA/PTEN* alterations is a useful preoperative marker for advanced neoplasia
Basar *et al*[75], 2018	United States	42	2015-2016/retrospective	Comparison between the tissue acquisition and diagnostic tissue yield of microforceps biopsy (MFB) with cyst fluid cytology	The cyst tissue acquisition yield for MFBs was 90%. Although cytology of cyst fluid and MFB were comparable in distinguishing mucinous and nonmucinous cysts and detecting cysts at high risk for malignancy, MFB was far superior to cytology for providing a specific cyst diagnosis
Kovacevic *et al*[76], 2018	Multicenter	28	NR/retrospective	Evaluation of the use of EUS-guided MFB in diagnosing pancreatic cystic lesions in a multicenter clinical setting	The use of the microforceps is feasible with acceptable rates of technical and clinical success
Mittal *et al*[110], 2018	United States	27	2016-2017/retrospective	Assessment of the technical feasibility, diagnostic yield, and safety of EUS-guided MFB for PCLs	MFBs were associated with high technical success, and an excellent safety profile and may be a useful adjunctive tool, complementing existing EUS-FNA sampling protocols for PCLs
Zhang *et al*[111], 2018	United States	48	2016-2017/retrospective	Comparing the diagnostic performance of the MFB with the current conventional analysis of PCF	PCF analysis and MFB have comparable performance in distinguishing between mucinous and non-mucinous cysts and for detecting high-risk cysts. However, MFB was found to be superior for diagnosing specific cyst subtypes, thus adding a significant value to preoperative patient management
Cheesman *et al*[112], 2019	United States	41	NR/retrospective	Comparing the diagnostic outcomes and changes in clinical management resulting from MFB and nCLE use in PCL	The combination of cyst fluid cytology/chemistry along with MFB and/or nCLE results in a significantly higher rate of a specific PCL diagnosis and has a major impact on changing clinical management decisions including need for continued surveillance or surgery. MFB and/or nCLE should thus be utilized with standard cyst fluid cytology/chemistry when performing EUS-FNA of PCL
Crinò*et al*[73], 2019	Italy	61	2016-2018/prospective	Evaluation of the diagnostic yield of EUS-guided through-the-needle MFB sampling of pancreatic cystic lesions according to the number of macroscopically visible samples retrieved	Two TTNB macroscopically visible specimens reached 100% histologic adequacy and a specific diagnosis in 74% of patients. The collection of a third specimen did not add any additional information and should be avoided to possibly decrease the risk of adverse events
Robles-Medranda and Olmos[113], 2019	Ecuador	36	2013-2018/retrospective	Defining the role of through-the-needle technologies such as nCLE and EUS- through-the-needle MFB in the diagnosis of pancreatic cyst malignancy	EUS-through-the-needle direct intracystic MFB and nCLE improves malignancy detection in pancreatic cysts
Samarasena *et al*[114], 2019	United States	15	NR/retrospective	Reporting the technical success and safety of EUS-guided through the needle biopsy (TTNB) for pancreatic cystic lesions	This technique has the potential to improve the diagnostic yield of EUS-FNA for pancreatic cystic neoplasms
Vestrup Rift *et al*[115], 2019	Denmark	27	2016-2017/retrospective	Analysis of the results of next-generation sequencing of microbiopsies from pancreatic cysts	Next-generation sequencing of microbiopsies may have the potential to improve diagnostic decision-making
Yang *et al*[116], 2019	United States	114	2016-2018/prospective	Comparing the yield of tissue acquired with EUS-guided TTNB with that of samples collected by EUS-guided FNA, and the accuracy of analysis of each sample type in the diagnosis of mucinous PCLs	TTNB collection of tissues for histologic analysis is safe and feasible, with an acquisition yield of 83.3%. Histologic analysis of samples collected by TTNB identified a larger proportion of mucinous PCLs compared with cytologic analysis of samples collected by FNA-even among samples categorized as equivocal, based on the level of carcinoembryonic antigen (CEA)
Wilen *et al*[117], 2019	United States	30	2016-2018/retrospective	Evaluation of the feasibility and added value of cyst wall biopsy using micro forceps in the diagnosis of pancreatic cysts	Cyst wall biopsy was able to make the diagnosis in 44% of cases where cytology was non-diagnostic and in 64% of cases when composite fluid markers and cytology was non-diagnostic. The high rate of histologic and IHC evaluation suggests that it offers

					the potential to incorporate tissue-based biomarkers in the diagnosis and management of pancreatic cysts
Krishna *et al*[118], 2020	United States	144	2015-2018/ Prospective	Comparing the accuracy of EUS with nCLE in differentiating mucinous from non-mucinous PCLs with that of measurement of CEA and cytology analysis	Analysis of cysts by nCLE identified mucinous cysts with greater accuracy than measurement of CEA and cytology analysis. EUS with nCLE can be used to differentiate mucinous from non-mucinous PCLs
Keane *et al*[119], 2019	United Kingdom	56	2014-2016/prospective	Defining the safety and efficacy of nCLE in diagnosis of indeterminate PCL	EUS-nCLE under conscious sedation in the day case setting is safe and provides additional information to standard EUS-FNA for diagnosing indeterminate PCL
Napoleon *et al*[67], 2019	France	78	2013-2016/prospective	Evaluation of the diagnostic performance of nCLE for large single non-communicating PCLs using surgical histopathology or EUS-FNA cytohistopathology as a reference diagnosis	nCLE had excellent diagnostic performance that surpassed that of CEA and EUS for the diagnosis of large single non-communicating PCLs. The nCLE procedure should be considered in patients with indeterminate PCLs to ensure a more specific diagnosis
Cheesman *et al*[120], 2020	United States	44	2016-2018/retrospective study	Comparing the diagnostic outcomes and changes in clinical management resulting from MFB and nCLE use in PCLs	MFB and nCLE led to significant improvements in specific PCL diagnosis, which in turn has major impacts in clinical management

IPMN: Intraductal papillary mucinous neoplasm; PCL: Pancreatic cystic lesion; EUS: Endoscopic ultrasonography; FNA: Fine-needle aspiration; NR: Not reported; nCLE: Needle-based confocal laser endomicroscopy; IHC: Immunohistochemistry.

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REFERENCES

1 **Abdelkader A**, Hunt B, Hartley CP, Panarelli NC, Giorgadze T. Cystic Lesions of the Pancreas: Differential Diagnosis and Cytologic-Histologic Correlation. *Arch Pathol Lab Med* 2020; **144**: 47-61 [PMID: 31538798 DOI: 10.5858/arpa.2019-0308-RA]
2 **Becker WF**, Welsh RA, Pratt HS. Cystadenoma and cystadenocarcinoma of the pancreas. *Ann Surg* 1965; **161**: 845-863 [PMID: 14295937 DOI: 10.1097/00000658-196506000-00005]
3 **Zhang XM**, Mitchell DG, Dohke M, Holland GA, Parker L. Pancreatic cysts: depiction on single-shot fast spin-echo MR images. *Radiology* 2002; **223**: 547-553 [PMID: 11997566 DOI: 10.1148/radiol.2232010815]
4 **Basturk O**, Coban I, Adsay NV. Pancreatic cysts: pathologic classification, differential diagnosis, and clinical implications. *Arch Pathol Lab Med* 2009; **133**: 423-438 [PMID: 19260748]
5 **Grützmann R**, Niedergethmann M, Pilarsky C, Klöppel G, Saeger HD. Intraductal papillary mucinous tumors of the pancreas: biology, diagnosis, and treatment. *Oncologist* 2010; **15**: 1294-1309 [PMID: 21147870 DOI: 10.1634/theoncologist.2010-0151]
6 **Jani N**, Bani Hani M, Schulick RD, Hruban RH, Cunningham SC. Diagnosis and management of cystic lesions of the pancreas. *Diagn Ther Endosc* 2011; **2011**: 478913 [PMID: 21904442 DOI: 10.1155/2011/478913]
7 **Kamata K**, Kitano M. Endoscopic diagnosis of cystic lesions of the pancreas. *Dig Endosc* 2019; **31**: 5-15 [PMID: 30085364 DOI: 10.1111/den.13257]
8 **Ge N**, Brugge WR, Saxena P, Sahai A, Adler DG, Giovannini M, Pausawasdi N, Santo E, Mishra G, Tam W, Kida M, de la Mora-Levy JG, Sharma M, Umar M, Katanuma A, Lee L, Garg PK, Eloubeidi MA, Yu HK, Raijman I, Arturo Arias BL, Bhutani M, Carrara S, Rai P, Mukai S, Palazzo L, Dietrich CF, Nguyen NQ, El-Nady M, Poley JW, Guaraldi S, Kalaitzakis E, Sabbagh LC, Lariño-Noia J, Gress FG, Lee YT, Rana SS, Fusaroli P, Hocke M, Dhir V, Lakhtakia S, Ratanachu-Ek T, Chalapathi Rao AS, Vilmann P, Okasha HH, Irisawa A, Ponnudurai R, Leong AT, Artifon E, Iglesias-Garcia J, Saftoiu A, Larghi A, Robles-Medranda C, Sun S. An international, multi-institution survey of the use of EUS in the diagnosis of pancreatic cystic lesions. *Endosc Ultrasound* 2019; **8**: 418-427 [PMID: 31552915 DOI: 10.4103/eus.eus_61_19]
9 **Okasha HH**, Ashry M, Imam HM, Ezzat R, Naguib M, Farag AH, Gemeie EH, Khattab HM. Role of endoscopic ultrasound-guided fine needle aspiration and ultrasound-guided fine-needle aspiration in diagnosis of cystic pancreatic lesions. *Endosc Ultrasound* 2015; **4**: 132-136 [PMID: 26020048 DOI: 10.4103/2303-9027.156742]
10 **Okasha H**, E Behiry M, Ramadan N, Ezzat R, Yamany A, El-Kholi S, Ahmed G. Endoscopic

ultrasound-guided fine needle aspiration in diagnosis of cystic pancreatic lesions. *Arab J Gastroenterol* 2019; **20**: 86-90 [PMID: 31182342 DOI: 10.1016/j.ajg.2019.05.008]

11 **Barthet M**, Giovannini M, Lesavre N, Boustiere C, Napoleon B, Koch S, Gasmi M, Vanbiervliet G, Gonzalez JM. Endoscopic ultrasound-guided radiofrequency ablation for pancreatic neuroendocrine tumors and pancreatic cystic neoplasms: a prospective multicenter study. *Endoscopy* 2019; **51**: 836-842 [PMID: 30669161 DOI: 10.1055/a-0824-7067]

12 **Okasha HH**, Naga YM, El Sherbiny M. EUS-guided radiofrequency ablation: Where we are? *Endosc Ultrasound* 2020; **9**: 277-279 [PMID: 32655083 DOI: 10.4103/eus.eus_33_20]

13 **Hafez HA**, Okasha HH, Hashem AM, Hassany M, El-Nahaas SM. The Role of EUS and EUS-FNA in Detection of Small Sized Liver Metastatic Lesions in Patients with Pancreatic and Gastro-Intestinal Primary Malignancy. *Med J Cairo Univ* 2020; **88**: 1219-1225 [DOI: 10.21608/mjcu.2020.110872]

14 **Kim JA**, Blumenfeld JD, Chhabra S, Dutruel SP, Thimmappa ND, Bobb WO, Donahue S, Rennert HE, Tan AY, Giambrone AE, Prince MR. Pancreatic Cysts in Autosomal Dominant Polycystic Kidney Disease: Prevalence and Association with PKD2 Gene Mutations. *Radiology* 2016; **280**: 762-770 [PMID: 27046073 DOI: 10.1148/radiol.2016151650]

15 **Banks PA**, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS; Acute Pancreatitis Classification Working Group. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; **62**: 102-111 [PMID: 23100216 DOI: 10.1136/gutjnl-2012-302779]

16 **ASGE Standards of Practice Committee**, Muthusamy VR, Chandrasekhara V, Acosta RD, Bruining DH, Chathadi KV, Eloubeidi MA, Faulx AL, Fonkalsrud L, Gurudu SR, Khashab MA, Kothari S, Lightdale JR, Pasha SF, Saltzman JR, Shaukat A, Wang A, Yang J, Cash BD, DeWitt JM. The role of endoscopy in the diagnosis and treatment of cystic pancreatic neoplasms. *Gastrointest Endosc* 2016; **84**: 1-9 [PMID: 27206409 DOI: 10.1016/j.gie.2016.04.014]

17 **Gress F**, Gottlieb K, Cummings O, Sherman S, Lehman G. Endoscopic ultrasound characteristics of mucinous cystic neoplasms of the pancreas. *Am J Gastroenterol* 2000; **95**: 961-965 [PMID: 10763945 DOI: 10.1111/j.1572-0241.2000.01976.x]

18 **Agarwal B**, Abu-Hamda E, Molke KL, Correa AM, Ho L. Endoscopic ultrasound-guided fine needle aspiration and multidetector spiral CT in the diagnosis of pancreatic cancer. *Am J Gastroenterol* 2004; **99**: 844-850 [PMID: 15128348 DOI: 10.1111/j.1572-0241.2004.04177.x]

19 **Lewis JJ**, Kowalski TE. Endoscopic ultrasound and fine needle aspiration in pancreatic cancer. *Cancer J* 2012; **18**: 523-529 [PMID: 23187838 DOI: 10.1097/PPO.0b013e318277008e]

20 **Snozek CL**, Mascarenhas RC, O'Kane DJ. Use of cyst fluid CEA, CA19-9, and amylase for evaluation of pancreatic lesions. *Clin Biochem* 2009; **42**: 1585-1588 [PMID: 19576876 DOI: 10.1016/j.clinbiochem.2009.06.020]

21 **Linder JD**, Geenen JE, Catalano MF. Cyst fluid analysis obtained by EUS-guided FNA in the evaluation of discrete cystic neoplasms of the pancreas: a prospective single-center experience. *Gastrointest Endosc* 2006; **64**: 697-702 [PMID: 17055859 DOI: 10.1016/j.gie.2006.01.070]

22 **Brugge WR**, Lewandrowski K, Lee-Lewandrowski E, Centeno BA, Szydlo T, Regan S, del Castillo CF, Warshaw AL. Diagnosis of pancreatic cystic neoplasms: a report of the cooperative pancreatic cyst study. *Gastroenterology* 2004; **126**: 1330-1336 [PMID: 15131794 DOI: 10.1053/j.gastro.2004.02.013]

23 **Lanke G**, Lee JH. Similarities and differences in guidelines for the management of pancreatic cysts. *World J Gastroenterol* 2020; **26**: 1128-1141 [PMID: 32231418 DOI: 10.3748/wjg.v26.i11.1128]

24 **Brugge WR**. Diagnosis and management of cystic lesions of the pancreas. *J Gastrointest Oncol* 2015; **6**: 375-388 [PMID: 26261724 DOI: 10.3978/j.issn.2078-6891.2015.057]

25 **Wang W**, Zhang L, Chen L, Wei J, Sun Q, Xie Q, Zhou X, Zhou D, Huang P, Yang Q, Xie H, Zhou L, Zheng S. Serum carcinoembryonic antigen and carbohydrate antigen 19-9 for prediction of malignancy and invasiveness in intraductal papillary mucinous neoplasms of the pancreas: A meta-analysis. *Biomed Rep* 2015; **3**: 43-50 [PMID: 25469245 DOI: 10.3892/br.2014.376]

26 **Kim JR**, Jang JY, Kang MJ, Park T, Lee SY, Jung W, Chang J, Shin Y, Han Y, Kim SW. Clinical implication of serum carcinoembryonic antigen and carbohydrate antigen 19-9 for the prediction of malignancy in intraductal papillary mucinous neoplasm of pancreas. *J Hepatobiliary Pancreat Sci* 2015; **22**: 699-707 [PMID: 26178866 DOI: 10.1002/jhbp.275]

27 **Cizginer S**, Turner BG, Bilge AR, Karaca C, Pitman MB, Brugge WR. Cyst fluid carcinoembryonic antigen is an accurate diagnostic marker of pancreatic mucinous cysts. *Pancreas* 2011; **40**: 1024-1028 [PMID: 21775920 DOI: 10.1097/MPA.0b013e31821bd62f]

28 **van der Waaij LA**, van Dullemen HM, Porte RJ. Cyst fluid analysis in the differential diagnosis of pancreatic cystic lesions: a pooled analysis. *Gastrointest Endosc* 2005; **62**: 383-389 [PMID: 16111956 DOI: 10.1016/s0016-5107(05)01581-6]

29 **Al-Rashdan A**, Schmidt CM, Al-Haddad M, McHenry L, Leblanc JK, Sherman S, Dewitt J. Fluid analysis prior to surgical resection of suspected mucinous pancreatic cysts. A single centre experience. *J Gastrointest Oncol* 2011; **2**: 208-214 [PMID: 22811854 DOI: 10.3978/j.issn.2078-6891.2011.020]

30 **Fukukura Y**, Fujiyoshi F, Hamada H, Takao S, Aikou T, Hamada N, Yonezawa S, Nakajo M. Intraductal papillary mucinous tumors of the pancreas. Comparison of helical CT and MR imaging. *Acta Radiol* 2003; **44**: 464-471 [PMID: 14510751 DOI: 10.1034/j.1600-0455.2003.00111.x]

31 **European Study Group on Cystic Tumours of the Pancreas**. European evidence-based guidelines

on pancreatic cystic neoplasms. *Gut* 2018; **67**: 789-804 [PMID: 29574408 DOI: 10.1136/gutjnl-2018-316027]

32 **Repák R**, Rejchrt S, Bártová J, Malírová E, Tycová V, Bures J. Endoscopic ultrasonography (EUS) and EUS-guided fine-needle aspiration with cyst fluid analysis in pancreatic cystic neoplasms. *Hepatogastroenterology* 2009; **56**: 629-635 [PMID: 19621669]

33 **Dumonceau JM**, Deprez PH, Jenssen C, Iglesias-Garcia J, Larghi A, Vanbiervliet G, Aithal GP, Arcidiacono PG, Bastos P, Carrara S, Czakó L, Fernández-Esparrach G, Fockens P, Ginès À, Havre RF, Hassan C, Vilmann P, van Hooft JE, Polkowski M. Indications, results, and clinical impact of endoscopic ultrasound (EUS)-guided sampling in gastroenterology: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline - Updated January 2017. *Endoscopy* 2017; **49**: 695-714 [PMID: 28511234 DOI: 10.1055/s-0043-109021]

34 **Polkowski M**, Jenssen C, Kaye P, Carrara S, Deprez P, Gines A, Fernández-Esparrach G, Eisendrath P, Aithal GP, Arcidiacono P, Barthet M, Bastos P, Fornelli A, Napoleon B, Iglesias-Garcia J, Seicean A, Larghi A, Hassan C, van Hooft JE, Dumonceau JM. Technical aspects of endoscopic ultrasound (EUS)-guided sampling in gastroenterology: European Society of Gastrointestinal Endoscopy (ESGE) Technical Guideline - March 2017. *Endoscopy* 2017; **49**: 989-1006 [PMID: 28898917 DOI: 10.1055/s-0043-119219]

35 **Jais B**, Rebours V, Malleo G, Salvia R, Fontana M, Maggino L, Bassi C, Manfredi R, Moran R, Lennon AM, Zaheer A, Wolfgang C, Hruban R, Marchegiani G, Fernández Del Castillo C, Brugge W, Ha Y, Kim MH, Oh D, Hirai I, Kimura W, Jang JY, Kim SW, Jung W, Kang H, Song SY, Kang CM, Lee WJ, Crippa S, Falconi M, Gomatos I, Neoptolemos J, Milanetto AC, Sperti C, Ricci C, Casadei R, Bissolati M, Balzano G, Frigerio I, Girelli R, Delhaye M, Bernier B, Wang H, Jang KT, Song DH, Huggett MT, Oppong KW, Pererva L, Kopchak KV, Del Chiaro M, Segersvard R, Lee LS, Conwell D, Osvaldt A, Campos V, Aguero Garcete G, Napoleon B, Matsumoto I, Shinzeki M, Bolado F, Fernandez JM, Keane MG, Pereira SP, Acuna IA, Vaquero EC, Angiolini MR, Zerbi A, Tang J, Leong RW, Faccinetto A, Morana G, Petrone MC, Arcidiacono PG, Moon JH, Choi HJ, Gill RS, Pavey D, Ouaïssi M, Sastre B, Spandre M, De Angelis CG, Rios-Vives MA, Concepcion-Martin M, Ikeura T, Okazaki K, Frulloni L, Messina O, Lévy P. Serous cystic neoplasm of the pancreas: a multinational study of 2622 patients under the auspices of the International Association of Pancreatology and European Pancreatic Club (European Study Group on Cystic Tumors of the Pancreas). *Gut* 2016; **65**: 305-312 [PMID: 26045140 DOI: 10.1136/gutjnl-2015-309638]

36 **Visser BC**, Yeh BM, Qayyum A, Way LW, McCulloch CE, Coakley FV. Characterization of cystic pancreatic masses: relative accuracy of CT and MRI. *AJR Am J Roentgenol* 2007; **189**: 648-656 [PMID: 17715113 DOI: 10.2214/AJR.07.2365]

37 **Procacci C**, Biasiutti C, Carbognin G, Accordini S, Bicego E, Guarise A, Spoto E, Andreis IA, De Marco R, Megibow AJ. Characterization of cystic tumors of the pancreas: CT accuracy. *J Comput Assist Tomogr* 1999; **23**: 906-912 [PMID: 10589565 DOI: 10.1097/00004728-199911000-00014]

38 **Abraham AS**, Simon B, Eapen A, Sathyakumar K, Chandramohan A, Raju RS, Joseph P, Kodiatte TA, Gowri M. Role of Cross-sectional Imaging (CT/MRI) in Characterization and Distinguishing Benign from Malignant/Potentially Malignant Cystic Lesions of Pancreas. *J Clin Imaging Sci* 2020; **10**: 28 [PMID: 32494507 DOI: 10.25259/JCIS_15_2020]

39 **Fan Z**, Yan K, Wang Y, Qiu J, Wu W, Yang L, Chen M. Application of Contrast-Enhanced Ultrasound in Cystic Pancreatic Lesions Using a Simplified Classification Diagnostic Criterion. *Biomed Res Int* 2015; **2015**: 974621 [PMID: 26090467 DOI: 10.1155/2015/974621]

40 **Wang Y**, Wang Y, Fan Z, Shan J, Yan K. The Value of Contrast-Enhanced Ultrasound Classification in Diagnosis of Pancreatic Cystic Lesions. *Biomed Res Int* 2019; **2019**: 5698140 [PMID: 31737668 DOI: 10.1155/2019/5698140]

41 **Kimura W**, Moriya T, Hirai I, Hanada K, Abe H, Yanagisawa A, Fukushima N, Ohike N, Shimizu M, Hatori T, Fujita N, Maguchi H, Shimizu Y, Yamao K, Sasaki T, Naito Y, Tanno S, Tobita K, Tanaka M. Multicenter study of serous cystic neoplasm of the Japan pancreas society. *Pancreas* 2012; **41**: 380-387 [PMID: 22415666 DOI: 10.1097/MPA.0b013e31822a27db]

42 **Lewandrowski K**, Warshaw A, Compton C. Macrocystic serous cystadenoma of the pancreas: a morphologic variant differing from microcystic adenoma. *Hum Pathol* 1992; **23**: 871-875 [PMID: 1644432 DOI: 10.1016/0046-8177(92)90397-l]

43 **Compagno J**, Oertel JE. Microcystic adenomas of the pancreas (glycogen-rich cystadenomas): a clinicopathologic study of 34 cases. *Am J Clin Pathol* 1978; **69**: 289-298 [PMID: 637043 DOI: 10.1093/ajcp/69.1.289]

44 **Distler M**, Aust D, Weitz J, Pilarsky C, Grützmann R. Precursor lesions for sporadic pancreatic cancer: PanIN, IPMN, and MCN. *Biomed Res Int* 2014; **2014**: 474905 [PMID: 24783207 DOI: 10.1155/2014/474905]

45 **Castellano-Megías VM**, Andrés CI, López-Alonso G, Colina-Ruizdelgado F. Pathological features and diagnosis of intraductal papillary mucinous neoplasm of the pancreas. *World J Gastrointest Oncol* 2014; **6**: 311-324 [PMID: 25232456 DOI: 10.4251/wjgo.v6.i9.311]

46 **Cooper CL**, O'Toole SA, Kench JG. Classification, morphology and molecular pathology of premalignant lesions of the pancreas. *Pathology* 2013; **45**: 286-304 [PMID: 23442735 DOI: 10.1097/PAT.0b013e32835f2205]

47 **Binmoeller KF**, Soehendra N. Endoscopic ultrasonography in the diagnosis and treatment of pancreatic pseudocysts. *Gastrointest Endosc Clin N Am* 1995; **5**: 805-816 [PMID: 8535629]

48 **Pausawasdi N**, Ratanachu-Ek T. Endoscopic ultrasonography evaluation for pancreatic cysts:

Necessity or overkill? *Dig Endosc* 2017; **29**: 444-454 [PMID: 28321928 DOI: 10.1111/den.12873]
49 **Kamata K**, Kitano M, Kudo M, Sakamoto H, Kadosaka K, Miyata T, Imai H, Maekawa K, Chikugo T, Kumano M, Hyodo T, Murakami T, Chiba Y, Takeyama Y. Value of EUS in early detection of pancreatic ductal adenocarcinomas in patients with intraductal papillary mucinous neoplasms. *Endoscopy* 2014; **46**: 22-29 [PMID: 24218310 DOI: 10.1055/s-0033-1353603]
50 **Baba T**, Yamaguchi T, Ishihara T, Kobayashi A, Oshima T, Sakaue N, Kato K, Ebara M, Saisho H. Distinguishing benign from malignant intraductal papillary mucinous tumors of the pancreas by imaging techniques. *Pancreas* 2004; **29**: 212-217 [PMID: 15367887 DOI: 10.1097/00006676-200410000-00006]
51 **Maguchi H**, Osanai M, Yanagawa N, Takahashi K, Itoh H, Katanuma A, Obara T, Kohgo Y. Endoscopic ultrasonography diagnosis of pancreatic cystic disease. *Endoscopy* 1998; **30** Suppl 1: A108-A110 [PMID: 9765099 DOI: 10.1055/s-2007-1001488]
52 **Huang ES**, Turner BG, Fernandez-Del-Castillo C, Brugge WR, Hur C. Pancreatic cystic lesions: clinical predictors of malignancy in patients undergoing surgery. *Aliment Pharmacol Ther* 2010; **31**: 285-294 [PMID: 19845568 DOI: 10.1111/j.1365-2036.2009.04173.x]
53 **Buscaglia JM**, Giday SA, Kantsevoy SV, Jagannath SB, Magno P, Wolfgang CL, Daniels JA, Canto MI, Okolo Iii PI. Patient- and cyst-related factors for improved prediction of malignancy within cystic lesions of the pancreas. *Pancreatology* 2009; **9**: 631-638 [PMID: 19657218 DOI: 10.1159/000181173]
54 **Hawes RH**, Clancy J, Hasan MK. Endoscopic ultrasound-guided fine needle aspiration in cystic pancreatic lesions. *Clin Endosc* 2012; **45**: 128-131 [PMID: 22866252 DOI: 10.5946/ce.2012.45.2.128]
55 **Rogart JN**, Loren DE, Singu BS, Kowalski TE. Cyst wall puncture and aspiration during EUS-guided fine needle aspiration may increase the diagnostic yield of mucinous cysts of the pancreas. *J Clin Gastroenterol* 2011; **45**: 164-169 [PMID: 20818233 DOI: 10.1097/MCG.0b013e3181eed6d2]
56 **Atef E**, El Nakeeb A, El Hanafy E, El Hemaly M, Hamdy E, El-Geidie A. Pancreatic cystic neoplasms: predictors of malignant behavior and management. *Saudi J Gastroenterol* 2013; **19**: 45-53 [PMID: 23319038 DOI: 10.4103/1319-3767.105927]
57 **de Castro SM**, Houwert JT, van der Gaag NA, Busch OR, van Gulik TM, Gouma DJ. Evaluation of a selective management strategy of patients with primary cystic neoplasms of the pancreas. *Int J Surg* 2011; **9**: 655-658 [PMID: 21925294 DOI: 10.1016/j.ijsu.2011.08.007]
58 **Donahue TR**, Hines OJ, Farrell JJ, Tomlinson JS, Eibl G, Reber HA. Cystic neoplasms of the pancreas: results of 114 cases. *Pancreas* 2010; **39**: 1271-1276 [PMID: 20717069 DOI: 10.1097/MPA.0b013e3181e1d6f4]
59 **Grobmyer SR**, Cance WG, Copeland EM, Vogel SB, Hochwald SN. Is there an indication for initial conservative management of pancreatic cystic lesions? *J Surg Oncol* 2009; **100**: 372-374 [PMID: 19267387 DOI: 10.1002/jso.21260]
60 **Sawhney MS**, Al-Bashir S, Cury MS, Brown A, Chuttani R, Pleskow DK, Callery MP, Vollmer CM. International consensus guidelines for surgical resection of mucinous neoplasms cannot be applied to all cystic lesions of the pancreas. *Clin Gastroenterol Hepatol* 2009; **7**: 1373-1376 [PMID: 19577006 DOI: 10.1016/j.cgh.2009.06.026]
61 **Goh BK**, Tan YM, Thng CH, Cheow PC, Chung YF, Chow PK, Wong WK, Ooi LL. How useful are clinical, biochemical, and cross-sectional imaging features in predicting potentially malignant or malignant cystic lesions of the pancreas? *J Am Coll Surg* 2008; **206**: 17-27 [PMID: 18155564 DOI: 10.1016/j.jamcollsurg.2007.06.312]
62 **Lee CJ**, Scheiman J, Anderson MA, Hines OJ, Reber HA, Farrell J, Kochman ML, Foley PJ, Drebin J, Oh YS, Ginsberg G, Ahmad N, Merchant NB, Isbell J, Parikh AA, Stokes JB, Bauer T, Adams RB, Simeone DM. Risk of malignancy in resected cystic tumors of the pancreas < or =3 cm in size: is it safe to observe asymptomatic patients? *J Gastrointest Surg* 2008; **12**: 234-242 [PMID: 18040749 DOI: 10.1007/s11605-007-0381-y]
63 **Bournet B**, Vignolle-Vidoni A, Grand D, Roques C, Breibach F, Cros J, Muscari F, Carrère N, Selves J, Cordelier P, Buscail L. Endoscopic ultrasound-guided fine-needle aspiration plus KRAS and GNAS mutation in malignant intraductal papillary mucinous neoplasm of the pancreas. *Endosc Int Open* 2016; **4**: E1228-E1235 [PMID: 27995180 DOI: 10.1055/s-0042-117216]
64 **Khalid A**, Zahid M, Finkelstein SD, LeBlanc JK, Kaushik N, Ahmad N, Brugge WR, Edmundowicz SA, Hawes RH, McGrath KM. Pancreatic cyst fluid DNA analysis in evaluating pancreatic cysts: a report of the PANDA study. *Gastrointest Endosc* 2009; **69**: 1095-1102 [PMID: 19152896 DOI: 10.1016/j.gie.2008.07.033]
65 **Durkin C**, Krishna SG. Advanced diagnostics for pancreatic cysts: Confocal endomicroscopy and molecular analysis. *World J Gastroenterol* 2019; **25**: 2734-2742 [PMID: 31235996 DOI: 10.3748/wjg.v25.i22.2734]
66 **Napoleon B**, Lemaistre AI, Pujol B, Caillol F, Lucidarme D, Bourdariat R, Morellon-Mialhe B, Fumex F, Lefort C, Lepilliez V, Palazzo L, Monges G, Poizat F, Giovannini M. In vivo characterization of pancreatic cystic lesions by needle-based confocal laser endomicroscopy (nCLE): proposition of a comprehensive nCLE classification confirmed by an external retrospective evaluation. *Surg Endosc* 2016; **30**: 2603-2612 [PMID: 26428198 DOI: 10.1007/s00464-015-4510-5]
67 **Napoleon B**, Palazzo M, Lemaistre AI, Caillol F, Palazzo L, Aubert A, Buscail L, Maire F, Morellon BM, Pujol B, Giovannini M. Needle-based confocal laser endomicroscopy of pancreatic cystic lesions: a prospective multicenter validation study in patients with definite diagnosis.

Endoscopy 2019; **51**: 825-835 [PMID: 30347425 DOI: 10.1055/a-0732-5356]

68 **Karia K**, Waxman I, Konda VJ, Gress FG, Sethi A, Siddiqui UD, Sharaiha RZ, Kedia P, Jamal-Kabani A, Gaidhane M, Kahaleh M. Needle-based confocal endomicroscopy for pancreatic cysts: the current agreement in interpretation. *Gastrointest Endosc* 2016; **83**: 924-927 [PMID: 26382051 DOI: 10.1016/j.gie.2015.08.080]

69 **Zhong L**, Chai N, Linghu E, Li H, Yang J, Tang P. A Prospective Study on Contrast-Enhanced Endoscopic Ultrasound for Differential Diagnosis of Pancreatic Cystic Neoplasms. *Dig Dis Sci* 2019; **64**: 3616-3622 [PMID: 31290040 DOI: 10.1007/s10620-019-05718-z]

70 **Aparicio JR**, Martínez J, Niveiro M, Cabezas A, Ruiz F, De Madaria E, Casellas JA. Direct intracystic biopsy and pancreatic cystoscopy through a 19-gauge needle EUS (with videos). *Gastrointest Endosc* 2010; **72**: 1285-1288 [PMID: 20970789 DOI: 10.1016/j.gie.2010.08.036]

71 **Shakhatreh MH**, Naini SR, Brijbassie AA, Grider DJ, Shen P, Yeaton P. Use of a novel through-the-needle biopsy forceps in endoscopic ultrasound. *Endosc Int Open* 2016; **4**: E439-E442 [PMID: 27092324 DOI: 10.1055/s-0042-101941]

72 **Huelsen A**, Cooper C, Saad N, Gupta S. Endoscopic ultrasound-guided, through-the-needle forceps biopsy in the assessment of an incidental large pancreatic cystic lesion with prior inconclusive fine-needle aspiration. *Endoscopy* 2017; **49**: E109-E110 [PMID: 28192812 DOI: 10.1055/s-0043-100217]

73 **Crinò SF**, Bernardoni L, Brozzi L, Barresi L, Malleo G, Salvia R, Frulloni L, Sina S, Parisi A, Remo A, Larghi A, Gabbrielli A, Manfrin E. Association between macroscopically visible tissue samples and diagnostic accuracy of EUS-guided through-the-needle microforceps biopsy sampling of pancreatic cystic lesions. *Gastrointest Endosc* 2019; **90**: 933-943 [PMID: 31100310 DOI: 10.1016/j.gie.2019.05.009]

74 **Yang D**, Samarasena JB, Jamil LH, Chang KJ, Lee D, Ona MA, Lo SK, Gaddam S, Liu Q, Draganov PV. Endoscopic ultrasound-guided through-the-needle microforceps biopsy in the evaluation of pancreatic cystic lesions: a multicenter study. *Endosc Int Open* 2018; **6**: E1423-E1430 [PMID: 30574535 DOI: 10.1055/a-0770-2700]

75 **Basar O**, Yuksel O, Yang DJ, Samarasena J, Forcione D, DiMaio CJ, Wagh MS, Chang K, Casey B, Fernandez-Del Castillo C, Pitman MB, Brugge WR. Feasibility and safety of microforceps biopsy in the diagnosis of pancreatic cysts. *Gastrointest Endosc* 2018; **88**: 79-86 [PMID: 29510146 DOI: 10.1016/j.gie.2018.02.039]

76 **Kovacevic B**, Karstensen JG, Havre RF, Pham KD, Giovannini M, Dabizzi E, Arcidiacono P, Santo E, Sequeiros EV, Klausen P, Rift CV, Hasselby JP, Toxværd A, Kalaitzakis E, Hansen CP, Vilmann P. Initial experience with EUS-guided microbiopsy forceps in diagnosing pancreatic cystic lesions: A multicenter feasibility study (with video). *Endosc Ultrasound* 2018; **7**: 383-388 [PMID: 30168479 DOI: 10.4103/eus.eus_16_18]

77 **Hara T**, Yamaguchi T, Ishihara T, Tsuyuguchi T, Kondo F, Kato K, Asano T, Saisho H. Diagnosis and patient management of intraductal papillary-mucinous tumor of the pancreas by using peroral pancreatoscopy and intraductal ultrasonography. *Gastroenterology* 2002; **122**: 34-43 [PMID: 11781278 DOI: 10.1053/gast.2002.30337]

78 **Maire F**, Couvelard A, Hammel P, Ponsot P, Palazzo L, Aubert A, Degott C, Dancour A, Felce-Dachez M, O'toole D, Lévy P, Ruszniewski P. Intraductal papillary mucinous tumors of the pancreas: the preoperative value of cytologic and histopathologic diagnosis. *Gastrointest Endosc* 2003; **58**: 701-706 [PMID: 14595305 DOI: 10.1016/s0016-5107(03)02032-7]

79 **Azar C**, Van de Stadt J, Rickaert F, Devière M, Baize M, Klöppel G, Gelin M, Cremer M. Intraductal papillary mucinous tumours of the pancreas. Clinical and therapeutic issues in 32 patients. *Gut* 1996; **39**: 457-464 [PMID: 8949654 DOI: 10.1136/gut.39.3.457]

80 **Kitagawa Y**, Unger TA, Taylor S, Kozarek RA, Traverso LW. Mucus is a predictor of better prognosis and survival in patients with intraductal papillary mucinous tumor of the pancreas. *J Gastrointest Surg* 2003; **7**: 12-19 [PMID: 12559180 DOI: 10.1016/S1091-255X(02)00152-X]

81 **Yamaguchi T**, Shirai Y, Ishihara T, Sudo K, Nakagawa A, Ito H, Miyazaki M, Nomura F, Saisho H. Pancreatic juice cytology in the diagnosis of intraductal papillary mucinous neoplasm of the pancreas: significance of sampling by peroral pancreatoscopy. *Cancer* 2005; **104**: 2830-2836 [PMID: 16287152 DOI: 10.1002/cncr.21565]

82 **Miura T**, Igarashi Y, Okano N, Miki K, Okubo Y. Endoscopic diagnosis of intraductal papillary-mucinous neoplasm of the pancreas by means of peroral pancreatoscopy using a small-diameter videoscope and narrow-band imaging. *Dig Endosc* 2010; **22**: 119-123 [PMID: 20447205 DOI: 10.1111/j.1443-1661.2010.00926.x]

83 **Itoi T**, Sofuni A, Itokawa F, Kurihara T, Tsuchiya T, Ishii K, Tsuji S, Ikeuchi N, Arisaka Y, Moriyasu F. Initial experience of peroral pancreatoscopy combined with narrow-band imaging in the diagnosis of intraductal papillary mucinous neoplasms of the pancreas (with videos). *Gastrointest Endosc* 2007; **66**: 793-797 [PMID: 17905024 DOI: 10.1016/j.gie.2007.03.1096]

84 **Nagayoshi Y**, Aso T, Ohtsuka T, Kono H, Ideno N, Igarashi H, Takahata S, Oda Y, Ito T, Tanaka M. Peroral pancreatoscopy using the SpyGlass system for the assessment of intraductal papillary mucinous neoplasm of the pancreas. *J Hepatobiliary Pancreat Sci* 2014; **21**: 410-417 [PMID: 24123930 DOI: 10.1002/jhbp.44]

85 **Chen YK**. Preclinical characterization of the Spyglass peroral cholangiopancreatoscopy system for direct access, visualization, and biopsy. *Gastrointest Endosc* 2007; **65**: 303-311 [PMID: 17258991 DOI: 10.1016/j.gie.2006.07.048]

86 **Ramchandani M**, Reddy DN, Gupta R, Lakhtakia S, Tandan M, Darisetty S, Sekaran A, Rao GV. Role of single-operator peroral cholangioscopy in the diagnosis of indeterminate biliary lesions: a single-center, prospective study. *Gastrointest Endosc* 2011; **74**: 511-519 [PMID: 21737076 DOI: 10.1016/j.gie.2011.04.034]

87 **Draganov PV**, Chauhan S, Wagh MS, Gupte AR, Lin T, Hou W, Forsmark CE. Diagnostic accuracy of conventional and cholangioscopy-guided sampling of indeterminate biliary lesions at the time of ERCP: a prospective, long-term follow-up study. *Gastrointest Endosc* 2012; **75**: 347-353 [PMID: 22248602 DOI: 10.1016/j.gie.2011.09.020]

88 **Tanaka SA**, McKee JD, Conway WC. Intracystic Biopsy and Diagnosis of Intraductal Papillary Mucinous Neoplasm *via* SpyGlass Pancreatoscopy. *Ochsner J* 2015; **15**: 452-454 [PMID: 26730233]

89 **Elta GH**, Enestvedt BK, Sauer BG, Lennon AM. ACG Clinical Guideline: Diagnosis and Management of Pancreatic Cysts. *Am J Gastroenterol* 2018; **113**: 464-479 [PMID: 29485131 DOI: 10.1038/ajg.2018.14]

90 **Chaudhary S**, Sun SY. Endoscopic ultrasound-guided radiofrequency ablation in gastroenterology: New horizons in search. *World J Gastroenterol* 2017; **23**: 4892-4896 [PMID: 28785143 DOI: 10.3748/wjg.v23.i27.4892]

91 **Pai M**, Habib N, Senturk H, Lakhtakia S, Reddy N, Cicinnati VR, Kaba I, Beckebaum S, Drymousis P, Kahaleh M, Brugge W. Endoscopic ultrasound guided radiofrequency ablation, for pancreatic cystic neoplasms and neuroendocrine tumors. *World J Gastrointest Surg* 2015; **7**: 52-59 [PMID: 25914783 DOI: 10.4240/wjgs.v7.i4.52]

92 **Silviu UB**, Daniel P, Claudiu M, Săndulescu L, Simona F, Ştefan P, Valeriu Ş, Adrian S. Endoscopic ultrasound-guided radiofrequency ablation of the pancreas: An experimental study with pathological correlation. *Endosc Ultrasound* 2015; **4**: 330-335 [PMID: 26643702 DOI: 10.4103/2303-9027.170426]

93 **Ofosu A**, Ramai D, Adler DG. Endoscopic ultrasound-guided ablation of pancreatic cystic neoplasms: ready for prime time? *Ann Gastroenterol* 2019; **32**: 39-45 [PMID: 30598590 DOI: 10.20524/aog.2018.0331]

94 **Schönleben F**, Allendorf JD, Qiu W, Li X, Ho DJ, Ciau NT, Fine RL, Chabot JA, Remotti HE, Su GH. Mutational analyses of multiple oncogenic pathways in intraductal papillary mucinous neoplasms of the pancreas. *Pancreas* 2008; **36**: 168-172 [PMID: 18376308 DOI: 10.1097/MPA.0b013e318158a4d2]

95 **Satoh K**, Shimosegawa T, Moriizumi S, Koizumi M, Toyota T. K-ras mutation and p53 protein accumulation in intraductal mucin-hypersecreting neoplasms of the pancreas. *Pancreas* 1996; **12**: 362-368 [PMID: 8740403 DOI: 10.1097/00006676-199605000-00007]

96 **Schönleben F**, Qiu W, Remotti HE, Hohenberger W, Su GH. PIK3CA, KRAS, and BRAF mutations in intraductal papillary mucinous neoplasm/carcinoma (IPMN/C) of the pancreas. *Langenbecks Arch Surg* 2008; **393**: 289-296 [PMID: 18343945 DOI: 10.1007/s00423-008-0285-7]

97 **Khalid A**, Pal R, Sasatomi E, Swalsky P, Slivka A, Whitcomb D, Finkelstein S. Use of microsatellite marker loss of heterozygosity in accurate diagnosis of pancreaticobiliary malignancy from brush cytology samples. *Gut* 2004; **53**: 1860-1865 [PMID: 15542529 DOI: 10.1136/gut.2004.039784]

98 **Hamilton SR**. World Health Organization Classification of Tumors. Pathology and Genetics of Tumors of the Digestive System. IARC Press: Lyon, 2000

99 **Nagtegaal ID**, Odze RD, Klimstra D, Paradis V, Rugge M, Schirmacher P, Washington KM, Carneiro F, Cree IA; WHO Classification of Tumours Editorial Board. The 2019 WHO classification of tumours of the digestive system. *Histopathology* 2020; **76**: 182-188 [PMID: 31433515 DOI: 10.1111/his.13975]

100 **Z'graggen K**, Rivera JA, Compton CC, Pins M, Werner J, Fernández-del Castillo C, Rattner DW, Lewandrowski KB, Rustgi AK, Warshaw AL. Prevalence of activating K-ras mutations in the evolutionary stages of neoplasia in intraductal papillary mucinous tumors of the pancreas. *Ann Surg* 1997; **226**: 491-8; discussion 498 [PMID: 9351717 DOI: 10.1097/00000658-199710000-00010]

101 **Jones M**, Zheng Z, Wang J, Dudley J, Albanese E, Kadayifci A, Dias-Santagata D, Le L, Brugge WR, Fernandez-del Castillo C, Mino-Kenudson M, Iafrate AJ, Pitman MB. Impact of next-generation sequencing on the clinical diagnosis of pancreatic cysts. *Gastrointest Endosc* 2016; **83**: 140-148 [PMID: 26253016 DOI: 10.1016/j.gie.2015.06.047]

102 **Kadayifci A**, Atar M, Wang JL, Forcione DG, Casey BW, Pitman MB, Brugge WR. Value of adding GNAS testing to pancreatic cyst fluid KRAS and carcinoembryonic antigen analysis for the diagnosis of intraductal papillary mucinous neoplasms. *Dig Endosc* 2017; **29**: 111-117 [PMID: 27514845 DOI: 10.1111/den.12710]

103 **Rosenbaum MW**, Jones M, Dudley JC, Le LP, Iafrate AJ, Pitman MB. Next-generation sequencing adds value to the preoperative diagnosis of pancreatic cysts. *Cancer Cytopathol* 2017; **125**: 41-47 [PMID: 27647802 DOI: 10.1002/cncy.21775]

104 **Singhi AD**, Nikiforova MN, Fasanella KE, McGrath KM, Pai RK, Ohori NP, Bartholow TL, Brand RE, Chennat JS, Lu X, Papachristou GI, Slivka A, Zeh HJ, Zureikat AH, Lee KK, Tsung A, Mantha GS, Khalid A. Preoperative GNAS and KRAS testing in the diagnosis of pancreatic mucinous cysts. *Clin Cancer Res* 2014; **20**: 4381-4389 [PMID: 24938521 DOI: 10.1158/1078-0432.CCR-14-0513]

105 **Springer S**, Wang Y, Dal Molin M, Masica DL, Jiao Y, Kinde I, Blackford A, Raman SP, Wolfgang CL, Tomita T, Niknafs N, Douville C, Ptak J, Dobbyn L, Allen PJ, Klimstra DS, Schattner MA, Schmidt CM, Yip-Schneider M, Cummings OW, Brand RE, Zeh HJ, Singhi AD, Scarpa A, Salvia R,

Malleo G, Zamboni G, Falconi M, Jang JY, Kim SW, Kwon W, Hong SM, Song KB, Kim SC, Swan N, Murphy J, Geoghegan J, Brugge W, Fernandez-Del Castillo C, Mino-Kenudson M, Schulick R, Edil BH, Adsay V, Paulino J, van Hooft J, Yachida S, Nara S, Hiraoka N, Yamao K, Hijioka S, van der Merwe S, Goggins M, Canto MI, Ahuja N, Hirose K, Makary M, Weiss MJ, Cameron J, Pittman M, Eshleman JR, Diaz LA Jr, Papadopoulos N, Kinzler KW, Karchin R, Hruban RH, Vogelstein B, Lennon AM. A combination of molecular markers and clinical features improve the classification of pancreatic cysts. *Gastroenterology* 2015; **149**: 1501-1510 [PMID: 26253305 DOI: 10.1053/j.gastro.2015.07.041]

106 **Arner DM**, Corning BE, Ahmed AM, Ho HC, Weinbaum BJ, Siddiqui U, Aslanian H, Adams RB, Bauer TW, Wang AY, Shami VM, Sauer BG. Molecular analysis of pancreatic cyst fluid changes clinical management. *Endosc Ultrasound* 2018; **7**: 29-33 [PMID: 29451166 DOI: 10.4103/eus.eus_22_17]

107 **Wu J**, Matthaei H, Maitra A, Dal Molin M, Wood LD, Eshleman JR, Goggins M, Canto MI, Schulick RD, Edil BH, Wolfgang CL, Klein AP, Diaz LA Jr, Allen PJ, Schmidt CM, Kinzler KW, Papadopoulos N, Hruban RH, Vogelstein B. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. *Sci Transl Med* 2011; **3**: 92ra66 [PMID: 21775669 DOI: 10.1126/scitranslmed.3002543]

108 **Singhi AD**, McGrath K, Brand RE, Khalid A, Zeh HJ, Chennat JS, Fasanella KE, Papachristou GI, Slivka A, Bartlett DL, Dasyam AK, Hogg M, Lee KK, Marsh JW, Monaco SE, Ohori NP, Pingpank JF, Tsung A, Zureikat AH, Wald AI, Nikiforova MN. Preoperative next-generation sequencing of pancreatic cyst fluid is highly accurate in cyst classification and detection of advanced neoplasia. *Gut* 2018; **67**: 2131-2141 [PMID: 28970292 DOI: 10.1136/gutjnl-2016-313586]

109 **Zhang ML**, Pitman MB. Practical Applications of Molecular Testing in the Cytologic Diagnosis of Pancreatic Cysts. *J Mol Pathol* 2021; **2**: 11-22 [DOI: 10.3390/jmp2010002]

110 **Mittal C**, Obuch JC, Hammad H, Edmundowicz SA, Wani S, Shah RJ, Brauer BC, Attwell AR, Kaplan JB, Wagh MS. Technical feasibility, diagnostic yield, and safety of microforceps biopsies during EUS evaluation of pancreatic cystic lesions (with video). *Gastrointest Endosc* 2018; **87**: 1263-1269 [PMID: 29309781 DOI: 10.1016/j.gie.2017.12.025]

111 **Zhang ML**, Arpin RN, Brugge WR, Forcione DG, Basar O, Pitman MB. Moray micro forceps biopsy improves the diagnosis of specific pancreatic cysts. *Cancer Cytopathol* 2018; **126**: 414-420 [PMID: 29660844 DOI: 10.1002/cncy.21988]

112 **Cheesman AR**, Zhu H, Kumta NA. EUS-guided microforceps biopsy and needle-based confocal laser endomicroscopy significantly improve the diagnostic yield and have major impact on clinical management of pancreatic cystic lesions [abstract]. *Gastrointest Endosc* 2019; **89**: AB144

113 **Robles-Medranda C**, Olmos JI. EUS-through-the-needle technologies in the diagnosis and malignancy detection of pancreatic cysts: a comparative study between different technologies [abstract]. *Gastrointest Endosc* 2019; **89**: AB608-A609

114 **Samarasena J**, Yu A, Lee D, Hashimoto R, Lu Y, Thieu D, Mai D, Lee J, Chang K. EUS-guided through-the-needle biopsy for pancreatic cystic lesions. *VideoGIE* 2019; **4**: 436-439 [PMID: 31517173 DOI: 10.1016/j.vgie.2019.04.020]

115 **Vestrup Rift C**, Melchior LC, Kovacevic B, Toxvaerd A, Klausen P, Karstensen JG, Kalaitzakis E, Storkholm J, Palnaes Hansen C, Vilmann P, Preuss Hasselby J. Next-generation sequencing of endoscopic ultrasound guided microbiopsies from pancreatic cystic neoplasms. *Histopathology* 2019; **75**: 767-771 [PMID: 31278869 DOI: 10.1111/his.13949]

116 **Yang D**, Trindade AJ, Yachimski P, Benias P, Nieto J, Manvar A, Ho S, Esnakula A, Gamboa A, Sethi A, Gupte A, Khara HS, Diehl DL, El Chafic A, Shah J, Forsmark CE, Draganov PV. Histologic Analysis of Endoscopic Ultrasound-Guided Through the Needle Microforceps Biopsies Accurately Identifies Mucinous Pancreas Cysts. *Clin Gastroenterol Hepatol* 2019; **17**: 1587-1596 [PMID: 30471456 DOI: 10.1016/j.cgh.2018.11.027]

117 **Wilen J**, Visrodia K, Lan G. The feasibility and value of cyst wall biopsy using micro forceps in the diagnosis of pancreatic cysts [abstract]. *Gastrointest Endosc* 2019; **89**: AB605

118 **Krishna SG**, Hart PA, Malli A, Kruger AJ, McCarthy ST, El-Dika S, Walker JP, Dillhoff ME, Manilchuk A, Schmidt CR, Pawlik TM, Porter K, Arnold CA, Cruz-Monserrate Z, Conwell DL. Endoscopic Ultrasound-Guided Confocal Laser Endomicroscopy Increases Accuracy of Differentiation of Pancreatic Cystic Lesions. *Clin Gastroenterol Hepatol* 2020; **18**: 432-440. e6 [PMID: 31220640 DOI: 10.1016/j.cgh.2019.06.010]

119 **Keane MG**, Wehnert N, Perez-Machado M, Fusai GK, Thorburn D, Oppong KW, Carroll N, Metz AJ, Pereira SP. A prospective trial of CONfocal endomicroscopy in CYSTic lesions of the pancreas: CONCYST-01. *Endosc Int Open* 2019; **7**: E1117-E1122 [PMID: 31475228 DOI: 10.1055/a-0957-2976]

120 **Cheesman AR**, Zhu H, Liao X, Szporn AH, Kumta NA, Nagula S, DiMaio CJ. Impact of EUS-guided microforceps biopsy sampling and needle-based confocal laser endomicroscopy on the diagnostic yield and clinical management of pancreatic cystic lesions. *Gastrointest Endosc* 2020; **91**: 1095-1104 [PMID: 31881204 DOI: 10.1016/j.gie.2019.12.022]

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REVIEW

Status quo and future prospects of artificial neural network from the perspective of gastroenterologists

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Abstract

Artificial neural networks (ANNs) are one of the primary types of artificial intelligence and have been rapidly developed and used in many fields. In recent years, there has been a sharp increase in research concerning ANNs in gastrointestinal (GI) diseases. This state-of-the-art technique exhibits excellent performance in diagnosis, prognostic prediction, and treatment. Competitions between ANNs and GI experts suggest that efficiency and accuracy might be compatible in virtue of technique advancements. However, the shortcomings of ANNs are not negligible and may induce alterations in many aspects of medical practice. In this review, we introduce basic knowledge about ANNs and summarize the current achievements of ANNs in GI diseases from the perspective of gastroenterologists. Existing limitations and future directions are also proposed to optimize ANN's clinical potential. In consideration of barriers to interdisciplinary knowledge, sophisticated concepts are discussed using plain words and metaphors to make this review more easily understood by medical practitioners and the general public.

Key Words: Artificial neural network; Gastrointestinal disease; Diagnosis; Treatment; Prognosis; Endoscopy

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Core Tip: This review summarizes the current achievements and existing limitations of artificial neural networks (ANNs) used in gastrointestinal (GI) diseases. The future directions of ANNs are also discussed to provide references for promoting its clinical value. To make this review readable, we introduce the basic knowledge of ANN and illustrate the contents from the perspective of gastroenterologists. ANN is believed to play a critical role in clinical practice of GI diseases.

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INTRODUCTION

The concept of artificial intelligence (AI) was first proposed at Dartmouth Conferences in 1956. Although definitions of AI vary, it is universally accepted that AI is designed to perform tasks that were previously fulfilled only by human intelligence. The debate on future directions of AI continues today. In the past few decades, AI has only been used to construct complex mathematical models. With the concurrent advancement of hardware, data, operation, and algorithm, AI has exhibited incomparable significance in many fields, such as fingerprint identification, information retrieval, and language translation. From 2016 to 2017, Robot AlphaGo driven by AI continuously defeated two world class Go grandmasters, which improved the connatural cognizance towards computer functions and drove us to reconsider the relationship between AI and human beings in the future.

As a branch of computer science, the integration of the cross-curricular interests of AI provides a strong boost for medicine. Many studies have focused on AI-aided screening, prevention, diagnosis, treatment, and health management. Since the MYCIN system was invented for clinical decisions with regard to infectious diseases in the 1970s[1], multiple systems supported by AI have been produced to meet the needs of medical care. For example, the AlmeHealthCoach system can automatically arrange medication regimens for patients at home. MedicalGraph can analyze the medical records and relevant laboratory tests to predict patients' disease risk. These productions also performed well in the field of medical robots, imaging diagnosis, and research on new drugs. Such progress has broken through unwavering predicaments of current medicine.

Artificial neural networks (ANNs) belong to a subtype of AI and have been used in many subspecialties of clinical medicine, including pathology[2], radiology[3], cardiovasology[4], neurology[5], orthopaedics[6], and gastroenterology[7]. Gastrointes-tinal (GI) tract disorders are diseases of the human digestive system and the therapeutic regimens of GI diseases strongly rely on imaging examinations. Mass image data are burdensome to radiologists, potentially increasing the odds of inaccurate clinical decisions. Compelling evidence has suggested that ANNs could effectively solve this problem[7-9]. Conversely, the rapid improvement of ANNs demands that clinicians enrich their knowledge and fully understand the strengths and pitfalls of ANNs.

This review focuses on the application of ANNs in GI diseases. We first introduce the basic knowledge of ANNs to help readers with a foundation for learning. The current achievements of ANNs are systematically summarized based on their applic-ations. The characteristics, limitations, and future directions of research are then proposed from the perspective of gastroenterologists.

OVERVIEW OF ANN

Machine learning

Machine learning (ML) is a method that endows computers with the ability to actively analyze data. If AI is a metaphor for one person, ML is like the brain of that person. The birth of ML serves as the fruit of multidisciplinary crosstalk, including statistics, probability theory, approximation theory, and algorithm complexity. The intrinsic connection of variables can be deduced based on learning experiences, as with a reasoned detective to reconstruct the crime from known facts. The cultivation of a detective requires the accumulation of basic knowledge and experience in handling many experiences. The detective will be qualified to participate in cases only after he receives adequate training and passes exams. Similarly, ML algorithms receive many data with known answers, and the resulting operation formula can be constructed to manage specific problems. This process is defined as the training phase. Different from classical linear functions, ML is automatic and unobservable. After training, ML

algorithms will be tested with external datasets during validation phase, from which the test results are critical indicators of model efficiency and accuracy. Common algorithms for ML include ANNs, decision trees, naïve Bayes, support vector machines (SVMs), random forest, and expectation maximization.

Based on the learning approaches, ML can be typically divided into four subfamilies: (1) Supervised; (2) Unsupervised; (3) Semi-supervised; and (4) Reinforcement learning. Supervised ML is given a dataset with known correct output (*i.e.*, labelled data), and the accuracy of the models is gradually optimized *via* feedback of labelled data. For example, to construct a model for pathological classification between gastric cancer (GC) and normal tissues, the diagnostic results in the internal datasets should be labelled. Supervised ML aims to solve two primary tasks: (1) Classification, such as rapid review of chest X-rays; and (2) Regression, such as predicting the recurrence risk of cancer patients based on clinical indices. These two tasks are based on the exploration of potential rules among variables. Logistic regression, back propagation neural networks (BPNNs), and K-nearest neighbors are common ML algorithms. Notably, most studies involved in this review are supervised models.

Unsupervised ML refers to a learning dataset without effective labels. This learning approach is typically used for disorderly and unsystematic data. Clustering is the representative use of unsupervised ML and can help uncover innate models and groups based on exploratory data. Taking next-generation sequencing as an example, tens of thousands of gene expression levels are listed. Genes will be clustered based on expression similarities and the process does not require prelabelled data. Additionally, unsupervised ML can be used to group and label datasets for next-step supervised algorithms.

Semi-supervised learning serves as an important helper for pattern recognition. It combines supervised and unsupervised approaches that flexibly use labelled and unlabeled data, thus integrating the advantages of both types. During semi-supervised learning, unsupervised parts can save the cost of human labor for labelling or classifying mass data, as well as decrease the difficulties of data handling, while the supervised part can guarantee model accuracy. Self-training, transductive learning, and transductive SVM are all types of semi-supervised learning.

Reinforcement learning is copied from the psychological process of acquired behaviors. Like training pets, any preset data are not required. Algorithms are gradually fed by signals in each layer and then receive "rewards" or "penalties" instantaneously after evaluating errors between true values and output data. The efficiency of algorithms can be adjusted during learning, and the core rules of reinforcement learning is maximizing rewards and minimizing penalties. How to obtain optimal balances and reliability allocation remains the most challenging problem for investigators. Currently, computers can independently solve complex problems based on reinforcement learning, as in Robot AlphaGo.

ANN history and fundamentals

The concept of ANN was first proposed in 1943. The development of ANN models has complicatedly approached from then on. In the 1980s, the rapid progress of ANN algorithms boosted a modern revolution (Figure 1). The design of ANNs is based on the human brain's neural network. An ANN is primarily composed of many neurons. The data flow (*i.e.*, the signal) is passed and programmed across neural nodes. Neurons in the different layers have their own missions to solve problems, which can be analogous to factory production lines. The deconstruction of missions for data analysis provides the ability to find the optimal solution within the shortest time. As a type of parallel distributed system driven by mass data, ANNs are free from the requirements of logical or mathematical associations known beforehand.

The structure of an ANN can be divided into three parts (Figure 2). First, input layers are responsible for receiving signals from specific datasets and serve as the "eye" of ANN to view the external environments. Second, hidden layers are predominant stages of ANNs and hinge on associations between input and output data. Raw data from input layers will be processed by neurons, and the numbers of hidden layers are always more than one. Missions for ANNs can be subtly disassembled and distributed into several layers. The multilayer structure promotes the efficiency of data analysis. Neurons in layers have serial connections based on settled sequences rather than parallel or parallel-series connections. Last, output layers make the final decisions and output results.

Common terms that describe shapes and capabilities of ANNs are listed (Table 1). The depth determines the efficiency of ANNs. The simplest ANN is a single-layer network (*i.e.*, a perceptron neural network) and has one input layer, one output layer, and one hidden layer. A single-layer network can only be used to describe a single

Table 1 Terms commonly used to describe artificial neural network structures

Term of ANN	Specific meaning
Size	Number of neurons in the whole model
Width	Number of neurons in the one layer
Depth	Number of layers
Framework	Arrangement methods of layers and neurons
Capability	The reflection contents of reality by the specific model

ANN: Artificial neural networks.

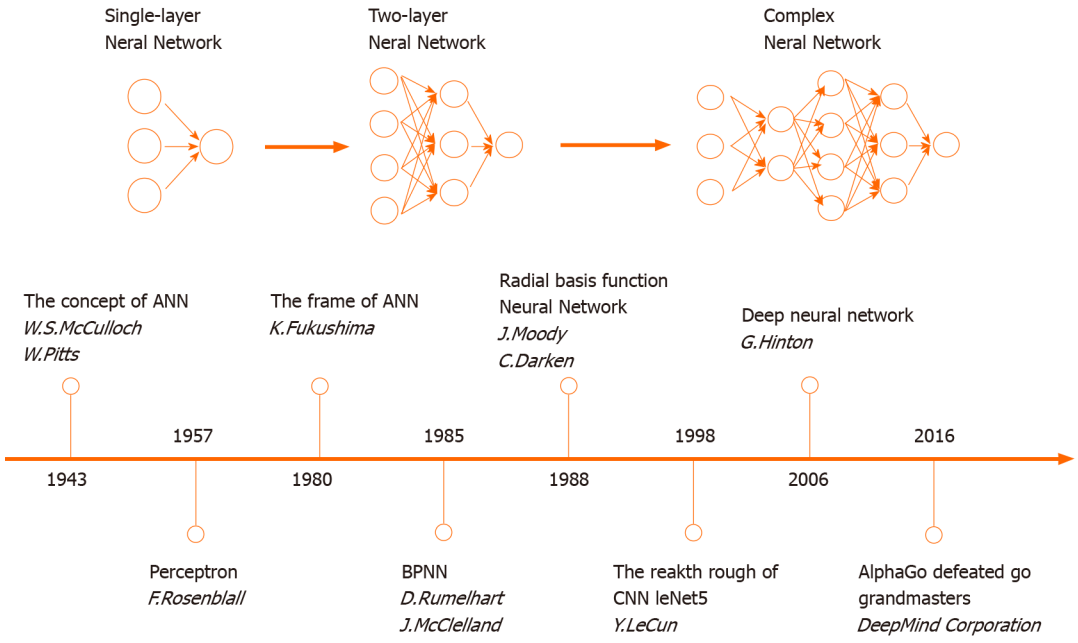


Figure 1 In the 1980s, the rapid progress of artificial neural network algorithms boosted a modern revolution. ANN: Artificial neural network; BPNN: Back propagation neural network; CNN: Convolutional neural network.

dichotomy based on a single factor, thus making most problems beyond its capabilities. Amplifying ANN depth can geometrically augment competence of fitting complicated features. Researchers began to create ANN models with multiple layers. Even in 2016, He *et al*[10] reported an ANN model with over 1000 layers[10]. However, excessive ANN depth may lead to system instability and loss of superficial learning abilities.

Each layer has a definite mission if the dataset volume and ANN depth are set. Width can be compared to the number of workers in a production line. Adequate workers will improve the qualities of tasks, while labor redundance will yield negative effects. A broad ANN width enhances the access to different data features, while a narrow width leads to low efficiency of feature extraction and excessive hardware burden. The concept of size describes the accumulation of both depth and width, which is an important index when evaluating an ANN's capability.

Additionally, ANNs can be divided into two major categories based on the directions of signal flow between hidden layers. There are many differences between the two types of ANNs (Table 2). In the feedforward neural network (FNN), data flow is unidirectional from the upper levels to the lower levels without feedback; no connections exist between neurons in any given layer. In terms of system learning, FNN is convenient for reprogramming and can process non-linear questions. The other one is feedback neural network. One neuron in feedback neural network can pass its output data to the other neurons in the same or upper layers. The algorithms will be simultaneously adjusted by signals from other neurons based on a pre-knowledge dataset. Repeated calibration contributes to the excellent robustness and

Table 2 Comparisons between feedforward and feedback neural network

Category	Feedforward neural network	Feedback neural network
Signal direction	Unidirectional	Unidirectional/bidirectional
Operation time	Short	Long
Feedback by output signal	No	Yes
Structural complexity	Simple	Complicated
Memory time	Short-term or none	Long-term
Applied ranges in medicine	Wide	Limited
Application	Perceptron network, back propagation network, radial basis function network	Recurrent neural network, Hopfieid network, Boltzmann machine

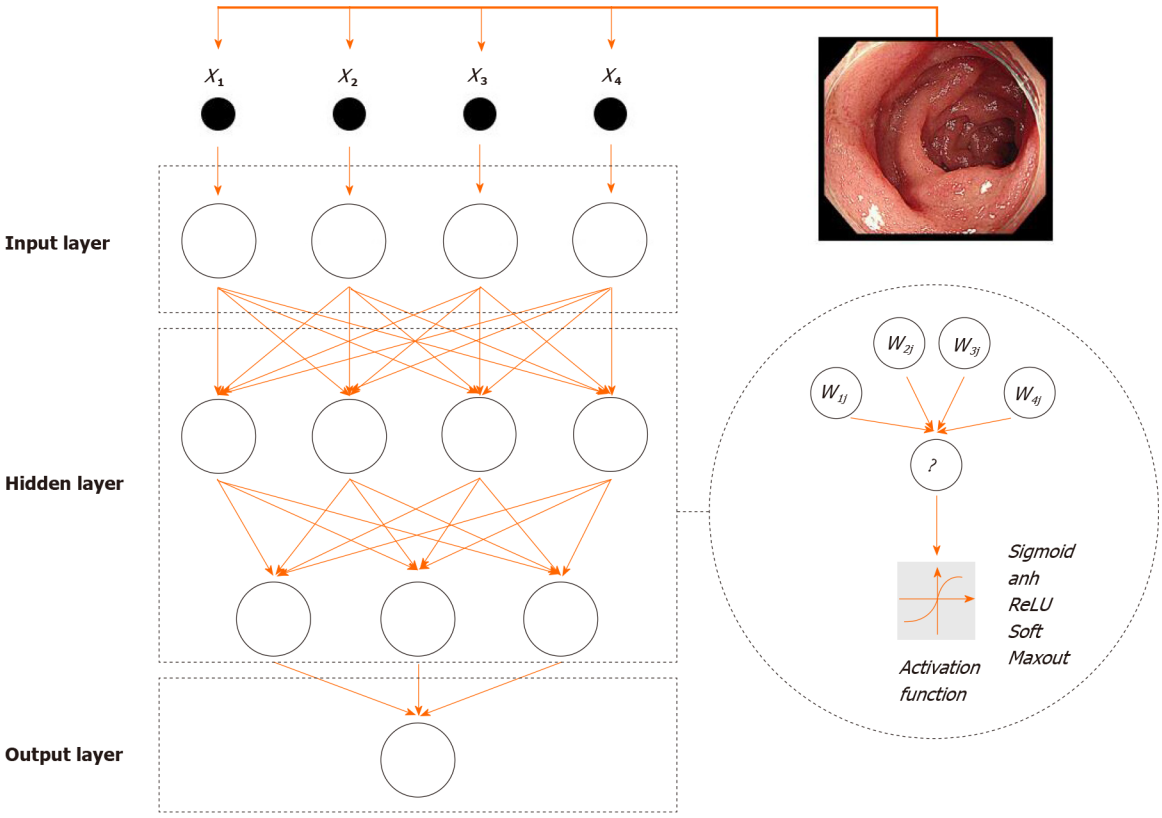


Figure 2 Structure of an artificial neural network.

accuracy of the ANN. A feedback neural network is typically used for image analysis, diagnosis, and outcome prediction.

Approaches of data processing in neurons can be defined as activation functions, and are like "all-or-none" principles of brain neurons. Activation functions primarily include sigmoid, relu, and tanh operations. Linear functions are excluded from activation functions because multiple overlays of linear functions can be compressed into one function. Accumulating studies have demonstrated that the nonlinear mapping ability of ANNs compensates for the boundedness of linear discriminant analysis. Otherwise, the "one-to-many" relationship between neurons guarantees the learning efficiency of the ANN. One neuron layer can simultaneously pass signals to more than one neuron. Importantly, signal flow is unequally allocated to each neuron in the next layer. The flow inequality makes the ANN adjustable to external environments. Before the computation of activation functions, data will be adjusted according to weight factors, each of which has a corresponding relationship with a specific neuron. Weight factors serve as the controllers of data flow. Larger weight factors have a greater influence on the output results. Conversely, setting a weight

factor equal to 0 can abrogate the functions of the corresponding neuron.

Categories of ANN

There are a wide range of ANN categories used in basic and clinical medicine, and each contains a specific algorithm that has distinct advantages over the others. Comprehension and discrimination of ANN categories can provide a good command of research frontiers, and ensure that ANNs are used in the correct situations. Brief introductions of common ANN methods are as follows.

Convolutional neural network: Convolutional neural networks (CNNs) are the most popular type of neural network nowadays due to their additional convolutional layers and pooling layers. Convolution and pooling are complex concepts in computer science. Briefly, convolutional layers can extract features from a dataset because neurons in this layer are responsible for collecting and analyzing information located in specific areas. These neurons can be analogue to cells in the visual cortex of a human brain and act as windows that look out across the external environment. Data is then sent to the pooling layers after convolution, where the features of the dataset are filtered and compressed. Pooling layers reduce background and texture information at the cost of partial data loss. Then, the data will be processed in classic neural networks.

The number of CNN layers is relatively flexible. The accuracy and performance, however, are limited in CNNs with shallow structures. The majority of CNNs belong to deep CNNs. CNNs are applicable in numerous fields, such as image classification, objective recognition, and natural language processing. These applications require learning features from mass data and being able to generalize results. Also, fine-grained recognition of medical images is the core difficulty of CNNs for feature extraction and can be finished using handcrafted or unsupervised CNNs. The extraction efficiency of unsupervised CNNs outperforms handcrafted CNNs[11].

BPNN: BPNN is a type of multi-layer FNN. BPNN algorithms can be divided into two stages: Forward propagation and back propagation. For forward propagation, signals are handled from input layers to hidden layers and then to output layers. If errors between the real output and expected output are not acceptable, the error values will undergo back propagation, where the error values will be reversely transmitted layer by layer and then equally split into all involved neurons. Neurons automatically use revisions based on feedback signals; thus, BPNN can learn and apply mapping relations without clarifying mathematical derivation beforehand. The simplicity and accuracy of BPNNs have allowed them to be widely used in medicine, particularly for the diagnosis and prediction of patient outcomes.

Bayesian neural network: The training phase of the ANN requires a certain amount of data, and small training datasets tend to impair the efficiency and practicability of most ANNs. However, sufficient sample size is not always available. Bayesian neural networks (BNNs) have been designed for such situations. BNNs are based on weight factors that describe probabilistic ranges that are subject to Gauss distribution instead of being constants. The parameters optimized by back propagation are the means and variances of the weight factors, and can increase the confidence of each item of data, increasing the dataset robustness. Recently, BNNs have exhibited great potential in analyzing image features and constructing disease models.

Recurrent neural network: Data are frequently independent in a dataset, and the input sequence makes no difference in neural networks. However, there are sometimes internally logical relations between input information. For example, a set of computed tomography (CT) images contain many layers, and the observation of images in order can dynamically describe the sizes and shapes of lesions. Thus, treating these images as independent data would lose much information. Recurrent neural network (RNN) was invented to overcome this limitation. As introduced before, the values in hidden layers are under the control of weight factors. In RNNs, weights are regulated by not only values of this time, both at the current time step and the last time step; thus, the output values of RNNs are affected by the input sequence of the dataset. RNNs have been widely used to manage datasets with successional subjects.

ACHIEVEMENTS OF ANN RESEARCH IN GI DISEASES

GI diseases primarily refer to acute and chronic disorders in the stomach and intestinal

tract. The diagnosis and prevention of GI diseases remain challenging for clinical practice based on three aspects: Anatomy, symptoms, and pathogenesis. First, regarding anatomy, the digestive tract is exposed to the external environment all the time; however, most of the digestive tract is beyond our visual observation. Thus, the GI tract is vulnerable to various irritants, but pathologic deterioration cannot be easily detected. Second, the symptoms of GI diseases are atypical. Patients with early-stage GI tumors tend to underestimate their disease severity and miss the best opportunities for treatment. Concurrently, symptoms are not qualified as critical indicators of diagnosis and outcome. Clinicians must use imaging examinations to further clarify disease conditions. Imaging interpretation is somewhat subjective, relying on experiences of radiologists. The rates of missed and delayed diagnosis remain high. Last, in terms of pathogenesis, GI diseases are multifactor diseases including heredity, food, microbes, and neuropsychic factors. Single preventive methods are ineffective for the treatment of GI diseases. However, the application of AI has gradually broken through this dilemma.

Pathological diagnosis

Pathological diagnosis is the gold standard for GI diseases, which has significant implications for diagnosis, therapeutic directions, and outcome prediction. This work is highly dependent on the subjective judgement of pathologists. Accuracy is susceptible to recognitive capability, professional experience, and fatigue levels. The misdiagnosis of pathological images remains beyond clinical requirements. With the rapid development of digital pathological sections, the transformation from qualitative to quantitative analysis has become a research topic. ANN models are effective at computer-aided pathological diagnosis. Karakitsos *et al*[12] tried to explore the potential of ANNs to discriminate benign from malignant gastric cells, and their model accuracy reached 97%[12]. Despite concentrating on cellular morphology, the capability of interpreting pathological images has been revealed.

Haematoxylin-eosin (H&E) staining is the most common method for discriminating benign and malignant lesions. For GC, most studies used CNN models to construct diagnostic systems for H&E staining[13-17]. In 2017, Sharma *et al*[17] reported the first study about computer-aided classification in H&E staining of GC. Regretfully, their system exhibited an accuracy of only 69.9%, far from the clinical standard[17]. Rapid progress has been made during the past three years. A CNN model fed by over 2000 H&E slide images achieved a near 100% sensitivity and 80.6% specificity[13]. Such optimal sensitivity showed that this model has potential in preliminary screening of H&E staining. Kosaraju *et al*[14] developed a novel CNN model called Deep-Hipo, which could extract and capture features at both high and low magnification levels. The overall accuracy of Deep-Hipo was 93.7%[14]. Deep learning model was also constructed for the prediction of microsatellite instability in colorectal cancer. The model significantly outperformed pathologists[18]. Importantly, models detecting multiscale receptive fields can be extended for survival prediction, subtype classification of GC, and selection of sensitive drugs.

Most studies concerning colorectal cancer (CRC) have also focused on distinguishing between benign and malignant tissues. Based on existing results, the accuracies or area under the curve (AUC) values of ANN-based models all exceed 80%[19-25]. Colon glands are important structures and indicators for pathological assessment[26]. However, the variation of glands in slides has been a barrier both for pathologists and algorithms. CNN models invented for it got excellent results about gland segmentation and classification[27-30]. Sirinukunwattana *et al*[31] showed a CNN model that automatically detects cell nuclei in CRC. The combination of spatially constrained CNN and softmax CNN might become a useful tool for exploring the tumor microenvironment[31]. Interestingly, a study investigated the correlation between genomic and epigenetic alterations in CRC. CNN algorithms were used to predict RNA expression classifiers from H&E images. This work lays the foundation for a comprehensive integration of morphology and molecular features[32]. Immunohistochemical staining and fluorescence microscopy belong to members of pathological examinations, and ANNs distinctly improved their diagnostic performance in GI tumors[33-35].

In addition to the auxiliary role of ANNs in pathological images, Wei *et al*[19] compared the accuracy of their model with that of pathologists and there was no significant difference between them[19]. The process from image input to result output could be finished within 2 s[20,36,37], and the time for ANN handling single image is evidently shorter than that for pathologists. ANNs can also complete repetitive processes with high-quality results. Based on the report of Yoon *et al*[20], the training time was only approximately 10 d[20], while the training time of a skilled pathologist

was based on the order of years. Thus, all evidence indicates the great potential of ANNs in fast pathological diagnosis.

Radiological examinations

X-ray, CT, and magnetic resonance imaging (MRI) are common radiological examinations. CT is accepted as first choice for GI diseases due to its high image quality, convenience, and price[38,39]. The increasing clinical demand for examinations places great burdens on radiologists. Experience-based diagnosis has innate limitations in accuracy and efficiency. This dilemma requires novel computer-aided models with the support of new techniques. Compelling evidence indicates that ANNs can provide outstanding values in auxiliary diagnosis[40,41], and are not limited to this field.

At the end of the last century, an Austrian group investigated the potential of ANNs in single-photon emission CT[42]. Despite the lack of further validation, this innovative research launched a sharp increase in related studies. Many of them aimed to detect primary lesions in CT images[43-47]. For example, a CNN-based model was trained with 288 CT images for polyp detection and achieved a sensitivity of 97%[47]. Studies published in earlier years used multiple types of ANNs, including BPNNs, massive-training ANNs, and BNNs[45,48-50]. In recent years, investigators have collectively turned to using CNNs[43,51,52]. This tacit alteration suggests that CNNs have fewer complications in automatic CT interpretations. The combination of ANNs and CT has also been used in the diagnosis of cancer metastasis and invasion depth[53-56], which are also important indicators of patient prognosis. A study even reported that CNN-based systems could distinguish benign and malignant lesions by CT[57]. More possible development directions of CT were discussed. In brief, the diagnostic value of CT has been tremendously strengthened with the support of ANNs.

MRI has a superior definition of abdominal viscera and presents more information to radiologists than CT. Most studies focused on the promotion of MRI in CRC. A CNN-based system created by Trebeschi *et al*[58] reached up to an AUC of 99% for advanced rectal cancer[58], and other recent studies all performed well in this field[59-61]. Certain studies have tried to explore new applications of MRI. CNNs could increase the diagnostic capabilities of 3D reconstruction[62]. Faster Region-based CNN was trained with 240 cases for evaluation of circumferential resection margins and achieved a 95.6% specificity[63]. Chemosensitivity prediction by MRI was also achieved with the support of algorithms[64,65]. KRAS mutation is an important biomarker of CRC targeted therapy[66,67]. Genetic testing is the gold standard of detecting KRAS mutation. Two Chinese groups demonstrated that deep radiomics signature serves as a promising tool for the prediction of KRAS mutations[68,69]. The progression of algorithms could also elevate the sensitivities of 3D Stomach MRI[70]. These achievements suggested the feasibility of non-invasive detection for medication guidance of GI tumors. Otherwise, the importance of ANNs in inflammatory bowel disease (IBD) diagnosis has been shown[71].

Endoscopic detection

Endoscopy is universally accepted as a revolutionary application in gastroenterology. Due to the unique advantages of the GI tract, endoscopists can visually observe diseased regions, obtain biopsies, and excise small lesions. The extensive application of endoscopy has effectively reduced mortality and economic burdens worldwide[72,73]. However, artificial discrimination and classification lead to a relatively high incidence of misdiagnosis. Conversely, endoscopy is widely used to screen and exclude diagnosis for health checks and suspected cases. Endoscopic sensitivity, therefore, must be improved. Thus, numerous studies have focused on the application of ANNs in endoscopy, and occupy the largest proportion of existing research.

The detection of GI tumors has become the mainstream direction of endoscopy. Many studies have developed endoscopic ANN-based systems. Like other methods of image analysis, investigators prefer CNNs to construct computer-aided models when managing endoscopic results[74-78]. For example, Hirasawa *et al*[74] trained a CNN model with 13584 endoscopic images and effectively captured GC lesions with a diameter of > 6 mm as well as all invasive GC[74]. Bagheri *et al*[79] proposed a CNN-based system to analyze the color levels during colonoscopies. The stratification of color levels significantly promoted diagnostic values, achieving a 93% precision and an 82% Dice score[79]. Otherwise, the fast speed is another advantage of ANN. Models can analyze thousands of images in a short time[74,80-83], which benefits from retrospective checks of images and facilitates real-time endoscopic diagnosis. For example, Zhang *et al*[80] developed a CNN model for the detection of gastric precancerous diseases in 2017[80]. After 2 years, they developed a novel CNN-based system

used for gastric polyps. This model achieved real-time analysis with 50 frames per second without a decline in diagnostic precision[84]. Intraoperative guidance and correction are urgently required. A deep CNN trained by 704 cases of colorectal adenomas, called ENDOANGEL, was published to help endoscopists monitor detection operations and avoid blind spots in real time[85]. This study is significant for computer-aided refinement of endoscopy.

Early screening has received much attention as people gradually attach importance to routine examinations. The atypical textures of lesions at an early stage pose a challenge for endoscopists. Numerous studies have aimed to improve the detection of GI polyps, precancerous lesions, and tumors at early stage using ANNs[9,78,86-92]. Interestingly, most research groups are from China and Japan, likely due to the regional characteristics of GI tumor incidence. The best performance of early detection was reported by Gao *et al*[78]. Their CNN system reached an overall accuracy of 98.7% and a specificity of 100%. ResNet50 stood out of five CNN models and performed the best in detecting CRC precancerous lesions with an accuracy of 93%[78]. Moreover, ANNs could assist in diagnosing *Helicobacter pylori* infection using the combination of blue laser imaging and linked color imaging[93-95]. This breakthrough undoubtedly expands the application ranges of endoscopy.

ANN application is not confined to traditional white-light endoscopy. Narrow-band imaging (NBI) is a new type of technique that can filter out broad-band spectrum in red, green, and blue colors, respectively. The concentrated layers of the GI mucosa can be altered by switching filtered wavelengths, endowing NBI endoscopy with unique strengths in detecting minor cancerous and even precancerous lesions. The integration of NBI and ANN has made amazing progress in early screening. Three studies respectively reported their models for the diagnosis of early GC[81,86,87]. For instance, Horiuchi *et al*[87] trained a CNN system that could differentiate between early GC and gastritis. This model achieved a high sensitivity but low specificity, yielding undesired accuracy[87]. In consideration of these fields of study, bias towards sensitivity is accepted. Trails concerning NBI colonoscopy were also performed and exhibited expectable results[7,96-98].

In recent years, wireless capsule endoscopy (WCE) has been developed for patient acceptance and the extension of detection zones. The small bowel, which is known as the blind spot of classical endoscopy, can be virtually examined only by WCE[99,100]. Thus, investigators use ANNs to enhance the capability of WCE diagnosis. A decade ago, Barbosa *et al*[101,102] began to develop an automatic detection system using a multilayer perceptron neural network[101,102]. Despite the limited size of the training dataset, this study proposed a novel direction for forthcoming models with great performance. With the development of WCE and algorithms, CNNs were shown to have sufficient potential to detect lesions in small-bowel WCE[8,83]. Particularly, Ding *et al*[8] trained a model with a large-sample dataset, achieving a sensitivity of 99.88%[8]. These achievements have changed the belief that the small bowel is the dead zone of GI tract examinations. However, two shortcomings of WCE still exist. First, WCE goes through a natural orifice, and its photograph angles are affected by GI peristalsis, body position, and contents within GI tract, presenting the possibility of losing sights. Second, during WCE movement, secreted mucus and chyme are likely to stick to WCE, decreasing image quality. ANNs may serve as an effective approach to solve these problems[103].

In addition to GI tumors, ANNs play an important role in the diagnosis of other acute and chronic GI diseases. Many studies concerning GI bleeding focused on WCE. The imaging features of serious GI bleeding are relatively distinct, while small lesions with bleeding are likely to be missed, particularly in WCE. The authors used CNN, BPNN, and probabilistic neural network (PNN) to optimize the WCE capabilities of instant diagnosis, which covered the stomach, small bowel, and colorectum. These ANN models were all reported to perform well[104-109]. Different from GI bleeding, progress on using ANNs to diagnose chronic atrophic gastritis is slow, likely because of atypical features and low rates of early screening. Two recent studies indicated the tremendous potential of CNNs in this field and showed that CNNs outperformed the expert endoscopists in identifying GI bleeding[110,111]. IBD is another indication of colonoscopy, and ANN was also used to improve the diagnostic value of endoscopy[112,113].

Prediction of risks and survival

A research group conducted a retrospective study of 521 GC patients in 2005. Three mining techniques, including ANN, decision tree, and Logistic regression, were used to analyze clinical information and predict postoperative complications. ANN outperformed the other two methods[114]. Since then, many studies have focused on the

clinical potential of ANNs in risk prediction. In particular, a large number of relevant reports have emerged in recent years, which is reflected in the rapid improvement of ML algorithms. ANN models for predicting cancer risks commonly include dozens of categorical variables, such as age, sex, oncology indicators, radiological tests, and therapeutic regimens[115-118]. Two Japanese groups combined clinical indicators and endoscopic images using CNNs to predict GC risks[94,119]. Genetic and microbial factors function as important regulators in carcinogenesis. Genomics and microbiome can exhibit the deep roots of GI tumors. Dadkhah *et al*[120] trained ANN models with the gut microbiome of 218 subjects to identify risks for colorectal polyps. Classification accuracy exceeded 75% based on home stool samples[120], which is expected as a novel method for convenient screening of colorectal polyps. mRNA profiles also have referential value in predicting the odds of malignant transformation[121,122]. The fruits of high-throughput screening techniques combined with deep analysis can depict the landscape of carcinogenesis. Subsequent etiology-based tests will contribute to revolutionary progress in precise prediction.

Metastasis is a critical index of therapeutic regimens and long-term prognosis. CT and MRI have detective limitations and only positive lymph nodes with swelling shapes can be observed, as well as distantly metastatic lesions. The results, however, always fall behind the real condition, misguiding surgical strategies and reducing survival benefits. Investigators have tried to use the advantages of ANNs to elevate the accuracy of the metastatic conditions in GI cancer. A German group used an FNN to predict lymph node metastasis of GC early in 2005[123], but the performance is poor, partly due to unadvanced algorithms and computers. With the development of ANNs, models included larger amount and more subtypes of clinical indicators. Their predictive efficiency has been augmented to reach clinical standards[124-127]. Two studies investigated ANNs used in identifying metastatic risks of CRC patients at T1 stage[128]. Although cases with metastatic T1 tumors are rare, the underlying mechanisms may be uncovered with the support of ANN. Kurokawa *et al*[128] first used BNN to construct a predictive model that involved a novel molecular target from basic medicine and showed great potential value[128]. The growth of novel biomarkers has recently provided more choices for GI diagnosis and prediction; however, few studies have emphasized the integration between these newly found targets and classical tumor biomarkers. Future development requires more investigation of ANNs to maximize the significance of existing fruits.

Survival time and quality of life are important factors that both patients and doctors consider. No efficient approaches have been developed to date, which is typically explained by disease heterogeneity, subpopulation differences, and medical experiences. These problems are likely based on one reason: Deficiency of data integration. Investigators rest their hopes for an accurate diagnosis on the excellent capabilities of information screening and automatic decision making. They used ANNs to weight many kinds of factors to quantify the survival outcome of cancer, GI bleeding, and IBD patients[106,129-133]. For GI cancer, certain studies compared ANN with TNM stage and models constructed by other ML methods. ANN typically exhibited better performance than Logistic regression, TNM stage, and even clinicians[134-136]. Competitions between different types of ANNs were also performed and the results were not consistent[137-139].

In addition to classical types of clinical information, certain novel biomarkers combined with ANNs have potential value in the prediction of GI disease risks and prognosis. Altomare *et al*[140] created a panel composed of exhaled volatile organic compounds (VOCs) from CRC patients. A PNN was used to synthetically analyze the levels of exhaled VOCs. This model could effectively identify CRC patients and predict the survival benefits from curative CRC surgery[140,141], which was a quantum leap in non-invasive screening. Several immune markers, including CD8, CD20, and CD68, combined with the proliferation marker Ki-67 by CNN showed great prognostic value in GC[135]. DNA aneuploidy, tumor-stroma ratio, and RNA sequencing were also proven to have great value in clinical prediction that were evacuated by ANN[142-144]. Otherwise, the guiding capabilities of ANNs in the prediction of both inpatient and outpatient services have been gradually revealed[145-148]. With the deep crosstalk between ANNs and medical needs, clinical tasks that were previously regarded as impossible or difficult are becoming feasible and easy.

Clinical decision support

Clinical decision making plays a conclusive role in the process. Numerous clinicians and scientists have been striving to promote standardized, precise, and individualized treatment. As the primary adjuvant regimens of GI cancer, chemotherapy should consider many factors, such as patient tolerance, pathological sensitivity, concrete

dosage, and regimens. Accumulating evidence has showed that ANNs could help overcome the therapeutic limitations caused by individual differences and mass data. Evaluation of chemotherapeutic sensitivity with ANN models is a hot topic. To establish reliable prediction systems for locally advanced and metastatic CRC, several ANN models have been used to integrate clinical indicators[149,150]. Their accuracies were significantly better than those of clinicians. Moreover, deep learning of radiological images exhibited its potential value in assessing chemosensitivity[64,65,151]. For example, a CNN system was trained using 202 cases with colorectal cancer liver metastases and was validated to have good accuracy for predicting responses to FOLFOX combined with bevacizumab regimens based on CT information[151]. The practicability of radiotherapy, anti-integrin therapy, traditional Chinese medicine, and immunotherapy could also be improved with the support of ANNs[152-155].

The quality control of surgical guidance and supervision is highly dependent on the skills of clinical teachers. It is difficult to cultivate doctors who receive normalized training and guarantee surgical processes free from interference of uncertainties. Kitaguchi *et al*[156,157] created a novel CNN-based deep learning approach fed by intraoperative videos. It could automatically recognize the surgical phase and action with high accuracies of 81% and 83.2%, respectively[156,157]. These achievements will initiate a novel field of ANN application. Clinicians, especially young surgeons, will reap benefits from these technological innovations. Regretfully, few studies have been reported due to ethic restriction and complicated surgical situations. More attention and investment should be paid for developing efficient guiding systems that precisely match skills and customs of surgeons.

Translation of basic medicine

Basic research is the frontier where ANNs have been widely used. Inherent logic and correlation between variables can be easily shown by ANNs, which may be too difficult for human intelligence. Many repetitive tasks can be finished within a short time. In the past 10 years, the rapid progress of ANNs has launched another wave of research (Table 3). Existing studies can be divided into three aspects: (1) Elucidation of mechanisms of GI carcinogenesis; (2) Guidance of drug selection; and (3) Integration of fundamental research data. First, high-throughput screening is the fundamental method for selecting critical molecules and pathways of GI cancer. High-throughput techniques will produce extremely large amounts of raw data. It is impossible to precisely choose potential targets only by manpower. Bidaut *et al*[158] began to use ANNs to characterize stomach stem cells based on microarray experiments[158]. This technique showed great value in the basic research of GI physiology. A protein-based ANN classifier was successfully constructed to identify the origins of tumor samples[159], which may promote the application of mass spectrometry in precise tumor diagnosis. Cell counting serves as a fundamental task in basic and clinical research. The low efficiency of classical manual detection cannot meet the requirement of many experiments. CNNs can significantly improve cell counting technology on their own merits[160]. A CNN-based cell counter has already been commercialized. Moreover, CNN showed great performance in discovering lncRNA-disease associations[161,162].

Development of new drugs is an important direction of research. Computer-aided target selection has gradually replaced inefficient artificial experiments. Joo *et al*[163] aimed to screen compounds targeting dysfunction proteins to find new drugs for CRC. Preliminary selection was performed using ANN, and then a benzimidazolone compound was selected out for further cell toxicity tests. This study provides a good example for subsequent studies that explore novel medications. A CNN-based model, DeepIC50, was trained with 27756 features at molecular levels. It was proven to effectively predict three drug responsiveness classes for GC patients[163]. Further validation is required to determine whether the applicable ranges of DeepIC50 can extend to other types of tumors. Furthermore, tankyrase inhibitors have been verified as potential anticancer drugs. To confirm the activity ranking of tankyrase inhibitors in CRC treatment, Berishvili *et al*[164] developed an affinity prediction model based on CNN. The Spearman correlation of the model achieved 0.73, which was superior to those of the other three virtual screening approaches[164].

With the development of molecular biology, many types of high-throughput screening techniques have been widely used, including RNA-seq, protein mass spectrometry, metabolomics, microbiome, and noncoding RNA chips. Diverse types of ANN were employed to evacuate efficient targets from sequenced datasets[120,144,165,166]. Most studies have focused on their diagnostic value. Bao *et al*[167] constructed an ANN model to identify expression profiles of a microsatellite

Table 3 Summary of studies concerning artificial neural network translation of basic achievements

Ref.	Disease	Type of data	ANN technique	Application direction	Outcome
Bao *et al*[167], 2020	CRC	Microsatellite instability from TCGA database	Multi-layer perceptron network	Prognostic prediction	100% accuracy
Coppedè *et al*[189], 2015	CRC	DNA methylation	AutoCM	Identification of connections between DNA methylation and CRC	A strong connection between the low methylation levels ofthe five *CRC* genes
Liu *et al*[190], 2004	CRC	Gene signature from GEDatasets	Multi-layer network	Identification of latent marker genes of CRC	91.94% accuracy
Berishvili *et al*[164], 2019	CRC	Approximately 4000 complexes for which the data on the target binding constants	CNN	Screening filter for compoundprioritization	73% Spearman rank correlation coefficient
Bloom *et al*[159], 2007	CRC and GC	MS	Multi-layer network	Differentiation between 6 common tumor types	87% accuracy
Dadkhah *et al*[120], 2019	colorectal polyp	Gut microbiome	ANN developed by Orange data mining tool	Early screening using collected stool	> 75% accuracy
Chang *et al*[166], 2011	CRC	miRNA profile	Not mentioned	Exploration of association between specific miRNAs and clinicopathological features	100% accuracy of miRNA panel
Chen *et al*[191], 2004	CRC	MS of serum protein pattern	Multi-layer perceptron network	Differentiation between CRC and healthy control	91% sensitivity; 93% specificity; 0.967 AUC
He *et al*[121], 2020	CRC and gastroesophageal cancer	Gene signature from TCGA database	Multi-layer network	Differentiation between types of cancer	CRC: 98.06% sensitivity; 96.88% precision. Gastroesophageal cancer: 94.89% sensitivity; 96.33% precision
Hu *et al*[192], 2015	CRC	Gene signature from database of NCBI NLM NIH	S-Kohonen neural network	Prediction of recurrence using gene expressions	91% accuracy
Kurokawa *et al*[128], 2005	CRC	Gene signature of nodal metastasis	BNN	Prediction of metastatic potential of CRC at stage I	88.0% sensitivity; 86.6% specificity; 0.904 AUC
Liu *et al*[160], 2019	Cancer cell	Synthetic microscopic images from two publicly datasets	CNN	Automated counting of cancer cells	-
Ronen *et al*[193], 2019	CRC	Gene signature from TCGA database	BNN	Stratification of CRC subtypes	-
Bilsland *et al*[194], 2015	CRC	A virtual library of compounds	Perceptron network	Screen of Benzimidazolone inhibitors for CRC treatment	CB-20903630 was selected out for further validation of CRC treatment
Maniruzzaman *et al*[195], 2019	CRC	Gene signature from patients	Fuzzy neural network	CRC classification	99.84% sensitivity; 99.75% specific; 99.81% accuracy; 0.9995 AUC
Inglese *et al*[196], 2017	CRC	3D MS	Deep neural network (unsupervised)	Identification of metabolic heterogeneity	Up to 0.6991 Pearson's correlation
Shi *et al*[197], 2020	CRC with liver metastasis	CT	ANN	Prediction of KRAS, NRAS and BRAF status	0.95 AUC
Jiang *et al*[198], 2020	GC	Two drug datasets	deep neural network	Prediction of drug-disease associations	17 kinds of drugs that were screened out by ANN had been confirmed as anti-tumor drugs
Bidaut *et al*[158], 2009	Stomach stem cell	Stemness signature	Perceptron network	Characterization of stem cells	-
Jing *et al*[168], 2019	Calibration of laboratory markers	CA-724	Radial basis function neural network	The effects of geographic factors on CA-724	CA724 reference values show spatial autocorrelation and regional variation

Xiao *et al*[122], 2018	GC	RNA-seq	Probabilistic neural networks (semi-supervised)	Diagnosis of cancer	96.23% accuracy; 99.08% precision
Hang *et al*[144], 2018	GC	MSI	Multi-layer perceptron network	Prognostic prediction	0.81 AUC
Xuan *et al*[161], 2019	GC	LncRNA profile	CNN	Prediction of GC	0.977 AUC
Joo *et al*[163], 2019	GC	Potential drugs from databases	CNN	Exploration of new drugs targeting	ANN-based model accurately predicts drug responsiveness as models previously reported
Liu *et al*[165], 2010	GC	MS from GC patients	Supervised neural network	Early screening	100% sensitivity; 75% specificity
Que *et al*[199], 2019	GC	MS from GC patients and clinicopathological parameters	Single-layer neural network	Prediction of long-term survival	0.82 AUC
Li *et al*[200], 2021	GC	Gene Expression Omnibus database	ANN	Differentiation between GC and healthy tissues	0.946 AUC

TCGA: The Cancer Genome Atlas; CRC: Colorectal cancer; GC: Gastric cancer; CNN: Convolutional neural network; BNN: Bayesian neural network; AUC: Area under the curve; MS: Mass spectroscopy; MSI: Microsatellite instability; ANN: Artificial neural networks; CT: Computed tomography; IHC: Immunohistochemistry; NCBI: National Center for Biotechnology Information; BRAF: V-raf murine sarcoma viral oncogene homolog B; NLM: Nonlinear-mirror.

status–related gene signature, which showed an accuracy of 100% in predicting the prognosis of CRC patient response to immunotherapy[167]. In addition to these novel targets, ANNs can optimize the diagnostic efficiency of clinical tumor biomarkers. According to the validation results of ANN, CA724 reference values are susceptible to geographical environmental factors, such as temperature, sunshine, and humidity[168]. This study suggested that indicators require precise calibration and ANN may become an effective tool. Briefly, ANNs have promising functions to integrate classical biomarkers and novel targets with optimum diagnostic weights.

ANN and GI noncancerous diseases

Although most studies paid their attentions to ANNs in GI cancer, the functions of ANNs in noncancerous GI diseases were also revealed. IBD is a collective name, covering ulcerative colitis and Crohn's disease. Recent studies manifested the potential value of ANNs applied in clinical practice of IBD (Table 4), which preliminarily focused on the following two parts: (1) Differential diagnosis. ANNs could help distinguish between IBD, irritable bowel syndrome, and healthy controls. The sensitivities reported by the studies were all over 75%[169-172]. The data suggested the translational potential in diagnostic assistance of ANNs; and (2) Prediction of therapeutic efficacy. Surgery, hormone therapy, immunosuppressors, and targeted drugs are alternative regimens for IBD therapy. ANN models were proved to predict the efficacies of classical drugs, infliximab, vedolizumab, and enterectomy by analyzing huge numbers of clinicopathological variables[154,173-175].

Moreover, ANNs exhibited remarkable detective abilities for GI bleeding and atrophic gastritis. These two distinct diseases have one similarity: Early detection has critical value while delayed diagnosis can lead to a poor prognosis. However, the rates of missed diagnosis for GI bleeding and atrophic gastritis are relatively high. ANNs may overcome this dilemma. According to the existing evidence, the detection accuracies of endoscopy were significantly elevated under the assistance of ANN models[111,176-178]. Collectively, these studies indicate the role of ANN in the clinical practice of GI noncancerous diseases.

FEATURES, LIMITATIONS, AND FUTURE PERSPECTIVES

Features of ANN

As mentioned above, accumulating studies have demonstrated that ANNs might have remarkable potential in diagnosis and treatment of GI diseases (Figure 3). ANN-based models commonly have optimal accuracies and AUC values. Some evaluation indexes

Table 4 Summary of existing studies of artificial neural networks applied in inflammatory bowel disease

Ref.	Disease	Aim	Number of samples	ANN technique	Included variables	Outcome
Ahmed *et al*[169], 2017	CD	Diagnosis	144 CD patients; 243 HC individuals	BPNN	103 variables	Accuracy 97.67%; sensitivity 96.07%; specificity 100%
Ananthakrishnan *et al*[154], 2017	UC and CD	Predicting treatment response to vedolizumab	43 UC patients; 42 CD patients	vedoNet	Gut microbiome	AUC of CD 88.1%; AUC of UC 85.3%
Anekboon *et al*[201], 2014	CD	Predicting single nucleotide polymorphisms	144 CD patients; 243 HC individuals	Multi-layer perceptron network	103 SNPs	Accuracy 90.4%; sensitivity 87.5%; specificity 92.2%
Dong *et al*[173], 2019	CD	Predicting the risk of surgical intervention in Chinese patients	83 patients with surgery; 83 patients without surgery	ANN	131 variables	Accuracy 90.89%; precision 46.83%; F1 score 0.5757
Fioravanti *et al*[202], 2018	IBD	Classification of metagenomics data	222 IBD patients; 38 HC individuals	CNN	Gut microbiota	-
Hardalaç *et al*[203], 2015	IBD	Predicting the effect of azathioprine on mucosal healing	129 IBD patients	BPNN	Age, age at diagnosis, usage of other medications prior to azathioprine use, smoking, sex, UC-CD	Accuracy 79.1%
Kirchberger-Tolstik *et al*[170], 2020	UC	Diagnosis	227 Raman maps with 567500 spectra	CNN	Images of Raman spectroscopy	sensitivity of 78%; specificity 93%
Klein *et al*[204], 2017	CD	Predicting the clinical phenotype	47 B1 patients; 19 B2 patients; 39 B3 patients	Two-layer FNN	H&E	B1 *vs* B2 phenotype: sensitivity 81%, specificity 74%, accuracy 75%, AUC 0.74; B1 *vs* B3 phenotype: sensitivity 69%, specificity 76%, accuracy 70.5%, AUC 0.78; B2 *vs* B3 phenotype: sensitivity 67%, specificity 72.5%, accuracy 69%, AUC 0.72
Lamash *et al*[71], 2019	CD	Visualization and quantitative estimation of CD	23 pediatric CD patients	CNN	MRI	DSCs of 75 ± 18%, 81 ± 8%, and 97 ± 2% for the lumen, wall, and background, respectively
Le *et al*[174], 2020	IBD	Predicting IBD and treatment status	68 CD patients; 53 UC patients; 34 HC individuals	Neural encoder-decoder (NED) network	Gut microbiota	CD *vs* HC: 95.2% AUC; UC *vs* HC: 92.5% AUC; CD *vs* UC: 81.8% AUC
Morilla *et al*[175], 2019	UC	Predicting treatment responses to infliximab for patients with acute severe UC	47 patients with acute severe ulcerative colitis	Deep neural network	MicroRNA profiles	84% accuracy; 0.82 AUC
Ozawa *et al*[112], 2019	UC	Identification of endoscopic inflammation severity	841 patients	CNN (GoogLeNet)	Colonoscopy images	0.86 AUC of Mayo 0; 0.98 AUC of Mayo 0-1
Peng *et al*[205], 2015	IBD	Predicting the frequency of relapse	569 UC patients; 332 CD patients	ANN	Meteorological data	High accuracy in predicting the frequency of relapse of IBD (MSE = 0.009, MAPE = 17.1 %)
Shepherd *et al*[171], 2014	IBD	Differential diagnosis between IBD and IBS	59 UC patients; 42 CD patients; 34 IBS patients; 46 HC individuals	Multi-layer perceptron neural network	Gas chromatograph coupled to a metal oxide sensor in stool samples	76% sensitivity, 88% specificity, 76% accuracy
Takayama *et al*[132], 2015	UC	Predicting treatment response to cytoapheresis	90 UC patients	Multi-layer perceptron neural network	13 clinical variables	96% sensitivity; 97% sensitivity
Tong *et al*[172], 2020	CD, UC and ITB	Differential diagnosis between CD, UC and ITB	5128 UC patients; 875 CD patients; ITB 396 patients	CNN	Differential features of endoscopic images between UC, CD and ITB	The precisions/recalls of UC-CD-ITB when employing the CNN were 0.99/0.97, 0.87/0.83, and 0.52/0.81, respectively

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; ITB: Intestinal tuberculosis; IBS: Irritable bowel syndrome; CNN: Convolutional neural network; BPNN: Back propagation neural network; FNN: Feedforward neural network; MSE: Mean square error; MAPE: Mean absolute percentage error; HC: Healthy control; AUC: Area under the curve; ANN: Artificial neural networks; SNPs: Single nucleotide polymorphisms; MRI: Magnetic resonance imaging; H&E: Haematoxylin-eosin.

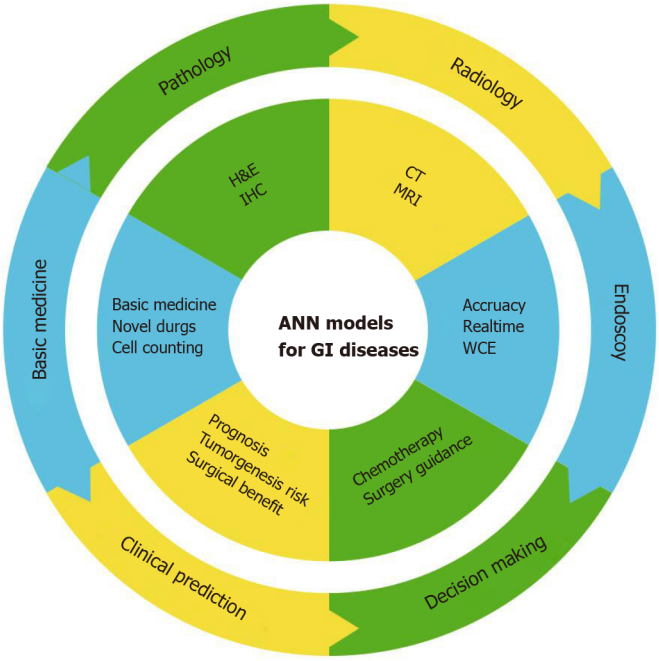


Figure 3 Artificial neural networks might have remarkable potential in diagnosis and treatment of gastrointestinal diseases. ANN: Artificial neural networks; GI: Gastrointestinal; CT: Computed tomography; MRI: Magnetic resonance imaging; IHC: Immunohistochemistry; WCE: Wireless capsule endoscopy; H&E: Haematoxylin-eosin.

of ANN models even achieved an accuracy of 100%[81,179]. For further validation of ANN metrics, comparisons were also performed and can be divided into three aspects based on the compared objects. First, regarding experts, studies involving comparisons with experts aimed at showing ANN superiority over artificial diagnosis[8,86,136]. The majority of ANNs exhibited faster speed and better accuracy than expert clinicians. Therefore, certain authors claimed that ANNs would become excellent tools for clinicians and scientists, perhaps even replacing humans in this capacity. Second, different ANN models have been studied. Researchers used several ANN algorithms that were trained with the same dataset and underwent validation phases [144,150,180]. The models with the best fitting degree were then selected out to process for further exploration. The comparisons exhibited the capabilities of different models in handling with clinical problems. Last, other reported data have shown that literature learning is another approach to determine the efficiency of developed models[106,181]. However, this type of comparative method is not reliable due to the inconsistency of research baselines, such as differences in datasets, hardware performance, and running time. These undefined bias may also affect comparative results. This method is thus not recommended for follow-up studies to further verify the advantages of their models. Generally, compelling evidence indicated that ANNs can lead the classification and deep analysis of GI clinical practice compared to linear statistical models and human labor.

Computers outperform humans in data processing, as shown in medical studies. The comprehensive diagnosis of GI diseases requires many types of examinations. GI clinicians must integrate clinical indicators to make diagnosis. However, existing guidelines and expert consensus cannot help GI clinicians manage the majority of complex diseases. ANNs can mitigate this limitation. Many types of patient characteristics and clinical indicators can be included in predictive models. Data stored in pictures is geometrically above that of textual data. CNNs can interpret radiological and endoscopic images. The analytic speed of CNNs is even fast enough to make real-time detection achievable. Some studies simultaneously included clinical indicators and picture information to analyze patient situations. This novel combination exceeds

the limitations of human capacity and provides optimal diagnostic and predictive accuracies.

Cost is also an important factor for clinical practice. Cultivating a qualified doctor demands considerable time and economic and social costs. However, ANN learning is a relatively simple process. Based on current reports, training ANN models with large-sample databases requires only several days[20]. ANNs can comprehensively analyze data features and adjust the weight values within an extremely short time. Furthermore, hardware is the basic requirement to run ANN algorithms, specifically graphics cards and central processing units, the two important data-processing elements. High-performance hardware costs a few thousand dollars, which is far cheaper than training a doctor. Collectively, the time and economic cost of training ANN models is much smaller than that of training doctors. It is believed that ANN will play a critical role in clinical practice in the near future.

Limitations and challenges

The application of ANNs appears to be a way to overcome existing problems for GI doctors. However, certain limitations should be taken seriously. First, training methods affect the efficiency of ANN models. Most research groups have performed small-sample studies with fewer than 1000 cases. For endoscopic detection, although the number of freezing frames could reach ten thousand, some were from the same video[8,182]. Thus, frame repeatability impaired their representativeness. The small size of dataset led to a higher likelihood of overfitting, which refers to models that receive too much training and overly fit the characteristics of internal datasets. Rigid observation of internal datasets will naturally reduce the model's flexibility to manage external data. For example, if the model is trained with a dataset of patients from country A, patients from country B may not be well described by this model due to various factors. Otherwise, retrospective datasets should be used as the primary sources of ANN training. Similar to common retrospective studies, biases also exist and affect the application of models. Selection bias is the most important factor. Patient data from medical histories could be selected based on research programmes. However, some studies used public databases to train their models[167,183]. The information of registered cases is inflexible, which means that researchers cannot exclude potential bias as they expected.

Second, the compromise between interpretability and accuracy should be emphasized. Models with optimal accuracy typically lack interpretability (*i.e.*, explainability). Conversely, models with nearly perfect interpretability are less accurate, like linear regression. During ANN training, algorithm modulation and data feedback are blind. Researchers do not know the activation functions in hidden layers, which are similar to a machine that hides its circuit in the box and places only switches and displayers outside; this feature is called as "black box". For important medical decisions, doctors and patients both have the right to know how decisions were made. An inability to understand how the model makes decisions will naturally reduce the patients' and doctors' confidence. Conversely, lack of explainability makes researchers unable to describe the deduction processes of ANN models, decreasing their referential value.

Third, sociological issues should be carefully considered for ANN applications, which could be divided into three aspects. First, regarding medical liability, mistakes and medical negligence are likely, despite the outstanding ANN performance in the validation phase. Thus, who should be responsible for decisions and how to distribute liability to pay for compensation are difficult to determine. Doctors, patients, and programmers are all seemingly innocent, and accidents primarily result from inherent shortcomings, not human carelessness. Second, regarding confidentiality, the operation of ANN models requires many types of data, some of which concern patient privacy. Doctors who betray privacies will receive punishment. However, ANN models are connected to the Internet or company intranets. It is difficult to distinguish the origins of a privacy disclosure. Furthermore, the capabilities of data extraction by ANNs are incomparable. The ranges of consent exemption thus require further argument. The balance between ANN research and patient privacy remains a challenge. Last, regarding legislation, multiparty participation is an important characteristic of ANNs, which should include hospitals, patients, product providers, and insurance companies. Regretfully, the rules of ANNs used in medical practice are still obscure. Existing legal norms cannot support the application and prospective studies of ANNs.

Future directions

The goal of ANN applications is to maximize medical benefits for patients, doctors, and society. To provide GI diagnosis and recommended treatment, ANNs must be further improved to bring them in line with clinical standards and inherent characteristics. Future directions are described below.

Enlargement of application ranges: ANNs have been used for GI diagnosis, prognosis and risk prediction, and therapeutic guidance, achieving relatively high accuracies. Significance is limited to constructing more models that have been proposed in the same fields[184,185]. Future studies should focus on expanding ANN application ranges. Treatment decisions and surgical guidance are two representative aspects of future development. They require including more factors and presupposing more complex situations. ANNs may become a promising tool to overcome the difficulties. Epidemiology is another field burdened with large quantities of data. Training ANN-based models will help investigators have a deep and quick command of epidemiologic features.

Development of unsupervised learning: Supervised learning is the mainstream direction of medical research. ANN models are primarily based on labelled datasets, which require considerable time and manpower. Future studies should try to use unsupervised and semi-supervised learning to reduce the training cost and develop comprehensive decision-making models. The unique advantages of unsupervised learning can break classical thoughts of clinical trials. It can automatically reveal the internal structures of mass data without grouping and statistical analysis. Also, unsupervised learning is "clear box" and can produce visual information that can be comprehended by humans, providing more convenient parameter adjustment and systematic optimization. Unsupervised ANN-based models should be created for data pre-treatment and association screening. Mature unsupervised systems for basic research are also required to find more diagnostic biomarkers and therapeutic targets.

Improvement of datasets: Large-sample prospective studies and real-world studies are encouraged. However, these studies will require high-quality datasets, including normalized inclusion criteria, number of cases, detailed degree, and rigorous check. Optimal sources of data can reduce the bias of ANN systems. Otherwise, larger sample sizes do not mean that enrolled cases should cover as many races and countries as possible. Heterogeneity is ubiquitous in medical care. There remains a lack of evidence that ANNs, perhaps even all AI algorithms, have the potential to generalize disease features throughout the world. It should be temporarily accepted to construct ANN models with functional, regional, or subpopulation characteristics rather than wide application. The qualities of the datasets determine the performance of the constructed models. More efforts should be paid to build full equipped registry systems and provide complete data for model training, which is typically neglected by constructors.

Emphasis on legislation: The nature of AI is totally different from human intelligence. It cannot accept liability by its own. Decision-making methods are still based on learning and experience. Also, ANNs are unable to correctly address problems that are beyond their knowledge. That is why commercial AI products are slightly clumsy when managing complex situations. The "black box" feature of ANNs means that all participants are precluded from the decision-making process. Once ANN models make mistakes, how to rationally distribute responsibilities should be carefully answered by legislation. The absence of reasonable rules also slowed clinical trials. Judgment criteria must be outlined to allow clinical trials to be launched. Problems that emerge will then guide legislation. Positive feedback will promote the progress of ANN application.

Progress in ethics: ANNs have been created to solve the specific problems, which naturally forms result-oriented operation patterns. However, this process is not always suitable for making decisions independently, particularly for GI diseases. For example, for an old patient who had severe ulcerative colitis and underwent many partial enterectomies, ANN models may show that he can benefit from the next surgery. Conversely, doctors will persuade patients to accept expectant treatment due to their tolerance, age, and emotion. The absence of empathy will make an ANN's decision "perfect" but impersonal, which is partially why ANNs cannot become a substitute for human decision makings. Regarding the substitutability of human labor, arguments on this topic have never been stopped. ANNs have gradually taken over simple tasks. Similar to the aforementioned statement, many models outperformed experts in

endoscopy, radiology, pathology, and gastroenterology[8,19,56,186]. Then, concerns about ANNs taking jobs from gastroenterologists in the future become more common. We hold the opposite view: The development of ANN techniques can reverse the transmission of pressure to improve the competence of gastroenterologists. Only in this way can pressure and injustice fundamentally be solved. Some issues of ethics and society will be answered by further application of ANNs.

Avoidance of overestimation: A misleading saying is that ANNs automatically produce analytic results after being fed raw data. Many studies emphasize construction methods but neglect the importance of data cleaning and conversion. Insufficient data pre-processing has serious impacts on the qualities of models. Rigorous criteria of data pre-processing should thus be developed. Coincidentally, ANNs have advantages regarding data labelling and classification. ANN models should be developed to help construct other ANN models for decision making. Although results showed that ANNs outperformed other AI methods, certain studies also highlighted their limitations[187,188]. An internal comparison between different ML methods is required.

CONCLUSION

The relationship between AI and medicine has received much attention. In this review, we introduced the basic knowledge of ANN, a primary method of AI and its application in the diagnosis, risk prediction and treatment of GI diseases. Considering interdisciplinary difficulties, the contents of this review were written from the perspective of gastroenterologists. Many vivid metaphors and brief conclusions reduced the comprehension threshold of GI clinicians and scientists. The characteristics, current limitations, and future directions were then illustrated. Undoubtedly, ANNs provide excellent value in clinical practice and even outperform GI experts and other types of AI techniques. However, there are still certain problems that hinder ANN applications, which require additional exploration in many fields. It is believed that ANN will become one of the most efficient tools for GI clinicians and benefit all participants in the future.

REFERENCES

1 **Sotos JG**. MYCIN and NEOMYCIN: two approaches to generating explanations in rule-based expert systems. *Aviat Space Environ Med* 1990; **61**: 950-954 [PMID: 2241738]

2 **Mungle T**, Tewary S, Das DK, Arun I, Basak B, Agarwal S, Ahmed R, Chatterjee S, Chakraborty C. MRF-ANN: a machine learning approach for automated ER scoring of breast cancer immunohistochemical images. *J Microsc* 2017; **267**: 117-129 [PMID: 28319275 DOI: 10.1111/jmi.12552]

3 **Wang JL**, Jin GL, Yuan ZG. Artificial neural network predicts hemorrhagic contusions following decompressive craniotomy in traumatic brain injury. *J Neurosurg Sci* 2021; **65**: 69-74 [PMID: 28884559 DOI: 10.23736/S0390-5616.17.04123-6]

4 **Zhang Y**, van der Werf NR, Jiang B, van Hamersvelt R, Greuter MJW, Xie X. Motion-corrected coronary calcium scores by a convolutional neural network: a robotic simulating study. *Eur Radiol* 2020; **30**: 1285-1294 [PMID: 31630233 DOI: 10.1007/s00330-019-06447-7]

5 **Li A**, Quan SF, Silva GE, Perfect MM, Roveda JM. A Novel Artificial Neural Network Based Sleep-Disordered Breathing Screening Tool. *J Clin Sleep Med* 2018; **14**: 1063-1069 [PMID: 29852901 DOI: 10.5664/jcsm.7182]

6 **Chen CY**, Chen YF, Chen HY, Hung CT, Shi HY. Artificial Neural Network and Cox Regression Models for Predicting Mortality after Hip Fracture Surgery: A Population-Based Comparison. *Medicina (Kaunas)* 2020; **56** [PMID: 32438724 DOI: 10.3390/medicina56050243]

7 **Chen PJ**, Lin MC, Lai MJ, Lin JC, Lu HH, Tseng VS. Accurate Classification of Diminutive Colorectal Polyps Using Computer-Aided Analysis. *Gastroenterology* 2018; **154**: 568-575 [PMID: 29042219 DOI: 10.1053/j.gastro.2017.10.010]

8 **Ding Z**, Shi H, Zhang H, Meng L, Fan M, Han C, Zhang K, Ming F, Xie X, Liu H, Liu J, Lin R, Hou X. Gastroenterologist-Level Identification of Small-Bowel Diseases and Normal Variants by Capsule Endoscopy Using a Deep-Learning Model. *Gastroenterology* 2019; **157**: 1044-1054. e5 [PMID: 31251929 DOI: 10.1053/j.gastro.2019.06.025]

9 **Ling T**, Wu L, Fu Y, Xu Q, An P, Zhang J, Hu S, Chen Y, He X, Wang J, Chen X, Zhou J, Xu Y, Zou X, Yu H. A deep learning-based system for identifying differentiation status and delineating the margins of early gastric cancer in magnifying narrow-band imaging endoscopy. *Endoscopy* 2020

[PMID: 32725617 DOI: 10.1055/a-1229-0920]

10 **He K**, Zhang X, Ren S, Sun J. Identity Mappings in Deep Residual Networks. In: Leibe B, Matas J, Sebe N, Welling M (eds). Computer Vision – ECCV 2016. Lecture Notes in Computer Science, vol 9908. Springer: Cham, 2016: 630-645 [DOI: 10.1007/978-3-319-46493-0_38]

11 **Fu J**, Zhong X, Li N, Van Dams R, Lewis J, Sung K, Raldow AC, Jin J, Qi XS. Deep learning-based radiomic features for improving neoadjuvant chemoradiation response prediction in locally advanced rectal cancer. *Phys Med Biol* 2020; **65**: 075001 [PMID: 32092710 DOI: 10.1088/1361-6560/ab7970]

12 **Karakitsos P**, Ioakim-Liossi A, Pouliakis A, Botsoli-Stergiou EM, Tzivras M, Archimandritis A, Kyrkou K. A comparative study of three variations of the learning vector quantizer in the discrimination of benign from malignant gastric cells. *Cytopathology* 1998; **9**: 114-125 [PMID: 9577738]

13 **Song Z**, Zou S, Zhou W, Huang Y, Shao L, Yuan J, Gou X, Jin W, Wang Z, Chen X, Ding X, Liu J, Yu C, Ku C, Liu C, Sun Z, Xu G, Wang Y, Zhang X, Wang D, Wang S, Xu W, Davis RC, Shi H. Clinically applicable histopathological diagnosis system for gastric cancer detection using deep learning. *Nat Commun* 2020; **11**: 4294 [PMID: 32855423 DOI: 10.1038/s41467-020-18147-8]

14 **Kosaraju SC**, Hao J, Koh HM, Kang M. Deep-Hipo: Multi-scale receptive field deep learning for histopathological image analysis. *Methods* 2020; **179**: 3-13 [PMID: 32442672 DOI: 10.1016/j.ymeth.2020.05.012]

15 **Qu J**, Hiruta N, Terai K, Nosato H, Murakawa M, Sakanashi H. Gastric Pathology Image Classification Using Stepwise Fine-Tuning for Deep Neural Networks. *J Healthc Eng* 2018; **2018**: 8961781 [PMID: 30034677 DOI: 10.1155/2018/8961781]

16 **Liang Q**, Nan Y, Coppola G, Zou K, Sun W, Zhang D, Wang Y, Yu G. Weakly Supervised Biomedical Image Segmentation by Reiterative Learning. *IEEE J Biomed Health Inform* 2019; **23**: 1205-1214 [PMID: 29994489 DOI: 10.1109/JBHI.2018.2850040]

17 **Sharma H**, Zerbe N, Klempert I, Hellwich O, Hufnagl P. Deep convolutional neural networks for automatic classification of gastric carcinoma using whole slide images in digital histopathology. *Comput Med Imaging Graph* 2017; **61**: 2-13 [PMID: 28676295 DOI: 10.1016/j.compmedimag.2017.06.001]

18 **Yamashita R**, Long J, Longacre T, Peng L, Berry G, Martin B, Higgins J, Rubin DL, Shen J. Deep learning model for the prediction of microsatellite instability in colorectal cancer: a diagnostic study. *Lancet Oncol* 2021; **22**: 132-141 [PMID: 33387492 DOI: 10.1016/S1470-2045(20)30535-0]

19 **Wei JW**, Suriawinata AA, Vaickus LJ, Ren B, Liu X, Lisovsky M, Tomita N, Abdollahi B, Kim AS, Snover DC, Baron JA, Barry EL, Hassanpour S. Evaluation of a Deep Neural Network for Automated Classification of Colorectal Polyps on Histopathologic Slides. *JAMA Netw Open* 2020; **3**: e203398 [PMID: 32324237 DOI: 10.1001/jamanetworkopen.2020.3398]

20 **Yoon H**, Lee J, Oh JE, Kim HR, Lee S, Chang HJ, Sohn DK. Tumor Identification in Colorectal Histology Images Using a Convolutional Neural Network. *J Digit Imaging* 2019; **32**: 131-140 [PMID: 30066123 DOI: 10.1007/s10278-018-0112-9]

21 **Graham S**, Chen H, Gamper J, Dou Q, Heng PA, Snead D, Tsang YW, Rajpoot N. MILD-Net: Minimal information loss dilated network for gland instance segmentation in colon histology images. *Med Image Anal* 2019; **52**: 199-211 [PMID: 30594772 DOI: 10.1016/j.media.2018.12.001]

22 **Graham S**, Vu QD, Raza SEA, Azam A, Tsang YW, Kwak JT, Rajpoot N. Hover-Net: Simultaneous segmentation and classification of nuclei in multi-tissue histology images. *Med Image Anal* 2019; **58**: 101563 [PMID: 31561183 DOI: 10.1016/j.media.2019.101563]

23 **Zhang S**, Han F, Liang Z, Tan J, Cao W, Gao Y, Pomeroy M, Ng K, Hou W. An investigation of CNN models for differentiating malignant from benign lesions using small pathologically proven datasets. *Comput Med Imaging Graph* 2019; **77**: 101645 [PMID: 31454710 DOI: 10.1016/j.compmedimag.2019.101645]

24 **Sena P**, Fioresi R, Faglioni F, Losi L, Faglioni G, Roncucci L. Deep learning techniques for detecting preneoplastic and neoplastic lesions in human colorectal histological images. *Oncol Lett* 2019; **18**: 6101-6107 [PMID: 31788084 DOI: 10.3892/ol.2019.10928]

25 **Wang KS**, Yu G, Xu C, Meng XH, Zhou J, Zheng C, Deng Z, Shang L, Liu R, Su S, Zhou X, Li Q, Li J, Wang J, Ma K, Qi J, Hu Z, Tang P, Deng J, Qiu X, Li BY, Shen WD, Quan RP, Yang JT, Huang LY, Xiao Y, Yang ZC, Li Z, Wang SC, Ren H, Liang C, Guo W, Li Y, Xiao H, Gu Y, Yun JP, Huang D, Song Z, Fan X, Chen L, Yan X, Huang ZC, Huang J, Luttrell J, Zhang CY, Zhou W, Zhang K, Yi C, Wu C, Shen H, Wang YP, Xiao HM, Deng HW. Accurate diagnosis of colorectal cancer based on histopathology images using artificial intelligence. *BMC Med* 2021; **19**: 76 [PMID: 33752648 DOI: 10.1186/s12916-021-01942-5]

26 **Awan R**, Sirinukunwattana K, Epstein D, Jefferyes S, Qidwai U, Aftab Z, Mujeeb I, Snead D, Rajpoot N. Glandular Morphometrics for Objective Grading of Colorectal Adenocarcinoma Histology Images. *Sci Rep* 2017; **7**: 16852 [PMID: 29203775 DOI: 10.1038/s41598-017-16516-w]

27 **Kainz P**, Pfeiffer M, Urschler M. Segmentation and classification of colon glands with deep convolutional neural networks and total variation regularization. *PeerJ* 2017; **5**: e3874 [PMID: 29018612 DOI: 10.7717/peerj.3874]

28 **Xu Y**, Li Y, Wang Y, Liu M, Fan Y, Lai M, Chang EI. Gland Instance Segmentation Using Deep Multichannel Neural Networks. *IEEE Trans Biomed Eng* 2017; **64**: 2901-2912 [PMID: 28358671 DOI: 10.1109/TBME.2017.2686418]

29 **Van Eycke YR**, Balsat C, Verset L, Debeir O, Salmon I, Decaestecker C. Segmentation of glandular epithelium in colorectal tumours to automatically compartmentalise IHC biomarker quantification: A

deep learning approach. *Med Image Anal* 2018; **49**: 35-45 [PMID: 30081241 DOI: 10.1016/j.media.2018.07.004]

30 **Xu J**, Luo X, Wang G, Gilmore H, Madabhushi A. A Deep Convolutional Neural Network for segmenting and classifying epithelial and stromal regions in histopathological images. *Neurocomputing* 2016; **191**: 214-223 [PMID: 28154470 DOI: 10.1016/j.neucom.2016.01.034]

31 **Sirinukunwattana K**, Ahmed Raza SE, Yee-Wah Tsang, Snead DR, Cree IA, Rajpoot NM. Locality Sensitive Deep Learning for Detection and Classification of Nuclei in Routine Colon Cancer Histology Images. *IEEE Trans Med Imaging* 2016; **35**: 1196-1206 [PMID: 26863654 DOI: 10.1109/TMI.2016.2525803]

32 **Sirinukunwattana K**, Domingo E, Richman SD, Redmond KL, Blake A, Verrill C, Leedham SJ, Chatzipli A, Hardy C, Whalley CM, Wu CH, Beggs AD, McDermott U, Dunne PD, Meade A, Walker SM, Murray GI, Samuel L, Seymour M, Tomlinson I, Quirke P, Maughan T, Rittscher J, Koelzer VH; S:CORT consortium. Image-based consensus molecular subtype (imCMS) classification of colorectal cancer using deep learning. *Gut* 2021; **70**: 544-554 [PMID: 32690604 DOI: 10.1136/gutjnl-2019-319866]

33 **Matsumoto T**, Niioka H, Kumamoto Y, Sato J, Inamori O, Nakao R, Harada Y, Konishi E, Otsuji E, Tanaka H, Miyake J, Takamatsu T. Deep-UV excitation fluorescence microscopy for detection of lymph node metastasis using deep neural network. *Sci Rep* 2019; **9**: 16912 [PMID: 31729459 DOI: 10.1038/s41598-019-53405-w]

34 **Shiraishi T**, Shinto E, Nearchou IP, Tsuda H, Kajiwara Y, Einama T, Caie PD, Kishi Y, Ueno H. Prognostic significance of mesothelin expression in colorectal cancer disclosed by area-specific four-point tissue microarrays. *Virchows Arch* 2020; **477**: 409-420 [PMID: 32107600 DOI: 10.1007/s00428-020-02775-y]

35 **Luo XJ**, Zhang B, Li JG, Luo XA, Yang LF. Autofluorescence spectroscopy for evaluating dysplasia in colorectal tissues. *Z Med Phys* 2012; **22**: 40-47 [PMID: 22112637 DOI: 10.1016/j.zemedi.2011.10.010]

36 **Jia Z**, Huang X, Chang EI, Xu Y. Constrained Deep Weak Supervision for Histopathology Image Segmentation. *IEEE Trans Med Imaging* 2017; **36**: 2376-2388 [PMID: 28692971 DOI: 10.1109/TMI.2017.2724070]

37 **Qaiser T**, Tsang YW, Taniyama D, Sakamoto N, Nakane K, Epstein D, Rajpoot N. Fast and accurate tumor segmentation of histology images using persistent homology and deep convolutional features. *Med Image Anal* 2019; **55**: 1-14 [PMID: 30991188 DOI: 10.1016/j.media.2019.03.014]

38 **Su H**, Ruan J, Chen T, Lin E, Shi L. CT-assessed sarcopenia is a predictive factor for both long-term and short-term outcomes in gastrointestinal oncology patients: a systematic review and meta-analysis. *Cancer Imaging* 2019; **19**: 82 [PMID: 31796090 DOI: 10.1186/s40644-019-0270-0]

39 **Umezawa S**, Nagata N, Arimoto J, Uchiyama S, Higurashi T, Nakano K, Ishii N, Sakurai T, Moriyasu S, Takeda Y, Nagase H, Komatsu H, Nakajima A, Mizuki A. Contrast-enhanced CT for Colonic Diverticular Bleeding before Colonoscopy: A Prospective Multicenter Study. *Radiology* 2018; **288**: 755-761 [PMID: 29893642 DOI: 10.1148/radiol.2018172910]

40 **Mehdy MM**, Ng PY, Shair EF, Saleh NIM, Gomes C. Artificial Neural Networks in Image Processing for Early Detection of Breast Cancer. *Comput Math Methods Med* 2017; **2017**: 2610628 [PMID: 28473865 DOI: 10.1155/2017/2610628]

41 **Wang CJ**, Hamm CA, Savic LJ, Ferrante M, Schobert I, Schlachter T, Lin M, Weinreb JC, Duncan JS, Chapiro J, Letzen B. Deep learning for liver tumor diagnosis part II: convolutional neural network interpretation using radiologic imaging features. *Eur Radiol* 2019; **29**: 3348-3357 [PMID: 31093705 DOI: 10.1007/s00330-019-06214-8]

42 **Knoll P**, Mirzaei S, Müllner A, Leitha T, Koriska K, Köhn H, Neumann M. An artificial neural net and error backpropagation to reconstruct single photon emission computerized tomography data. *Med Phys* 1999; **26**: 244-248 [PMID: 10076982 DOI: 10.1118/1.598511]

43 **Zheng L**, Zhang X, Hu J, Gao Y, Zhang M, Li S, Zhou X, Niu T, Lu Y, Wang D. Establishment and Applicability of a Diagnostic System for Advanced Gastric Cancer T Staging Based on a Faster Region-Based Convolutional Neural Network. *Front Oncol* 2020; **10**: 1238 [PMID: 32850373 DOI: 10.3389/fonc.2020.01238]

44 **Suzuki K**, Rockey DC, Dachman AH. CT colonography: advanced computer-aided detection scheme utilizing MTANNs for detection of "missed" polyps in a multicenter clinical trial. *Med Phys* 2010; **37**: 12-21 [PMID: 20175461 DOI: 10.1118/1.3263615]

45 **Suzuki K**, Zhang J, Xu J. Massive-training artificial neural network coupled with Laplacian-eigenfunction-based dimensionality reduction for computer-aided detection of polyps in CT colonography. *IEEE Trans Med Imaging* 2010; **29**: 1907-1917 [PMID: 20570766 DOI: 10.1109/TMI.2010.2053213]

46 **Liu X**, Guo S, Zhang H, He K, Mu S, Guo Y, Li X. Accurate colorectal tumor segmentation for CT scans based on the label assignment generative adversarial network. *Med Phys* 2019; **46**: 3532-3542 [PMID: 31087327 DOI: 10.1002/mp.13584]

47 **Chen Y**, Ren Y, Fu L, Xiong J, Larsson R, Xu X, Sun J, Zhao J. A 3D Convolutional Neural Network Framework for Polyp Candidates Detection on the Limited Dataset of CT Colonography. *Annu Int Conf IEEE Eng Med Biol Soc* 2018; **2018**: 678-681 [PMID: 30440487 DOI: 10.1109/EMBC.2018.8512305]

48 **Jerebko AK**, Malley JD, Franaszek M, Summers RM. Multiple neural network classification scheme for detection of colonic polyps in CT colonography data sets. *Acad Radiol* 2003; **10**: 154-

160 [PMID: 12583566 DOI: 10.1016/s1076-6332(03)80039-9]
49 **Jerebko AK**, Summers RM, Malley JD, Franaszek M, Johnson CD. Computer-assisted detection of colonic polyps with CT colonography using neural networks and binary classification trees. *Med Phys* 2003; **30**: 52-60 [PMID: 12557979 DOI: 10.1118/1.1528178]
50 **Suzuki K**, Yoshida H, Näppi J, Dachman AH. Massive-training artificial neural network (MTANN) for reduction of false positives in computer-aided detection of polyps: Suppression of rectal tubes. *Med Phys* 2006; **33**: 3814-3824 [PMID: 17089846 DOI: 10.1118/1.2349839]
51 **Men K**, Boimel P, Janopaul-Naylor J, Zhong H, Huang M, Geng H, Cheng C, Fan Y, Plastaras JP, Ben-Josef E, Xiao Y. Cascaded atrous convolution and spatial pyramid pooling for more accurate tumor target segmentation for rectal cancer radiotherapy. *Phys Med Biol* 2018; **63**: 185016 [PMID: 30109986 DOI: 10.1088/1361-6560/aada6c]
52 **Roth HR**, Lu L, Liu J, Yao J, Seff A, Cherry K, Kim L, Summers RM. Improving Computer-Aided Detection Using Convolutional Neural Networks and Random View Aggregation. *IEEE Trans Med Imaging* 2016; **35**: 1170-1181 [PMID: 26441412 DOI: 10.1109/TMI.2015.2482920]
53 **Gao Y**, Zhang ZD, Li S, Guo YT, Wu QY, Liu SH, Yang SJ, Ding L, Zhao BC, Lu Y. Deep neural network-assisted computed tomography diagnosis of metastatic lymph nodes from gastric cancer. *Chin Med J (Engl)* 2019; **132**: 2804-2811 [PMID: 31856051 DOI: 10.1097/CM9.0000000000000532]
54 **Huang Z**, Liu D, Chen X, Yu P, Wu J, Song B, Hu J, Wu B. Retrospective imaging studies of gastric cancer: Study protocol clinical trial (SPIRIT Compliant). *Medicine (Baltimore)* 2020; **99**: e19157 [PMID: 32080093 DOI: 10.1097/MD.0000000000019157]
55 **Hou Z**, Yang Y, Li S, Yan J, Ren W, Liu J, Wang K, Liu B, Wan S. Radiomic analysis using contrast-enhanced CT: predict treatment response to pulsed low dose rate radiotherapy in gastric carcinoma with abdominal cavity metastasis. *Quant Imaging Med Surg* 2018; **8**: 410-420 [PMID: 29928606 DOI: 10.21037/qims.2018.05.01]
56 **Khalili K**, Lawlor RL, Pourafkari M, Lu H, Tyrrell P, Kim TK, Jang HJ, Johnson SA, Martel AL. Convolutional neural networks versus radiologists in characterization of small hypoattenuating hepatic nodules on CT: a critical diagnostic challenge in staging of colorectal carcinoma. *Sci Rep* 2020; **10**: 15248 [PMID: 32943654 DOI: 10.1038/s41598-020-71364-5]
57 **Tan J**, Gao Y, Liang Z, Cao W, Pomeroy MJ, Huo Y, Li L, Barish MA, Abbasi AF, Pickhardt PJ. 3D-GLCM CNN: A 3-Dimensional Gray-Level Co-Occurrence Matrix-Based CNN Model for Polyp Classification *via* CT Colonography. *IEEE Trans Med Imaging* 2020; **39**: 2013-2024 [PMID: 31899419 DOI: 10.1109/TMI.2019.2963177]
58 **Trebeschi S**, van Griethuysen JJM, Lambregts DMJ, Lahaye MJ, Parmar C, Bakers FCH, Peters NHGM, Beets-Tan RGH, Aerts HJWL. Deep Learning for Fully-Automated Localization and Segmentation of Rectal Cancer on Multiparametric MR. *Sci Rep* 2017; **7**: 5301 [PMID: 28706185 DOI: 10.1038/s41598-017-05728-9]
59 **Panic J**, Defeudis A, Mazzetti S, Rosati S, Giannetto G, Vassallo L, Regge D, Balestra G, Giannini V. A Convolutional Neural Network based system for Colorectal cancer segmentation on MRI images. *Annu Int Conf IEEE Eng Med Biol Soc* 2020; **2020**: 1675-1678 [PMID: 33018318 DOI: 10.1109/EMBC44109.2020.9175804]
60 **Wang J**, Lu J, Qin G, Shen L, Sun Y, Ying H, Zhang Z, Hu W. Technical Note: A deep learning-based autosegmentation of rectal tumors in MR images. *Med Phys* 2018; **45**: 2560-2564 [PMID: 29663417 DOI: 10.1002/mp.12918]
61 **Wang M**, Xie P, Ran Z, Jian J, Zhang R, Xia W, Yu T, Ni C, Gu J, Gao X, Meng X. Full convolutional network based multiple side-output fusion architecture for the segmentation of rectal tumors in magnetic resonance images: A multi-vendor study. *Med Phys* 2019; **46**: 2659-2668 [PMID: 30972763 DOI: 10.1002/mp.13541]
62 **Soomro MH**, Coppotelli M, Conforto S, Schmid M, Giunta G, Del Secco L, Neri E, Caruso D, Rengo M, Laghi A. Automated Segmentation of Colorectal Tumor in 3D MRI Using 3D Multiscale Densely Connected Convolutional Neural Network. *J Healthc Eng* 2019; **2019**: 1075434 [PMID: 30838121 DOI: 10.1155/2019/1075434]
63 **Wang D**, Xu J, Zhang Z, Li S, Zhang X, Zhou Y, Lu Y. Evaluation of Rectal Cancer Circumferential Resection Margin Using Faster Region-Based Convolutional Neural Network in High-Resolution Magnetic Resonance Images. *Dis Colon Rectum* 2020; **63**: 143-151 [PMID: 31842158 DOI: 10.1097/DCR.0000000000001519]
64 **Shi L**, Zhang Y, Nie K, Sun X, Niu T, Yue N, Kwong T, Chang P, Chow D, Chen JH, Su MY. Machine learning for prediction of chemoradiation therapy response in rectal cancer using pre-treatment and mid-radiation multi-parametric MRI. *Magn Reson Imaging* 2019; **61**: 33-40 [PMID: 31059768 DOI: 10.1016/j.mri.2019.05.003]
65 **Nie K**, Shi L, Chen Q, Hu X, Jabbour SK, Yue N, Niu T, Sun X. Rectal Cancer: Assessment of Neoadjuvant Chemoradiation Outcome based on Radiomics of Multiparametric MRI. *Clin Cancer Res* 2016; **22**: 5256-5264 [PMID: 27185368 DOI: 10.1158/1078-0432.CCR-15-2997]
66 **Bangi E**, Ang C, Smibert P, Uzilov AV, Teague AG, Antipin Y, Chen R, Hecht C, Gruszczynski N, Yon WJ, Malyshev D, Laspina D, Selkridge I, Rainey H, Moe AS, Lau CY, Taik P, Wilck E, Bhardwaj A, Sung M, Kim S, Yum K, Sebra R, Donovan M, Misiukiewicz K, Schadt EE, Posner MR, Cagan RL. A personalized platform identifies trametinib plus zoledronate for a patient with KRAS-mutant metastatic colorectal cancer. *Sci Adv* 2019; **5**: eaav6528 [PMID: 31131321 DOI: 10.1126/sciadv.aav6528]

67 **Sartore-Bianchi A**, Trusolino L, Martino C, Bencardino K, Lonardi S, Bergamo F, Zagonel V, Leone F, Depetris I, Martinelli E, Troiani T, Ciardiello F, Racca P, Bertotti A, Siravegna G, Torri V, Amatu A, Ghezzi S, Marrapese G, Palmeri L, Valtorta E, Cassingena A, Lauricella C, Vanzulli A, Regge D, Veronese S, Comoglio PM, Bardelli A, Marsoni S, Siena S. Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial. *Lancet Oncol* 2016; **17**: 738-746 [PMID: 27108243 DOI: 10.1016/S1470-2045(16)00150-9]

68 **He K**, Liu X, Li M, Li X, Yang H, Zhang H. Noninvasive KRAS mutation estimation in colorectal cancer using a deep learning method based on CT imaging. *BMC Med Imaging* 2020; **20**: 59 [PMID: 32487083 DOI: 10.1186/s12880-020-00457-4]

69 **Wu X**, Li Y, Chen X, Huang Y, He L, Zhao K, Huang X, Zhang W, Dong M, Huang J, Xia T, Liang C, Liu Z. Deep Learning Features Improve the Performance of a Radiomics Signature for Predicting KRAS Status in Patients with Colorectal Cancer. *Acad Radiol* 2020; **27**: e254-e262 [PMID: 31982342 DOI: 10.1016/j.acra.2019.12.007]

70 **Huang L**, Li M, Gou S, Zhang X, Jiang K. Automated Segmentation Method for Low Field 3D Stomach MRI Using Transferred Learning Image Enhancement Network. *Biomed Res Int* 2021; **2021**: 6679603 [PMID: 33628806 DOI: 10.1155/2021/6679603]

71 **Lamash Y**, Kurugol S, Freiman M, Perez-Rossello JM, Callahan MJ, Bousvaros A, Warfield SK. Curved planar reformatting and convolutional neural network-based segmentation of the small bowel for visualization and quantitative assessment of pediatric Crohn's disease from MRI. *J Magn Reson Imaging* 2019; **49**: 1565-1576 [PMID: 30353957 DOI: 10.1002/jmri.26330]

72 **Laursen SB**, Leontiadis GI, Stanley AJ, Møller MH, Hansen JM, Schaffalitzky de Muckadell OB. Relationship between timing of endoscopy and mortality in patients with peptic ulcer bleeding: a nationwide cohort study. *Gastrointest Endosc* 2017; **85**: 936-944. e3 [PMID: 27623102 DOI: 10.1016/j.gie.2016.08.049]

73 **Ogura M**, Hikiba Y, Maeda S, Matsumura M, Okano K, Sassa R, Yoshida H, Kawabe T, Omata M. Mortality from gastric cancer in patients followed with upper gastrointestinal endoscopy. *Scand J Gastroenterol* 2008; **43**: 574-580 [PMID: 18415750 DOI: 10.1080/00365520701813954]

74 **Hirasawa T**, Aoyama K, Tanimoto T, Ishihara S, Shichijo S, Ozawa T, Ohnishi T, Fujishiro M, Matsuo K, Fujisaki J, Tada T. Application of artificial intelligence using a convolutional neural network for detecting gastric cancer in endoscopic images. *Gastric Cancer* 2018; **21**: 653-660 [PMID: 29335825 DOI: 10.1007/s10120-018-0793-2]

75 **Yoon HJ**, Kim S, Kim JH, Keum JS, Oh SI, Jo J, Chun J, Youn YH, Park H, Kwon IG, Choi SH, Noh SH. A Lesion-Based Convolutional Neural Network Improves Endoscopic Detection and Depth Prediction of Early Gastric Cancer. *J Clin Med* 2019; **8** [PMID: 31454949 DOI: 10.3390/jcm8091310]

76 **Zhu Y**, Wang QC, Xu MD, Zhang Z, Cheng J, Zhong YS, Zhang YQ, Chen WF, Yao LQ, Zhou PH, Li QL. Application of convolutional neural network in the diagnosis of the invasion depth of gastric cancer based on conventional endoscopy. *Gastrointest Endosc* 2019; **89**: 806-815. e1 [PMID: 30452913 DOI: 10.1016/j.gie.2018.11.011]

77 **Komeda Y**, Handa H, Watanabe T, Nomura T, Kitahashi M, Sakurai T, Okamoto A, Minami T, Kono M, Arizumi T, Takenaka M, Hagiwara S, Matsui S, Nishida N, Kashida H, Kudo M. Computer-Aided Diagnosis Based on Convolutional Neural Network System for Colorectal Polyp Classification: Preliminary Experience. *Oncology* 2017; **93** Suppl 1: 30-34 [PMID: 29258081 DOI: 10.1159/000481227]

78 **Gao J**, Guo Y, Sun Y, Qu G. Application of Deep Learning for Early Screening of Colorectal Precancerous Lesions under White Light Endoscopy. *Comput Math Methods Med* 2020; **2020**: 8374317 [PMID: 32952602 DOI: 10.1155/2020/8374317]

79 **Bagheri M**, Mohrekesh M, Tehrani M, Najarian K, Karimi N, Samavi S, Reza Soroushmehr SM. Deep Neural Network based Polyp Segmentation in Colonoscopy Images using a Combination of Color Spaces. *Annu Int Conf IEEE Eng Med Biol Soc* 2019; **2019**: 6742-6745 [PMID: 31947388 DOI: 10.1109/EMBC.2019.8856793]

80 **Zhang X**, Hu W, Chen F, Liu J, Yang Y, Wang L, Duan H, Si J. Gastric precancerous diseases classification using CNN with a concise model. *PLoS One* 2017; **12**: e0185508 [PMID: 28950010 DOI: 10.1371/journal.pone.0185508]

81 **Ueyama H**, Kato Y, Akazawa Y, Yatagai N, Komori H, Takeda T, Matsumoto K, Ueda K, Hojo M, Yao T, Nagahara A, Tada T. Application of artificial intelligence using a convolutional neural network for diagnosis of early gastric cancer based on magnifying endoscopy with narrow-band imaging. *J Gastroenterol Hepatol* 2021; **36**: 482-489 [PMID: 32681536 DOI: 10.1111/jgh.15190]

82 **Zhang R**, Zheng Y, Poon CCY, Shen D, Lau JYW. Polyp detection during colonoscopy using a regression-based convolutional neural network with a tracker. *Pattern Recognit* 2018; **83**: 209-219 [PMID: 31105338 DOI: 10.1016/j.patcog.2018.05.026]

83 **Saito H**, Aoki T, Aoyama K, Kato Y, Tsuboi A, Yamada A, Fujishiro M, Oka S, Ishihara S, Matsuda T, Nakahori M, Tanaka S, Koike K, Tada T. Automatic detection and classification of protruding lesions in wireless capsule endoscopy images based on a deep convolutional neural network. *Gastrointest Endosc* 2020; **92**: 144-151. e1 [PMID: 32084410 DOI: 10.1016/j.gie.2020.01.054]

84 **Zhang X**, Chen F, Yu T, An J, Huang Z, Liu J, Hu W, Wang L, Duan H, Si J. Real-time gastric polyp detection using convolutional neural networks. *PLoS One* 2019; **14**: e0214133 [PMID:

30908513 DOI: 10.1371/journal.pone.0214133]
85 **Gong D**, Wu L, Zhang J, Mu G, Shen L, Liu J, Wang Z, Zhou W, An P, Huang X, Jiang X, Li Y, Wan X, Hu S, Chen Y, Hu X, Xu Y, Zhu X, Li S, Yao L, He X, Chen D, Huang L, Wei X, Wang X, Yu H. Detection of colorectal adenomas with a real-time computer-aided system (ENDOANGEL): a randomised controlled study. *Lancet Gastroenterol Hepatol* 2020; **5**: 352-361 [PMID: 31981518 DOI: 10.1016/S2468-1253(19)30413-3]
86 **Li L**, Chen Y, Shen Z, Zhang X, Sang J, Ding Y, Yang X, Li J, Chen M, Jin C, Chen C, Yu C. Convolutional neural network for the diagnosis of early gastric cancer based on magnifying narrow band imaging. *Gastric Cancer* 2020; **23**: 126-132 [PMID: 31332619 DOI: 10.1007/s10120-019-00992-2]
87 **Horiuchi Y**, Aoyama K, Tokai Y, Hirasawa T, Yoshimizu S, Ishiyama A, Yoshio T, Tsuchida T, Fujisaki J, Tada T. Convolutional Neural Network for Differentiating Gastric Cancer from Gastritis Using Magnified Endoscopy with Narrow Band Imaging. *Dig Dis Sci* 2020; **65**: 1355-1363 [PMID: 31584138 DOI: 10.1007/s10620-019-05862-6]
88 **Sakai Y**, Takemoto S, Hori K, Nishimura M, Ikematsu H, Yano T, Yokota H. Automatic detection of early gastric cancer in endoscopic images using a transferring convolutional neural network. *Annu Int Conf IEEE Eng Med Biol Soc* 2018; **2018**: 4138-4141 [PMID: 30441266 DOI: 10.1109/EMBC.2018.8513274]
89 **Wu L**, Zhou W, Wan X, Zhang J, Shen L, Hu S, Ding Q, Mu G, Yin A, Huang X, Liu J, Jiang X, Wang Z, Deng Y, Liu M, Lin R, Ling T, Li P, Wu Q, Jin P, Chen J, Yu H. A deep neural network improves endoscopic detection of early gastric cancer without blind spots. *Endoscopy* 2019; **51**: 522-531 [PMID: 30861533 DOI: 10.1055/a-0855-3532]
90 **Hwang M**, Wang D, Kong XX, Wang Z, Li J, Jiang WC, Hwang KS, Ding K. An automated detection system for colonoscopy images using a dual encoder-decoder model. *Comput Med Imaging Graph* 2020; **84**: 101763 [PMID: 32805673 DOI: 10.1016/j.compmedimag.2020.101763]
91 **Wang W**, Tian J, Zhang C, Luo Y, Wang X, Li J. An improved deep learning approach and its applications on colonic polyp images detection. *BMC Med Imaging* 2020; **20**: 83 [PMID: 32698839 DOI: 10.1186/s12880-020-00482-3]
92 **Xu M**, Zhou W, Wu L, Zhang J, Wang J, Mu G, Huang X, Li Y, Yuan J, Zeng Z, Wang Y, Huang L, Liu J, Yu H. Artificial intelligence in diagnosis of gastric precancerous conditions by image-enhanced endoscopy: a multicenter, diagnostic study (with video). *Gastrointest Endosc* 2021 [PMID: 33722576 DOI: 10.1016/j.gie.2021.03.013]
93 **Nakashima H**, Kawahira H, Kawachi H, Sakaki N. Artificial intelligence diagnosis of *Helicobacter pylori* infection using blue laser imaging-bright and linked color imaging: a single-center prospective study. *Ann Gastroenterol* 2018; **31**: 462-468 [PMID: 29991891 DOI: 10.20524/aog.2018.0269]
94 **Nakashima H**, Kawahira H, Kawachi H, Sakaki N. Endoscopic three-categorical diagnosis of Helicobacter pylori infection using linked color imaging and deep learning: a single-center prospective study (with video). *Gastric Cancer* 2020; **23**: 1033-1040 [PMID: 32382973 DOI: 10.1007/s10120-020-01077-1]
95 **Itoh T**, Kawahira H, Nakashima H, Yata N. Deep learning analyzes Helicobacter pylori infection by upper gastrointestinal endoscopy images. *Endosc Int Open* 2018; **6**: E139-E144 [PMID: 29399610 DOI: 10.1055/s-0043-120830]
96 **Zorron Cheng Tao Pu L**, Maicas G, Tian Y, Yamamura T, Nakamura M, Suzuki H, Singh G, Rana K, Hirooka Y, Burt AD, Fujishiro M, Carneiro G, Singh R. Computer-aided diagnosis for characterization of colorectal lesions: comprehensive software that includes differentiation of serrated lesions. *Gastrointest Endosc* 2020; **92**: 891-899 [PMID: 32145289 DOI: 10.1016/j.gie.2020.02.042]
97 **Zheng Y**, Zhang R, Yu R, Jiang Y, Mak TWC, Wong SH, Lau JYW, Poon CCY. Localisation of Colorectal Polyps by Convolutional Neural Network Features Learnt from White Light and Narrow Band Endoscopic Images of Multiple Databases. *Annu Int Conf IEEE Eng Med Biol Soc* 2018; **2018**: 4142-4145 [PMID: 30441267 DOI: 10.1109/EMBC.2018.8513337]
98 **Lai LL**, Blakely A, Invernizzi M, Lin J, Kidambi T, Melstrom KA, Yu K, Lu T. Separation of color channels from conventional colonoscopy images improves deep neural network detection of polyps. *J Biomed Opt* 2021; **26** [PMID: 33442965 DOI: 10.1117/1.JBO.26.1.015001]
99 **Voderholzer WA**, Beinhoelzl J, Rogalla P, Murrer S, Schachschal G, Lochs H, Ortner MA. Small bowel involvement in Crohn's disease: a prospective comparison of wireless capsule endoscopy and computed tomography enteroclysis. *Gut* 2005; **54**: 369-373 [PMID: 15710985 DOI: 10.1136/gut.2004.040055]
100 **Fritscher-Ravens A**, Scherbakov P, Bufler P, Torroni F, Ruuska T, Nuutinen H, Thomson M, Tabbers M, Milla P. The feasibility of wireless capsule endoscopy in detecting small intestinal pathology in children under the age of 8 years: a multicentre European study. *Gut* 2009; **58**: 1467-1472 [PMID: 19625281 DOI: 10.1136/gut.2009.177774]
101 **Barbosa DJ**, Ramos J, Lima CS. Detection of small bowel tumors in capsule endoscopy frames using texture analysis based on the discrete wavelet transform. *Annu Int Conf IEEE Eng Med Biol Soc* 2008; **2008**: 3012-3015 [PMID: 19163340 DOI: 10.1109/IEMBS.2008.4649837]
102 **Barbosa DJ**, Ramos J, Correia JH, Lima CS. Automatic detection of small bowel tumors in capsule endoscopy based on color curvelet covariance statistical texture descriptors. *Annu Int Conf IEEE Eng Med Biol Soc* 2009; **2009**: 6683-6686 [PMID: 19964706 DOI: 10.1109/IEMBS.2009.5334013]
103 **Gayathri Devi K**, Radhakrishnan R. Automatic segmentation of colon in 3D CT images and

removal of opacified fluid using cascade feed forward neural network. *Comput Math Methods Med* 2015; **2015**: 670739 [PMID: 25838838 DOI: 10.1155/2015/670739]

104 **Majid A**, Khan MA, Yasmin M, Rehman A, Yousafzai A, Tariq U. Classification of stomach infections: A paradigm of convolutional neural network along with classical features fusion and selection. *Microsc Res Tech* 2020; **83**: 562-576 [PMID: 31984630 DOI: 10.1002/jemt.23447]

105 **Hwang Y**, Lee HH, Park C, Tama BA, Kim JS, Cheung DY, Chung WC, Cho YS, Lee KM, Choi MG, Lee S, Lee BI. Improved classification and localization approach to small bowel capsule endoscopy using convolutional neural network. *Dig Endosc* 2020 [PMID: 32640059 DOI: 10.1111/den.13787]

106 **Tan Q**, Ma AJ, Deng H, Wong VW, Tse YK, Yip TC, Wong GL, Ching JY, Chan FK, Yuen PC. A Hybrid Residual Network and Long Short-Term Memory Method for Peptic Ulcer Bleeding Mortality Prediction. *AMIA Annu Symp Proc* 2018; **2018**: 998-1007 [PMID: 30815143]

107 **Hajabdollahi M**, Esfandiarpoor R, Najarian K, Karimi N, Samavi S, Reza Soroushmehr SM. Low Complexity CNN Structure for Automatic Bleeding Zone Detection in Wireless Capsule Endoscopy Imaging. *Annu Int Conf IEEE Eng Med Biol Soc* 2019; **2019**: 7227-7230 [PMID: 31947501 DOI: 10.1109/EMBC.2019.8857751]

108 **Hung CY**, Lin CH, Chang CS, Li JL, Lee CC. Predicting Gastrointestinal Bleeding Events from Multimodal In-Hospital Electronic Health Records Using Deep Fusion Networks. *Annu Int Conf IEEE Eng Med Biol Soc* 2019; **2019**: 2447-2450 [PMID: 31946393 DOI: 10.1109/EMBC.2019.8857244]

109 **Li XL**, Zhang H, Zhang XL, Liu H, Xie GT. Exploring transfer learning for gastrointestinal bleeding detection on small-size imbalanced endoscopy images. *Annu Int Conf IEEE Eng Med Biol Soc* 2017; **2017**: 1994-1997 [PMID: 29060286 DOI: 10.1109/EMBC.2017.8037242]

110 **Guimarães P**, Keller A, Fehlmann T, Lammert F, Casper M. Deep-learning based detection of gastric precancerous conditions. *Gut* 2020; **69**: 4-6 [PMID: 31375599 DOI: 10.1136/gutjnl-2019-319347]

111 **Zhang Y**, Li F, Yuan F, Zhang K, Huo L, Dong Z, Lang Y, Zhang Y, Wang M, Gao Z, Qin Z, Shen L. Diagnosing chronic atrophic gastritis by gastroscopy using artificial intelligence. *Dig Liver Dis* 2020; **52**: 566-572 [PMID: 32061504 DOI: 10.1016/j.dld.2019.12.146]

112 **Ozawa T**, Ishihara S, Fujishiro M, Saito H, Kumagai Y, Shichijo S, Aoyama K, Tada T. Novel computer-assisted diagnosis system for endoscopic disease activity in patients with ulcerative colitis. *Gastrointest Endosc* 2019; **89**: 416-421. e1 [PMID: 30367878 DOI: 10.1016/j.gie.2018.10.020]

113 **Aoki T**, Yamada A, Aoyama K, Saito H, Tsuboi A, Nakada A, Niikura R, Fujishiro M, Oka S, Ishihara S, Matsuda T, Tanaka S, Koike K, Tada T. Automatic detection of erosions and ulcerations in wireless capsule endoscopy images based on a deep convolutional neural network. *Gastrointest Endosc* 2019; **89**: 357-363. e2 [PMID: 30670179 DOI: 10.1016/j.gie.2018.10.027]

114 **Chien CW**, Lee YC, Ma T, Lee TS, Lin YC, Wang W, Lee WJ. The application of artificial neural networks and decision tree model in predicting post-operative complication for gastric cancer patients. *Hepatogastroenterology* 2008; **55**: 1140-1145 [PMID: 18705347]

115 **Wang YH**, Nguyen PA, Islam MM, Li YC, Yang HC. Development of Deep Learning Algorithm for Detection of Colorectal Cancer in EHR Data. *Stud Health Technol Inform* 2019; **264**: 438-441 [PMID: 31437961 DOI: 10.3233/SHTI190259]

116 **Hsieh MH**, Sun LM, Lin CL, Hsieh MJ, Sun K, Hsu CY, Chou AK, Kao CH. Development of a Prediction Model for Colorectal Cancer among Patients with Type 2 Diabetes Mellitus Using a Deep Neural Network. *J Clin Med* 2018; **7** [PMID: 30213141 DOI: 10.3390/jcm7090277]

117 **Chau R**, Jenkins MA, Buchanan DD, Ait Ouakrim D, Giles GG, Casey G, Gallinger S, Haile RW, Le Marchand L, Newcomb PA, Lindor NM, Hopper JL, Win AK. Determining the familial risk distribution of colorectal cancer: a data mining approach. *Fam Cancer* 2016; **15**: 241-251 [PMID: 26681340 DOI: 10.1007/s10689-015-9860-6]

118 **Nartowt BJ**, Hart GR, Roffman DA, Llor X, Ali I, Muhammad W, Liang Y, Deng J. Scoring colorectal cancer risk with an artificial neural network based on self-reportable personal health data. *PLoS One* 2019; **14**: e0221421 [PMID: 31437221 DOI: 10.1371/journal.pone.0221421]

119 **Nakahira H**, Ishihara R, Aoyama K, Kono M, Fukuda H, Shimamoto Y, Nakagawa K, Ohmori M, Iwatsubo T, Iwagami H, Matsuno K, Inoue S, Matsuura N, Shichijo S, Maekawa A, Kanesaka T, Yamamoto S, Takeuchi Y, Higashino K, Uedo N, Matsunaga T, Tada T. Stratification of gastric cancer risk using a deep neural network. *JGH Open* 2020; **4**: 466-471 [PMID: 32514455 DOI: 10.1002/jgh3.12281]

120 **Dadkhah E**, Sikaroodi M, Korman L, Hardi R, Baybick J, Hanzel D, Kuehn G, Kuehn T, Gillevet PM. Gut microbiome identifies risk for colorectal polyps. *BMJ Open Gastroenterol* 2019; **6**: e000297 [PMID: 31275588 DOI: 10.1136/bmjgast-2019-000297]

121 **He B**, Zhang Y, Zhou Z, Wang B, Liang Y, Lang J, Lin H, Bing P, Yu L, Sun D, Luo H, Yang J, Tian G. A Neural Network Framework for Predicting the Tissue-of-Origin of 15 Common Cancer Types Based on RNA-Seq Data. *Front Bioeng Biotechnol* 2020; **8**: 737 [PMID: 32850691 DOI: 10.3389/fbioe.2020.00737]

122 **Xiao Y**, Wu J, Lin Z, Zhao X. A semi-supervised deep learning method based on stacked sparse auto-encoder for cancer prediction using RNA-seq data. *Comput Methods Programs Biomed* 2018; **166**: 99-105 [PMID: 30415723 DOI: 10.1016/j.cmpb.2018.10.004]

123 **Hensler K**, Waschulzik T, Mönig SP, Maruyama K, Hölscher AH, Bollschweiler E. Quality-assured Efficient Engineering of Feedforward Neural Networks (QUEEN) -- pretherapeutic estimation of

lymph node status in patients with gastric carcinoma. *Methods Inf Med* 2005; **44**: 647-654 [PMID: 16400373]

124 **Mekicar J**, Omejc M. Preoperative prediction of lymph node status in gastric cancer patients with the help of computer analysis. *Dig Surg* 2009; **26**: 256-261 [PMID: 19590202 DOI: 10.1159/000227296]

125 **Spelt L**, Nilsson J, Andersson R, Andersson B. Artificial neural networks--a method for prediction of survival following liver resection for colorectal cancer metastases. *Eur J Surg Oncol* 2013; **39**: 648-654 [PMID: 23514791 DOI: 10.1016/j.ejso.2013.02.024]

126 **Jagric T**, Potrc S, Jagric T. Prediction of liver metastases after gastric cancer resection with the use of learning vector quantization neural networks. *Dig Dis Sci* 2010; **55**: 3252-3261 [PMID: 20186483 DOI: 10.1007/s10620-010-1155-z]

127 **Kwak MS**, Lee HH, Yang JM, Cha JM, Jeon JW, Yoon JY, Kim HI. Deep Convolutional Neural Network-Based Lymph Node Metastasis Prediction for Colon Cancer Using Histopathological Images. *Front Oncol* 2020; **10**: 619803 [PMID: 33520727 DOI: 10.3389/fonc.2020.619803]

128 **Kurokawa S**, Arimura Y, Yamamoto H, Adachi Y, Endo T, Sato T, Suga T, Hosokawa M, Shinomura Y, Imai K. Tumour matrilysin expression predicts metastatic potential of stage I (pT1) colon and rectal cancers. *Gut* 2005; **54**: 1751-1758 [PMID: 16284286 DOI: 10.1136/gut.2005.071035]

129 **Skrede OJ**, De Raedt S, Kleppe A, Hveem TS, Liestøl K, Maddison J, Askautrud HA, Pradhan M, Nesheim JA, Albregtsen F, Farstad IN, Domingo E, Church DN, Nesbakken A, Shepherd NA, Tomlinson I, Kerr R, Novelli M, Kerr DJ, Danielsen HE. Deep learning for prediction of colorectal cancer outcome: a discovery and validation study. *Lancet* 2020; **395**: 350-360 [PMID: 32007170 DOI: 10.1016/S0140-6736(19)32998-8]

130 **Gohari MR**, Biglarian A, Bakhshi E, Pourhoseingholi MA. Use of an artificial neural network to determine prognostic factors in colorectal cancer patients. *Asian Pac J Cancer Prev* 2011; **12**: 1469-1472 [PMID: 22126483]

131 **Lee J**, An JY, Choi MG, Park SH, Kim ST, Lee JH, Sohn TS, Bae JM, Kim S, Lee H, Min BH, Kim JJ, Jeong WK, Choi DI, Kim KM, Kang WK, Kim M, Seo SW. Deep Learning-Based Survival Analysis Identified Associations Between Molecular Subtype and Optimal Adjuvant Treatment of Patients With Gastric Cancer. *JCO Clin Cancer Inform* 2018; **2**: 1-14 [PMID: 30652558 DOI: 10.1200/CCI.17.00065]

132 **Takayama T**, Okamoto S, Hisamatsu T, Naganuma M, Matsuoka K, Mizuno S, Bessho R, Hibi T, Kanai T. Computer-Aided Prediction of Long-Term Prognosis of Patients with Ulcerative Colitis after Cytoapheresis Therapy. *PLoS One* 2015; **10**: e0131197 [PMID: 26111148 DOI: 10.1371/journal.pone.0131197]

133 **Li Z**, Wu X, Gao X, Shan F, Ying X, Zhang Y, Ji J. Development and validation of an artificial neural network prognostic model after gastrectomy for gastric carcinoma: An international multicenter cohort study. *Cancer Med* 2020; **9**: 6205-6215 [PMID: 32666682 DOI: 10.1002/cam4.3245]

134 **Liu MM**, Wen L, Liu YJ, Cai Q, Li LT, Cai YM. Application of data mining methods to improve screening for the risk of early gastric cancer. *BMC Med Inform Decis Mak* 2018; **18**: 121 [PMID: 30526601 DOI: 10.1186/s12911-018-0689-4]

135 **Meier A**, Nekolla K, Hewitt LC, Earle S, Yoshikawa T, Oshima T, Miyagi Y, Huss R, Schmidt G, Grabsch HI. Hypothesis-free deep survival learning applied to the tumour microenvironment in gastric cancer. *J Pathol Clin Res* 2020; **6**: 273-282 [PMID: 32592447 DOI: 10.1002/cjp2.170]

136 **Bottaci L**, Drew PJ, Hartley JE, Hadfield MB, Farouk R, Lee PW, Macintyre IM, Duthie GS, Monson JR. Artificial neural networks applied to outcome prediction for colorectal cancer patients in separate institutions. *Lancet* 1997; **350**: 469-472 [PMID: 9274582 DOI: 10.1016/S0140-6736(96)11196-X]

137 **Korhani Kangi A**, Bahrampour A. Predicting the Survival of Gastric Cancer Patients Using Artificial and Bayesian Neural Networks. *Asian Pac J Cancer Prev* 2018; **19**: 487-490 [PMID: 29480983 DOI: 10.22034/APJCP.2018.19.2.487]

138 **Amiri Z**, Mohammad K, Mahmoudi M, Parsaeian M, Zeraati H. Assessing the effect of quantitative and qualitative predictors on gastric cancer individuals survival using hierarchical artificial neural network models. *Iran Red Crescent Med J* 2013; **15**: 42-48 [PMID: 23486933 DOI: 10.5812/ircmj.4122]

139 **Bollschweiler EH**, Mönig SP, Hensler K, Baldus SE, Maruyama K, Hölscher AH. Artificial neural network for prediction of lymph node metastases in gastric cancer: a phase II diagnostic study. *Ann Surg Oncol* 2004; **11**: 506-511 [PMID: 15123460 DOI: 10.1245/ASO.2004.04.018]

140 **Altomare DF**, Di Lena M, Porcelli F, Trizio L, Travaglio E, Tutino M, Dragonieri S, Memeo V, de Gennaro G. Exhaled volatile organic compounds identify patients with colorectal cancer. *Br J Surg* 2013; **100**: 144-150 [PMID: 23212621 DOI: 10.1002/bjs.8942]

141 **Altomare DF**, Di Lena M, Porcelli F, Travaglio E, Longobardi F, Tutino M, Depalma N, Tedesco G, Sardaro A, Memeo R, de Gennaro G. Effects of Curative Colorectal Cancer Surgery on Exhaled Volatile Organic Compounds and Potential Implications in Clinical Follow-up. *Ann Surg* 2015; **262**: 862-6; discussion 866 [PMID: 26583677 DOI: 10.1097/SLA.0000000000001471]

142 **Gemoll T**, Strohkamp S, Schillo K, Thorns C, Habermann JK. MALDI-imaging reveals thymosin beta-4 as an independent prognostic marker for colorectal cancer. *Oncotarget* 2015; **6**: 43869-43880 [PMID: 26556858 DOI: 10.18632/oncotarget.6103]

143 **Zhao K**, Li Z, Yao S, Wang Y, Wu X, Xu Z, Wu L, Huang Y, Liang C, Liu Z. Artificial intelligence quantified tumour-stroma ratio is an independent predictor for overall survival in resectable colorectal cancer. *EBioMedicine* 2020; **61**: 103054 [PMID: 33039706 DOI: 10.1016/j.ebiom.2020.103054]

144 **Hang X**, Li D, Wang J, Wang G. Prognostic significance of microsatellite instabilityassociated pathways and genes in gastric cancer. *Int J Mol Med* 2018; **42**: 149-160 [PMID: 29717769 DOI: 10.3892/ijmm.2018.3643]

145 **Francis NK**, Luther A, Salib E, Allanby L, Messenger D, Allison AS, Smart NJ, Ockrim JB. The use of artificial neural networks to predict delayed discharge and readmission in enhanced recovery following laparoscopic colorectal cancer surgery. *Tech Coloproctol* 2015; **19**: 419-428 [PMID: 26084884 DOI: 10.1007/s10151-015-1319-0]

146 **Lee SM**, Kang JO, Suh YM. Comparison of hospital charge prediction models for colorectal cancer patients: neural network vs. decision tree models. *J Korean Med Sci* 2004; **19**: 677-681 [PMID: 15483343 DOI: 10.3346/jkms.2004.19.5.677]

147 **Wang J**, Li M, Hu YT, Zhu Y. Comparison of hospital charge prediction models for gastric cancer patients: neural network vs. decision tree models. *BMC Health Serv Res* 2009; **9**: 161 [PMID: 19751514 DOI: 10.1186/1472-6963-9-161]

148 **Maslekar S**, Gardiner AB, Monson JR, Duthie GS. Artificial neural networks to predict presence of significant pathology in patients presenting to routine colorectal clinics. *Colorectal Dis* 2010; **12**: 1254-1259 [PMID: 19604289 DOI: 10.1111/j.1463-1318.2009.02005.x]

149 **Bibault JE**, Giraud P, Housset M, Durdux C, Taieb J, Berger A, Coriat R, Chaussade S, Dousset B, Nordlinger B, Burgun A. Deep Learning and Radiomics predict complete response after neo-adjuvant chemoradiation for locally advanced rectal cancer. *Sci Rep* 2018; **8**: 12611 [PMID: 30135549 DOI: 10.1038/s41598-018-30657-6]

150 **Huang CM**, Huang MY, Huang CW, Tsai HL, Su WC, Chang WC, Wang JY, Shi HY. Machine learning for predicting pathological complete response in patients with locally advanced rectal cancer after neoadjuvant chemoradiotherapy. *Sci Rep* 2020; **10**: 12555 [PMID: 32724164 DOI: 10.1038/s41598-020-69345-9]

151 **Maaref A**, Romero FP, Montagnon E, Cerny M, Nguyen B, Vandenbroucke F, Soucy G, Turcotte S, Tang A, Kadoury S. Predicting the Response to FOLFOX-Based Chemotherapy Regimen from Untreated Liver Metastases on Baseline CT: a Deep Neural Network Approach. *J Digit Imaging* 2020; **33**: 937-945 [PMID: 32193665 DOI: 10.1007/s10278-020-00332-2]

152 **Ahn SH**, Yeo AU, Kim KH, Kim C, Goh Y, Cho S, Lee SB, Lim YK, Kim H, Shin D, Kim T, Kim TH, Youn SH, Oh ES, Jeong JH. Comparative clinical evaluation of atlas and deep-learning-based auto-segmentation of organ structures in liver cancer. *Radiat Oncol* 2019; **14**: 213 [PMID: 31775825 DOI: 10.1186/s13014-019-1392-z]

153 **Lin YC**, Huang WT, Ou SC, Hung HH, Cheng WZ, Lin SS, Lin HJ, Huang ST. Neural network analysis of Chinese herbal medicine prescriptions for patients with colorectal cancer. *Complement Ther Med* 2019; **42**: 279-285 [PMID: 30670255 DOI: 10.1016/j.ctim.2018.12.001]

154 **Ananthakrishnan AN**, Luo C, Yajnik V, Khalili H, Garber JJ, Stevens BW, Cleland T, Xavier RJ. Gut Microbiome Function Predicts Response to Anti-integrin Biologic Therapy in Inflammatory Bowel Diseases. *Cell Host Microbe* 2017; **21**: 603-610. e3 [PMID: 28494241 DOI: 10.1016/j.chom.2017.04.010]

155 **Hildebrand LA**, Pierce CJ, Dennis M, Paracha M, Maoz A. Artificial Intelligence for Histology-Based Detection of Microsatellite Instability and Prediction of Response to Immunotherapy in Colorectal Cancer. *Cancers (Basel)* 2021; **13** [PMID: 33494280 DOI: 10.3390/cancers13030391]

156 **Kitaguchi D**, Takeshita N, Matsuzaki H, Oda T, Watanabe M, Mori K, Kobayashi E, Ito M. Automated laparoscopic colorectal surgery workflow recognition using artificial intelligence: Experimental research. *Int J Surg* 2020; **79**: 88-94 [PMID: 32413503 DOI: 10.1016/j.ijsu.2020.05.015]

157 **Kitaguchi D**, Takeshita N, Matsuzaki H, Takano H, Owada Y, Enomoto T, Oda T, Miura H, Yamanashi T, Watanabe M, Sato D, Sugomori Y, Hara S, Ito M. Real-time automatic surgical phase recognition in laparoscopic sigmoidectomy using the convolutional neural network-based deep learning approach. *Surg Endosc* 2020; **34**: 4924-4931 [PMID: 31797047 DOI: 10.1007/s00464-019-07281-0]

158 **Bidaut G**, Stoeckert CJ Jr. Characterization of unknown adult stem cell samples by large scale data integration and artificial neural networks. *Pac Symp Biocomput* 2009; 356-367 [PMID: 19209714 DOI: 10.1142/9789812836939_0034]

159 **Bloom GC**, Eschrich S, Zhou JX, Coppola D, Yeatman TJ. Elucidation of a protein signature discriminating six common types of adenocarcinoma. *Int J Cancer* 2007; **120**: 769-775 [PMID: 17131332 DOI: 10.1002/ijc.22041]

160 **Liu Q**, Junker A, Murakami K, Hu P. Automated Counting of Cancer Cells by Ensembling Deep Features. *Cells* 2019; **8** [PMID: 31480740 DOI: 10.3390/cells8091019]

161 **Xuan P**, Pan S, Zhang T, Liu Y, Sun H. Graph Convolutional Network and Convolutional Neural Network Based Method for Predicting lncRNA-Disease Associations. *Cells* 2019; **8** [PMID: 31480350 DOI: 10.3390/cells8091012]

162 **Xuan P**, Sheng N, Zhang T, Liu Y, Guo Y. CNNDLP: A Method Based on Convolutional Autoencoder and Convolutional Neural Network with Adjacent Edge Attention for Predicting lncRNA-Disease Associations. *Int J Mol Sci* 2019; **20** [PMID: 31480319 DOI:

10.3390/ijms20174260]
163 **Joo M**, Park A, Kim K, Son WJ, Lee HS, Lim G, Lee J, Lee DH, An J, Kim JH, Ahn T, Nam S. A Deep Learning Model for Cell Growth Inhibition IC50 Prediction and Its Application for Gastric Cancer Patients. *Int J Mol Sci* 2019; **20** [PMID: 31842404 DOI: 10.3390/ijms20246276]
164 **Berishvili VP**, Perkin VO, Voronkov AE, Radchenko EV, Syed R, Venkata Ramana Reddy C, Pillay V, Kumar P, Choonara YE, Kamal A, Palyulin VA. Time-Domain Analysis of Molecular Dynamics Trajectories Using Deep Neural Networks: Application to Activity Ranking of Tankyrase Inhibitors. *J Chem Inf Model* 2019; **59**: 3519-3532 [PMID: 31276400 DOI: 10.1021/acs.jcim.9b00135]
165 **Liu W**, Gao X, Cai Q, Li J, Zhu Z, Li C, Yao X, Yang Q, Xiang M, Yan M. Identification of novel serum biomarkers for gastric cancer by magnetic bead. *Front Biosci (Elite Ed)* 2010; **2**: 961-971 [PMID: 20515767 DOI: 10.2741/e155]
166 **Chang KH**, Miller N, Kheirelseid EA, Lemetre C, Ball GR, Smith MJ, Regan M, McAnena OJ, Kerin MJ. MicroRNA signature analysis in colorectal cancer: identification of expression profiles in stage II tumors associated with aggressive disease. *Int J Colorectal Dis* 2011; **26**: 1415-1422 [PMID: 21739196 DOI: 10.1007/s00384-011-1279-4]
167 **Bao X**, Zhang H, Wu W, Cheng S, Dai X, Zhu X, Fu Q, Tong Z, Liu L, Zheng Y, Zhao P, Fang W, Liu F. Analysis of the molecular nature associated with microsatellite status in colon cancer identifies clinical implications for immunotherapy. *J Immunother Cancer* 2020; **8** [PMID: 33028695 DOI: 10.1136/jitc-2020-001437]
168 **Jing J**, Ge M, Yang Z, Li P. Spatial distribution characteristics of tumor marker CA724 reference values in China. *Cancer Med* 2019; **8**: 4465-4474 [PMID: 31199587 DOI: 10.1002/cam4.2176]
169 **Ahmed SS**, Dey N, Ashour AS, Sifaki-Pistolla D, Bălas-Timar D, Balas VE, Tavares JM. Effect of fuzzy partitioning in Crohn's disease classification: a neuro-fuzzy-based approach. *Med Biol Eng Comput* 2017; **55**: 101-115 [PMID: 27106754 DOI: 10.1007/s11517-016-1508-7]
170 **Kirchberger-Tolstik T**, Pradhan P, Vieth M, Grunert P, Popp J, Bocklitz TW, Stallmach A. Towards an Interpretable Classifier for Characterization of Endoscopic Mayo Scores in Ulcerative Colitis Using Raman Spectroscopy. *Anal Chem* 2020; **92**: 13776-13784 [PMID: 32965101 DOI: 10.1021/acs.analchem.0c02163]
171 **Shepherd SF**, McGuire ND, de Lacy Costello BP, Ewen RJ, Jayasena DH, Vaughan K, Ahmed I, Probert CS, Ratcliffe NM. The use of a gas chromatograph coupled to a metal oxide sensor for rapid assessment of stool samples from irritable bowel syndrome and inflammatory bowel disease patients. *J Breath Res* 2014; **8**: 026001 [PMID: 24674940 DOI: 10.1088/1752-7155/8/2/026001]
172 **Tong Y**, Lu K, Yang Y, Li J, Lin Y, Wu D, Yang A, Li Y, Yu S, Qian J. Can natural language processing help differentiate inflammatory intestinal diseases in China? *BMC Med Inform Decis Mak* 2020; **20**: 248 [PMID: 32993636 DOI: 10.1186/s12911-020-01277-w]
173 **Dong Y**, Xu L, Fan Y, Xiang P, Gao X, Chen Y, Zhang W, Ge Q. A novel surgical predictive model for Chinese Crohn's disease patients. *Medicine (Baltimore)* 2019; **98**: e17510 [PMID: 31725605 DOI: 10.1097/MD.0000000000017510]
174 **Le V**, Quinn TP, Tran T, Venkatesh S. Deep in the Bowel: Highly Interpretable Neural Encoder-Decoder Networks Predict Gut Metabolites from Gut Microbiome. *BMC Genomics* 2020; **21**: 256 [PMID: 32689932 DOI: 10.1186/s12864-020-6652-7]
175 **Morilla I**, Uzzan M, Laharie D, Cazals-Hatem D, Denost Q, Daniel F, Belleannee G, Bouhnik Y, Wainrib G, Panis Y, Ogier-Denis E, Treton X. Colonic MicroRNA Profiles, Identified by a Deep Learning Algorithm, That Predict Responses to Therapy of Patients With Acute Severe Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2019; **17**: 905-913 [PMID: 30223112 DOI: 10.1016/j.cgh.2018.08.068]
176 **Li B**, Meng MQ. Computer-aided detection of bleeding regions for capsule endoscopy images. *IEEE Trans Biomed Eng* 2009; **56**: 1032-1039 [PMID: 19174349 DOI: 10.1109/TBME.2008.2010526]
177 **Li B**, Meng MQ. Computer-based detection of bleeding and ulcer in wireless capsule endoscopy images by chromaticity moments. *Comput Biol Med* 2009; **39**: 141-147 [PMID: 19147126 DOI: 10.1016/j.compbiomed.2008.11.007]
178 **Kanai M**, Togo R, Ogawa T, Haseyama M. Chronic atrophic gastritis detection with a convolutional neural network considering stomach regions. *World J Gastroenterol* 2020; **26**: 3650-3659 [PMID: 32742133 DOI: 10.3748/wjg.v26.i25.3650]
179 **Wuestemann J**, Hupfeld S, Kupitz D, Genseke P, Schenke S, Pech M, Kreissl MC, Grosser OS. Analysis of Bone Scans in Various Tumor Entities Using a Deep-Learning-Based Artificial Neural Network Algorithm-Evaluation of Diagnostic Performance. *Cancers (Basel)* 2020; **12** [PMID: 32957650 DOI: 10.3390/cancers12092654]
180 **Zhu L**, Luo W, Su M, Wei H, Wei J, Zhang X, Zou C. Comparison between artificial neural network and Cox regression model in predicting the survival rate of gastric cancer patients. *Biomed Rep* 2013; **1**: 757-760 [PMID: 24649024 DOI: 10.3892/br.2013.140]
181 **Billah M**, Waheed S. Gastrointestinal polyp detection in endoscopic images using an improved feature extraction method. *Biomed Eng Lett* 2018; **8**: 69-75 [PMID: 30603191 DOI: 10.1007/s13534-017-0048-x]
182 **Ozawa T**, Ishihara S, Fujishiro M, Kumagai Y, Shichijo S, Tada T. Automated endoscopic detection and classification of colorectal polyps using convolutional neural networks. *Therap Adv Gastroenterol* 2020; **13**: 1756284820910659 [PMID: 32231710 DOI: 10.1177/1756284820910659]
183 **Al-Bahrani R**, Agrawal A, Choudhary A. Survivability prediction of colon cancer patients using

neural networks. *Health Informatics J* 2019; **25**: 878-891 [PMID: 28927314 DOI: 10.1177/1460458217720395]

184 **Mohan BP**, Khan SR, Kassab LL, Ponnada S, Chandan S, Ali T, Dulai PS, Adler DG, Kochhar GS. High pooled performance of convolutional neural networks in computer-aided diagnosis of GI ulcers and/or hemorrhage on wireless capsule endoscopy images: a systematic review and meta-analysis. *Gastrointest Endosc* 2021; **93**: 356-364. e4 [PMID: 32721487 DOI: 10.1016/j.gie.2020.07.038]

185 **Aziz M**, Fatima R, Dong C, Lee-Smith W, Nawras A. The impact of deep convolutional neural network-based artificial intelligence on colonoscopy outcomes: A systematic review with meta-analysis. *J Gastroenterol Hepatol* 2020; **35**: 1676-1683 [PMID: 32267558 DOI: 10.1111/jgh.15070]

186 **Dolgobrodov SG**, Moore P, Marshall R, Bittern R, Steele RJ, Cuschieri A. Artificial neural network: predicted vs observed survival in patients with colonic cancer. *Dis Colon Rectum* 2007; **50**: 184-191 [PMID: 17180250 DOI: 10.1007/s10350-006-0779-8]

187 **Cueto-López N**, García-Ordás MT, Dávila-Batista V, Moreno V, Aragonés N, Alaiz-Rodríguez R. A comparative study on feature selection for a risk prediction model for colorectal cancer. *Comput Methods Programs Biomed* 2019; **177**: 219-229 [PMID: 31319951 DOI: 10.1016/j.cmpb.2019.06.001]

188 **Zhang B**, Liang XL, Gao HY, Ye LS, Wang YG. Models of logistic regression analysis, support vector machine, and back-propagation neural network based on serum tumor markers in colorectal cancer diagnosis. *Genet Mol Res* 2016; **15** [PMID: 27323037 DOI: 10.4238/gmr.15028643]

189 **Coppedè F**, Grossi E, Lopomo A, Spisni R, Buscema M, Migliore L. Application of artificial neural networks to link genetic and environmental factors to DNA methylation in colorectal cancer. *Epigenomics* 2015; **7**: 175-186 [PMID: 25942531 DOI: 10.2217/epi.14.77]

190 **Liu B**, Cui Q, Jiang T, Ma S. A combinational feature selection and ensemble neural network method for classification of gene expression data. *BMC Bioinformatics* 2004; **5**: 136 [PMID: 15450124 DOI: 10.1186/1471-2105-5-136]

191 **Chen YD**, Zheng S, Yu JK, Hu X. Artificial neural networks analysis of surface-enhanced laser desorption/ionization mass spectra of serum protein pattern distinguishes colorectal cancer from healthy population. *Clin Cancer Res* 2004; **10**: 8380-8385 [PMID: 15623616 DOI: 10.1158/1078-0432.CCR-1162-03]

192 **Hu HP**, Niu ZJ, Bai YP, Tan XH. Cancer classification based on gene expression using neural networks. *Genet Mol Res* 2015; **14**: 17605-17611 [PMID: 26782405 DOI: 10.4238/2015.December.21.33]

193 **Ronen J**, Hayat S, Akalin A. Evaluation of colorectal cancer subtypes and cell lines using deep learning. *Life Sci Alliance* 2019; **2** [PMID: 31792061 DOI: 10.26508/lsa.201900517]

194 **Bilsland AE**, Pugliese A, Liu Y, Revie J, Burns S, McCormick C, Cairney CJ, Bower J, Drysdale M, Narita M, Sadaie M, Keith WN. Identification of a Selective G1-Phase Benzimidazolone Inhibitor by a Senescence-Targeted Virtual Screen Using Artificial Neural Networks. *Neoplasia* 2015; **17**: 704-715 [PMID: 26476078 DOI: 10.1016/j.neo.2015.08.009]

195 **Maniruzzaman M**, Jahanur Rahman M, Ahammed B, Abedin MM, Suri HS, Biswas M, El-Baz A, Bangeas P, Tsoulfas G, Suri JS. Statistical characterization and classification of colon microarray gene expression data using multiple machine learning paradigms. *Comput Methods Programs Biomed* 2019; **176**: 173-193 [PMID: 31200905 DOI: 10.1016/j.cmpb.2019.04.008]

196 **Inglese P**, McKenzie JS, Mroz A, Kinross J, Veselkov K, Holmes E, Takats Z, Nicholson JK, Glen RC. Deep learning and 3D-DESI imaging reveal the hidden metabolic heterogeneity of cancer. *Chem Sci* 2017; **8**: 3500-3511 [PMID: 28507724 DOI: 10.1039/c6sc03738k]

197 **Shi R**, Chen W, Yang B, Qu J, Cheng Y, Zhu Z, Gao Y, Wang Q, Liu Y, Li Z, Qu X. Prediction of KRAS, NRAS and BRAF status in colorectal cancer patients with liver metastasis using a deep artificial neural network based on radiomics and semantic features. *Am J Cancer Res* 2020; **10**: 4513-4526 [PMID: 33415015]

198 **Jiang HJ**, Huang YA, You ZH. SAEROF: an ensemble approach for large-scale drug-disease association prediction by incorporating rotation forest and sparse autoencoder deep neural network. *Sci Rep* 2020; **10**: 4972 [PMID: 32188871 DOI: 10.1038/s41598-020-61616-9]

199 **Que SJ**, Chen QY, Qing-Zhong, Liu ZY, Wang JB, Lin JX, Lu J, Cao LL, Lin M, Tu RH, Huang ZN, Lin JL, Zheng HL, Li P, Zheng CH, Huang CM, Xie JW. Application of preoperative artificial neural network based on blood biomarkers and clinicopathological parameters for predicting long-term survival of patients with gastric cancer. *World J Gastroenterol* 2019; **25**: 6451-6464 [PMID: 31798281 DOI: 10.3748/wjg.v25.i43.6451]

200 **Li C**, Yu H, Sun Y, Zeng X, Zhang W. Identification of the hub genes in gastric cancer through weighted gene co-expression network analysis. *PeerJ* 2021; **9**: e10682 [PMID: 33717664 DOI: 10.7717/peerj.10682]

201 **Anekboon K**, Lursinsap C, Phimoltares S, Fucharoen S, Tongsima S. Extracting predictive SNPs in Crohn's disease using a vacillating genetic algorithm and a neural classifier in case-control association studies. *Comput Biol Med* 2014; **44**: 57-65 [PMID: 24377689 DOI: 10.1016/j.compbiomed.2013.09.017]

202 **Fioravanti D**, Giarratano Y, Maggio V, Agostinelli C, Chierici M, Jurman G, Furlanello C. Phylogenetic convolutional neural networks in metagenomics. *BMC Bioinformatics* 2018; **19**: 49 [PMID: 29536822 DOI: 10.1186/s12859-018-2033-5]

203 **Hardalaç F**, Başaranoğlu M, Yüksel M, Kutbay U, Kaplan M, Özderin Özin Y, Kılıç ZM, Demirbağ AE, Coşkun O, Aksoy A, Gangarapu V, Örmeci N, Kayaçetin E. The rate of mucosal

healing by azathioprine therapy and prediction by artificial systems. *Turk J Gastroenterol* 2015; **26**: 315-321 [PMID: 26039001 DOI: 10.5152/tjg.2015.0199]

204 **Klein A**, Mazor Y, Karban A, Ben-Itzhak O, Chowers Y, Sabo E. Early histological findings may predict the clinical phenotype in Crohn's colitis. *United European Gastroenterol J* 2017; **5**: 694-701 [PMID: 28815033 DOI: 10.1177/2050640616676435]

205 **Peng JC**, Ran ZH, Shen J. Seasonal variation in onset and relapse of IBD and a model to predict the frequency of onset, relapse, and severity of IBD based on artificial neural network. *Int J Colorectal Dis* 2015; **30**: 1267-1273 [PMID: 25976931 DOI: 10.1007/s00384-015-2250-6]

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REVIEW

Molecular alterations in pancreatic tumors

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Abstract

Genetic alterations in pancreatic tumors can usually be classified in: (1) Mutational activation of oncogenes; (2) Inactivation of tumor suppressor genes; and (3) Inactivation of genome maintenance genes controlling the repair of DNA damage. Endoscopic ultrasound-guided fine-needle aspiration has improved pre-operative diagnosis, but the management of patients with a pancreatic lesion is still challenging. Molecular testing could help mainly in solving these "inconclusive" specimens. The introduction of multi-gene analysis approaches, such as next-generation sequencing, has provided a lot of useful information on the molecular characterization of pancreatic tumors. Different types of pancreatic tumors (*e.g.*, pancreatic ductal adenocarcinomas, intraductal papillary mucinous neoplasms, solid pseudopapillary tumors) are characterized by specific molecular alterations. The aim of this review is to summarize the main molecular alterations found in pancreatic tumors.

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Core Tip: To date, in patients with pancreatic cancer there are no targetable molecules for personalized treatment in clinical practice. The introduction of multi-gene analysis approaches, such as massive parallel sequencing, has provided a lot of useful information regarding the molecular characterization of pancreatic tumors. However, a huge amount of data needs to be properly managed to determine the information that is useful and correct. A deeper knowledge of the molecular alterations characterizing pancreatic neoplasms may lead to new potential therapeutic targets for these tumors.

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INTRODUCTION

Genetic alterations in pancreatic tumors are usually classified in: (1) Tumors with activation of oncogenes (*e.g.*, *KRAS* mutation—more than 90% of pancreatic tumors); (2) Tumors harboring inactivation of tumor suppressor genes (*e.g.*, *p16/CDKN2A*, *TP53*, and *SMAD4*); and (3) Tumors with inactivation of genes controlling the repair of DNA damage (*e.g.*, *hMLH1* and *MSH2*)[1].

KRAS gene mutations inhibit the ability of KRAS protein to hydrolyze GTP (guanosine-5'-triphosphate), leaving the protein constitutively active, mediating cell survival and differentiation. *KRAS* is the most common marker used for single-gene testing. Its use is strongly limited by the identification of mutations also in low-grade pancreaticobiliary dysplasia or chronic pancreatitis (about 10%)[2-5]. To date, the "The Papanicolaou Society of Cytopathology guidelines" does not encourage testing of *KRAS* in bile duct strictures and solid pancreatic masses as a useful "single-gene" test.

P53 protein is involved in cell-cycle regulation, the apoptotic process, and plays a crucial role in the maintenance of genomic stability. Mutations in the *TP53* gene lead to inactivation of the normal protein function. In the presence of DNA damage, the functional loss of the p53 protein enhances cellular survival, facilitating the accumulation of further genetic mutations[6]. *TP53* is mostly inactivated by single-point mutations[7]. *TP53* gene inactivation is a very common event in pancreatic cancer (50% to 75% of pancreatic cancers harbor *TP53* mutations)[7-9].

CDKN2A/p16 maps on chromosome 9p and encodes the protein $p14^{ARF}$, an activator of p53 protein, and $p16^{INK4a}$ protein. This protein inhibits the progression of the cell cycle at the G1-S checkpoint binding of cyclin-dependent kinases (CDKs), as CDK4 and CDK6[10]. *CDKN2A/p16* was the first tumor suppressor gene that was shown to undergo silencing and promoter hypermethylation in pancreatic cancer[11]. The Rb/p16 pathway is down-regulated in most pancreatic cancers, almost all through p16 gene inactivation[11]. Mutations in the *CDKN2A* gene are associated with an increased risk of cancers. Moreover, *CDKN2A* alterations are also frequently observed in cancer cell lines.

SMAD4/DPC4 gene is located on chromosome 18q and the protein acts in the signal transduction cascade, involving transforming growth factor β (TGF-β). A loss of SMAD4 protein leads to unregulated cellular proliferation[12].

The aim of this review is to summarize the main molecular alterations found in pancreatic tumors, both in solid (*e.g.*, pancreatic ductal adenocarcinoma (PDAC), Table 1) and in cystic neoplasms (*e.g.*, intraductal papillary mucinous neoplasms, IPMN).

Table 1 Main genetic alterations detectable in pancreatic ductal adenocarcinoma

Type of pancreatic lesion	Genetic alteration	Reported frequency (%)	Type of alterations	Role in clinical practice
PDAC	*KRAS*	70-90	Point mutations	Diagnostic/prognostic
	TP53	50-75	Point mutations/LOH	Prognostic
	CDKN2A/p16	90-98	Point mutations/LOH	Prognostic/genetic surveillance
	SMAD4	40-60	Point mutations/LOH	Prognostic
	BRCA1/2	5-10	Point mutations	Predictive/genetic surveillance
	NTRK1-3	< 1	Gene fusions	Predictive
	MSI	< 2	LOF	Predictive

The percentages quoted are estimated from the literature cited in the paper. Diagnostic role appears mainly in preoperative material. In bold those markers recommended for clinical practice by National Comprehensive Cancer Network 2019 guidelines. PDAC: Pancreatic ductal adenocarcinoma; LOH: Loss of heterozygosity.

PDAC

Wide genome analysis

According to data obtained by whole-exome sequencing analysis, PDAC harbors an average of about 60 genetic alterations, and most of them are point mutations[1,8,13]. Based on these alterations, 12 cellular pathways genetically altered in pancreatic neoplasia have been identified[8]. Massive sequencing studies carried out on PDACs revealed that alterations may be found in some well-known genes (*e.g.*, *KRAS*, *CDKN2A*, *TP53*, *ARID1A*, *SMAD4*) or in novel genes, that may be involved in DNA damage repair (*e.g.*, *ATM*), chromatin modification (*e.g.*, *EPC1* and *ARID2*), or in neoplastic carcinogenesis (*e.g.*, *KDM6A* and *PREX2*)[14]. Whole-exome sequencing analysis has defined some putative therapeutic targets (*e.g.*, RBM10) associated with longer survival in patients with pancreatic cancers, others associated with improved survival (*e.g.*, KRAS p.Q61H mutation), and others defining sensitivity to target therapies in PDAC models (*e.g.*, BRAF mutations as sensitivity markers for treatment with vemurafenib)[15].

An expression analysis study led to the cluster of PDAC in 4 different subtypes: (1) Squamous; (2) Pancreatic progenitor; (3) Immunogenic; and (4) Aberrantly differentiated endocrine exocrine[16]. Also analysis of the structural genomic alterations in PDACs have been classified into four different subtypes: (1) "Stable", when PDACs contain less than 50 structural variations; (2) "Locally rearranged", when PDACs exhibit a focal event on one or two chromosomes; (3) "Scattered subtype", when PDACs show a fewer number of chromosomal damages and less than 200 structural variations; and (4) "Unstable", when PDACs exhibit more than 200 structural events[17].

KRAS

KRAS is the most frequently mutated oncogene in pancreatic cancers (> 95%) and the most frequent gene mutated in PDAC (from 70% to 95%)[8,15,18,19]. The acquisition of a *KRAS* mutation represents an early and initiating event in PDACs. However, the low frequency of progression of precursor lesions to PDAC suggests that additional alterations are needed for neoplastic progression[20]. In PDAC, the mutations in *KRAS* are not located only in exon 2, but they have also been found in other exons[14,15,21]. However, the mutations harbored in exon 2 exhibited a similar association with survival, while cases mutated in exon 3 seem to have a remarkably favorable prognosis[15]. Coexistent *KRAS* mutations were detected in the same pancreatic neoplastic mass more frequently than in other tumors[21-23].

KRAS analysis may be of particular interest in the case of doubtful or inconclusive diagnoses (*e.g.*, specimens with cytological atypia or acellular specimens). It is well-established that cytopathology together with *KRAS* analysis allows improved diagnosis of PDAC in endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) specimens[24-31]. Finding a *KRAS* mutation in EUS-FNA material may: (1) Indicate that a re-evaluation of the cytopathology report is needed (mainly if doubtful); (2) Indicate a second FNA or surgery[21]; and (3) Lead to a reduction in

false-negative diagnoses[32].

A worse prognosis has been observed in patients with tumors harboring coexistent *KRAS* mutations together with TP53 alterations and/or loss of SMAD4 protein[33,34]. KRAS p.G12C variant is a mutation that could be targeted by KRAS-G12C-specific inhibitors[35]. PDAC which also harbors the p.G12C alteration may benefit from this treatment. KRAS-wild type PDACs represent a distinct molecular subtype of pancreatic cancer that could benefit from tailored treatments, including BRAF antagonists and MAPK inhibitors[36].

TP53

EUS-FNA sensitivity in the diagnosis of pancreatic malignant lesions can be improved by also implementing the evaluation of the *TP53* gene[37-39]. P53 protein overexpression has been detected in specimens with pancreatic cancer but not in those with chronic pancreatitis. TP53 alterations have been detected in 50%-75% of PDACs. Combining the evaluation of p53 protein expression with histological examination improves the sensitivity of diagnosis of pancreatic cancers, with a high specificity [37,38]. The sensitivity of EUS-FNA in the diagnosis of PDAC is further improved by combining p53 and Ki67 staining[38].

The combination of p53 and CA19.9 increases the sensitivity of cytology but it can negatively affect the specificity[39]. A worse patient prognosis has been correlated with loss of p53 protein, mainly if p53 loss is combined with KRAS alterations and loss of SMAD4 protein expression[33,34]. In a recent study based on next-generation sequencing (NGS) results from the CONKO-001 phase III trial, it has been demonstrated that patients with *TP53* mutated PDAC tumors benefit from adjuvant gemcitabine treatment[40].

SMAD4

Approximately 50% of pancreatic cancers show SMAD4 protein inactivation, due to homozygous deletion and intragenic mutations[10,41-43]. Loss of SMAD4 generally occurs late in pancreatic carcinogenesis, and it has been frequently observed in pancreatic adenocarcinomas, but not in extra-pancreatic lesions[44]. In reactive and inflammatory diseases of the pancreas (*e.g.*, chronic pancreatitis) SMAD4 activity is usually preserved. Loss of SMAD4 protein has also been linked with an increased risk of developing metastases and worse prognosis[33,34,43,45,46]. In PDAC, *SMAD4* mutations lead to the prevention of the normal transduction of TGF-β signals; TGF-β inhibitor has shown efficacy in a preclinical investigation[47].

A study by Hsieh *et al*[48] demonstrated that SMAD4 deficiency in PDAC cells led to higher sensitivity to gemcitabine. On the contrary, *SMAD4* mutated cells were sensitive to gemcitabine similar to that in cells with wild-type SMAD4[48]. These data suggest that the *SMAD4* copy number may be a therapeutic marker for PDAC treatment with gemcitabine[49]. In this study, it was observed that SMAD4 deficiency led to upregulation of cell cycle-related genes, such as CDK1, with consequent higher sensitivity to other agents modulating the cell cycle such as clofarabine, cytarabine, darinaparsin, and olaparib[48,49].

CDKN2A

More than 95% of sporadic pancreatic carcinomas harbor a *CDKN2A* gene inactivation due to intragenic mutation, usually coupled with the loss of the other allele, promoter hypermethylation, or homozygous deletion of both alleles[50-52]. *CDKN2A* is involved in familial pancreatic cancer, although *CDKN2A* germline mutations in patients with pancreatic cancer occur rarely (0.6%)[53,54]. Patients harboring *CDKN2A* mutations are more likely to report a family history of pancreatic cancer than those without *CDKN2A* alterations[54]. Surveillance protocols from age 40 are recommended for *CDKN2A* mutation carriers[55].

Decreased expression of p16 protein has been associated with a tendency for the tumor to be larger than in those with normal p16 expression levels. Pancreatic neoplasms with loss of p16 expression, due to mutations and/or promoter hypermethylation, are significantly larger. Moreover, the survival period in these patients was significantly shorter when compared to those with a pancreatic tumor characterized by intact p16 functions[33,56,57]. CDK4/6 is a potential target in CDKN2A-deficient tumors and the efficacy of CDK4 inhibitors has been confirmed in PDAC preclinical models[58].

BRCA1-2

Alterations in the BRCA pathway and defects in DNA maintenance (such as genomic

instability and the BRCA mutational signature) may have implications for the therapeutic selection of patients with pancreatic tumors[17]. *BRCA1/2* mutation frequencies range from 5% to 10%, and BRCA alterations have been detected both in sporadic and familial PDAC[1,59]. Patients with PDAC harboring germline *BRCA1* or *BRCA2* mutations showed a longer progression-free survival if treated with a PARP inhibitor[60,61]. According to the recommendation from the International Cancer of the Pancreas Screening Consortium, for *BRCA1* a consensus of 69.9% was reached for recommending that *BRCA1* mutation carriers undergo surveillance, whereas no consensus was reached on family history criteria for *BRCA1* mutation carriers[55]. For carriers of *BRCA2* mutations, the consensus (agreement of 93%) was to recommend surveillance for mutation carriers who have a blood relative with pancreatic cancer[55]. For *BRCA2* mutation carriers with a germline variant (deleterious), the recommended age to initiate surveillance is generally 50 years[55]. With regard to the surveillance protocols for high-risk individuals, the consensus is that pancreatic imaging with magnetic resonance imaging (MRI)/magnetic retrograde cholangiopancreatography and/or EUS should be the first-line test for pancreatic surveillance[55]. Pancreatic computed tomography is reserved for individuals unable to undergo MRI or EUS[55].

Other alterations

Even if uncommon, MSI/dMMR (Microsatellite Instability/defective DNA mismatch repair) has been described in about 1%-2% of PDAC[62,63]. PDACs with MSI are usually associated with medullary histology and are rarely mutated in *KRAS* or *TP53* genes[62,64]. National Comprehensive Cancer Network (NCCN) guidelines recommend the MSI and MMR tests in locally advanced and metastatic pancreatic carcinomas[65]. NCCN recommends the treatment of PDAC with the PD-1 (programm-ed cell death-1) inhibitor, Pembrolizumab, only in those patients with MSI-H (high-MSI) or dMMR advanced/metastatic pancreatic cancer[65].

BRAF mutations are an uncommon event in PDAC[1]. Some evidence suggests that patients with pancreas tumors harboring *BRAFV600E* mutations may benefit from treatment with RAF-MEK-targeted therapy[66].

The *MGMT* promoter can be hypermethylated in PDAC[34,67]. In 1998, treatment with temozolomide in advanced pancreatic cancer has been tried in a phase II study, but no relevant clinical response was observed[68]. *ARID1A* oncosuppressor protein deficiency was significantly associated with poor outcome in PDAC patients[15]. Amplifications and copy-number gains of oncogenes such as *ERBB2*, *MET*, and *FGFR1* may be detected in pancreatic tumors[17]. The inactivation of several genes (*e.g.*, *ROBO1*, *ROBO2*, *SLIT2*, and *RNF43*) leads to an aberrant WNT (Wingless-related integration site) signaling[15,17]. Unresectable non-metastatic pancreatic carcinomas may also harbor mutations in *GRM8* and *TRIM33* genes[69], while only a small fraction (approximately 5%) of pancreatic adenocarcinomas showed Cyclin E overexpression[70]. Different to solid pseudopapillary neoplasia (SPN), *CTNNB1* mutations are uncommon in PDAC[15,71]. In PDAC, the frequency of *NTRK* (neurotrophic tropomyosin receptor kinase) fusion is very low (about 0.3%), but clinical trials revealed that the selective TRK (tropomyosin receptor kinase) inhibitors are also effective in PDAC harboring *NTRK* rearrangement[58]. NCCN guidelines suggest the use of a TRK inhibitor in *NTRK* gene fusion-positive advanced/metastatic pancreatic cancer[61,65].

Organoids are a preclinical model that is becoming increasingly important for studying tumor behavior because they can simulate metastases, microtumors, and the tumor microenvironment better than "classical" monolayer culture systems[72]. An interesting preclinical model for the study of PDAC is the set-up of three-dimensional (3D)-tumoroids *in vitro* culture systems[72-78]. These organoids can also be generated from a resected PDAC and are amenable to therapeutic screening as well as genetic and biochemical perturbation[77]. In a study by Boj *et al*[76], organoids derived from murine and human PDAC generated lesions similar to pancreatic intraepithelial neoplasia (PanIN) and progressed to invasive PDAC. Intriguingly, the expression of mutated KRAS protein (KRASG12D) in PDAC organoids was sufficient to induce a preinvasive neoplasm[76]. PDAC tumoroid cultures retain the capacity to maintain tumor stroma and characteristics of the primary tumor including the long-term (> 44 d) production of CEA (carcinoembryonic antigen) and CA19-9 (carbohydrate antigen 19-9)[74]. This 3D cell culture model of PDAC would help the diagnostics, investigation of genetic drivers, and identification of novel therapeutic targets[72]. Moreover, this culture could also allow clarification on how the immunosuppressive mechanism affects the growth and stasis of tumors[74]. Moreover, another important aspect is that organoids are suitable for storage in biobanks and used for further

research, ensuring access to relevant sample numbers[78].

CYSTIC PANCREATIC TUMORS

IPMN

KRAS mutations are harbored by over 90% of low-grade PanIN[79], and mutant *KRAS* is sufficient to initiate the development of PanINs and IPMNs[20,80-83] (Table 2).

The distinction between IPMNs and mucinous cystic neoplasms (MCNs) from non-neoplastic pancreatic cysts may be helped by analysis of pancreatic cyst fluid. In fact, a *KRAS* mutation is highly specific for mucinous differentiation, but not for identifying MCNs[84]. *KRAS* alterations have a very high specificity but low sensitivity for MCNs and IPMNs (approximately 15% and 70%, respectively). If *KRAS* analysis is combined with that of *GNAS*, the sensitivity increases[85,86]. The differential diagnosis of cystic mucinous lesions (IPMN and MCN), mainly when the pre-operative cytology is non-diagnostic or when the CEA cyst fluid levels are indeterminate, may be helped by the analysis of *KRAS* and Loss-of-Heterozygosity (LOH)[87].

IPMNs rarely harbor mutations in the *TP53* gene (approximately 10%). The overexpression of *TP53* was more commonly observed in IPMNs of the pancreatobiliary type with invasion[88]. In a cohort of IPMN patients, the overexpression of TP53, together with loss of function of SMAD4, was strongly associated with patient survival[88]. In IPMNs, SMAD4 loss of function was rarely detected; SMAD4 loss is more common in IPMNs of the pancreatobiliary type with invasion[88].

The *GNAS* gene encodes the α-subunit of the stimulatory G-protein (Gαs). This subunit regulates the adenylate cyclase activity through Gαs-coupled receptors. Alterations in GNAS may determine the characteristic IPMN phenotype[89]. *GNAS* activating mutations are reported prevalently in IPMN (approximately 40%-60%) [85,88,90-92] and invasive pancreatic cancers, only if arising in association with an IPMN[79,93]. In the majority of IPMNs (approximately 90%), at least one of the *KRAS* or *GNAS* genes harbor mutations[94], and in about half of IPMN (approximately 40%), a *GNAS* alteration coexists with a *KRAS* mutation[90]. The combination of *KRAS* and *GNAS* mutations helps in distinguishing between a serous cystic neoplasm (SCN) and an IPMN with high sensitivity and specificity. In fact, if most IPMNs have a *GNAS* and/or a *KRAS* alteration, no SCNs harbor either mutation. Besides, detecting a mutation in the *GNAS* gene in cyst fluid may help to distinguish IPMNs from MCNs[95].

RNF43 mutation frequency in IPMN is about 25%, ranging from 10% to 75%[93,96]. These mutations are often inactivating alterations and are found in association with LOH.

CDKN2A/p16 inactivating mutations have also been found in IPMNs with high-grade dysplasia, other than in pancreatic adenocarcinoma[97].

Germline mutations of *STK11/LKB1* genes have been associated with IPMNs and invasive pancreatic cancer[98,99]. Besides, somatic mutations of *STK11/LKB1* are observed in about 5% of patients with sporadic IPMNs and pancreatic cancers[98,99].

A high amount of DNA and high-amplitude mutations in the pancreatic cyst fluid may be indicators of malignancy, helping to identifying malignant cystic lesions[86].

The use of NGS in pancreatic cyst fluid allows high sensitivity and specificity in classifying pancreatic cancers, mainly for the diagnosis of IPMN with advanced neoplasia[100].

MCN

MCNs harbor alterations also commonly found in PDAC. MCNs frequently have *KRAS* gene mutations, mainly in MCNs with high-grade dysplasia[101]. The diagnosis of mucinous cysts may be helped by the presence of a *KRAS* mutation in cyst fluid[86]. p16/CDKN2A expression is altered in MCN, even if in a lower percentage (about 15%) if compared to that of PDAC[102]. P53 has been reported with aberrant expression in MCN with high-grade dysplasia[103]. *TP53* alterations are often associated with aggressiveness and seem to be involved in progression to PDAC[92]. Mutations of *RNF43* have been reported in MCN[92]. *PIK3CA* alterations were described with very low frequency in MCN and in association with high-grade dysplasia and invasive adenocarcinoma[104]. As in IPMN, no *VHL* mutations were detected in MCN[92], but, different to IPMN, the *GNAS* gene is not mutated in MCN[96]. The LOH of *Dpc4/Smad4* contributes to MCN progression in mice with *KRAS-G12D* mutation[105], confirming that the *SMAD4* gene acts as a PDAC tumor suppressor[106]. Whole-exome sequencing performed on a cohort of MCN revealed

Table 2 Main genetic alterations detectable in cystic pancreatic lesions

Type of pancreatic lesion	Genetic alteration	Reported frequency (%)	Type of alterations	Role in clinical practice
IPMN	*KRAS*	90	Point mutations	Diagnostic
	TP53	10	Point mutations/LOH	Prognostic
	GNAS	40-60	Point mutations	Diagnostic
	RNF43	25	Point mutations/LOH	Diagnostic
MCN	*KRAS*	0-25% (LG); 50-90% (HG)	Point mutations	Diagnostic
	CDKN2A/p16	0-10 (LG); 50 (HG)	Point mutations/LOH	Prognostic
	TP53	0 (LG); 20-50 (HG)	Point mutations/LOH	Prognostic
SCN	*VHL*	40-60	Point mutations/LOH	Diagnostic

The percentages quoted are estimated from the literature cited in the paper. Diagnostic role appears mainly in preoperative material. IPMN: Intraductal papillary mucinous neoplasms; MCN: Mucinous cystic neoplasia; LG: Low-grade mucinous cystic neoplasia; HG: High-grade mucinous cystic neoplasia; SCN: Serous cystic neoplasm; LOH: Loss of heterozygosity.

that the tumors harbored about 16 somatic mutations per tumor, lower than the number of mutations observed in IPMN (about 27 per tumor)[92].

SCN

VHL mutations are frequently reported in SCN (from 40% up to 60% of cases)[100,107]. SCNs usually do not harbor alterations in genes frequently mutated in IPMN, or MCN (*i.e.*, *KRAS*, *GNAS*, *TP53*), helping to distinguish SCNs from the other mucinous neoplasia[100,108]. The lack of *CTNNB1* mutations allows the differentiation of SCN from SPN. Moreover, SCNs do not harbor mutations in genes frequently altered in neuroendocrine pancreatic tumors[109]. Whole exome sequencing analysis performed on a cohort of eight SCNs revealed that almost all tumors harbored a LOH on chromosome 3p[92]. An average of only 10 non-synonymous somatic mutations was detected in SCNs[92], far less than the average observed in PDAC.

OTHER PANCREATIC TUMORS

SPN

The *CTNNB1* gene (codons from 32 to 37) encodes for a region that plays a crucial role in the regulation of the β-catenin protein[110,111]. Alterations within this *CTNNB1* region usually block β-catenin phosphorylation, inhibiting degradation of the protein[112]. *CTNNB1* mutations are characteristic of pancreatic SPN[71,113,114]. Different to PDAC, SPNs are not mutated in *KRAS*, *TP53*, or *SMAD4* genes, and *CTNNB1* mutations are the main molecular alteration detected[92]. DNA array CGH (comparative genomic hybridization) performed on a pediatric case of SPN revealed a loss in chromosome band 11p15.5, a chromosomal region encoding for the *HRAS* gene[115]. As suggested by Selenica *et al*[116], even if inhibition of the Wnt pathway may be an intuitive therapeutic option for this disease, the evidence that clinically advanced Wnt pathway inhibitors target components upstreaming β-catenin activity is a clear limitation. For this reason, future drugs should be designed to target the β-catenin protein directly[116] (Table 3).

Pancreatic neuroendocrine tumors

Pancreatic neuroendocrine tumors (PanNETs) show a distinct landscape of molecular alterations if compared to the other pancreatic tumors. The mutation frequency in PanNET was lower than that observed in poorly differentiated neuroendocrine carcinomas (45% and 83%, respectively)[117], and the incidence of mutations was higher in PanNET with a high risk of progression than those with low risk[117]. *MEN1* alterations (both mutations and LOH) have been found in up to 70% of PanNETs and have been associated with a better prognosis[118,119]. PanNETs harbor alterations in those genes involved in the chromatin remodeling complex, such as loss of ATRX and DAXX proteins expression[118]. Mutations in the mTOR (mammalian target of rapamycin) pathway genes (*e.g.*, *PIK3CA*, *PTEN*, and *TSC2*) have been detected in

Table 3 Main genetic alterations detectable in other pancreatic tumors

Type of pancreatic lesion	Genetic alteration	Reported frequency (%)	Type of alterations	Role in clinical practice
SPN	*CTNNB1*	90-100	Point mutations	Diagnostic
PanNET	*MEN1*	70	Point mutations/LOH	Diagnostic/prognostic
	VHL	25	Point mutations/LOH	Diagnostic
AAC	*CTNNB1*	5-25	Point mutationsLOF	Diagnostic
	MSI	5-15	LOF	Predictive?

The percentages quoted are estimated from the literature cited in the paper. Diagnostic role appears mainly in preoperative material. SPN: Solid pseudopapillary neoplasm; PanNET: Pancreatic neuroendocrine tumor; AAC: Acinic adenocarcinoma; LOH: Loss of heterozygosity; LOF: Loss of function.

PanNET[118]. For example, *PTEN* and *TSC2* genes have been observed as inactivating in primary PanNET and their low protein levels were associated with shorter overall and disease-free survival[120]. *VHL* inactivation, due to deletion or methylation, was also observed in up to 25% of PanNETs[121,122].

Acinar cell carcinomas

Activating mutations in *CTNNB1* and inactivating mutations in *APC* genes have been observed in up to 25% of acinar cell carcinomas (ACCs). *KRAS* mutations are an uncommon event in this type of neoplasia[123]. Nevertheless, *TP53* mutations were also found only in a low fraction of ACCs[123-125], deletion of the *TP53* region (chromosome band 17p13.1) was detected by FISH in about half of a cohort of 54 ACCs[125]. Alterations found in IPMN (*e.g.*, *GNAS* and *RNF43*) or in PanNET (*e.g.*, *MEN1*) were rarely found in ACC[126] A genome-wide analysis performed on ACCs revealed a median number of 137 point mutations per tumor and *COL12A1*, *FRY*, *FRYL*, and *PLB1* were the most frequently mutated genes[124]. Intriguingly, a genome-wide analysis identified no recurrent point mutations in ACCs[124]. Microsatellite instability was detected in a fraction of ACC ranging from 7% to 14% of cases[127]. ACC with MSI did not exhibit distinct morphological or clinical features[128].

CONCLUSION

To date, there are no targetable molecules available for personalized patient treatment of pancreatic tumors in clinical practice, as stated by the current European Society for Medical Oncology (ESMO) guidelines[129]. EUS-FNA has improved pre-operative diagnosis[130-132], but the management of patients with a pancreatic lesion is still challenging. In fact, in a subset of cases, such as lesions with atypical/suspicious cytopathologic features, the pre-operative diagnosis remains inconclusive[133]. Performing a molecular characterization could help mainly in solving these "inconclusive" specimens. The introduction of multi-gene analysis approaches, such as NGS, has provided a lot of useful information regarding the molecular characterization of pancreatic tumors[134]. EUS-FNAC (FNA cytology) is a useful diagnostic tool for pancreatic lesions[135] and molecular analysis can be successfully performed on cytological smears[21]. However, it has becoming increasingly crucial to have sufficient material for histological, immunohistochemical, and molecular characterization. Pancreatic FNAB (FNA biopsy) provides enough material to allow proper histological assessment, immunostaining, and molecular techniques [*e.g.*, NGS, digital polymerase chain reaction (PCR)][136-138].

A huge amount of data needs to be properly managed to determine the information that is useful and correct[139]. The recent ESMO guidelines have outlined their indications for the use of NGS in the characterization of metastatic cancers. As regards PDAC, it is not currently recommended to perform multigene NGS in daily practice[140]. However, ESMO encourages multigene sequencing in order to get access to innovative drugs. Moreover, NGS can be an alternative technique to PCR-based assays if it is not associated with extra cost for the public health care system if the patient is informed about the putative benefits of this analysis[140]. In conclusion, a deeper knowledge of the molecular alterations characterizing pancreatic neoplasms may lead to new potential therapeutic targets for these tumors.

REFERENCES

1 **Cancer Genome Atlas Research Network**. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. *Cancer Cell* 2017; **32**: 185-203. e13 [PMID: 28810144 DOI: 10.1016/j.ccell.2017.07.007]

2 **Layfield LJ**, Ehya H, Filie AC, Hruban RH, Jhala N, Joseph L, Vielh P, Pitman MB; Papanicolaou Society of Cytopathology. Utilization of ancillary studies in the cytologic diagnosis of biliary and pancreatic lesions: the Papanicolaou Society of Cytopathology guidelines for pancreatobiliary cytology. *Diagn Cytopathol* 2014; **42**: 351-362 [PMID: 24639398 DOI: 10.1002/dc.23093]

3 **Lüttges J**, Reinecke-Lüthge A, Möllmann B, Menke MA, Clemens A, Klimpfinger M, Sipos B, Klöppel G. Duct changes and K-ras mutations in the disease-free pancreas: analysis of type, age relation and spatial distribution. *Virchows Arch* 1999; **435**: 461-468 [PMID: 10592048 DOI: 10.1007/s004280050428]

4 **Löhr M**, Klöppel G, Maisonneuve P, Lowenfels AB, Lüttges J. Frequency of K-ras mutations in pancreatic intraductal neoplasias associated with pancreatic ductal adenocarcinoma and chronic pancreatitis: a meta-analysis. *Neoplasia* 2005; **7**: 17-23 [PMID: 15720814 DOI: 10.1593/neo.04445]

5 **Löhr M**, Maisonneuve P, Lowenfels AB. K-Ras mutations and benign pancreatic disease. *Int J Pancreatol* 2000; **27**: 93-103 [PMID: 10862508 DOI: 10.1385/IJGC:27:2:093]

6 **Vogelstein B**, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004; **10**: 789-799 [PMID: 15286780 DOI: 10.1038/nm1087]

7 **Forbes SA**, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, Ding M, Bamford S, Cole C, Ward S, Kok CY, Jia M, De T, Teague JW, Stratton MR, McDermott U, Campbell PJ. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res* 2015; **43**: D805-D811 [PMID: 25355519 DOI: 10.1093/nar/gku1075]

8 **Jones S**, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008; **321**: 1801-1806 [PMID: 18772397 DOI: 10.1126/science.1164368]

9 **Scarpa A**, Capelli P, Mukai K, Zamboni G, Oda T, Iacono C, Hirohashi S. Pancreatic adenocarcinomas frequently show p53 gene mutations. *Am J Pathol* 1993; **142**: 1534-1543 [PMID: 8494051]

10 **Maitra A**, Hruban RH. Pancreatic cancer. *Annu Rev Pathol* 2008; **3**: 157-188 [PMID: 18039136 DOI: 10.1146/annurev.pathmechdis.3.121806.154305]

11 **Schutte M**, Hruban RH, Geradts J, Maynard R, Hilgers W, Rabindran SK, Moskaluk CA, Hahn SA, Schwarte-Waldhoff I, Schmiegel W, Baylin SB, Kern SE, Herman JG. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. *Cancer Res* 1997; **57**: 3126-3130 [PMID: 9242437]

12 **Siegel PM**, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003; **3**: 807-821 [PMID: 14557817 DOI: 10.1038/nrc1208]

13 **Wang L**, Tsutsumi S, Kawaguchi T, Nagasaki K, Tatsuno K, Yamamoto S, Sang F, Sonoda K, Sugawara M, Saiura A, Hirono S, Yamaue H, Miki Y, Isomura M, Totoki Y, Nagae G, Isagawa T, Ueda H, Murayama-Hosokawa S, Shibata T, Sakamoto H, Kanai Y, Kaneda A, Noda T, Aburatani H. Whole-exome sequencing of human pancreatic cancers and characterization of genomic instability caused by MLH1 haploinsufficiency and complete deficiency. *Genome Res* 2012; **22**: 208-219 [PMID: 22156295 DOI: 10.1101/gr.123109.111]

14 **Biankin AV**, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, Miller DK, Wilson PJ, Patch AM, Wu J, Chang DK, Cowley MJ, Gardiner BB, Song S, Harliwong I, Idrisoglu S, Nourse C, Nourbakhsh E, Manning S, Wani S, Gongora M, Pajic M, Scarlett CJ, Gill AJ, Pinho AV, Rooman I, Anderson M, Holmes O, Leonard C, Taylor D, Wood S, Xu Q, Nones K, Fink JL, Christ A, Bruxner T, Cloonan N, Kolle G, Newell F, Pinese M, Mead RS, Humphris JL, Kaplan W, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chou A, Chin VT, Chantrill LA, Mawson A, Samra JS, Kench JG, Lovell JA, Daly RJ, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N; Australian Pancreatic Cancer Genome Initiative, Kakkar N, Zhao F, Wu YQ, Wang M, Muzny DM, Fisher WE, Brunicardi FC, Hodges SE, Reid JG, Drummond J, Chang K, Han Y, Lewis LR, Dinh H, Buhay CJ, Beck T, Timms L, Sam M, Begley K, Brown A, Pai D, Panchal A, Buchner N, De Borja R, Denroche RE, Yung CK, Serra S, Onetto N, Mukhopadhyay D, Tsao MS, Shaw PA, Petersen GM, Gallinger S, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Schulick RD, Wolfgang CL, Morgan RA, Lawlor RT, Capelli P, Corbo V, Scardoni M, Tortora G, Tempero MA, Mann KM, Jenkins NA, Perez-Mancera PA, Adams DJ, Largaespada DA, Wessels LF, Rust AG, Stein LD, Tuveson DA, Copeland NG, Musgrove EA, Scarpa A, Eshleman JR, Hudson TJ, Sutherland RL, Wheeler DA, Pearson JV, McPherson JD, Gibbs RA, Grimmond SM. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature* 2012; **491**: 399-405 [PMID: 23103869 DOI: 10.1038/nature11547]

15 **Witkiewicz AK**, McMillan EA, Balaji U, Baek G, Lin WC, Mansour J, Mollaee M, Wagner KU, Koduru P, Yopp A, Choti MA, Yeo CJ, McCue P, White MA, Knudsen ES. Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. *Nat Commun* 2015;

6: 6744 [PMID: 25855536 DOI: 10.1038/ncomms7744]

16 **Bailey P**, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, Miller DK, Christ AN, Bruxner TJ, Quinn MC, Nourse C, Murtaugh LC, Harliwong I, Idrisoglu S, Manning S, Nourbakhsh E, Wani S, Fink L, Holmes O, Chin V, Anderson MJ, Kazakoff S, Leonard C, Newell F, Waddell N, Wood S, Xu Q, Wilson PJ, Cloonan N, Kassahn KS, Taylor D, Quek K, Robertson A, Pantano L, Mincarelli L, Sanchez LN, Evers L, Wu J, Pinese M, Cowley MJ, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chantrill LA, Mawson A, Humphris J, Chou A, Pajic M, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Lovell JA, Merrett ND, Toon CW, Epari K, Nguyen NQ, Barbour A, Zeps N, Moran-Jones K, Jamieson NB, Graham JS, Duthie F, Oien K, Hair J, Grützmann R, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Rusev B, Capelli P, Salvia R, Tortora G, Mukhopadhyay D, Petersen GM; Australian Pancreatic Cancer Genome Initiative, Munzy DM, Fisher WE, Karim SA, Eshleman JR, Hruban RH, Pilarsky C, Morton JP, Sansom OJ, Scarpa A, Musgrove EA, Bailey UM, Hofmann O, Sutherland RL, Wheeler DA, Gill AJ, Gibbs RA, Pearson JV, Biankin AV, Grimmond SM. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* 2016; **531**: 47-52 [PMID: 26909576 DOI: 10.1038/nature16965]

17 **Waddell N**, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, Johns AL, Miller D, Nones K, Quek K, Quinn MC, Robertson AJ, Fadlullah MZ, Bruxner TJ, Christ AN, Harliwong I, Idrisoglu S, Manning S, Nourse C, Nourbakhsh E, Wani S, Wilson PJ, Markham E, Cloonan N, Anderson MJ, Fink JL, Holmes O, Kazakoff SH, Leonard C, Newell F, Poudel B, Song S, Taylor D, Waddell N, Wood S, Xu Q, Wu J, Pinese M, Cowley MJ, Lee HC, Jones MD, Nagrial AM, Humphris J, Chantrill LA, Chin V, Steinmann AM, Mawson A, Humphrey ES, Colvin EK, Chou A, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Pettitt JA, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N, Jamieson NB, Graham JS, Niclou SP, Bjerkvig R, Grützmann R, Aust D, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Falconi M, Zamboni G, Tortora G, Tempero MA; Australian Pancreatic Cancer Genome Initiative, Gill AJ, Eshleman JR, Pilarsky C, Scarpa A, Musgrove EA, Pearson JV, Biankin AV, Grimmond SM. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 2015; **518**: 495-501 [PMID: 25719666 DOI: 10.1038/nature14169]

18 **Hruban RH**, van Mansfeld AD, Offerhaus GJ, van Weering DH, Allison DC, Goodman SN, Kensler TW, Bose KK, Cameron JL, Bos JL. K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. *Am J Pathol* 1993; **143**: 545-554 [PMID: 8342602]

19 **Aung KL**, Fischer SE, Denroche RE, Jang GH, Dodd A, Creighton S, Southwood B, Liang SB, Chadwick D, Zhang A, O'Kane GM, Albaba H, Moura S, Grant RC, Miller JK, Mbabaali F, Pasternack D, Lungu IM, Bartlett JMS, Ghai S, Lemire M, Holter S, Connor AA, Moffitt RA, Yeh JJ, Timms L, Krzyzanowski PM, Dhani N, Hedley D, Notta F, Wilson JM, Moore MJ, Gallinger S, Knox JJ. Genomics-Driven Precision Medicine for Advanced Pancreatic Cancer: Early Results from the COMPASS Trial. *Clin Cancer Res* 2018; **24**: 1344-1354 [PMID: 29288237 DOI: 10.1158/1078-0432.CCR-17-2994]

20 **Morris JP 4th**, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer* 2010; **10**: 683-695 [PMID: 20814421 DOI: 10.1038/nrc2899]

21 **de Biase D**, Visani M, Baccarini P, Polifemo AM, Maimone A, Fornelli A, Giuliani A, Zanini N, Fabbri C, Pession A, Tallini G. Next generation sequencing improves the accuracy of KRAS mutation analysis in endoscopic ultrasound fine needle aspiration pancreatic lesions. *PLoS One* 2014; **9**: e87651 [PMID: 24504548 DOI: 10.1371/journal.pone.0087651]

22 **Gormally E**, Caboux E, Vineis P, Hainaut P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. *Mutat Res* 2007; **635**: 105-117 [PMID: 17257890 DOI: 10.1016/j.mrrev.2006.11.002]

23 **Visani M**, de Biase D, Baccarini P, Fabbri C, Polifemo AM, Zanini N, Pession A, Tallini G. Multiple KRAS mutations in pancreatic adenocarcinoma: molecular features of neoplastic clones indicate the selection of divergent populations of tumor cells. *Int J Surg Pathol* 2013; **21**: 546-552 [PMID: 23426962 DOI: 10.1177/1066896912475073]

24 **Bournet B**, Souque A, Senesse P, Assenat E, Barthet M, Lesavre N, Aubert A, O'Toole D, Hammel P, Levy P, Ruszniewski P, Bouisson M, Escourrou J, Cordelier P, Buscail L. Endoscopic ultrasound-guided fine-needle aspiration biopsy coupled with KRAS mutation assay to distinguish pancreatic cancer from pseudotumoral chronic pancreatitis. *Endoscopy* 2009; **41**: 552-557 [PMID: 19533561 DOI: 10.1055/s-0029-1214717]

25 **Bournet B**, Selves J, Grand D, Danjoux M, Hanoun N, Cordelier P, Buscail L. Endoscopic ultrasound-guided fine-needle aspiration biopsy coupled with a KRAS mutation assay using allelic discrimination improves the diagnosis of pancreatic cancer. *J Clin Gastroenterol* 2015; **49**: 50-56 [PMID: 24798941 DOI: 10.1097/MCG.0000000000000053]

26 **Ginestà MM**, Mora J, Mayor R, Farré A, Peinado MA, Busquets J, Serrano T, Capellá G, Fabregat J. Genetic and epigenetic markers in the evaluation of pancreatic masses. *J Clin Pathol* 2013; **66**: 192-197 [PMID: 23135349 DOI: 10.1136/jclinpath-2012-201123]

27 **Maluf-Filho F**, Kumar A, Gerhardt R, Kubrusly M, Sakai P, Hondo F, Matuguma SE, Artifon E, Monteiro da Cunha JE, César Machado MC, Ishioka S, Forero E. Kras mutation analysis of fine

needle aspirate under EUS guidance facilitates risk stratification of patients with pancreatic mass. *J Clin Gastroenterol* 2007; **41**: 906-910 [PMID: 18090159 DOI: 10.1097/MCG.0b013e31805905e9]

28 **Ogura T**, Yamao K, Sawaki A, Mizuno N, Hara K, Hijioka S, Niwa Y, Tajika M, Kondo S, Shimizu Y, Bhatia V, Higuchi K, Hosoda W, Yatabe Y. Clinical impact of K-ras mutation analysis in EUS-guided FNA specimens from pancreatic masses. *Gastrointest Endosc* 2012; **75**: 769-774 [PMID: 22284089 DOI: 10.1016/j.gie.2011.11.012]

29 **Pellisé M**, Castells A, Ginès A, Solé M, Mora J, Castellví-Bel S, Rodríguez-Moranta F, Fernàndez-Esparrach G, Llach J, Bordas JM, Navarro S, Piqué JM. Clinical usefulness of KRAS mutational analysis in the diagnosis of pancreatic adenocarcinoma by means of endosonography-guided fine-needle aspiration biopsy. *Aliment Pharmacol Ther* 2003; **17**: 1299-1307 [PMID: 12755843 DOI: 10.1046/j.1365-2036.2003.01579.x]

30 **Tada M**, Komatsu Y, Kawabe T, Sasahira N, Isayama H, Toda N, Shiratori Y, Omata M. Quantitative analysis of K-ras gene mutation in pancreatic tissue obtained by endoscopic ultrasonography-guided fine needle aspiration: clinical utility for diagnosis of pancreatic tumor. *Am J Gastroenterol* 2002; **97**: 2263-2270 [PMID: 12358243 DOI: 10.1111/j.1572-0241.2002.05980.x]

31 **Takahashi K**, Yamao K, Okubo K, Sawaki A, Mizuno N, Ashida R, Koshikawa T, Ueyama Y, Kasugai K, Hase S, Kakumu S. Differential diagnosis of pancreatic cancer and focal pancreatitis by using EUS-guided FNA. *Gastrointest Endosc* 2005; **61**: 76-79 [PMID: 15672060 DOI: 10.1016/s0016-5107(04)02224-2]

32 **Bournet B**, Gayral M, Torrisani J, Selves J, Cordelier P, Buscail L. Role of endoscopic ultrasound in the molecular diagnosis of pancreatic cancer. *World J Gastroenterol* 2014; **20**: 10758-10768 [PMID: 25152579 DOI: 10.3748/wjg.v20.i31.10758]

33 **Oshima M**, Okano K, Muraki S, Haba R, Maeba T, Suzuki Y, Yachida S. Immunohistochemically detected expression of 3 major genes (CDKN2A/p16, TP53, and SMAD4/DPC4) strongly predicts survival in patients with resectable pancreatic cancer. *Ann Surg* 2013; **258**: 336-346 [PMID: 23470568 DOI: 10.1097/SLA.0b013e3182827a65]

34 **Masetti M**, Acquaviva G, Visani M, Tallini G, Fornelli A, Ragazzi M, Vasuri F, Grifoni D, Di Giacomo S, Fiorino S, Lombardi R, Tuminati D, Ravaioli M, Fabbri C, Bacchi-Reggiani ML, Pession A, Jovine E, de Biase D. Long-term survivors of pancreatic adenocarcinoma show low rates of genetic alterations in KRAS, TP53 and SMAD4. *Cancer Biomark* 2018; **21**: 323-334 [PMID: 29103024 DOI: 10.3233/CBM-170464]

35 **Gillson J**, Ramaswamy Y, Singh G, Gorfe AA, Pavlakis N, Samra J, Mittal A, Sahni S. Small Molecule KRAS Inhibitors: The Future for Targeted Pancreatic Cancer Therapy? *Cancers (Basel)* 2020; **12** [PMID: 32456277 DOI: 10.3390/cancers12051341]

36 **Luchini C**, Paolino G, Mattiolo P, Piredda ML, Cavaliere A, Gaule M, Melisi D, Salvia R, Malleo G, Shin JI, Cargnin S, Terrazzino S, Lawlor RT, Milella M, Scarpa A. KRAS wild-type pancreatic ductal adenocarcinoma: molecular pathology and therapeutic opportunities. *J Exp Clin Cancer Res* 2020; **39**: 227 [PMID: 33115526 DOI: 10.1186/s13046-020-01732-6]

37 **Itoi T**, Takei K, Sofuni A, Itokawa F, Tsuchiya T, Kurihara T, Nakamura K, Moriyasu F, Tsuchida A, Kasuya K. Immunohistochemical analysis of p53 and MIB-1 in tissue specimens obtained from endoscopic ultrasonography-guided fine needle aspiration biopsy for the diagnosis of solid pancreatic masses. *Oncol Rep* 2005; **13**: 229-234 [PMID: 15643503]

38 **Jahng AW**, Reicher S, Chung D, Varela D, Chhablani R, Dev A, Pham B, Nieto J, Venegas RJ, French SW, Stabile BE, Eysselein VE. Staining for p53 and Ki-67 increases the sensitivity of EUS-FNA to detect pancreatic malignancy. *World J Gastrointest Endosc* 2010; **2**: 362-368 [PMID: 21173913 DOI: 10.4253/wjge.v2.i11.362]

39 **Mu DQ**, Wang GF, Peng SY. p53 protein expression and CA19.9 values in differential cytological diagnosis of pancreatic cancer complicated with chronic pancreatitis and chronic pancreatitis. *World J Gastroenterol* 2003; **9**: 1815-1818 [PMID: 12918127 DOI: 10.3748/wjg.v9.i8.1815]

40 **Sinn M**, Sinn BV, Treue D, Keilholz U, Damm F, Schmuck R, Lohneis P, Klauschen F, Striefler JK, Bahra M, Bläker H, Bischoff S, Pelzer U, Oettle H, Riess H, Budczies J, Denkert C. *TP53* Mutations Predict Sensitivity to Adjuvant Gemcitabine in Patients with Pancreatic Ductal Adenocarcinoma: Next-Generation Sequencing Results from the CONKO-001 Trial. *Clin Cancer Res* 2020; **26**: 3732-3739 [PMID: 32234756 DOI: 10.1158/1078-0432.CCR-19-3034]

41 **Iacobuzio-Donahue CA**, Wilentz RE, Argani P, Yeo CJ, Cameron JL, Kern SE, Hruban RH. Dpc4 protein in mucinous cystic neoplasms of the pancreas: frequent loss of expression in invasive carcinomas suggests a role in genetic progression. *Am J Surg Pathol* 2000; **24**: 1544-1548 [PMID: 11075857 DOI: 10.1097/00000478-200011000-00011]

42 **Hahn SA**, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996; **271**: 350-353 [PMID: 8553070 DOI: 10.1126/science.271.5247.350]

43 **Tascilar M**, Skinner HG, Rosty C, Sohn T, Wilentz RE, Offerhaus GJ, Adsay V, Abrams RA, Cameron JL, Kern SE, Yeo CJ, Hruban RH, Goggins M. The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2001; **7**: 4115-4121 [PMID: 11751510]

44 **Schutte M**, Hruban RH, Hedrick L, Cho KR, Nadasdy GM, Weinstein CL, Bova GS, Isaacs WB, Cairns P, Nawroz H, Sidransky D, Casero RA Jr, Meltzer PS, Hahn SA, Kern SE. DPC4 gene in various tumor types. *Cancer Res* 1996; **56**: 2527-2530 [PMID: 8653691]

45 **Blackford A**, Serrano OK, Wolfgang CL, Parmigiani G, Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Eshleman JR, Goggins M, Jaffee EM, Iacobuzio-Donahue CA, Maitra A, Cameron JL,

Olino K, Schulick R, Winter J, Herman JM, Laheru D, Klein AP, Vogelstein B, Kinzler KW, Velculescu VE, Hruban RH. SMAD4 gene mutations are associated with poor prognosis in pancreatic cancer. *Clin Cancer Res* 2009; **15**: 4674-4679 [PMID: 19584151 DOI: 10.1158/1078-0432.CCR-09-0227]

46 **Singh P**, Srinivasan R, Wig JD. SMAD4 genetic alterations predict a worse prognosis in patients with pancreatic ductal adenocarcinoma. *Pancreas* 2012; **41**: 541-546 [PMID: 22504380 DOI: 10.1097/MPA.0b013e318247d6af]

47 **Shi L**, Sheng J, Wang M, Luo H, Zhu J, Zhang B, Liu Z, Yang X. Combination Therapy of TGF-β Blockade and Commensal-derived Probiotics Provides Enhanced Antitumor Immune Response and Tumor Suppression. *Theranostics* 2019; **9**: 4115-4129 [PMID: 31281535 DOI: 10.7150/thno.35131]

48 **Hsieh YY**, Liu TP, Chou CJ, Chen HY, Lee KH, Yang PM. Integration of Bioinformatics Resources Reveals the Therapeutic Benefits of Gemcitabine and Cell Cycle Intervention in SMAD4-Deleted Pancreatic Ductal Adenocarcinoma. *Genes (Basel)* 2019; **10** [PMID: 31569425 DOI: 10.3390/genes10100766]

49 **Dardare J**, Witz A, Merlin JL, Gilson P, Harlé A. SMAD4 and the TGFβ Pathway in Patients with Pancreatic Ductal Adenocarcinoma. *Int J Mol Sci* 2020; **21** [PMID: 32429474 DOI: 10.3390/ijms21103534]

50 **Bartsch D**, Shevlin DW, Tung WS, Kisker O, Wells SA Jr, Goodfellow PJ. Frequent mutations of CDKN2 in primary pancreatic adenocarcinomas. *Genes Chromosomes Cancer* 1995; **14**: 189-195 [PMID: 8589035 DOI: 10.1002/gcc.2870140306]

51 **Caldas C**, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, Weinstein CL, Hruban RH, Yeo CJ, Kern SE. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet* 1994; **8**: 27-32 [PMID: 7726912 DOI: 10.1038/ng0994-27]

52 **Rozenblum E**, Schutte M, Goggins M, Hahn SA, Panzer S, Zahurak M, Goodman SN, Sohn TA, Hruban RH, Yeo CJ, Kern SE. Tumor-suppressive pathways in pancreatic carcinoma. *Cancer Res* 1997; **57**: 1731-1734 [PMID: 9135016]

53 **Bartsch DK**, Sina-Frey M, Lang S, Wild A, Gerdes B, Barth P, Kress R, Grützmann R, Colombo-Benkmann M, Ziegler A, Hahn SA, Rothmund M, Rieder H. CDKN2A germline mutations in familial pancreatic cancer. *Ann Surg* 2002; **236**: 730-737 [PMID: 12454511 DOI: 10.1097/00000658-200212000-00005]

54 **McWilliams RR**, Wieben ED, Rabe KG, Pedersen KS, Wu Y, Sicotte H, Petersen GM. Prevalence of CDKN2A mutations in pancreatic cancer patients: implications for genetic counseling. *Eur J Hum Genet* 2011; **19**: 472-478 [PMID: 21150883 DOI: 10.1038/ejhg.2010.198]

55 **Goggins M**, Overbeek KA, Brand R, Syngal S, Del Chiaro M, Bartsch DK, Bassi C, Carrato A, Farrell J, Fishman EK, Fockens P, Gress TM, van Hooft JE, Hruban RH, Kastrinos F, Klein A, Lennon AM, Lucas A, Park W, Rustgi A, Simeone D, Stoffel E, Vasen HFA, Cahen DL, Canto MI, Bruno M; International Cancer of the Pancreas Screening (CAPS) consortium. Management of patients with increased risk for familial pancreatic cancer: updated recommendations from the International Cancer of the Pancreas Screening (CAPS) Consortium. *Gut* 2020; **69**: 7-17 [PMID: 31672839 DOI: 10.1136/gutjnl-2019-319352]

56 **Ohtsubo K**, Watanabe H, Yamaguchi Y, Hu YX, Motoo Y, Okai T, Sawabu N. Abnormalities of tumor suppressor gene p16 in pancreatic carcinoma: immunohistochemical and genetic findings compared with clinicopathological parameters. *J Gastroenterol* 2003; **38**: 663-671 [PMID: 12898359 DOI: 10.1007/s00535-003-1119-6]

57 **Gerdes B**, Ramaswamy A, Ziegler A, Lang SA, Kersting M, Baumann R, Wild A, Moll R, Rothmund M, Bartsch DK. p16INK4a is a prognostic marker in resected ductal pancreatic cancer: an analysis of p16INK4a, p53, MDM2, an Rb. *Ann Surg* 2002; **235**: 51-59 [PMID: 11753042 DOI: 10.1097/00000658-200201000-00007]

58 **Qian Y**, Gong Y, Fan Z, Luo G, Huang Q, Deng S, Cheng H, Jin K, Ni Q, Yu X, Liu C. Molecular alterations and targeted therapy in pancreatic ductal adenocarcinoma. *J Hematol Oncol* 2020; **13**: 130 [PMID: 33008426 DOI: 10.1186/s13045-020-00958-3]

59 **Wong W**, Raufi AG, Safyan RA, Bates SE, Manji GA. BRCA Mutations in Pancreas Cancer: Spectrum, Current Management, Challenges and Future Prospects. *Cancer Manag Res* 2020; **12**: 2731-2742 [PMID: 32368150 DOI: 10.2147/CMAR.S211151]

60 **Golan T**, Hammel P, Reni M, Van Cutsem E, Macarulla T, Hall MJ, Park JO, Hochhauser D, Arnold D, Oh DY, Reinacher-Schick A, Tortora G, Algül H, O'Reilly EM, McGuinness D, Cui KY, Schlienger K, Locker GY, Kindler HL. Maintenance Olaparib for Germline *BRCA*-Mutated Metastatic Pancreatic Cancer. *N Engl J Med* 2019; **381**: 317-327 [PMID: 31157963 DOI: 10.1056/NEJMoa1903387]

61 **Sohal DPS**, Kennedy EB, Cinar P, Conroy T, Copur MS, Crane CH, Garrido-Laguna I, Lau MW, Johnson T, Krishnamurthi S, Moravek C, O'Reilly EM, Philip PA, Pant S, Shah MA, Sahai V, Uronis HE, Zaidi N, Laheru D. Metastatic Pancreatic Cancer: ASCO Guideline Update. *J Clin Oncol* 2020; JCO2001364 [PMID: 32755482 DOI: 10.1200/JCO.20.01364]

62 **Luchini C**, Brosens LAA, Wood LD, Chatterjee D, Shin JI, Sciammarella C, Fiadone G, Malleo G, Salvia R, Kryklyva V, Piredda ML, Cheng L, Lawlor RT, Adsay V, Scarpa A. Comprehensive characterisation of pancreatic ductal adenocarcinoma with microsatellite instability: histology, molecular pathology and clinical implications. *Gut* 2021; **70**: 148-156 [PMID: 32350089 DOI: 10.1136/gutjnl-2020-320726]

63 **Hu ZI**, Shia J, Stadler ZK, Varghese AM, Capanu M, Salo-Mullen E, Lowery MA, Diaz LA Jr, Mandelker D, Yu KH, Zervoudakis A, Kelsen DP, Iacobuzio-Donahue CA, Klimstra DS, Saltz LB, Sahin IH, O'Reilly EM. Evaluating Mismatch Repair Deficiency in Pancreatic Adenocarcinoma: Challenges and Recommendations. *Clin Cancer Res* 2018; **24**: 1326-1336 [PMID: 29367431 DOI: 10.1158/1078-0432.CCR-17-3099]

64 **Bazzichetto C**, Luchini C, Conciatori F, Vaccaro V, Di Cello I, Mattiolo P, Falcone I, Ferretti G, Scarpa A, Cognetti F, Milella M. Morphologic and Molecular Landscape of Pancreatic Cancer Variants as the Basis of New Therapeutic Strategies for Precision Oncology. *Int J Mol Sci* 2020; **21** [PMID: 33266496 DOI: 10.3390/ijms21228841]

65 **National Comprehensive Cancer Network®**. NCCN guidelines for patients: Pancreatic Cancer. National Comprehensive Cancer Network®, 2019

66 **Pishvaian MJ**, Blais EM, Brody JR, Lyons E, DeArbeloa P, Hendifar A, Mikhail S, Chung V, Sahai V, Sohal DPS, Bellakbira S, Thach D, Rahib L, Madhavan S, Matrisian LM, Petricoin EF 3rd. Overall survival in patients with pancreatic cancer receiving matched therapies following molecular profiling: a retrospective analysis of the Know Your Tumor registry trial. *Lancet Oncol* 2020; **21**: 508-518 [PMID: 32135080 DOI: 10.1016/S1470-2045(20)30074-7]

67 **Peng DF**, Kanai Y, Sawada M, Ushijima S, Hiraoka N, Kitazawa S, Hirohashi S. DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. *Carcinogenesis* 2006; **27**: 1160-1168 [PMID: 16537562 DOI: 10.1093/carcin/bgi361]

68 **Moore MJ**, Feld R, Hedley D, Oza A, Siu LL. A phase II study of temozolomide in advanced untreated pancreatic cancer. *Invest New Drugs* 1998; **16**: 77-79 [PMID: 9740547 DOI: 10.1023/a:1006043332368]

69 **Valero V 3rd**, Saunders TJ, He J, Weiss MJ, Cameron JL, Dholakia A, Wild AT, Shin EJ, Khashab MA, O'Broin-Lennon AM, Ali SZ, Laheru D, Hruban RH, Iacobuzio-Donahue CA, Herman JM, Wolfgang CL. Reliable Detection of Somatic Mutations in Fine Needle Aspirates of Pancreatic Cancer With Next-generation Sequencing: Implications for Surgical Management. *Ann Surg* 2016; **263**: 153-161 [PMID: 26020105 DOI: 10.1097/SLA.0000000000001156]

70 **Calhoun ES**, Jones JB, Ashfaq R, Adsay V, Baker SJ, Valentine V, Hempen PM, Hilgers W, Yeo CJ, Hruban RH, Kern SE. BRAF and FBXW7 (CDC4, FBW7, AGO, SEL10) mutations in distinct subsets of pancreatic cancer: potential therapeutic targets. *Am J Pathol* 2003; **163**: 1255-1260 [PMID: 14507635 DOI: 10.1016/S0002-9440(10)63485-2]

71 **Zeng G**, Germinaro M, Micsenyi A, Monga NK, Bell A, Sood A, Malhotra V, Sood N, Midda V, Monga DK, Kokkinakis DM, Monga SP. Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma. *Neoplasia* 2006; **8**: 279-289 [PMID: 16756720 DOI: 10.1593/neo.05607]

72 **Doctor A**, Seifert V, Ullrich M, Hauser S, Pietzsch J. Three-Dimensional Cell Culture Systems in Radiopharmaceutical Cancer Research. *Cancers (Basel)* 2020; **12** [PMID: 32993034 DOI: 10.3390/cancers12102765]

73 **Lee JH**, Kim SK, Khawar IA, Jeong SY, Chung S, Kuh HJ. Microfluidic co-culture of pancreatic tumor spheroids with stellate cells as a novel 3D model for investigation of stroma-mediated cell motility and drug resistance. *J Exp Clin Cancer Res* 2018; **37**: 4 [PMID: 29329547 DOI: 10.1186/s13046-017-0654-6]

74 **Finnberg NK**, Gokare P, Lev A, Grivennikov SI, MacFarlane AW 4th, Campbell KS, Winters RM, Kaputa K, Farma JM, Abbas AE, Grasso L, Nicolaides NC, El-Deiry WS. Application of 3D tumoroid systems to define immune and cytotoxic therapeutic responses based on tumoroid and tissue slice culture molecular signatures. *Oncotarget* 2017; **8**: 66747-66757 [PMID: 28977993 DOI: 10.18632/oncotarget.19965]

75 **Ehlen L**, Arndt J, Treue D, Bischoff P, Loch FN, Hahn EM, Kotsch K, Klauschen F, Beyer K, Margonis GA, Kreis ME, Kamphues C. Novel methods for *in vitro* modeling of pancreatic cancer reveal important aspects for successful primary cell culture. *BMC Cancer* 2020; **20**: 417 [PMID: 32404074 DOI: 10.1186/s12885-020-06929-8]

76 **Boj SF**, Hwang CI, Baker LA, Chio II, Engle DD, Corbo V, Jager M, Ponz-Sarvise M, Tiriac H, Spector MS, Gracanin A, Oni T, Yu KH, van Boxtel R, Huch M, Rivera KD, Wilson JP, Feigin ME, Öhlund D, Handly-Santana A, Ardito-Abraham CM, Ludwig M, Elyada E, Alagesan B, Biffi G, Yordanov GN, Delcuze B, Creighton B, Wright K, Park Y, Morsink FH, Molenaar IQ, Borel Rinkes IH, Cuppen E, Hao Y, Jin Y, Nijman IJ, Iacobuzio-Donahue C, Leach SD, Pappin DJ, Hammell M, Klimstra DS, Basturk O, Hruban RH, Offerhaus GJ, Vries RG, Clevers H, Tuveson DA. Organoid models of human and mouse ductal pancreatic cancer. *Cell* 2015; **160**: 324-338 [PMID: 25557080 DOI: 10.1016/j.cell.2014.12.021]

77 **Baker LA**, Tiriac H, Tuveson DA. Generation and Culture of Human Pancreatic Ductal Adenocarcinoma Organoids from Resected Tumor Specimens. *Methods Mol Biol* 2019; **1882**: 97-115 [PMID: 30378047 DOI: 10.1007/978-1-4939-8879-2_9]

78 **Drost J**, Clevers H. Organoids in cancer research. *Nat Rev Cancer* 2018; **18**: 407-418 [PMID: 29692415 DOI: 10.1038/s41568-018-0007-6]

79 **Kanda M**, Matthaei H, Wu J, Hong SM, Yu J, Borges M, Hruban RH, Maitra A, Kinzler K, Vogelstein B, Goggins M. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology* 2012; **142**: 730-733. e9 [PMID: 22226782 DOI: 10.1053/j.gastro.2011.12.042]

80 **Guerra C**, Schuhmacher AJ, Cañamero M, Grippo PJ, Verdaguer L, Pérez-Gallego L, Dubus P,

Sandgren EP, Barbacid M. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell* 2007; **11**: 291-302 [PMID: 17349585 DOI: 10.1016/j.ccr.2007.01.012]

81 **Hingorani SR**, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003; **4**: 437-450 [PMID: 14706336 DOI: 10.1016/s1535-6108(03)00309-x]

82 **Pylayeva-Gupta Y**, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* 2011; **11**: 761-774 [PMID: 21993244 DOI: 10.1038/nrc3106]

83 **Seidler B**, Schmidt A, Mayr U, Nakhai H, Schmid RM, Schneider G, Saur D. A Cre-loxP-based mouse model for conditional somatic gene expression and knockdown *in vivo* by using avian retroviral vectors. *Proc Natl Acad Sci USA* 2008; **105**: 10137-10142 [PMID: 18621715 DOI: 10.1073/pnas.0800487105]

84 **Nikiforova MN**, Khalid A, Fasanella KE, McGrath KM, Brand RE, Chennat JS, Slivka A, Zeh HJ, Zureikat AH, Krasinskas AM, Ohori NP, Schoedel KE, Navina S, Mantha GS, Pai RK, Singhi AD. Integration of KRAS testing in the diagnosis of pancreatic cystic lesions: a clinical experience of 618 pancreatic cysts. *Mod Pathol* 2013; **26**: 1478-1487 [PMID: 23743931 DOI: 10.1038/modpathol.2013.91]

85 **Molin MD**, Matthaei H, Wu J, Blackford A, Debeljak M, Rezaee N, Wolfgang CL, Butturini G, Salvia R, Bassi C, Goggins MG, Kinzler KW, Vogelstein B, Eshleman JR, Hruban RH, Maitra A. Clinicopathological correlates of activating GNAS mutations in intraductal papillary mucinous neoplasm (IPMN) of the pancreas. *Ann Surg Oncol* 2013; **20**: 3802-3808 [PMID: 23846778 DOI: 10.1245/s10434-013-3096-1]

86 **Khalid A**, Zahid M, Finkelstein SD, LeBlanc JK, Kaushik N, Ahmad N, Brugge WR, Edmundowicz SA, Hawes RH, McGrath KM. Pancreatic cyst fluid DNA analysis in evaluating pancreatic cysts: a report of the PANDA study. *Gastrointest Endosc* 2009; **69**: 1095-1102 [PMID: 19152896 DOI: 10.1016/j.gie.2008.07.033]

87 **Al-Haddad M**, DeWitt J, Sherman S, Schmidt CM, LeBlanc JK, McHenry L, Coté G, El Chafic AH, Luz L, Stuart JS, Johnson CS, Klochan C, Imperiale TF. Performance characteristics of molecular (DNA) analysis for the diagnosis of mucinous pancreatic cysts. *Gastrointest Endosc* 2014; **79**: 79-87 [PMID: 23845445 DOI: 10.1016/j.gie.2013.05.026]

88 **Kuboki Y**, Shimizu K, Hatori T, Yamamoto M, Shibata N, Shiratori K, Furukawa T. Molecular biomarkers for progression of intraductal papillary mucinous neoplasm of the pancreas. *Pancreas* 2015; **44**: 227-235 [PMID: 25423558 DOI: 10.1097/MPA.0000000000000253]

89 **Komatsu H**, Tanji E, Sakata N, Aoki T, Motoi F, Naitoh T, Katayose Y, Egawa S, Unno M, Furukawa T. A GNAS mutation found in pancreatic intraductal papillary mucinous neoplasms induces drastic alterations of gene expression profiles with upregulation of mucin genes. *PLoS One* 2014; **9**: e87875 [PMID: 24498386 DOI: 10.1371/journal.pone.0087875]

90 **Amato E**, Molin MD, Mafficini A, Yu J, Malleo G, Rusev B, Fassan M, Antonello D, Sadakari Y, Castelli P, Zamboni G, Maitra A, Salvia R, Hruban RH, Bassi C, Capelli P, Lawlor RT, Goggins M, Scarpa A. Targeted next-generation sequencing of cancer genes dissects the molecular profiles of intraductal papillary neoplasms of the pancreas. *J Pathol* 2014; **233**: 217-227 [PMID: 24604757 DOI: 10.1002/path.4344]

91 **Rosenbaum MW**, Jones M, Dudley JC, Le LP, Iafrate AJ, Pitman MB. Next-generation sequencing adds value to the preoperative diagnosis of pancreatic cysts. *Cancer Cytopathol* 2017; **125**: 41-47 [PMID: 27647802 DOI: 10.1002/cncy.21775]

92 **Wu J**, Jiao Y, Dal Molin M, Maitra A, de Wilde RF, Wood LD, Eshleman JR, Goggins MG, Wolfgang CL, Canto MI, Schulick RD, Edil BH, Choti MA, Adsay V, Klimstra DS, Offerhaus GJ, Klein AP, Kopelovich L, Carter H, Karchin R, Allen PJ, Schmidt CM, Naito Y, Diaz LA Jr, Kinzler KW, Papadopoulos N, Hruban RH, Vogelstein B. Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. *Proc Natl Acad Sci USA* 2011; **108**: 21188-21193 [PMID: 22158988 DOI: 10.1073/pnas.1118046108]

93 **Furukawa T**, Kuboki Y, Tanji E, Yoshida S, Hatori T, Yamamoto M, Shibata N, Shimizu K, Kamatani N, Shiratori K. Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. *Sci Rep* 2011; **1**: 161 [PMID: 22355676 DOI: 10.1038/srep00161]

94 **Singhi AD**, Nikiforova MN, Fasanella KE, McGrath KM, Pai RK, Ohori NP, Bartholow TL, Brand RE, Chennat JS, Lu X, Papachristou GI, Slivka A, Zeh HJ, Zureikat AH, Lee KK, Tsung A, Mantha GS, Khalid A. Preoperative GNAS and KRAS testing in the diagnosis of pancreatic mucinous cysts. *Clin Cancer Res* 2014; **20**: 4381-4389 [PMID: 24938521 DOI: 10.1158/1078-0432.CCR-14-0513]

95 **Wu J**, Matthaei H, Maitra A, Dal Molin M, Wood LD, Eshleman JR, Goggins M, Canto MI, Schulick RD, Edil BH, Wolfgang CL, Klein AP, Diaz LA Jr, Allen PJ, Schmidt CM, Kinzler KW, Papadopoulos N, Hruban RH, Vogelstein B. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. *Sci Transl Med* 2011; **3**: 92ra66 [PMID: 21775669 DOI: 10.1126/scitranslmed.3002543]

96 **Lee JH**, Kim Y, Choi JW, Kim YS. KRAS, GNAS, and RNF43 mutations in intraductal papillary mucinous neoplasm of the pancreas: a meta-analysis. *Springerplus* 2016; **5**: 1172 [PMID: 27512631 DOI: 10.1186/s40064-016-2847-4]

97 **Abe T**, Fukushima N, Brune K, Boehm C, Sato N, Matsubayashi H, Canto M, Petersen GM, Hruban RH, Goggins M. Genome-wide allelotypes of familial pancreatic adenocarcinomas and familial and sporadic intraductal papillary mucinous neoplasms. *Clin Cancer Res* 2007; **13**: 6019-6025 [PMID: 17947463 DOI: 10.1158/1078-0432.CCR-07-0471]

98 **Su GH**, Hruban RH, Bansal RK, Bova GS, Tang DJ, Shekher MC, Westerman AM, Entius MM, Goggins M, Yeo CJ, Kern SE. Germline and somatic mutations of the STK11/LKB1 Peutz-Jeghers gene in pancreatic and biliary cancers. *Am J Pathol* 1999; **154**: 1835-1840 [PMID: 10362809 DOI: 10.1016/S0002-9440(10)65440-5]

99 **Sato N**, Rosty C, Jansen M, Fukushima N, Ueki T, Yeo CJ, Cameron JL, Iacobuzio-Donahue CA, Hruban RH, Goggins M. STK11/LKB1 Peutz-Jeghers gene inactivation in intraductal papillary-mucinous neoplasms of the pancreas. *Am J Pathol* 2001; **159**: 2017-2022 [PMID: 11733352 DOI: 10.1016/S0002-9440(10)63053-2]

100 **Singhi AD**, McGrath K, Brand RE, Khalid A, Zeh HJ, Chennat JS, Fasanella KE, Papachristou GI, Slivka A, Bartlett DL, Dasyam AK, Hogg M, Lee KK, Marsh JW, Monaco SE, Ohori NP, Pingpank JF, Tsung A, Zureikat AH, Wald AI, Nikiforova MN. Preoperative next-generation sequencing of pancreatic cyst fluid is highly accurate in cyst classification and detection of advanced neoplasia. *Gut* 2018; **67**: 2131-2141 [PMID: 28970292 DOI: 10.1136/gutjnl-2016-313586]

101 **Yoshizawa K**, Nagai H, Sakurai S, Hironaka M, Morinaga S, Saitoh K, Fukayama M. Clonality and K-ras mutation analyses of epithelia in intraductal papillary mucinous tumor and mucinous cystic tumor of the pancreas. *Virchows Arch* 2002; **441**: 437-443 [PMID: 12447672 DOI: 10.1007/s00428-002-0645-6]

102 **Kim SG**, Wu TT, Lee JH, Yun YK, Issa JP, Hamilton SR, Rashid A. Comparison of epigenetic and genetic alterations in mucinous cystic neoplasm and serous microcystic adenoma of pancreas. *Mod Pathol* 2003; **16**: 1086-1094 [PMID: 14614047 DOI: 10.1097/01.MP.0000094088.37888.A6]

103 **Jimenez RE**, Warshaw AL, Z'graggen K, Hartwig W, Taylor DZ, Compton CC, Fernández-del Castillo C. Sequential accumulation of K-ras mutations and p53 overexpression in the progression of pancreatic mucinous cystic neoplasms to malignancy. *Ann Surg* 1999; **230**: 501-9; discussion 509 [PMID: 10522720 DOI: 10.1097/00000658-199910000-00006]

104 **Garcia-Carracedo D**, Chen ZM, Qiu W, Huang AS, Tang SM, Hruban RH, Su GH. PIK3CA mutations in mucinous cystic neoplasms of the pancreas. *Pancreas* 2014; **43**: 245-249 [PMID: 24518503 DOI: 10.1097/MPA.0000000000000034]

105 **Izeradjene K**, Combs C, Best M, Gopinathan A, Wagner A, Grady WM, Deng CX, Hruban RH, Adsay NV, Tuveson DA, Hingorani SR. Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas. *Cancer Cell* 2007; **11**: 229-243 [PMID: 17349581 DOI: 10.1016/j.ccr.2007.01.017]

106 **Bardeesy N**, Cheng KH, Berger JH, Chu GC, Pahler J, Olson P, Hezel AF, Horner J, Lauwers GY, Hanahan D, DePinho RA. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. *Genes Dev* 2006; **20**: 3130-3146 [PMID: 17114584 DOI: 10.1101/gad.1478706]

107 **Springer S**, Wang Y, Dal Molin M, Masica DL, Jiao Y, Kinde I, Blackford A, Raman SP, Wolfgang CL, Tomita T, Niknafs N, Douville C, Ptak J, Dobbyn L, Allen PJ, Klimstra DS, Schattner MA, Schmidt CM, Yip-Schneider M, Cummings OW, Brand RE, Zeh HJ, Singhi AD, Scarpa A, Salvia R, Malleo G, Zamboni G, Falconi M, Jang JY, Kim SW, Kwon W, Hong SM, Song KB, Kim SC, Swan N, Murphy J, Geoghegan J, Brugge W, Fernandez-Del Castillo C, Mino-Kenudson M, Schulick R, Edil BH, Adsay V, Paulino J, van Hooft J, Yachida S, Nara S, Hiraoka N, Yamao K, Hijioka S, van der Merwe S, Goggins M, Canto MI, Ahuja N, Hirose K, Makary M, Weiss MJ, Cameron J, Pittman M, Eshleman JR, Diaz LA Jr, Papadopoulos N, Kinzler KW, Karchin R, Hruban RH, Vogelstein B, Lennon AM. A combination of molecular markers and clinical features improve the classification of pancreatic cysts. *Gastroenterology* 2015; **149**: 1501-1510 [PMID: 26253305 DOI: 10.1053/j.gastro.2015.07.041]

108 **Wood LD**, Hruban RH. Pathology and molecular genetics of pancreatic neoplasms. *Cancer J* 2012; **18**: 492-501 [PMID: 23187835 DOI: 10.1097/PPO.0b013e31827459b6]

109 **Charville GW**, Kao CS. Serous Neoplasms of the Pancreas: A Comprehensive Review. *Arch Pathol Lab Med* 2018; **142**: 1134-1140 [PMID: 30141993 DOI: 10.5858/arpa.2017-0195-RS]

110 **Nelson WJ**, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004; **303**: 1483-1487 [PMID: 15001769 DOI: 10.1126/science.1094291]

111 **Rubinfeld B**, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P. Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 1997; **275**: 1790-1792 [PMID: 9065403 DOI: 10.1126/science.275.5307.1790]

112 **Tanaka Y**, Kato K, Notohara K, Hojo H, Ijiri R, Miyake T, Nagahara N, Sasaki F, Kitagawa N, Nakatani Y, Kobayashi Y. Frequent beta-catenin mutation and cytoplasmic/nuclear accumulation in pancreatic solid-pseudopapillary neoplasm. *Cancer Res* 2001; **61**: 8401-8404 [PMID: 11731417]

113 **Abraham SC**, Klimstra DS, Wilentz RE, Yeo CJ, Conlon K, Brennan M, Cameron JL, Wu TT, Hruban RH. Solid-pseudopapillary tumors of the pancreas are genetically distinct from pancreatic ductal adenocarcinomas and almost always harbor beta-catenin mutations. *Am J Pathol* 2002; **160**: 1361-1369 [PMID: 11943721 DOI: 10.1016/s0002-9440(10)62563-1]

114 **Kubota Y**, Kawakami H, Natsuizaka M, Kawakubo K, Marukawa K, Kudo T, Abe Y, Kubo K, Kuwatani M, Hatanaka Y, Mitsuhashi T, Matsuno Y, Sakamoto N. CTNNB1 mutational analysis of solid-pseudopapillary neoplasms of the pancreas using endoscopic ultrasound-guided fine-needle

aspiration and next-generation deep sequencing. *J Gastroenterol* 2015; **50**: 203-210 [PMID: 24700283 DOI: 10.1007/s00535-014-0954-y]

115 **Kempski HM**, Austin N, Chatters SJ, Toomey SM, Chalker J, Anderson J, Sebire NJ. Previously unidentified complex cytogenetic changes found in a pediatric case of solid-pseudopapillary neoplasm of the pancreas. *Cancer Genet Cytogenet* 2006; **164**: 54-60 [PMID: 16364763 DOI: 10.1016/j.cancergencyto.2005.06.017]

116 **Selenica P**, Raj N, Kumar R, Brown DN, Arqués O, Reidy D, Klimstra D, Snuderl M, Serrano J, Palmer HG, Weigelt B, Reis-Filho JS, Scaltriti M. Solid pseudopapillary neoplasms of the pancreas are dependent on the Wnt pathway. *Mol Oncol* 2019; **13**: 1684-1692 [PMID: 30972907 DOI: 10.1002/1878-0261.12490]

117 **Vijayvergia N**, Boland PM, Handorf E, Gustafson KS, Gong Y, Cooper HS, Sheriff F, Astsaturov I, Cohen SJ, Engstrom PF. Molecular profiling of neuroendocrine malignancies to identify prognostic and therapeutic markers: a Fox Chase Cancer Center Pilot Study. *Br J Cancer* 2016; **115**: 564-570 [PMID: 27482646 DOI: 10.1038/bjc.2016.229]

118 **Jiao Y**, Shi C, Edil BH, de Wilde RF, Klimstra DS, Maitra A, Schulick RD, Tang LH, Wolfgang CL, Choti MA, Velculescu VE, Diaz LA Jr, Vogelstein B, Kinzler KW, Hruban RH, Papadopoulos N. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science* 2011; **331**: 1199-1203 [PMID: 21252315 DOI: 10.1126/science.1200609]

119 **Corbo V**, Dalai I, Scardoni M, Barbi S, Beghelli S, Bersani S, Albarello L, Doglioni C, Schott C, Capelli P, Chilosi M, Boninsegna L, Becker KF, Falconi M, Scarpa A. MEN1 in pancreatic endocrine tumors: analysis of gene and protein status in 169 sporadic neoplasms reveals alterations in the vast majority of cases. *Endocr Relat Cancer* 2010; **17**: 771-783 [PMID: 20566584 DOI: 10.1677/ERC-10-0028]

120 **Missiaglia E**, Dalai I, Barbi S, Beghelli S, Falconi M, della Peruta M, Piemonti L, Capurso G, Di Florio A, delle Fave G, Pederzoli P, Croce CM, Scarpa A. Pancreatic endocrine tumors: expression profiling evidences a role for AKT-mTOR pathway. *J Clin Oncol* 2010; **28**: 245-255 [PMID: 19917848 DOI: 10.1200/JCO.2008.21.5988]

121 **Schmitt AM**, Schmid S, Rudolph T, Anlauf M, Prinz C, Klöppel G, Moch H, Heitz PU, Komminoth P, Perren A. VHL inactivation is an important pathway for the development of malignant sporadic pancreatic endocrine tumors. *Endocr Relat Cancer* 2009; **16**: 1219-1227 [PMID: 19690016 DOI: 10.1677/ERC-08-0297]

122 **Scarpa A**, Chang DK, Nones K, Corbo V, Patch AM, Bailey P, Lawlor RT, Johns AL, Miller DK, Mafficini A, Rusev B, Scardoni M, Antonello D, Barbi S, Sikora KO, Cingarlini S, Vicentini C, McKay S, Quinn MC, Bruxner TJ, Christ AN, Harliwong I, Idrisoglu S, McLean S, Nourse C, Nourbakhsh E, Wilson PJ, Anderson MJ, Fink JL, Newell F, Waddell N, Holmes O, Kazakoff SH, Leonard C, Wood S, Xu Q, Nagaraj SH, Amato E, Dalai I, Bersani S, Cataldo I, Dei Tos AP, Capelli P, Davì MV, Landoni L, Malpaga A, Miotto M, Whitehall VL, Leggett BA, Harris JL, Harris J, Jones MD, Humphris J, Chantrill LA, Chin V, Nagrial AM, Pajic M, Scarlett CJ, Pinho A, Rooman I, Toon C, Wu J, Pinese M, Cowley M, Barbour A, Mawson A, Humphrey ES, Colvin EK, Chou A, Lovell JA, Jamieson NB, Duthie F, Gingras MC, Fisher WE, Dagg RA, Lau LM, Lee M, Pickett HA, Reddel RR, Samra JS, Kench JG, Merrett ND, Epari K, Nguyen NQ, Zeps N, Falconi M, Simbolo M, Butturini G, Van Buren G, Partelli S, Fassan M; Australian Pancreatic Cancer Genome Initiative, Khanna KK, Gill AJ, Wheeler DA, Gibbs RA, Musgrove EA, Bassi C, Tortora G, Pederzoli P, Pearson JV, Biankin AV, Grimmond SM. Whole-genome landscape of pancreatic neuroendocrine tumours. *Nature* 2017; **543**: 65-71 [PMID: 28199314 DOI: 10.1038/nature21063]

123 **Rigaud G**, Moore PS, Zamboni G, Orlandini S, Taruscio D, Paradisi S, Lemoine NR, Klöppel G, Scarpa A. Allelotype of pancreatic acinar cell carcinoma. *Int J Cancer* 2000; **88**: 772-777 [PMID: 11072247 DOI: 10.1002/1097-0215(20001201)88:5<772::aid-ijc14>3.0.co;2-w]

124 **Jäkel C**, Bergmann F, Toth R, Assenov Y, van der Duin D, Strobel O, Hank T, Klöppel G, Dorrell C, Grompe M, Moss J, Dor Y, Schirmacher P, Plass C, Popanda O, Schmezer P. Genome-wide genetic and epigenetic analyses of pancreatic acinar cell carcinomas reveal aberrations in genome stability. *Nat Commun* 2017; **8**: 1323 [PMID: 29109526 DOI: 10.1038/s41467-017-01118-x]

125 **La Rosa S**, Bernasconi B, Frattini M, Tibiletti MG, Molinari F, Furlan D, Sahnane N, Vanoli A, Albarello L, Zhang L, Notohara K, Casnedi S, Chenard MP, Adsay V, Asioli S, Capella C, Sessa F. TP53 alterations in pancreatic acinar cell carcinoma: new insights into the molecular pathology of this rare cancer. *Virchows Arch* 2016; **468**: 289-296 [PMID: 26586531 DOI: 10.1007/s00428-015-1882-9]

126 **Jiao Y**, Yonescu R, Offerhaus GJ, Klimstra DS, Maitra A, Eshleman JR, Herman JG, Poh W, Pelosof L, Wolfgang CL, Vogelstein B, Kinzler KW, Hruban RH, Papadopoulos N, Wood LD. Whole-exome sequencing of pancreatic neoplasms with acinar differentiation. *J Pathol* 2014; **232**: 428-435 [PMID: 24293293 DOI: 10.1002/path.4310]

127 **Thompson ED**, Wood LD. Pancreatic Neoplasms With Acinar Differentiation: A Review of Pathologic and Molecular Features. *Arch Pathol Lab Med* 2020; **144**: 808-815 [PMID: 31869246 DOI: 10.5858/arpa.2019-0472-RA]

128 **Liu W**, Shia J, Gönen M, Lowery MA, O'Reilly EM, Klimstra DS. DNA mismatch repair abnormalities in acinar cell carcinoma of the pancreas: frequency and clinical significance. *Pancreas* 2014; **43**: 1264-1270 [PMID: 25058881 DOI: 10.1097/MPA.0000000000000190]

129 **Ducreux M**, Cuhna AS, Caramella C, Hollebecque A, Burtin P, Goéré D, Seufferlein T,

Haustermans K, Van Laethem JL, Conroy T, Arnold D; ESMO Guidelines Committee. Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2015; **26** Suppl 5: v56-v68 [PMID: 26314780 DOI: 10.1093/annonc/mdv295]

130 **Hong SK**, Loren DE, Rogart JN, Siddiqui AA, Sendecki JA, Bibbo M, Coben RM, Meckes DP, Kowalski TE. Targeted cyst wall puncture and aspiration during EUS-FNA increases the diagnostic yield of premalignant and malignant pancreatic cysts. *Gastrointest Endosc* 2012; **75**: 775-782 [PMID: 22317883 DOI: 10.1016/j.gie.2011.12.015]

131 **Dumonceau JM**, Polkowski M, Larghi A, Vilmann P, Giovannini M, Frossard JL, Heresbach D, Pujol B, Fernández-Esparrach G, Vazquez-Sequeiros E, Ginès A; European Society of Gastrointestinal Endoscopy. Indications, results, and clinical impact of endoscopic ultrasound (EUS)-guided sampling in gastroenterology: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline. *Endoscopy* 2011; **43**: 897-912 [PMID: 21842456 DOI: 10.1055/s-0030-1256754]

132 **Jenssen C**, Hocke M, Fusaroli P, Gilja OH, Buscarini E, Havre RF, Ignee A, Saftoiu A, Vilmann P, Burmester E, Nolsøe CP, Nürnberg D, D'Onofrio M, Lorentzen T, Piscaglia F, Sidhu PS, Dietrich CF. EFSUMB Guidelines on Interventional Ultrasound (INVUS), Part IV - EUS-guided Interventions: General aspects and EUS-guided sampling (Long Version). *Ultraschall Med* 2016; **37**: E33-E76 [PMID: 26515966 DOI: 10.1055/s-0035-1553785]

133 **Varadarajulu S**, Fockens P, Hawes RH. Best practices in endoscopic ultrasound-guided fine-needle aspiration. *Clin Gastroenterol Hepatol* 2012; **10**: 697-703 [PMID: 22475740 DOI: 10.1016/j.cgh.2012.03.017]

134 **Pishvaian MJ**, Bender RJ, Halverson D, Rahib L, Hendifar AE, Mikhail S, Chung V, Picozzi VJ, Sohal D, Blais EM, Mason K, Lyons EE, Matrisian LM, Brody JR, Madhavan S, Petricoin EF 3rd. Molecular Profiling of Patients with Pancreatic Cancer: Initial Results from the Know Your Tumor Initiative. *Clin Cancer Res* 2018; **24**: 5018-5027 [PMID: 29954777 DOI: 10.1158/1078-0432.CCR-18-0531]

135 **Ko SH**, Pyo JS, Son BK, Lee HY, Oh IW, Chung KH. Comparison between Conventional Smear and Liquid-Based Preparation in Endoscopic Ultrasonography-Fine Needle Aspiration Cytology of Pancreatic Lesions. *Diagnostics (Basel)* 2020; **10** [PMID: 32397572 DOI: 10.3390/diagnostics10050293]

136 **Fabbri C**, Fornelli A, Fuccio L, Giovanelli S, Tarantino I, Antonini F, Liotta R, Frazzoni L, Gusella P, La Marca M, Barresi L, Macarri G, Traina M, De Biase D, Fiorino S, Jovine E, Larghi A, Cennamo V. High diagnostic adequacy and accuracy of the new 20G procore needle for EUS-guided tissue acquisition: Results of a large multicentre retrospective study. *Endosc Ultrasound* 2019; **8**: 261-268 [PMID: 31115386 DOI: 10.4103/eus.eus_14_19]

137 **Sho S**, Court CM, Kim S, Braxton DR, Hou S, Muthusamy VR, Watson RR, Sedarat A, Tseng HR, Tomlinson JS. Digital PCR Improves Mutation Analysis in Pancreas Fine Needle Aspiration Biopsy Specimens. *PLoS One* 2017; **12**: e0170897 [PMID: 28125707 DOI: 10.1371/journal.pone.0170897]

138 **de Biase D**, Visani M, Acquaviva G, Fornelli A, Masetti M, Fabbri C, Pession A, Tallini G. The Role of Next-Generation Sequencing in the Cytologic Diagnosis of Pancreatic Lesions. *Arch Pathol Lab Med* 2018; **142**: 458-464 [PMID: 29565213 DOI: 10.5858/arpa.2017-0215-RA]

139 **de Biase D**, Fassan M, Malapelle U. Next-Generation Sequencing in Tumor Diagnosis and Treatment. *Diagnostics (Basel)* 2020; **10** [PMID: 33212911 DOI: 10.3390/diagnostics10110962]

140 **Mosele F**, Remon J, Mateo J, Westphalen CB, Barlesi F, Lolkema MP, Normanno N, Scarpa A, Robson M, Meric-Bernstam F, Wagle N, Stenzinger A, Bonastre J, Bayle A, Michiels S, Bièche I, Rouleau E, Jezdic S, Douillard JY, Reis-Filho JS, Dienstmann R, André F. Recommendations for the use of next-generation sequencing (NGS) for patients with metastatic cancers: a report from the ESMO Precision Medicine Working Group. *Ann Oncol* 2020; **31**: 1491-1505 [PMID: 32853681 DOI: 10.1016/j.annonc.2020.07.014]

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REVIEW

Toward a new era of hepatitis B virus therapeutics: The pursuit of a functional cure

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Abstract

Hepatitis B virus (HBV) infection, although preventable by vaccination, remains a global health problem and a major cause of chronic liver disease. Although current treatment strategies suppress viral replication very efficiently, the optimal endpoint of hepatitis B surface antigen (HBsAg) clearance is rarely achieved. Moreover, the thorny problems of persistent chromatin-like covalently closed circular DNA and the presence of integrated HBV DNA in the host genome are ignored. Therefore, the scientific community has focused on developing innovative therapeutic approaches to achieve a functional cure of HBV, defined as undetectable HBV DNA and HBsAg loss over a limited treatment period. A deeper understanding of the HBV life cycle has led to the introduction of novel direct-acting antivirals that exert their function through multiple mechanisms, including inhibition of viral entry, transcriptional silencing, epigenetic manipulation, interference with capsid assembly, and disruption of HBsAg release. In parallel, another category of new drugs aims to restore dysregulated immune function in chronic hepatitis B accompanied by lethargic cellular and humoral responses. Stimulation of innate immunity by pattern-recognition receptor agonists leads to upregulation of antiviral cytokine expression and appears to contribute to HBV containment. Immune checkpoint inhibitors and adoptive transfer of genetically engineered T cells are breakthrough technologies currently being explored that may elicit potent HBV-specific T-cell responses. In addition, several clinical trials are attempting to clarify the role of therapeutic vaccination in this setting. Ultimately, it is increasingly recognized that elimination of HBV requires a treatment regimen based on a combination of multiple drugs. This review describes the rationale for progressive therapeutic interventions and discusses the latest findings in the field of HBV therapeutics.

Key Words: Chronic hepatitis B; Functional cure; Direct-acting antivirals; Gene silencing;

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Core Tip: Despite preventive vaccination and effective antiviral drugs, approximately 300 million people worldwide are at risk of liver-related morbidity and mortality from hepatitis B virus (HBV) infection. In the search for more effective strategies, research has focused on two broad categories of therapeutic intervention. First, direct-acting antivirals that interrupt various stages of the HBV life cycle are yielding promising results in clinical trials that show suppression of the HBV antigen load. Second, the need to address depleted immune responses and revive the tolerogenic liver microenvironment has brought immunotherapies to the forefront. It is likely that an upcoming treatment that combines agents from both classes will achieve HBV elimination.

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INTRODUCTION

Despite the proliferation of effective vaccine programs and the introduction of well tolerated antiviral drugs, hepatitis B virus (HBV) infection remains a public health problem of international concern. The global hepatitis B surface antigen (HBsAg) seroprevalence is estimated to be 3.9%, corresponding to approximately 300 million infections[1]. HBV is a noncytopathic hepatotropic virus that causes acute or chronic infection leading to hepatic complications such as cirrhosis or hepatocellular carcinoma (HCC) and is responsible for more than 884000 deaths annually[2]. Halting the progression of end-stage liver disease and preventing the resulting liver-related mortality is the fundamental goal of HBV treatment. Current therapeutic agents, namely nucleoside or nucleotide drugs (NUCs) and interferon-α (IFN-α), have as their main goal the sustained suppression of viral replication and consequently the regression of hepatic necroinflammatory activity. Achieving undetectable hepatitis B e antigen (HBeAg) levels, with or without hepatitis B e antibody seroconversion, is an important goal that indicates partial immune control of chronic infection. Sustained loss of HBsAg is an optimal endpoint demonstrating profound suppression of viral transcription[3]. NUCs inhibit reverse transcription and are highly effective in suppressing serum HBV DNA levels, even below the limit of detection, providing a high barrier to viral resistance. However, HBsAg clearance is rarely observed even after long-term treatment (10%), and treatment discontinuation is associated with significant rates of virological or clinical relapse[4]. On the other hand, alternative IFN-based monotherapy, although providing a more durable response after treatment, is fraught with significant side effects, making it suitable only for some patient subgroups[5]. Obviously, a functional cure, defined as undetectable HBV DNA levels and loss of HBsAg over a finite treatment period, is the exception rather than the rule of the current standard of care[6].

The inefficiency of NUCs and IFN-α in eradicating HBV infection is primarily a result of their inability to eliminate episomal covalently closed circular DNA (cccDNA) and the integrated viral genome[7]. Notably, intrahepatic HBV DNA synthesis has been reported to persist despite the administration of NUCs, thus maintaining the cccDNA pool either by intracellular replenishment or by new rounds of infection caused by residual viremia[8,9]. Apparently, the propensity of HBV to chronify is closely related to the stability and persistence of cccDNA, which serves as a transcriptional template for viral replication and viral antigen production[10]. Moreover, integrated HBV DNA has been confirmed as the dominant source of HBsAg in HBe-negative individuals, revealing an additional viral strategy to maintain infectivity[11]. In parallel, HBV exploits the tolerogenic microenvironment of the liver to secrete large amounts of antigens that cause CD4+ and CD8+ T-cell and B-cell anergy or

exhaustion. The HBV-induced immunosuppressive cascade and associated dysregulation of innate and adaptive immune responses contribute not only to the establishment of chronic infection but also to fibrosis progression and HCC development[12].

The track record in the field of anti-HCV therapy has reinvigorated research directed toward development of novel anti-HBV drugs aimed at improving cure rates by direct action on cccDNA. In this review, we briefly examine the HBV life cycle (Figure 1) to elucidate the mechanisms of action of emerging direct-acting antivirals (DAAs), which are then further analyzed. We also discuss the other major categories of agents in development that constitute immunotherapies to restore HBV-specific host responses (Figure 2).

METHODOLOGY

We conducted a comprehensive review of all drug classes in development for chronic hepatitis B (CHB) treatment. The Medline database was searched for English articles published up to December 2020. The search words and strategy were "chronic hepatitis B" AND ("therapy" OR "treatment") AND ("investigational" OR "experimental" OR "pilot"). The database search yielded 942 articles. The Drug Watch List on the Hepatitis B Foundation website (https://www.hepb.org) was accessed to identify developing therapies in that setting. Abstracts presented at major hepatology conferences through December 2020 were reviewed and summarized. In addition, the reference lists of all the above materials were reviewed for relevant articles. The Clinicaltrial.gov database was accessed to identify clinical trials investigating novel anti-HBV therapeutics. Electronic and manual searches were performed independently by two of the authors (Tsounis EP and Tourkochristou E). Beyond an all-inclusive search, emphasis was placed on drug candidates investigated in late-stage clinical trials or on agents showing potential for functional cure.

HBV LIFECYCLE

HBV is a relatively small (3.2 kb) DNA virus characterized by a compact organization of its genome, which contains four partially or completely overlapping open reading frames (ORFs). The HBV genome encodes seven proteins; L-, M-, S-HBsAg, core, polymerase and the non-structural precore/HBeAg and HBV X protein (HBx). The infectious virion or Dane particle consists of an outer shell of HBsAg floating in a lipid bilayer around the viral nucleocapsid that encases the partially double-stranded, relaxed, circular HBV DNA (rcDNA) with a copy of its polymerase. Initially, the interaction of HBV particles with the carbohydrate side chains of hepatocyte heparan sulfate proteoglycans (HSPGs) appears to initiate the attachment process[13]. The role of glypican-5, which is an additional factor facilitating viral attachment to the host cell, has also been described[14]. Virus entry into the host cell is mediated by human sodium taurocholate co-transport peptide (NTCP), a bile acid transporter specifically expressed in human liver cells, thus conferring HBV its hepatotropic property[15]. The N-terminal end of the PreS1 domain of L-HBsAg is essential for docking to the NTCP receptor[16]. The aforementioned domain also interacts with the clathrin heavy chain and the corresponding adaptor protein 2, suggesting clathrin-mediated endocytosis of the NTCP-HBV complex[17]. After fusion has occurred, the nucleocapsid, detached from its outer shell, floats in the cytoplasm and is transferred to the perinuclear region by mechanisms that remain to be elucidated. Upon approaching the nuclear pore complex, the interaction of the nucleocapsid with nucleoporin 153, an integral component of transport *via* importin β, enables translocation of the viral genome across the nuclear membrane[18]. After the capsid disintegrates at the nuclear pore, the rcDNA and attached polymerase are released into the nucleoplasm[19]. The host repair apparatus is then recruited to convert rcDNA into cccDNA, which resembles a nucleosomally bound mini-chromosome[20]. In this structure, the viral genome is characterized by a long half-life and serves as a transcriptional template. Several epigenetic factors that modify histone acetylation and methylation, together with HBx and core proteins (Cp), appear to orchestrate transcriptional regulation[21]. The host RNA polymerase II and supporting hepatocyte-specific transcription factors [hepatocyte nuclear factor (HNF) 1α, HNF4α] use cccDNA to synthesize the 5'-capped and 3'-polyadenylated viral transcripts, which are subsequently exported to the cytoplasm for translation[22]. The largest ORF generates greater-than-genome-length

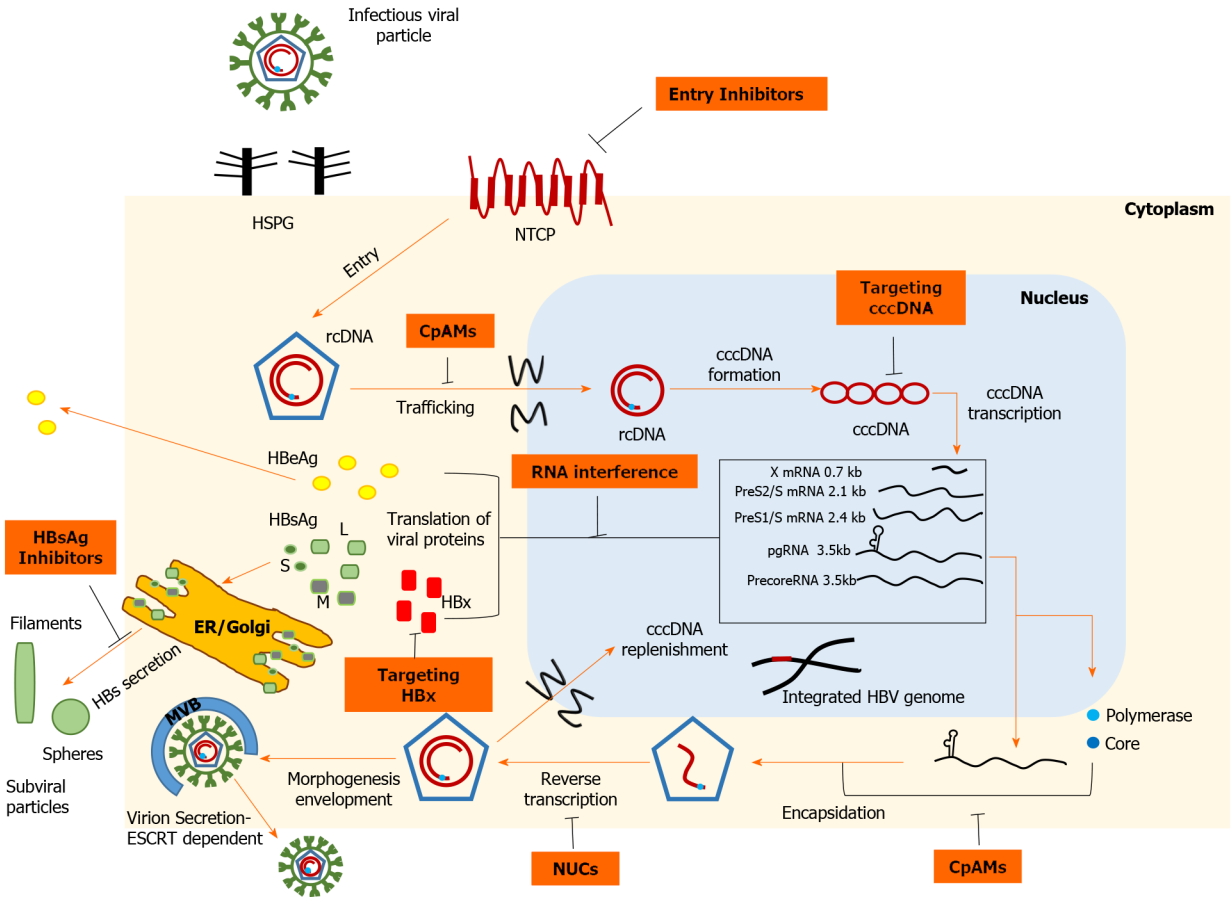


Figure 1 Schematic representation of the hepatitis B virus life cycle and the targets of direct-acting antivirals. Viral entry can be prevented, either by disrupting viral interaction with heparan sulfate proteoglycans or by inhibiting high-affinity binding to the sodium taurocholate cotransporting polypeptide receptor. Strategies targeting covalently closed circular DNA (cccDNA) include: Prevention of cccDNA formation, cccDNA degradation or destabilization, gene editing tools to cause sequence-specific damage, and epigenetic manipulations to functionally silence cccDNA. Inhibition of the interplay between hepatitis B virus (HBV) X protein and host proteins leads to transcriptional silencing. Therapeutics based on RNA interference target viral transcripts and block HBV protein expression. Nucleoside or nucleotide drugs are approved inhibitors of reverse transcriptase. Core protein allosteric modulators interfere with the kinetics of nucleocapsid assembly/disassembly and can affect the various functions of the core protein. Hepatitis B surface antigen (HBsAg) release inhibitors limit the circulating HBsAg load. cccDNA: Covalently closed circular DNA; CpAMs: Core protein allosteric modulators; ER: Endoplasmic reticulum; ESCRT: Endosomal sorting complexes required for transport; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HBx: Hepatitis B virus X protein; HSPG: Heparan sulfate proteoglycans; NTCP: Sodium taurocholate cotransporting polypeptide; NUCs: Nucleot(s)ide analogues; rcDNA: Relaxed circular DNA.

pregenomic RNA (pgRNA), which not only encodes polymerase and Cp but is also reverse transcribed to generate HBV DNA copies. The encapsidation signal ε, located at the 5'-end of pgRNA, recruits RNA polymerase and triggers encapsidation of the duplex, a process realized by the 5'-cap arrangement, the arginine-rich C-terminal domain of Cp, and host eIF4E and heat shock proteins[22-24]. After rcDNA synthesis by reverse transcription, the mature nucleocapsid can follow two different pathways. In early stages of infection, nucleocapsids return to the nuclear basket to amplify the cccDNA pool[25]. However, when a sufficient amount of HBsAg has been synthesized, the nucleocapsids bud through the endoplasmic reticulum (ER) membrane, where envelope glycoproteins settle to acquire their outer envelope[22,26]. Unlike noninfectious subviral particles (SVPs), which accumulate in the lumen of the ER-Golgi intermediate compartment before release from the cell, secretion of mature virions is dependent on the endosomal sorting complex and associated multivesicular bodies required for transport[27].

Entry inhibitors

Recent data suggest that in the absence of reinfection, proliferating hepatocytes significantly decrease the intranuclear cccDNA reservoir even without the use of cytolytic mechanisms[28]. In this setting, efficient prevention of HBV entry into the host cell could significantly curb HBV infection. Currently, the only approved agent that inhibits HBV entry is hepatitis B immunoglobulin (HBIG), a mixture of polyclonal antibodies derived from pooled human plasma from immunized donors. Antibody

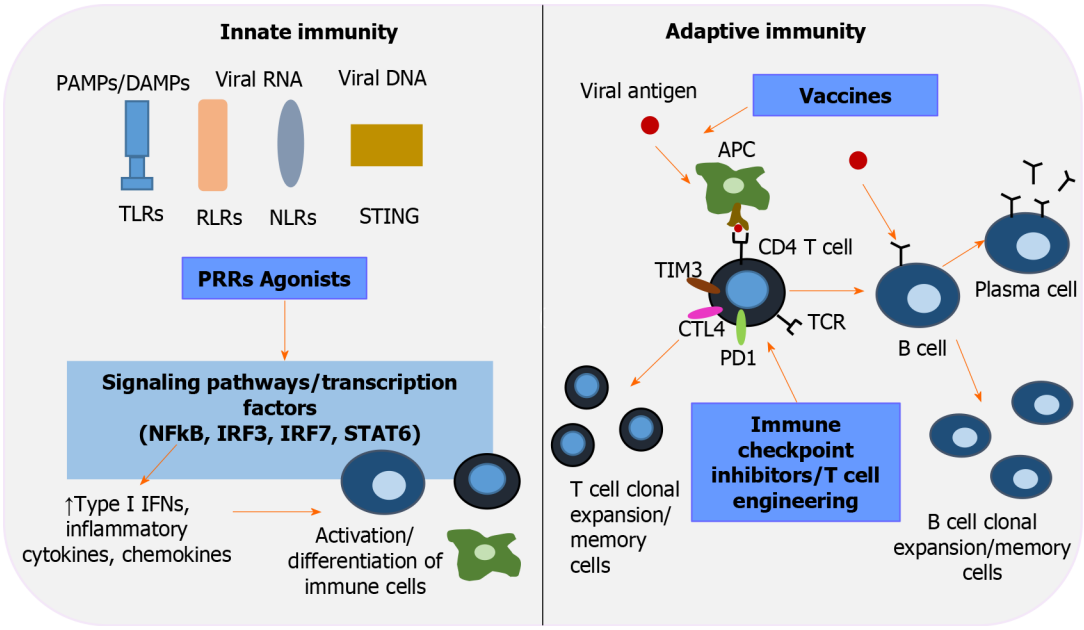


Figure 2 Immunotherapeutic interventions to revive host immunity in chronic hepatitis B virus infection. Pattern-recognition receptors (PRRs), including toll-like receptors, retinoic acid-inducible gene-I-like receptors, nucleotide-binding oligomerization domain-like receptors, stimulator of interferon genes, are key players in innate immunity and the first line of defense that recognizes pathogen-associated molecular patterns/damage-associated molecular patterns. Activation of PRRs by their corresponding agonists triggers transduction signals and transcription factors [nuclear factor-κB, interferon regulatory factor (IRF) 3, IRF 7, signal transducer and activator of transcription 6], which in turn upregulate type interferons, inflammatory cytokines and chemokines, leading to well-orchestrated immune cell differentiation. Immune checkpoint inhibitors aim to restore T-cell function by inhibiting negative regulators of T-cell activation (programmed cell death protein 1, cytotoxic T-lymphocyte-associated protein 4, T-cell immunoglobulin and mucin domain-3). Adoptive transfer of genetically engineered T -cells is an alternative strategy to elicit potent hepatitis B virus - specific T-cell responses. The role of therapeutic vaccination in overcoming exhausted cellular and humoral responses is currently under investigation. An efficient vaccine repairs function and induces antigen- presenting cells to activate the two arms of adaptive immunity: polyclonal and multispecific CD4+ and CD8+ T-cell responses, and well-regulated B cells that differentiate into plasma cells and secrete neutralizing antibodies. IFNs: Interferons; IRF: Interferon regulatory factor; NF-κB: Nuclear factor-κB; NLRs: Nucleotide-binding oligomerization domain-like receptors; PAMPS: Pathogen-associated molecular patterns; PD-1: Programmed cell death protein 1; PRRs: Pattern-recognition receptors; RLRs: Retinoic acid-inducible gene-I-like receptors; STAT6: Signal transducer and activator of transcription 6; STING: Stimulator of interferon genes; TIM3: T-cell immunoglobulin and mucin domain-3; TLRs: Toll-like receptors.

binding to infectivity regions within the envelope proteins, *i.e.* the antigenic loop of the S domain and the receptor binding site of the preS1 domain, causes antibody-mediated neutralization of virions and prevents infection of hepatocytes[29]. Nevertheless, HBIG is produced only in limited quantities by an expensive and time-consuming purification process, limiting its clinical use only for prophylaxis. In an attempt to overcome these barriers, research has focused on the development of several monoclonal antibodies (HzKR359-1, HzKR127-3.2) targeting specific epitopes of surface proteins, and preclinical studies show promising results[30]. GC1102 is a recombinant anti-HBs monoclonal antibody that is being studied in combination with NUC in a Phase II trial (NCT03801798)[31]. The early interaction of a virion with low affinity for HSPGs represents a potential point for therapeutic intervention. Indeed, heparin and suramin bind to HBV envelope proteins and disrupt viral binding to HSPGs, whereas synthetic antilipopolysaccharide peptides bind directly to HSPGs and block them[29,32,33]. Interestingly, an alternative pathway has recently been described for IFN-α to attenuate viral infectivity by secretion of soluble factors that alter HSPG function[34]. Proanthocyanidin and its oligomeric flavonoid analogs may have a therapeutic role by targeting the preS1 region of the large surface protein and inhibiting HBV uptake from the NTCP receptor without exerting serious cytotoxic effects[35]. Agents that block the NTCP receptor represent realistic and achievable treatment options and can be divided into two groups, NTCP substrates and agents that bind to NTCP without being transported[29]. Indeed, conjugated bile acids, including taurochol, ursodeoxychol, and tauroursodeoxychol, have shown antiviral activity in cell culture systems by limiting HBV/hepatitis delta virus (HDV) infection[36]. However, efficient inhibition requires bile acid concentrations well beyond the physiological range, so long-term administration could be associated with side effects. Moreover, *in vitro* experiments have shown that NTCP-mediated HBV entry into hepatocytes could be limited by already approved therapeutic agents, namely the antihypertensive drug irbesartan and the hypolipidemic agent ezetimibe[37,38]. On the other hand, cyclosporine A analogs and peptides derived

from the HBV preS1 region constitute a second group of nonsubstrate ligands that inhibit NTCP. Although cyclosporine A, a commonly used immunosuppressant, has significant anti-HBV activity, it was associated with severe dysregulation of bile acid uptake in hepatocytes[39]. Nevertheless, the antiviral effect of cyclosporine A derivatives (SCY450, SCY995) has been demonstrated, which may be functionally separate from bile acid transport[40]. Bulevirtide, formerly known as Myrcludex B, is a synthetic myristoylated peptide derived from the preS1 domain of L-HBsAg that irreversibly blocks NTCP at nonsaturating concentrations[29]. Bulevirtide induced HBV DNA decline and contributed to alanine aminotransferase (ALT) normalization in an open-label, randomized, Phase Ib/IIa study including 48 HBeAg-negative patients with CHB (NCT02881008). However, HBsAg responses were weak, so research has primarily focused on investigating its value regarding HBV/HDV coinfection; a Phase III clinical trial of bulevirtide in this setting is ongoing (NCT03852719). A recent study found that administration of high-dose bulevirtide in patients with HBV/HDV coinfection was safe and well tolerated. Thirty patients were randomized 1:1 to receive 10 mg of bulevirtide plus pegylated interferon-alpha (Peg-IFN-α) or tenofovir for 48 wk. At week 48, remarkable HDV RNA suppression was observed in both treatment arms (median reduction of 4 $\log_{10}$IU/mL); 86.7% of patients in the Peg-IFN group had undetectable HDV RNA levels and 40% of participants receiving tenofovir achieved ALT normalization[41].

Targeting cccDNA

The plasmid-like intranuclear cccDNA plays a central role in the HBV life cycle, serving as a template for the production of viral antigens and thus for the progeny of virions. Therefore, treatment strategies that impede cccDNA formation, promote its degradation, or support transcriptional silencing may be of great importance[42]. IFN-α and lymphotoxin-β receptor agonists upregulate the nuclear deaminases apolipoprotein B mRNA-editing enzyme and catalytic polypeptide-like (APOBEC) 3A/3B, thus causing noncytolytic degradation of cccDNA, but without complete depletion of the reservoir[43]. A similar mechanism of action has been reported for various interferons and antiviral cytokines (IFN-γ, IFN-β, IFN-λ1/λ2, TNF-α)[44,45]. Moreover, cccDNA degradation can be enhanced by destabilizing molecules. In that regard, oral administration of agent ccc_R08, derived from small molecule library research, achieved sustained declines of HBsAg and cccDNA by cccDNA destabilization in a mouse model of HBV infection[46]. Genetic editing technology provides new tools to inactivate or eliminate cccDNA. Meganucleases, zinc finger nucleases, transcription activator-like effector nucleases, and CRISPR-associated (Cas) nucleases are cleaving enzymes that target specific DNA sequences. Subsequent misrepair of break sites leads to an increased mutation rate and undermines cccDNA stability. In experimental models, cleavage proteins have shown significant antiviral activity[47-49]. *In vitro* experiments of the CRISPR/Cas9 system show significant functional inactivation of cccDNA by mutagenesis, inducing suppression of viral replication, and potent synergy with NUCs[50,51]. Recent evidence suggests that additive modulation of host DNA repair pathways enhances the antiviral activity of CRISPR/Cas9 and causes profound reduction of intracellular pgRNA[52]. However, fragments of viral DNA appear to be repaired in transcriptionally active "small cccDNA" formations[51]. In addition, identifying conserved cccDNA regions, ensuring effective transfer into hepatocytes, and minimizing the risk of host genetic damage from HBV DNA integration are important hurdles that must be overcome before this technology can be applied to humans. The chromatin-based structure of cccDNA is amenable to epigenetic manipulations that allow intervention at the transcriptional level. Indeed, IFN-α triggers histone hypoacetylation of cccDNA, inhibits binding of the transcription factor signal transducer and activator of transcription (STAT) 1 and STAT2 to active cccDNA, and collects corepressors, suggesting that IFN-associated antiviral activity is exerted to some extent by epigenetic mechanisms[53]. Similarly, suppression of cccDNA transcription has been observed in cell culture models following administration of the small molecule C646, which is a selective inhibitor of the histone acetyltransferases CREB binding protein and p300[54]. GS-5801 is a prodrug of a lysine demethylase-5 inhibitor and has shown antiviral activity in HBV-infected primary human hepatocytes by remodeling the chromatin methylation pattern[55]. In addition, the selective synthetic farnesoid X receptor agonist EYP001, originally developed as a therapeutic for nonalcoholic steatohepatitis, inhibits cccDNA transcription *in vitro* and had a satisfactory safety profile when given to CHB patients[56]. A Phase II trial of EYP001 in combination with NUCs against CHB(NCT04465916) is currently recruiting participants.

Targeting HBx

The HBx protein is essential for HBV replication by causing epigenetic regulation of the extrachromosomal viral genome, making it an attractive target for attenuating transcription. HBx promotes ubiquitin-mediated degradation of structural maintenance of chromosomes 5/6 (Smc5/6), a restriction factor of cccDNA transcription[57]. Nitazoxide, a thiazolid-derived antiprotozoal agent, blocks the interaction of HBx and host protein DNA damage-binding protein 1, which is essential for ubiquitination of Smc5/6, and reduces viral antigen production in the HBV minicircle system[58]. Administration of nitazoxide in humans has produced favorable results, with serum HBV DNA undetectable in 8 of 9 patients after 4 to 20 wk of treatment[59]. A 12-mo, placebo-controlled, Phase II trial of nitazoxanide in virologically suppressed HBeAg-negative patients is ongoing to evaluate the efficacy of nitazoxide and the pharmacokinetic relationships with standard NUC treatment (NCT03905655). In the same context, pevonedistat inhibits activation of neural precursor cell expressed, developmentally downregulated 8 (NEDD-8), a ubiquitin-like protein critical for the ubiquitin-proteosome pathway, and minimizes cccDNA transcription by restoring Smc5/6 levels[60]. In addition, HBx promotes overexpression of the histone deacetylase sirtuin-2, which in turn promotes HBV replication and has been linked to hepatocarcinogenesis. AGK2 is a selective inhibitor of sirtuin-2, and was found to reduce HBV antigen production in both *in vivo* and *in vitro* studies[61]. Finally, significant antiviral activity was observed in a transgenic mouse model using CRV431, a cyclophilin inhibitor that blocks the formation of the HBx-cyclophilin A complex in a dose-dependent manner[62]. Genetic engineering has made it possible to knock down HBx gene expression using CRISPR/Cas9 technology. Specifically, a custom CRISPR/Cas9 system targeting a specific sequence of the HBx gene has been developed and tested in HCC cells containing an integrated HBV genome. Apart from decreasing HBx and HBsAg production, HBx/CRISPR reduced the epithelial-mesenchymal transition capacity and stem-cell properties of HCC cells, revealing a perspective for controlling HBV-associated tumor progression[63].

RNA interference

HBV hijacks host translational machinery to produce proteins that ensure survival and replication. It is becoming clear that agents that accelerate the degradation of viral transcripts or silence their expression could be a useful means of controlling the viral antigen load. Reduced viral replication and the emerging stimulation of the host immune response could therefore pave the way for a functional cure. RNA therapeutics have gained popularity on the battlefield against HBV, with two basic approaches being used in clinical trials, double-stranded small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs)[64]. siRNA interacts with the RNA-induced silencing complex (RISC) and the endonuclease argonaut 2 (AGO2). When siRNA docks with RISC, its two strands are separated and the coding strand is destroyed. The antisense strand binds to the corresponding sequence of the target RNA, which is cleaved by AGO2, and the residues are degraded by exonucleases[64]. All HBV transcripts, including subgenomic and pgRNA, have an identical 3' terminus that is a suitable target for simultaneous silencing of all viral transcripts. Several siRNAs have been developed and tested in animal models, and some of them have found their way from the laboratory bench to clinical trials. The first anti-HBV RNAi-based therapeutic to be administered in humans was ARC-520, which contains two different siRNAs conjugated to cholesterol that promotes uptake into hepatocytes and a polymer-based excipient (ARC -EX1) that enhances endosomal release into the cytoplasm. A significant reduction in HBsAg was observed (mean reduction of 0.38 $\log_{10}$ IU/mL in HBeAg-negative and 0.54 $\log_{10}$ IU/mL and in HBeAg-positive patients) that persisted for at least 80 d after the last dose[65]. Nevertheless, in HBeAg-negative and NUC-experienced individuals, a substantial portion of HBsAg is thought to be derived from integrated HBV DNA[10]. Deletion of HBV DNA regions upon integration resulted in truncated mRNAs lacking the sequence recognized by siRNAs, which reduced the efficiency of RNAi in the aforementioned patients[65].

JNJ-3989 (formerly ARO-HBV), which consists of two siRNAs that target hepatocytes and the entire HBV transcriptome was developed in an attempt to circumvent that problem and target both cccDNA and integrated transcripts[66]. Regardless of HBe serostatus, three monthly doses of JNJ-3989 on days 1, 27, and 57, combined with daily NUC treatment, resulted in profound HBsAg reduction along with significant reductions of HBV DNA, HBV RNA, and hepatitis B core antigen (HBcAg). Remarkably, 22/39 (56%) of patients achieved a sustained HBsAg response (defined as ≥ 1.0 $\log_{10}$ IU/mL reduction) even 9 mo after the last JNJ-3989 injection[66].

VIR-2218 (formerly ALN-HBV) is another a liver-targeted pangenotypic siRNA. In a Phase II clinical trial, 24 CHB patients undergoing NUC treatment were administered two monthly doses of VIR-2218 on days 1 and 29. Preliminary results found a pivotal HBsAg decline that persisted in a subset of patients for 28 wk after treatment (a mean decline of 1.0 $\log_{10}$IU/mL) and suggest that different patterns of response may underestimate the ultimate therapeutic effect of VIR-2218[67]. The lipid nanoparticle platform-based delivery technology ARB-1467 consists of three siRNAs directed against all HBV transcripts. Single and multiple doses of ARB-1467 induced significant HBsAg decline in a Phase II study, independent of HBeAg status[68]. An alternative delivery technology utilizes the RNAi therapeutic AB-729 conjugated to a trimer of N-acetylgalactosamine (GalNAc) ligand that promotes uptake into hepatocytes *via* the asialoglycoprotein receptor (ASGR). Preliminary results of a phase I study show that a single dose of AB-729 resulted in a profound HBsAg decline in CHB patients on stable NUC therapy that persisted for 12 wk after treatment[69]. RG6346 also uses GalNAc-RNAi technology and has demonstrated a reduction in markers of viral activity and stimulation of the immune-mediated host response in a Phase Ib/IIa study, as evidenced by self-resolving ALT relapses. No serious adverse events were noted and 11 of 12 (92%) NUC-suppressed CHB patients receiving four monthly doses of RG6346 achieved an HBsAg drop ≥ 1.0 $\log_{10}$IU/mL[70].

ASOs are small single-stranded DNA or RNA oligomers that silence the target mRNA by various mechanisms including inhibition of 5' cap formation, steric disruption of translation, or activation of RNase H[64]. GSK3389404 (Ionis-HBV-LRx) is an ASO-GalNAc conjugate that targets all four ORFs of HBV and exerts its function *via* its active metabolite GSK3228836 (Ionis-HBVRx), which binds to the complementary target mRNA. The ASO/RNA duplex is degraded in the nucleus *via* an RNAase H1-dependent pathway[71]. The results of a recent double-blind, placebo-controlled Phase II trial in which CHB patients were treated with multiple doses of GSK3228836 over a 4-wk period showed a significant reduction in HBsAg in patients treated with NUC (mean decrease of 2.5 $\log_{10}$IU/mL) and in NUC-naïve patients (mean decrease of 1.6 $\log_{10}$IU/mL)[72]. The simultaneous suppression of HBcAg and HBV RNA suggests a remarkable downregulation of cccDNA transcriptional activity[73]. ALG-020572 and ALG-020576 are GalNAc-conjugated ASOs that have superior antiviral efficacy compared with GSK3228836 in mouse models. Promisingly, the combination of the two agents in a 1:1 ratio shows potent antiviral activity with pangenotypic coverage even in viral genome integration, suggesting a synergism with a variety of anti-HBV therapeutics[74]. Similarly, RO7062931 is a single-stranded oligonucleotide based on locked nucleic acid (LNA) technology. It was tested in a phase I clinical trial in which 59 CHB patients on established NUC therapy were randomized to receive either placebo or 2-5 injections of the compound. A dose-dependent reduction in HBsAg was observed and was independent of HBe serostatus, but no sustained response to treatment was observed[75]. GapmeR is another antisense LNA that benefits from hyperconserved regions at the 5' end of the HBx gene and efficiently suppresses HBV expression *in vitro*[76]. Reducing off-target toxicity, improving hepatocyte availability, and increasing affinity for a wide range of quasispecies are essential for the new generation of RNAi therapeutics. However, the inability to directly eradicate cccDNA and evolutionary pressures leading to increased HBV mutation rates diminish their efficacy and may limit their application as only a component of upcoming combination therapy.

Small molecule drug candidates that silence gene expression have performed well in preclinical studies. RG7834 is the first orally bioavailable, highly selective dihydroquinolizinone-based small molecule to produce significant HBV DNA suppression and HBsAg regression in animal models[77]. The proposed mechanism of action involves accelerated intranuclear viral mRNA degradation, which requires the posttranscriptional regulatory element of HBV rather than inhibition of transcription[78].

Core protein allosteric modulators

The Cp is a relatively small protein comprising 183 residues and is the basic element of the nucleocapsid. The N-terminal region is responsible for the assembly function and associated homodimer structure of Cp. Multimerization of 120 core dimers generates the icosahedral structure of the mature nucleocapsid. However, Cp is not only a static carrier of the HBV genome but also has a multifunctional role. The arginine-rich C-terminal domain is required for pgRNA capsidation, coordinates reverse transcription initiation, and regulates intracellular transport pathways *via* nuclear localization signals. In addition, Cp makes epigenetic modifications to cccDNA and interacts with HBsAg to promote virion morphogenesis[79]. Imprinting of the nucleocapsid at

various points in the viral life cycle implies that allosteric modulation of Cp may be an effective therapeutic option. Nuclear protein allosteric modulators (CpAMs) interfere with the assembly process by binding to a hydrophobic pocket within the dimer-dimer interface, thereby triggering changes in the quaternary structure[80]. Based on their pharmacological properties, we recognize two classes of CpAMs. Type I CpAMs, typically heteroaryldihydropyrimidine analogs (HAP), alter the kinetics of Cp oligomerization and promote the formation of large aberrant, nonencapsulated polymers that are prone to degradation or intracellular aggregation. Phenylpropenamides and sulfamoylbenzamides (SBAs) are the major II type CpAMs and cause acceleration of the assembly process, resulting in nascent capsids that lack the pgRNA polymerase complex-important for HBV replication[80]. In addition to limiting rcDNA new synthesis, certain CpAMs inhibit de novo cccDNA formation by disrupting nucleocapsid uncoating during the initial steps of the viral life cycle[81].

Numerous pharmaceuticals targeting Cp have been tested in clinical trials. RO7049389 is an orally administered compound that belongs to class I of CpAMs and has shown a satisfactory safety and tolerability profile. When administered to patients with CHB over a 28-d period, RO7049389 resulted in a significant reduction in HBV DNA (median decrease of 2.66-3.2 $\log_{10}$IU/mL). Remarkably, HBV DNA decreased below the limit of detection in 13/16 HBeAg-negative patients[82]. Currently, RO7049389 is being studied in a Phase II trial as part of a combination therapy that also includes an immunomodulator and an NUC (NCT04225715). GLS4 and KL060332, which exhibit potent antiviral activity and low cytotoxic effects in experimental models, are among the HAP derivatives that promote defective capsid assembly and have successfully entered clinical development[83,84].

The first SBA derivative NVR 3-778 was administered in a placebo-controlled, multicenter Phase I study to 73 HBeAg-negative patients over a treatment period of 4 wk. Significant reductions in HBV DNA and RNA were observed at daily doses > 1000 mg, but HBsAg levels were unaffected. The mean HBV DNA change was more pronounced in patients receiving NVR 3-778 plus Peg-IFN (−1.97 $\log_{10}$IU/mL) than in patients receiving NVR 3-778 monotherapy (−1.43 $\log_{10}$IU/mL) or Peg-IFN monotherapy (−1.06 $\log_{10}$IU/mL)[85]. After meeting safety standards, JNJ-6379, another potent SBA derivative that manipulates nuclear protein to form empty capsids and affects the kinetics of nucleocapsid degradation, was enrolled in a Phase II clinical trial[86]. Preliminary data from 88 unpretreated and 84 virologically suppressed patients randomized to receive 75 mg or 250 mg of JNJ-6379 plus NUC or placebo plus NUC support more pronounced HBV DNA and RNA suppression in untreated patients receiving dual therapy. Importantly, a median HBsAg reduction of 0.4 $\log_{10}$ IU/mL was observed in untreated HBeAg-positive patients receiving 250 mg JNJ-6379 plus NUC[87]. JNJ-6379 is under investigation in a Phase II trial evaluating the safety and efficacy of a triple combination of anti-HBV therapies along with a NUC and the RNAi-based JNJ-3989 (NCT03982186). AB-423, AB-506 and EDP-514 are II CpAMs that have entered clinical trials (Table 1). AB-506 was discontinued due to 2 cases of acute hepatitis during a 28-d phase Ia study in healthy volunteers (ACTRN-12618000 987268).

Data from two phase II studies of the first-generation core inhibitor ABI-H0731 strongly support further development of that agent. The first is an ongoing, double-blind, placebo-controlled study in virologically suppressed patients randomized to receive either ABI-H0731 (300 mg daily) plus NUC or placebo plus NUC (NCT03576066). After 24 wk of treatment, a higher proportion of patients treated with combination therapy achieved undetectable HBV DNA, as measured by high-sensitivity PCR, compared with those receiving placebo plus NUC [15/16 (94%) *vs* 7/10 (70%)][88]. The second study was an open-label extension study (NCT03780543) that demonstrated sustained reductions in HBV DNA, RNA, and viral antigens in patients treated with ABI-H0731 plus NUC for more than 1 year[89]. ABI-H2158 is a second-generation core inhibitor. Recently, data were published from a phase Ib study in which HBeAg-positive patients were blindly randomized to receive 100, 300, or 500 mg ABI-H2158 or placebo once daily for 14 d. The changes in HBV DNA from baseline to day 15 were −2.3 $\log_{10}$IU/mL, −2.5 $\log_{10}$IU/mL, −2.7 $\log_{10}$IU/mL, and −0.1 $\log_{10}$ IU/mL, respectively[90]. QL-007, ZM-H1505R, ALG-001075, ALG-000184, GLP-26, AT-61, AT-130, and phthalazinone derivatives are other CpAMs in the pipeline for an HBV cure (Table 1).

HBsAg inhibitors

In the sera of infected patients, in addition to infectious Dane particles, we find an excess (even 10000-fold) of empty SVPs in the form of spheres and filaments composed of envelope proteins. SVPs transport most of the circulating HBsAg, interfere with

Table 1 Direct-acting antivirals in the pipeline for chronic hepatitis B virus infection

	Compound	Class and action	Phase of development	Ref. or trial number
Entry Inhibitors	HBIG	Polyclonal antibodies neutralizing HBsAg	Available	Herrscher *et al*[29]
	GC1102	Monoclonal antibody neutralizing HBsAg	Phase II	NCT03801798
	HzKR359-1, HzKR127-3.2	Anti-preS1 monoclonal antibodies	Preclinical	Wi *et al*[30]
	Heparin, Suramin	Inhibition of HBV-HSPGs interaction	Preclinical	Herrscher *et al*[29] and Petcu *et al*[33]
	SALPs	Inhibition of HBV-HSPGs interaction	Preclinical	Krepstakies *et al*[32]
	PAC and analogs	Anti-preS1 oligomeric flavonoid analogs	Preclinical	Tsukuda *et al*[35]
	Conjugated bile acids (TCA, UDCA, TUDCA)	NTCP inhibitors	Preclinical	Yan *et al*[36]
	Ezetimibe, Irbesartan	NTCP inhibitors	Available	Lucifora *et al*[37] and Ko *et al*[38]
	Cyclosporine A analogs (SCY450, SCY995)	NTCP inhibitors	Preclinical	Watashi *et al*[39] and Shimura *et al*[40]
	Bulevirtide(Myrcludex B)	NTCP inhibitor	Phase III	Wedemeyer *et al*[41]
TargetingcccDNA	Interferons, TNF-α, Lymphotoxin-β receptor agonists	cccDNA degradation	Available	Bockmann *et al*[44] and Xia *et al*[45]
	ccc-R08	cccDNA destabilizer	Preclinical	Wang *et al*[46]
	Zinc finger nucleases	Gene editing technology	Preclinical	Cradick *et al*[47]
	TALENs	Gene editing technology	Preclinical	Chen *et al*[49]
	CRISPR□associated (Cas) nucleases	Gene editing technology	Preclinical	Ramanan *et al*[48], Seeger and Sohn[50], and Martinez *et al*[51]
	C646	CBP and p300 inhibitorEpigenetic silencing	Preclinical	Cougot *et al*[54]
	GS-5801	Lysine demethylase 5 inhibitorEpigenetic silencing	Phase I	Gilmore *et al*[55]
	EYP001	FXR agonist	Phase II	Erken *et al*[56]
TargetingHBx	Nitazoxide	HBx-DDB1 interaction inhibitor	Phase II	Sekiba *et al*[58], Rossignol and Bréchot[59]
	Pevonedistat	NEDD8-activating enzyme inhibitor	Preclinical	Sekiba *et al*[60]
	AGK2	SIRT-2 inhibitor	Preclinical	Yu *et al*[61]
	CRV431	Cyclophilin inhibitor	Phase I	NCT03596697
RNA interference	JNJ-3989	siRNA	Phase II	Gane *et al*[66]
	VIR-2218 (or ALN-HBV)	siRNA	Phase II	Gane *et al*[67]
	ARB-1467	siRNA	Phase II	Streinu-Cerce *et al*[68]
	ARB-1740	siRNA	Phase I	Thi *et al*[191]
	AB-729	siRNA	Phase I	Yuen *et al*[69]
	RG6346 (or DCR-HBVS)	siRNA	Phase II	Yuen *et al*[70]
	GSK3228836, GSK33389404	ASOs	Phase II	Han *et al*[71] and Yuen *et al*[72]
	ALG-020572, ALG-020576	ASOs	Preclinical	Hong *et al*[74]
	RO7062931 (or RG6004)	Antisense LNA	Phase I	Yuen *et al*[75]

	Gapmers	Antisense LNA	Preclinical	Cortese *et al*[76]
	RG7834	RNA destabilizer	Preclinical	Mueller *et al*[77] and Zhou *et al*[78]
Core protein allosteric modulators (CpAMs)	RO7049389 (or RG7907)	Class I CpAM (HAP derivative)	Phase II	Gane *et al*[82]
	GLS4 (or morphothiadine)	Class I CpAM (HAP derivative)	Phase II	Zhao *et al*[83]
	KL060332	Class I CpAM (HAP derivative)	Phase I	Tai *et al*[84]
	NVR3-778	Class II CpAM (SBA derivative)	Phase I	Yuen *et al*[85]
	JNJ-6379	Class II CpAM (SBA derivative)	Phase II	Berke *et al*[86] and Janssen *et al*[87]
	AB-423	Class II CpAM (SBA derivative)	Phase I	Mani *et al*[192]
	EDP-514	Class II CpAM	Phase I	NCT04470388
	ABI-H0731	CpAM	Phase II	Fung *et al*[88] and Yuen *et al*[89]
	ABI-H2158	CpAM	Phase II	Agarwal *et al*[90]
	ABI-H3733	CpAM	Phase I	NCT04271592
	QL-007	CpAM	Phase I	NCT03770624
	ZM-H1505R	CpAM (Pyrazole derivative)	Phase I	NCT04220801
	ALG-001075, ALG-000184	Class II CpAMs	Preclinical	Zhang *et al*[193]
	GLP-26	Class II CpAM (SBA derivative)	Preclinical	Amblard *et al*[194]
	AT-61, AT-130	Class II CpAMs (PPA derivatives)	Preclinical	Delaney *et al*[195]
	Phthalazinone derivatives	CpAMs	Preclinical	Chen *et al*[196]
HBsAg inhibitors	REP 2139, REP 2156	NAPsSVP release inhibitors	Phase II	Bazinet *et al*[95]
	ALG-010133	STOPS	Phase I	Nie *et al*[94] and Gohil *et al*[96]

ASOs: Antisense oligonucleotides; CBP: CREB binding protein; cccDNA: Covalently closed circular DNA; CpAM: Core protein allosteric modulators; DDB1: DNA damage-binding protein 1; FXR: Farnesoid X receptor; HAP: Heteroaryldihydropyrimidine; HBIG: Hepatitis B immunoglobulin; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HBx: Hepatitis B virus X protein; HSPGs: Heparan sulfate proteoglycans; LNA: Locked nucleic acid; NEDD8: Neural precursor cell expressed, developmentally downregulated-regulated 8; NTCP: Na+ - taurocholate cotransporting polypeptide; PAC: Proanthocyanidins; PPA: Phenylpropenamide; SALPs: Synthetic anti-lipopolysaccharide peptides; SBA: Sulfamoylbenzamide; siRNA: Small interfering RNA; SIRT-2: Sirtuin 2; STOPS: S-antigen transport inhibitory oligonucleotide polymers; SVP: Subviral particles; TALENs: Transcription activator-like effector nucleases; TCA: Taurocholic acid; TUDCA: Tauroursodeoxycholic acid; UDCA: Ursodeoxycholic acid.

signaling pathways of innate and adaptive immunity, and thus contribute to viral persistence[91]. Nucleic acid polymers (NAPs) act through phosphorothioated oligonucleotides to disrupt apolipoprotein interactions involved in SVP assembly and secretion[92]. Analysis of the relative changes in HBsAg isoforms during NAP administration in CHB patients suggests a stronger effect on S-HBsAg. As S-HBsAg is the major component of SVPs, it can be inferred that NAPs act by selectively inhibiting SVP release[93]. S-antigen transport inhibitory oligonucleotide polymers (STOPS) share structural similarities with NAPs, implying a comparable mechanism of action. Moreover, STOPs are characterized by distinct chemical properties that provide enhanced efficacy in different HBV cell lines, including those with a high degree of HBV integration[94]. Therefore, elimination of HBsAg by inhibition of SVP secretion and consequent abrogation of immunosuppressive effect reflects a significant therapeutic opportunity.

REP-401 was an open-label phase II study to validate the safety and efficacy of the NAPs REP 2139-Mg and REP 2156-Mg in HBeAg-negative patients with CHB. After 24 wk of NUC treatment, 40 subjects were randomized to two groups; the first group received experimental treatment (NUC + Peg-IFN + REP 2139-Mg or REP 2165-Mg) for

48 wk. The second group received control therapy (NUC + Peg-IFN) for 24 wk followed by experimental treatment for 48 wk. The addition of NAPs to Peg-IFN + NUC therapy resulted in remarkable HBsAg clearance and seroconversion without serious adverse effects. Importantly, at the 48-wk post-treatment follow-up, 14 of 40 patients achieved a functional cure with durable HBsAg seroconversion. Efficacy and safety parameters between REP 2139-Mg and REP 2165-Mg were equivalent[95]. As for STOPS, ALG-010133 is the leading candidate that has progressed to clinical testing after demonstrating minimal genotoxic effects and favorable pharmacokinetic properties in mice and monkeys (NCT04485663)[96].

Restoring host immunity in HBV infection

Control of HBV infection requires an alert innate immune system and coordinated specific humoral and cellular anti-HBV responses. Resolution of acute HBV infection in adults reflects an optimal host response involving early control of viral replication possibly by IFN-mediated non-cytolytic mechanisms. The human immune system is equipped with a series of pattern-recognition receptors (PRRs) that recognize HBV and trigger signal transduction pathways leading to the production of proinflammatory cytokines and IFN by effector cells. In parallel, functional antigen-presenting cells (APCs) activate the two arms of adaptive immunity, polyclonal and multispecific CD4+ and CD8+ T-cell responses, and properly regulated B cells that differentiate into plasma cells that secrete neutralizing antibodies. Their relative contribution to viral clearance remains to be elucidated. In a chimpanzee model, effective CD4+ T-cell priming and cytotoxic T-cell activity were shown to be essential for limiting HBV infection[97,98]. On the other hand, the importance of humoral defense was highlighted in patients with previously cleared HBV infection who received rituximab for treatment of malignancies. In those patients, anti-HBs seropositivity was highly protective against viral reactivation[99]. It remains controversial whether HBV is a "stealth" virus that evades detection or acts as a suppressor of the innate defense system. Interestingly, coinfection with other hepatotropic viruses stimulates the host response and suppresses HBV replication, suggesting that agents that activate innate immunity offer a therapeutic prospect[100]. In established infection, HBV polarizes monocytes/macrophages toward an anti-inflammatory phenotype by stimulating the synthesis of TGF-β and IL-10 and recruiting myeloid derived suppressive cells (MDSCs) with inhibitory functions. *In vitro*, MDSCs secrete arginase that downregulates IFN-γ expression by HBV-specific T cells and promotes regulatory T-cell activity. Under those circumstances, HBV educates natural killer (NK) cell differentiation to regulatory NK cells and promotes the creation of deficient and tolerogenic dendritic cells (DCs). Moreover, CD4+ and CD8+ T cells respond inadequately with decreased cytokine secretion and weak cytotoxicity, whereas expression of programmed cell death protein 1 (PD-1) and other negative signaling pathways indicate T-cell exhaustion in CHB resulting from long-term exposure to a high antigen load[12]. A better understanding of HBV viral and host immune responses has led to attempts to develop therapeutic factors that target a dysfunctional immune system. A variety of immunomodulatory strategies are currently being investigated, including PRR agonists, agents targeting immune checkpoint proteins, T-cell engineering, and therapeutic vaccination to promote efficient and long-term anti-HBV activity (Table 2).

Toll-like receptor agonists

Toll-like receptors (TLRs) are a distinct class of PRRs that recognize pathogen-associated molecular patterns and damage-associated molecular patterns. Activation of TLRs initiates a cascade of signaling pathways leading to the production of proinflammatory cytokines, chemokines, type I IFNs, and antimicrobial peptides[101,102]. In CHB, abnormal (mainly downregulated) expression and impaired function of TLRs have been reported in both *in vivo* and *in vitro* studies [103-106]. Moreover, *in vivo* and *in vitro* studies have shown that TLR-mediated innate immune responses can lead to inhibition of HBV replication[107-110]. Therefore, TLRs represent a promising target for stimulating the immune system and rebalancing immune responses during chronic infection. GS-9620, also known as vesatolimod, is an orally bioavailable TLR-7 agonist that has shown promising results in preclinical studies by increasing IFN production, activating immune cells, and reducing viral DNA in serum and liver[109,111]. Although phase II studies with vesatolimod have shown a dose-dependent induction of IFN-stimulated gene expression, no significant decrease in HBsAg has been observed[112,113]. Attempts to optimize hepatoselectivity led to development of APR002, a novel TLR-7 agonist that has a better serum-to-liver ratio than GS-9620 because its uptake is mediated by the organic-anion-transporting polypeptide 1B1/3 transporters, which are highly expressed in the sinusoidal

Table 2 Candidate immunotherapies for chronic hepatitis B virus infection

	Compound	Class and action	Phase of development	Ref. or trial number
TLR agonists	GS-9620 (or Vesatolimod)	TLR-7 agonist	Phase II	Janssen *et al*[112] and Boni *et al*[113]
	RO7020531 (RG7854)	TLR-7 agonist	Phase II	Luk *et al*[116]
	JNJ-64794964	TLR-7 agonist	Phase I	Wu *et al*[117]
	APR002	TLR-7 agonist	Preclinical	Korolowizc *et al*[115]
	T7-EA	TLR-7 agonist	Preclinical	Hu *et al*[118]
	GS-9688 (or Selgantolimod)	TLR-8 agonist	Phase II	Mackman *et al*[121] and Janssen *et al*[122]
	ImmunoTAC™	ASGR1-TLR8 agonist conjugate	Preclinical	Baum *et al*[123]
RIG-I, NOD agonists	SB9200 (or Inarigivir)	RIG-I/NOD-2 agonist	Phase II	Yuen *et al*[129]
STING agonists	DMXAA (or Vadimezan or ASA404)	STING agonist	Preclinical	Guo *et al*[132]
T-cell engineering	TCR-engineered T cells	TCR-specific for MHC-I restricted HBV epitopes	Phase I	NCT03899415 NCT02719782 NCT02686372
	S-CAR engineered T cells	CAR targeting envelope proteins	Preclinical	Festag *et al*[145]
	IMC-I109V	ImmTAV targeting HLA-A restricted HBV epitopes	Phase II	EudraCT2019-004212-64
	ImmTAV-HLA-E	ImmTAV targeting HBV epitopes presented by HLA-E	Preclinical	Leonard *et al*[151]
Immune checkpoint inhibitors	Nivolumab	Monoclonal antibodyPD-1 inhibitor	Phase Ib	Gane *et al*[155]
	ASC22 (Envafolimab)	Monoclonal antibodyPD-L1 inhibitor	Phase II	NCT04465890
	HSV-1 glycoprotein D (gD)	BTLA-HVEM pathway inhibitor	Preclinical	Hasanpourghadi *et al*[158]
	APG-1387	IAP antagonist	Phase II	Zhang *et al*[159]
Vaccines	INO-1800	DNA vaccine	Phase I	NCT02431312
	HB110	DNA vaccine	Phase I	Yoon *et al*[164]
	JNJ-64300535	DNA vaccine	Phase I	NCT03463369
	AIC649	Viral vector vaccine (iPPVO)	Phase I	Addy *et al*[167]
	TG1050	Viral vector vaccine (Ad5)	Phase Ib	Zoulim *et al*[168]
	VTP-300	Viral vector vaccine (ChAdOx1)	Phase I	Vaccitech[169]
	HepTcell	T cell vaccine	Phase II	Lim *et al*[171] and Altimmune[172]
	GS-4774	Yeast-based, T-cell vaccine	Phase II	Boni *et al*[173]
	Chimigen® HBV (C-HBV)	Immune complex vaccine	Preclinical	Ma *et al*[177]
	YIC (HBsAg-HBIG)	Vaccine based on yeast-derived immune complexes	Phase II	Zhou *et al*[178] and Xu *et al*[179]
	ABX-203 (or Nasvac)	HBsAg and HBcAg	Phase III	Al Mahtab *et al*[183] and Yoshida *et al*[184]
	VBI-2601 (or BRII-179)	eVLP-based vaccine	Phase II	ACTRN-12619001210167
	NP-preS1	Nanoparticle-based vaccine	Preclinical	Wang *et al*[190]

Ad5: Adenovirus serotype 5; ASGR1: Asialoglycoprotein receptor 1; BTLA-HVEM: B and T lymphocyte attenuator/herpes virus entry mediator; CAR: Chimeric antigen receptor; ChAdOx1: Chimpanzee adenovirus Oxford 1; eVLP: enveloped virus-like particle; HBcAg: Hepatitis B core antigen; HBsAg: Hepatitis B surface antigen ; HBV: Hepatitis B Virus; HLA: Human leukocyte antigen; HSV: Herpes simplex virus; IAP: Inhibitors of apoptosis protein;

ImmTAV: Immunomobilizing monoclonal T cell receptors against virus; iPPVO: Inactivated Parapoxvirus ovis; MHC: Major histocompatibility complex; NOD: Nucleotide-binding and oligomerization domain; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; RIG-I: Retinoic acid-inducible gene I; STING: Stimulator of IFN genes; TCR: T cell receptor; YIC: Yeast-derived immune complexes

membrane of hepatocytes[114]. A weekly oral dose of APR002 in combination with entecavir over a 12-wk period contributed to sustained immune-mediated suppression of CHB and cccDNA elimination by inducing antibody production and stimulating IFN gene expression[115]. RO7020531 is another TLR-7 agonist that has shown a favorable safety profile in clinical trials. A phase II trial evaluating the safety and efficacy of triple anti-HBV therapy with RO7020531 along with NUC and the CpAM RO7049389 (NCT04225715) is ongoing[116]. Single and multiple doses of JNJ-64794964 (JNJ-4964), which is a TLR-7 agonist, were administered to healthy adult volunteers to clarify its pharmacokinetic/pharmacodynamic properties. The bioavailability and safety parameters of a weekly dosing regimen and JNJ-4964-mediated immune stimulation as demonstrated by IFN-α, IFN-γ-inducible protein 10, IFN-stimulated gene 15 (ISG15), and neopterin changes, support further development of this agent[117]. Interestingly, a novel TLR-7 agonist (T7-EA) has been developed as a component of a therapeutic vaccine that is under investigation. T7-EA has demonstrated the ability to reverse immune tolerance to the HBV by inducing HBsAg-specific antibody production and Th1-type immune responses in healthy and HBV-infected mice[118].

In contrast to TLR-7 agonists, TLR-8 activation induces mainly proinflammatory cytokines and chemokines [IL-12, and TNF-α, macrophage inflammatory protein (MIP)- 1α] rather than IFN production because of the different receptor expression pattern between different populations of immune cells[119]. GS-9688 or selgantolimod is a selective TLR-8 agonist that has shown potent antiviral activity in preclinical studies, reducing HBV RNA, HBV DNA, HBsAg, and HBeAg[120,121]. A phase II multicenter, double-blind study was conducted to evaluate the safety and efficacy of GS-9688 in 67 (39 HBeAg-positive) viremic CHB patients randomized (2:2:1) to receive 3 mg or 1.5 mg of GS-9688 or placebo once weekly for 24 wk in combination with daily NUC therapy. Oral administration of GS-9688 up to 3 mg once weekly for 24 wk was safe, well tolerated and elicited dose-dependent changes in cytokine/cytokine receptor levels (IL-12p40, IL-1Rα and IFN-γ) and shifts in immune populations. Although the primary endpoint of an HBsAg reduction ≥ 1 $\log_{10}$IU/mL was not met, participants who showed a reduction ≥ 0.5 $\log_{10}$IU/mL (n = 3) were exclusively in the GS-9688 treatment group. Stronger HBsAg responses were associated with higher baseline ALT and IFN-γ levels[122]. A novel ImmunoTAC™ therapeutic is now available, consisting of a TLR-8 agonist bound to a monoclonal antibody directed against liver-specific ASGR1, and shown to result in hepatoselective immune activation. In cell cultures, the ASGR1-TLR8 conjugate strongly stimulates myeloid cells, which subsequently upregulate IFN-γ expression and activate B cells. In HBV-infected mice, this compound exhibited minimal hepatotoxicity and induced significant HBsAg seroconversion by enhancing anti-HBs and anti-HBc responses of IFNγ+ T cells and triggering anti-HBs B-cell responses[123].

Retinoic acid-inducible gene I-, nucleotide-binding oligomerization domain-like receptors-, and stimulator of IFN genes-like receptor agonists

Retinoic acid-inducible gene I (RIG-I)-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors are members of the PRR receptor family. When stimulated by viral cytosolic RNA, RIG-I-like receptors (RLRs) undergo conformational changes that trigger intracellular signaling pathways and transcription factors [nuclear factor-κB, interferon regulatory factor (IRF) 3, IRF7] that activate ISGs to produce IFN-α and other cytokines[124]. Similarly, NOD-like receptors (NLRs) are important coordinators of innate immunity involved in inflammasome activation, cell death regulation, antigen presentation, and differentiation of adaptive immunity[125]. Evidence suggests that HBV has evolved multiple mechanisms to evade RLR- and NLR-mediated responses[126,127]. In addition to direct inhibition of the HBV replication complex, the orally administered dinucleotide inarigivir (SB9200) acts preferentially *via* stimulation of RIG-I and NOD-2 receptors and has demonstrated a suitable pharmacokinetic profile and robust antiviral efficacy in a marmoset HBV model[128]. Final results from the phase II ACHIEVE study, in which 80 patients were randomized 4:1 to receive inarigivir (25 mg, 50 mg, 100 mg, and 200 mg) or placebo for 12 wk followed by 12 wk of tenofovir administration in both arms, reported a dose-

dependent decline in HBV DNA independent of HBeAg serostatus. The efficacy and safety of inarigivir were highlighted at a daily dose of up to 200 mg. Further studies investigating higher doses or additional inarigivir treatment in NUC-suppressed patients are ongoing (NCT03434353)[129].

The stimulator of IFN genes (STING) downstream signaling pathway is an integral component of innate immunity and recognizes cyclic dinucleotides that are products of aberrant cytosolic DNA. STING recruits and activates the transcription factors IRF-3 and STAT-6 and induces the expression of type I IFNs and STAT-6-dependent chemokines[130]. Preclinical studies have focused on manipulating the STING antiviral drive to overcome tolerance in CHB. The STING-ligand cyclic guanosine monophosphate adenosine monophosphate (cGAMP) was used as an adjuvant for an HBsAg-derived vaccine and tested in HBV-infected mice. Indeed, cGAMP enhanced the effect of the vaccine on humoral and cellular immunity. Specifically, the STING cascade upregulated cytokines that triggered the synthesis of specific anti-HBV antigens, activated APCs in lymphoid tissues, and elicited strong CD4+ and CD8+ T-cell responses[131]. The STING agonist DMXAA (also known as vadimezan or ASA404) has shown positive results in curbing HBV infection *in vivo*. DMXAA induced macrophages to produce antiviral cytokines with a unique profile, resulting in a significant reduction of HBV DNA and cytoplasmic HBV capsids in infected mice[132].

T-cell engineering

T cells are key players in the coordination of the adaptive immune response. CD4+ T cells contribute to the activation of B cells, regulate their differentiation into antibody-producing plasma cells, promote the formation of memory CD8+ T cells, and trigger the proliferation of CD8+ T cells directed against pathogen-infected cells. CD4+ T cells secrete a variety of cytokines and generate specific environmental stimuli responsible for the activation of APCs and the development of specific types of T effector cells[133]. CD4+ regulatory T cells (Tregs) downregulate immune responses and provide tolerance to self-antigens by maintaining immune homeostasis[134]. Although a vigorous immune response against the HBV virus is induced during acute infection, T-cell exhaustion or anergy is observed in chronically infected patients[135]. Exhausted CD4+ and CD8+ T cells are no longer effectively stimulated by their respective ligands, and are characterized by upregulated co-inhibitory receptors (PD-1, cytotoxic T-lymphocyte-associated protein 4, T-cell immunoglobulin and mucin domain, CD244)[136,137], death receptors and pro-apoptotic molecules (tumor necrosis factor-related apoptosis-inducing ligand-2, bcl-2-interacting mediator of cell death)[138,139]. As a result, clonal expansion of HBV-specific T cells is impeded, pro-apoptotic signals are triggered, and antiviral cytokines are downregulated, leading to a weak anti-HBV response[135,140]. To reprogram immune function, a new concept is emerging using autologous transfer of genetically restored HBV-specific T cells. Indeed, adoptive immunity transfer has been demonstrated in CHB patients who achieved HBsAg clearance after receiving allogeneic bone marrow transplantation from donors with cleared HBV infection, indicating that efficient donor HBV-specific T cells are able to overcome immunological exhaustion or anergy[141].

T-cell engineering is a revolutionary strategy to repair the function of HBV-specific T cells. In this method, T cells are engineered with a chimeric antigen receptor (CAR) that targets HBV envelope proteins[142] or with a natural T-cell receptor (TCR) specific for HBV epitopes restricted to major histocompatibility complex (MHC) class I[143]. To generate a genetically engineered T-cell population, T cells are isolated from peripheral blood and activated *in vitro* to receive a transgene consisting of a CAR or a TCR using viral transduction techniques. Subsequently, CAR or TCR-manipulated T cells are cultured for clonal expansion and transfused back to the patient after a quality assessment process[144]. *In vivo* experiments have been performed to investigate the antiviral effect of CAR or TCR-manipulated T cells in HBV-infected animals. Festag *et al*[145] developed an HBV mouse model that received human CAR receptor targeting protein S (S-CAR) containing a human B-cell-derived single-chain antibody fragment, a human immunoglobulin G (IgG) spacer, and CD28- and CD3-signaling immunogenic domains. Specifically, murine CD8+ T cells were manipulated to express S-CAR and transferred into HBV transgenic mice. S-CAR T cells exhibited robust antiviral activity and achieved significant decreases in serum HBsAg, HBeAg, and HBV DNA and increases in anti-HBs without therapy-associated side effects. Since human-derived domains of S-CAR could induce rejection of transplanted cells in immunocompetent organisms, further technical manipulations were performed to ensure sustained expansion and persistence of S-CAR T cells[145].

Irradiation of immunocompetent HBV mice and specific S-CAR tolerance treatment [involving transfer of nonfunctional, signal-deficient S-Decoy (Δ) CAR T cells] were essential for maintaining the long-lasting antiviral efficacy of S-CAR T cells. Similarly, a preclinical study using T cells engineered to carry HBV-specific TCRs addressed to HBV core or envelope proteins yielded positive results. CD4+ and CD8+ T cells derived from either healthy donors or CHB patients, after transplantation of the TCR into HBV-infected mice, showed potent antiviral effects and achieved significant reductions in HBsAg, HBeAg, HBV DNA (4 $\log_{10}$ mL/IU) and pgRNA. Importantly, the engineered T cells induced clearance of HBV-infected hepatocytes without significant toxicity to healthy liver tissue. Coadministration with bulevirtide provided durable control of HBV infection[146]. Although preclinical models suggest T-cell engineering as a promising strategy for efficient elimination of HBV virus, further research is needed to clarify the immunological implications and safety parameters of genetically engineered T cells. Adverse events associated with the binding of transplanted T cells to other cognate or "off-target" antigens have been reported in oncology patients[147,148]. Achieving sustained activity of transplanted T cells and defining the optimal mechanism of T-cell delivery, cell volume, and frequency of infusions required are among the challenges that need to be addressed[149]. Research is ongoing and three clinical trials (NCT03899415, NCT02719782, NCT02686372) investigating the safety and efficacy of TCR-engineered T cells in patients with HBV-related HCC are currently recruiting participants.

Redirection of endogenous, non-HBV-specific T cells to eliminate HBV-infected cells can be mediated by immunomobilizing monoclonal TCRs against virus (ImmTAV) molecules. ImmTAV molecules are bispecific molecules that combine a TCR with increased affinity that targets HBV-specific human leukocyte antigen (HLA)-limited epitopes and a recombinant anti-CD3 antibody that triggers T-cell activation. ImmTAV molecules designed for HLA-A*02:01-restricted HBV antigen epitopes generated T cells that specifically lyse *in vitro* HCC cells containing integrated HBV DNA and HBV-infected cells[150]. A phase II study is currently evaluating the safety, antiviral activity, and pharmacokinetics of the ImmTAV molecule IMC-I109V in HLA-A*02:01-positive patients with CHB (EudraCT 2019-004212-64). However, the highly polymorphic nature of HLA class I complexes compromises the efficacy of ImmTAV therapeutics. Leonard *et al*[151] used HLA-E, which is universally expressed and highly conserved, to overcome HLA-related limitations and developed an ImmTAV platform that recognizes HBsAg and HBV Cps presented by HLA-E alleles (HLA-E*01:01 and 01:03). ImmTAV targeting HLA-E has demonstrated potent antiviral efficacy *in vitro* without significant cytotoxic effects on non-infected cells, joining the long line of immunotherapeutic agents pursuing HBV eradication[151].

Immune checkpoint inhibitors

T-cell exhaustion has been associated with upregulation of inhibitory receptors on the cell surface, resulting in a weak cellular response[152]. Therefore, the design of immune checkpoint inhibitors that target these receptors and suppress their activity may be a useful means of restoring T-cell function in HBV infection.

Nivolumab is an immune checkpoint inhibitor that has been clinically tested in certain cancers and has shown a favorable safety profile[153,154]. It is a genetically engineered human IgG4 monoclonal antibody that binds to PD-1. By inhibiting the PD-1 interaction with its respective ligands (PD-L1 or PD-L2), nivolumab blocks the signal transduction pathway, resulting in functionally active T cells[154]. A phase Ib study (ACTRN12615001133527) evaluated the efficacy of this immunomodulator alone or in combination with the immunostimulatory vaccine GS-4774 (discussed later in the text) in virally suppressed CHB patients. Patients received a single intravenous dose of nivolumab, either 0.1 mg/kg (n = 2) or 0.3 mg/kg (n = 12). In addition, 10 patients received 40 yeast units (YU) of GS-4774 subcutaneously at baseline and 40 YU of GS-4774 plus 0.3 mg/kg nivolumab at week 4. Mean changes in HBsAg levels were estimated 12 wk after nivolumab administration, while safety and immunological outcomes were assessed at week 24 (end of follow-up). Sustained and strong binding of nivolumab to the PD-1 receptor was observed in all patients and a significant decrease in HBsAg levels (0.5 $\log_{10}$) was observed in patients in the higher dose group. Nivolumab also induced HBsAg-specific T-cell responses without serious adverse events until the end of follow-up, results that support further clinical testing of this agent[155].

In addition, ACS22 (Envafolimab) is a single-domain antibody generated by a fusion of the PD-L1 domain with the Fc fragment of the human IgG1 antibody. This chimeric molecule binds with high affinity to PD-L1 and inhibits the PD-1/PD-L1 pathway, thereby improving T-cell function[156]. A phase II trial is ongoing to evaluate the

safety, tolerability and efficacy of ASC22 in CHB patients (NCT04465890).

Herpes simplex virus-1 glycoprotein D (gD) is one of the checkpoint inhibitors of the B and T lymphocyte attenuator/herpes virus entry mediator pathway that revives T-cell response and antigen recognition by CD8+ T cells[157]. A viral vector-based vaccine expressing HBc and polymerase antigens in genetic combination with gD was tested in a mouse model. Vaccination resulted in a reduction of HBV genome copies by approximately 2 $\log_{10}$ IU/mL for a period of more than 8 wk. The use of gD expanded the antigen repertoire recognized by CD8+ T cells and optimized their response. If these results are confirmed in clinical trials, a shift toward subdominant epitopes may be necessary to circumvent irreversibly depleted T cells targeting immunodominant HBV epitopes[158].

Apoptosis is an additional biological checkpoint process that promotes the elimination of pathogen-infected cells and contributes to viral control. Inhibitors of apoptosis proteins (IAPs) have been associated with impaired clearance of HBV virus by preventing IFN-mediated death of virus-infected cells and are currently being investigated as potential targets of anti-HBV therapeutics. APG-1387 is an IAP antagonist that shows robust anti-HBV activity *in vivo*. Intravenous or intraperitoneal injection of 20 mg/kg APG-1387 once weekly for 4-20 wk in HBV mice induced clearance of serum HBsAg, HBeAg, HBV DNA, and elimination of liver HBcAg and achieved sustained HBV clearance after treatment. These effects on HBV markers mediated by APG-1387 were attributed to activation of intrahepatic HBV-specific CD4+ and CD8+ T-cell responses and stimulation of immune-related genes[159]. A phase II open-label dose-escalation study to evaluate the safety, tolerability, and pharmacodynamics of APG-1387 in CHB patients is ongoing (NCT04568265).

Vaccination

Vaccination against HBV virus is a safe, effective, and globally accepted prevention strategy that is part of routine childhood immunization programs[160]. To date, attempts to use vaccination as part of anti-HBV treatment against established infection have not met the expectations raised by experimental models[161]. The basic mechanism of action of the vaccine involves the injection of an inactive compound of the target pathogen that is recognized by APCs, which in turn activate T and B cells. This activation results in the production of specific antibodies, lysis of pathogen-infected cells, and clonal expansion of T and B cells including memory subpopulations that contribute to long-term immunity against the virus[162]. Several categories of HBV vaccines are being tested, including DNA vaccines, viral vector-based vaccines, T-cell epitope peptides, and immune complex (IC) vaccines[163].

DNA vaccines: INO-1800 is a DNA-based vaccine containing plasmids encoding surface and core antigens. A phase I dose-escalation clinical trial (NCT02431312) was conducted to evaluate the safety and immunogenicity of INO-1800 either alone or in combination with INO-9112 (DNA plasmid expressing human IL-12) as an adjuvant in 90 virally suppressed CHB patients. This study is complete and final results are awaited. HB-110 is an investigational vaccine equipped with a broader antigenic arsenal consisting of three DNA plasmids (pGX10-S/L, pGX10-C/P and pGX10-hIL-12m) expressing HBV envelope proteins (S, L), Cp/polymerase and human IL-12, respectively. Although they elicit potent T-cell and antibody-mediated responses in animal models, the effect of HB-110 was weak in 27 Korean patients receiving NUC[164]. A phase I study is ongoing to evaluate the safety and efficacy of an additional DNA vaccine JNJ-64300535 in virally suppressed CHB patients receiving NUC therapy (NCT03463369). Intracellular delivery of the aforementioned DNA vaccines, instead of viral vectors, is based on electroporation, a technology that disrupts cell permeability by applying a potential difference across the membrane. Optimization of electroporation platforms is critical to transfect larger cell populations and improve vaccine immunogenicity[165].

Viral vaccines: AIC649 is an immunostimulatory vaccine consisting of inactivated parapoxvirus particles. In animal models, AIC649 induced significant upregulation of Th1 cytokines, activation of APCs, and HBV-specific T-cell responses, resulting in a remarkable decrease in HBV DNA comparable to that induced by tenofovir or entecavir[166]. Single intravenous doses of AIC649 were tested in CHB patients in a randomized, placebo-controlled trial. The study included 32 patients divided into four ascending dose groups (6 AIC649:2 placebo in each group) and followed for 3 mo. A single dose of this agent was reported to stimulate innate immunity, as evidenced by increased plasma levels of IL-1β, IL-6, IL-8, IFN-γ, and reduced levels of IL-10. In addition, a significant expansion of the CD4+ T-cell memory population was observed,

whereas HBsAg levels did not change significantly[167].

The vector-based vaccine TG1050 uses a non-replicative serotype 5 adenovirus expressing a large fusion protein containing the polymerase and domains of envelope and core antigens. Forty-eight virally suppressed CHB patients were randomized in a 1:1:1 ratio to three dose levels (10^9, 10^{10}, 10^{11} viral particles) of TG1050 in a phase I study; in the first phase, 12 participants were assigned to a single-dose cohort (9 vaccine:3 placebo) and the remaining participants were assigned to a multiple (3) dose cohort (27 vaccine:9 placebo). TG1050 demonstrated a favorable safety profile and induced IFN-γ-producing T cells that reacted to HBV antigens. Although HBcAg was undetectable in 6 patients, the decrease in HBsAg was not significant[168].

VTP-300 is based on a viral vector platform and consists of replication-deficient chimpanzee adenovirus oxford 1, which encodes three full-length HBV antigens and is designed to elicit specific CD4+ and CD8+ T-cell responses[169]. A phase I study to evaluate the safety and efficacy of VTP-300 in viral suppressed CHB patients is ongoing (NCT04297917).

T-cell vaccines: The initiation of T-cell-mediated eradication of a virus-infected cell depends on the interaction of the TCR with the target antigen presented by the MHC. T-cell vaccines consist of HBV epitope peptides that target specific TCR receptors and aim to elicit T-cell responses against HBV antigens[170].

The HepTcell vaccine is a synthetic immunomodulator consisting of nine peptides designed to induce CD4+ and CD8+ T-cell responses against multiple HBV epitopes. A formulation of these nine peptides in combination with a TLR-9 agonist adjuvant was prepared to enhance HBV-specific immune responses. Lim *et al*[171] evaluated the safety and efficacy of HepTcell against HBV infection in a placebo-controlled phase Ib study. A total of 60 HBeAg-negative CHB patients on established NUC treatment were randomized to three dose-escalating cohorts of HepTcell with or without IC31. They received 3 injections with a 28-d injection interval and were followed up for 6 mo[171]. The results support further development of this immunomodulator in a phase II trial[172], as HepTcell administration was safe and induced virus-specific cellular immune responses, especially in combination with TLR9 adjuvant[171].

GS-4774 is a therapeutic vaccine containing HBx, core and envelope proteins together with recombinant *Saccharomyces cerevisiae* as a natural adjuvant to elicit HBV-specific T-cell responses. In a multicenter, randomized phase II trial, 169 HBsAg-positive, treatment-naïve patients were recruited. Participants were randomized 1:2:2:2 into groups receiving oral TDF (300 mg) alone or in combination with 2, 10, or 40 GS-4774 YU. Vaccination was administered subcutaneously in six monthly doses, while oral administration of NUCs continued until the end of follow-up (week 48). The addition of GS-4774 to NUC treatment was well tolerated, induced a reduction of Tregs, and stimulated IFN-γ, TNF-α, and IL-2-producing CD8+ T cells directed against specific HBV epitopes. However, HBsAg levels remained stable and CD4+ T-cell responses were anemic[173]. Therefore, restoration of T-cell immune responses by vaccination is an achievable strategy, but clinical benefits may occur in the context of combination treatment.

IC vaccines: Research aimed at HBV eradication has used IC-based therapies to increase host immune activation. ICs, which consist of immunoglobulins bound to their cognate antigens, are able to exert significant immunoregulatory effects. Specifically, ICs can activate APCs *via* binding of their Fc fragment to Fc receptors (FcγR) expressed on these cells[174]. Activation of FcγR-mediated signaling in DCs, as well as the effect of ICs on cross-presentation, leads to upregulation of antigen delivery to MHC-II molecules, resulting in increased antibody production and clonal expansion of immune memory cells[175]. In addition, ICs trigger molecular signaling pathways associated with phagocyte action (ITAM/Syk) and other signal transduction pathways that regulate cellular processes, tumor suppression, and inflammation[176].

Chimigen® HBV is a therapeutic vaccine based on a fusion molecule consisting of HBV antigens (PreS1 and PreS2 peptide fragments and Cp) and a murine Fc fragment targeting FcγRII (CD32) and mannose receptor (CD206). The Fc-receptor interaction leads to internalization of the chimeric molecule by immature DCs and promotes MHC-I and -II surface expression. In preclinical studies, vaccination induced clonal expansion of HBV-specific T cells, upregulated IFN-γ, TNF-α, perforin, and granzyme B expression by CD4+/CD8+ T cells, and induced apoptosis of Tregs[177].

Yeast-derived immune complexes (YIC) of HBsAg in combination with high-titer immunoglobulin (HBsAg-HBIG) were developed to facilitate antigen uptake by APCs and initiate strong adaptive immune responses. The immunomodulatory effects of ICs were illustrated in a pilot study in which 44 HBsAg- and HBeAg-positive CHB

patients were randomly assigned to receive intramuscular injections of YIC, alum, or normal saline as adjuvants to adenofovir. Patients treated with YIC experienced a relative increase in Th1 and Th2 CD4+ T cells and an increase in Tc1 and Tc17 CD8+ T cells, whereas the number of Tregs decreased. Regarding the cytokine profile, IL-2 and IFN-γ were significantly upregulated, whereas the inhibitory cytokines IL-10 and TGF-β, and transcription factor Foxp3 were reduced[178]. Although the primary endpoint was not met, administration of YIC showed strong antiviral efficacy with respect to HBeAg seroconversion in a phase IIb study[179].

Not to be neglected are the possible implications of ICs in immunopathological reactions. It has been described that an inability of phagocytes to clear ICs leads to deposition, which can trigger inflammation and tissue damage by activating complement receptors on innate immune cells. Furthermore, paradoxical enhancement of bacterial infections can occur when non-neutralizing IgG-antibody complexes activate FcγR signaling in infected monocytes/macrophages (intrinsic antibody-dependent enhancement)[180,181]. The investigation of ICs in HBV vaccinology has just begun and further studies are needed to evaluate IC-associated effects and assess their potential as part of combination therapies[182].

Optimizing drug delivery in HBV vaccination

Breakthroughs in biomedical engineering have contributed to improved drug delivery systems involving either routes of administration or delivery vehicles. The ABX-203 vaccine (Nasvac), which contains HBsAg and HBcAg antigens, was developed for nasal administration. Nasvac has been enrolled in a phase III open-label, randomized clinical trial (NCT01374308) comparing the therapeutic efficacy of Nasvac to Peg-IFN in therapy-naïve CHB patients. A total of 160 patients were assigned 1:1 to either Nasvac or Peg-IFN. Nasvac was administered *via* nasal spray (1 mL) (baseline to week 12) or *via* nasal spray and subcutaneously (1 mL) (from week 12 to week 24). Patients in the Peg-IFN group received 180 μg Peg-IFN once weekly for 48 wk. A 24-wk follow-up period was conducted. Nasvac showed a better safety profile in terms of adverse effects and induced a higher HBV DNA drop compared to Peg-IFN. In parallel, transient ALT flares were more frequent in the Nasvac group than in the Peg-IFN group (85% *vs* 30%), suggesting that vaccination induces a higher rate of positive immune activation. In addition, Nasvac administration prevented progression of liver cirrhosis, as measured by liver stiffness, and induced HBeAg seroconversion more frequently[183]. The efficacy of Nasvac was also evaluated in a clinical trial of 71 CHB patients (including 29 participants on a stable NUC regimen) who received ten doses of the drug once every two weeks. Nasal administration of Nasvac was associated with sustained reductions in HBsAg and increases in anti-HBs in both groups, and notably, 6 of 45 patients monitored 18 mo after treatment achieved functional recovery[184].

Optimization of vaccine delivery by using innovative viral vectors (chimapanzee adenovirus, ChAd or modified vaccinia Ankara) encoding HBV antigens was investigated in normal and HBV transgenic mice. Stimulation of HBV-specific T-cell responses in the liver and increased production of HBc- and HBs-specific IgG were observed without significant hepatotoxicity, providing new insights for clinical investigations[185,186].

VBI-2601 (or BRII-179) is a protein-based vaccine candidate that has entered a phase II trial (ACTRN12619001210167) using enveloped virus-like particle (eVLP) technology. Briefly, eVLPs are noninfectious nanostructures that mimic *bona fide* virions and carry viral antigenic epitopes to elicit immune responses[187]. In the case of VBI-2601, the immunogenic preS1 domain was selected to trigger immune-mediated clearance of HBV-infected cells. Nanoparticles are a novel strategy for vaccine delivery, as ferritin-based nanoparticles have been found to elicit B-cell responses and antibody production in Epstein-Barr virus or influenza virus infections[188,189]. A nanoparticle vaccine was developed that delivers the pre-S1 domain of HBsAg to specific DCs and lymphoid sinus-associated macrophages that activate follicular helper T cells and B cells, respectively. Encouragingly, administration of ferritin nanoparticle pre-S1 vaccine in HBV-infected mice resulted in significant decreases or even undetectable HBV DNA and HBsAg. Moreover, vaccination resulted in impressive rates of HBsAg seroconversion and cccDNA reduction[190]. Clearly, among the new therapeutics targeting functional cure, nanoparticle vaccines represent promising candidates worthy of future evaluation.

DISCUSSION

So far, the introduction of NUCs has been the hallmark of therapeutic interventions against HBV infection, achieving suppression of viral replication and thus significantly improving the prognosis of patients. Nevertheless, the impact of existing treatment regimens on cccDNA stability is negligible, implying that the intranuclear cccDNA pool is constantly renewed, leading to chronification of HBV infection. Although HBsAg clearance has long been an endpoint of clinical trials, the performance of NUCs and IFN-based therapies has been more or less subpar. Considering that HBsAg can originate from integrated viral DNA, it is clear that despite highly efficient drugs that achieve functional cure, hepatocytes harboring reservoirs of HBV DNA may be responsible for persistent subclinical antigen expression after resolution of infection or residual risk for HCC. In parallel, with current therapeutics, there is little or no reversal of defective innate immune responses and depletion of adaptive immunity effectors; *ergo*, HBV establishes and replicates by exploiting the tolerogenic microenvironment of the liver.

Innovative advances in biotechnology, as well as a better understanding of the complex life cycle of HBV and its intricate effects on the host immune system, have reinvigorated the field of HBV therapeutics. A number of drug candidates with antiviral activity through various mechanisms have entered phase II/III clinical trials, demonstrating comparable or greater reductions in HBV DNA and/or RNA compared to NUCs. The breakthrough entry inhibitor bulevirtide has demonstrated synergistic activity with Peg-IFN, resulting in significant viral suppression and HBsAg reduction in HBV/HDV-coinfected patients. Consistent reduction of HBV DNA was achieved with CpAMs, and remarkably, coadministration with NUCs resulted in even more profound inhibition of viral replication. RNAi-based therapeutics with the lead agents JNJ-3989 and VIR-2218, as well as NAPs, provided more exciting results and achieved sustained HBsAg loss off-treatment in a substantial proportion of patients. In contrast, gene editing technology, although providing direct cccDNA eradication, has not yet been tested in clinical trials. Therefore, a sterilizing cure that results in elimination of cccDNA and integrated DNA still seems a long way off, as it would require genome-targeting tools.

In the field of immunotherapies, TLR agonists and other PRR agonists that stimulate innate immunity have induced a substantial decrease in HBV DNA with weak effects on HBsAg serostatus, whereas immune checkpoint inhibitors and especially the PD-1 blocker nivolumab induced stronger HBsAg responses in clinical trials. T-cell depletion due to activation-induced cell death or T-cell exhaustion or anergy, caused by high levels of viral antigens circulating in soluble form in patient sera, and the concomitant inability to elicit potent cytotoxic responses, are possibly the reasons why a conventional therapeutic HBV vaccine is elusive. Moreover, chronic inflammation leads to a tolerogenic intrahepatic environment and suppresses T-cell effector functions, which compromises vaccine efficacy. Nonetheless, efforts to improve immunogenicity, the use of new vectors, and the introduction of innovative delivery platforms have optimized HBV vaccine efficacy. Several vaccines have elicited HBV-specific immune responses in clinical trials, and results from a phase III trial of Nasvac offer the possibility of achieving a functional cure through immunotherapy. However, enhancement of host immunity is a risk factor for induction of uncontrollable immune activation and severe liver injury. Due to the variable hepatic inflammatory status and the different pattern of intrinsic immune responses, different degrees of hepatotoxicity may occur when the same treatment strategy is applied. Therefore, the implementation of strict criteria is imperative to ensure the safety of clinical trials evaluating immunotherapies.

Obviously, a functional cure is more likely in the context of a combination therapy that simultaneously targets HBV through multifaceted mechanisms. In parallel, an immunomodulator could be used to restore innate immunity, while vaccination or T-cell engineering could reprogram and enhance HBV multispecific cellular and humoral responses. However, the extent to which the combination of different therapeutic agents has an additive or synergistic effect on HBV suppression remains to be elucidated. Therefore, it is important to design clinical trials that examine not only the efficacy of concomitantly administered drugs, but also the resulting toxicity or potential drug-drug interactions. Due to altered bioavailability and underlying comorbidities, cirrhotic patients are more susceptible to hepatotoxicity and may be harmed by hepatic flares, so this population should receive special consideration.

Interestingly, phase II trials are already underway investigating concurrent administration of three DAAs (RNAi (JNJ-3989) plus CpAM (JNJ-6379) plus NUC) (NCT04129554) or two DAAs together with an immunomodulator (NUC plus CpAM

(RO7049389) plus TLR7 agonist (RO7020531)) (NCT04225715). IFNs, either in their native form or modified to increase their half-life, remain a major weapon in the armamentarium against HBV providing antiviral efficacy not only through direct mechanism (cccDNA degradation or epigenetic silencing) but also by exerting robust immunoregulatory functions. IFNs due to their distinct mechanism of action retain a position as part of "add-on" or "switch" to IFN treatment strategies in selected patients on long-term NUCs to enhance HBsAg clearance[5]. Accordingly, numerous clinical trials investigating experimental combination therapies include Peg-IFN. PENGUIN (NCT04667104) and Piranga (NCT04225715) are ongoing phase II trials assessing the efficacy of quadruple (RNAi (JNJ-3989) plus CpAM (JNJ-6379) plus NUC plus Peg-IFN) and triple (siRNA (RO7445482) plus Peg-IFN plus NUC) combination regimens, respectively, in virologically suppressed participants with CHB. Apart from add-on strategies, the use of sequential treatment regimens could repair step by step the dysregulated immunity and eventually enable HBV eradication. A randomized, multicenter, phase II study is intended to evaluate if sequential ASO (GSK3228836) and Peg-IFN treatment increases the rate of HBV surface antigen (HBsAg) loss in participants on stable NUC therapy (NCT04676724). Undoubtedly, further studies are needed to divide patients into groups that would benefit most from each combination therapy.

CONCLUSION

We are in the midst of a storm of evolving pharmaceutical agents that tip the balance toward a functional cure for HBV infection. First, a number of DAA drugs have provided encouraging results targeting multiple points in the HBV life cycle; inhibition of viral entry into the host cell, suppression of gene expression, blocking HBsAg release, or even direct elimination of cccDNA are among the features of antiviral drugs in the pipeline. Second, immune-based therapies have entered clinical trials, and sustained restoration of innate immunity and enhancement of HBV-specific cellular and humoral responses without induction of uncontrolled flares seems more realistic than ever. There is a growing consensus that a future treatment could consist of a NUC-based backbone, at least one novel DAA, and one or more immunomodulators. Further studies are needed to clarify the parameters of overlapping toxicity and to establish the most efficient combination strategy.

REFERENCES

1 **Polaris Observatory Collaborators**. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol* 2018; **3**: 383-403 [PMID: 29599078 DOI: 10.1016/S2468-1253(18)30056-6]

2 **World Health Organization**. Hepatitis B. WHO Fact sheets. 27 Jul 2020. [cited 10 January 2021]. Available from: https://www.who.int/news-room/fact-sheets/detail/hepatitis-b

3 **European Association for the Study of the Liver**. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]

4 **Suk-Fong Lok A**. Hepatitis B Treatment: What We Know Now and What Remains to Be Researched. *Hepatol Commun* 2019; **3**: 8-19 [PMID: 30619990 DOI: 10.1002/hep4.1281]

5 **Viganò M**, Grossi G, Loglio A, Lampertico P. Treatment of hepatitis B: Is there still a role for interferon? *Liver Int* 2018; **38** Suppl 1: 79-83 [PMID: 29427498 DOI: 10.1111/liv.13635]

6 **Cornberg M**, Lok AS, Terrault NA, Zoulim F; 2019 EASL-AASLD HBV Treatment Endpoints Conference Faculty. Guidance for design and endpoints of clinical trials in chronic hepatitis B - Report from the 2019 EASL-AASLD HBV Treatment Endpoints Conference‡. *J Hepatol* 2020; **72**: 539-557 [PMID: 31730789 DOI: 10.1016/j.jhep.2019.11.003]

7 **Fanning GC**, Zoulim F, Hou J, Bertoletti A. Therapeutic strategies for hepatitis B virus infection: towards a cure. *Nat Rev Drug Discov* 2019; **18**: 827-844 [PMID: 31455905 DOI: 10.1038/s41573-019-0037-0]

8 **Burdette D**, Cathcart A, Shauf A, Win R, Zaboli S, Hedskog C, Svarovskaia SE, Barry V, Inzunza D, Flaherty JF, Gaggar A, Fletcher S, Chan H, Mo H, Lazerwith S, Feierbach B, Delaney B. Evidence for the presence of infectious virus in the serum from chronic hepatitis B patients suppressed on nucleos (t) ide therapy with detectable but not quantifiable HBV DNA. *J Hepatol* 2019; **70**: e95 [DOI: 10.1016/S0618-8278(19)30168-9]

9 **Boyd A**, Lacombe K, Lavocat F, Maylin S, Miailhes P, Lascoux-Combe C, Delaugerre C, Girard PM, Zoulim F. Decay of ccc-DNA marks persistence of intrahepatic viral DNA synthesis under tenofovir in HIV-HBV co-infected patients. *J Hepatol* 2016; **65**: 683-691 [PMID: 27210429 DOI:

10.1016/j.jhep.2016.05.014]
10 **Nassal M**. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. *Gut* 2015; **64**: 1972-1984 [PMID: 26048673 DOI: 10.1136/gutjnl-2015-309809]
11 **Wooddell CI**, Yuen MF, Chan HL, Gish RG, Locarnini SA, Chavez D, Ferrari C, Given BD, Hamilton J, Kanner SB, Lai CL, Lau JYN, Schluep T, Xu Z, Lanford RE, Lewis DL. RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg. *Sci Transl Med* 2017; **9** [PMID: 28954926 DOI: 10.1126/scitranslmed.aan0241]
12 **Li TY**, Yang Y, Zhou G, Tu ZK. Immune suppression in chronic hepatitis B infection associated liver disease: A review. *World J Gastroenterol* 2019; **25**: 3527-3537 [PMID: 31367154 DOI: 10.3748/wjg.v25.i27.3527]
13 **Schulze A**, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology* 2007; **46**: 1759-1768 [PMID: 18046710 DOI: 10.1002/hep.21896]
14 **Verrier ER**, Colpitts CC, Bach C, Heydmann L, Weiss A, Renaud M, Durand SC, Habersetzer F, Durantel D, Abou-Jaoudé G, López Ledesma MM, Felmlee DJ, Soumillon M, Croonenborghs T, Pochet N, Nassal M, Schuster C, Brino L, Sureau C, Zeisel MB, Baumert TF. A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses. *Hepatology* 2016; **63**: 35-48 [PMID: 26224662 DOI: 10.1002/hep.28013]
15 **Yan H**, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 2012; **1**: e00049 [PMID: 23150796 DOI: 10.7554/eLife.00049]
16 **Blanchet M**, Sureau C. Infectivity determinants of the hepatitis B virus pre-S domain are confined to the N-terminal 75 amino acid residues. *J Virol* 2007; **81**: 5841-5849 [PMID: 17376925 DOI: 10.1128/JVI.00096-07]
17 **Huang HC**, Chen CC, Chang WC, Tao MH, Huang C. Entry of hepatitis B virus into immortalized human primary hepatocytes by clathrin-dependent endocytosis. *J Virol* 2012; **86**: 9443-9453 [PMID: 22740403 DOI: 10.1128/JVI.00873-12]
18 **Schmitz A**, Schwarz A, Foss M, Zhou L, Rabe B, Hoellenriegel J, Stoeber M, Panté N, Kann M. Nucleoporin 153 arrests the nuclear import of hepatitis B virus capsids in the nuclear basket. *PLoS Pathog* 2010; **6**: e1000741 [PMID: 20126445 DOI: 10.1371/journal.ppat.1000741]
19 **Rabe B**, Delaleau M, Bischof A, Foss M, Sominskaya I, Pumpens P, Cazenave C, Castroviejo M, Kann M. Nuclear entry of hepatitis B virus capsids involves disintegration to protein dimers followed by nuclear reassociation to capsids. *PLoS Pathog* 2009; **5**: e1000563 [PMID: 19714236 DOI: 10.1371/journal.ppat.1000563]
20 **Xia Y**, Carpentier A, Cheng X, Block PD, Zhao Y, Zhang Z, Protzer U, Liang TJ. Human stem cell-derived hepatocytes as a model for hepatitis B virus infection, spreading and virus-host interactions. *J Hepatol* 2017; **66**: 494-503 [PMID: 27746336 DOI: 10.1016/j.jhep.2016.10.009]
21 **Koumbi L**, Karayiannis P. The Epigenetic Control of Hepatitis B Virus Modulates the Outcome of Infection. *Front Microbiol* 2015; **6**: 1491 [PMID: 26779147 DOI: 10.3389/fmicb.2015.01491]
22 **Karayiannis P**. Hepatitis B virus: virology, molecular biology, life cycle and intrahepatic spread. *Hepatol Int* 2017; **11**: 500-508 [PMID: 29098564 DOI: 10.1007/s12072-017-9829-7]
23 **Kim S**, Wang H, Ryu WS. Incorporation of eukaryotic translation initiation factor eIF4E into viral nucleocapsids *via* interaction with hepatitis B virus polymerase. *J Virol* 2010; **84**: 52-58 [PMID: 19776122 DOI: 10.1128/JVI.01232-09]
24 **Nassal M**. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. *J Virol* 1992; **66**: 4107-4116 [PMID: 1602535 DOI: 10.1128/JVI.66.7.4107-4116.1992]
25 **Lentz TB**, Loeb DD. Roles of the envelope proteins in the amplification of covalently closed circular DNA and completion of synthesis of the plus-strand DNA in hepatitis B virus. *J Virol* 2011; **85**: 11916-11927 [PMID: 21900164 DOI: 10.1128/JVI.05373-11]
26 **Gerelsaikhan T**, Tavis JE, Bruss V. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. *J Virol* 1996; **70**: 4269-4274 [PMID: 8676448 DOI: 10.1128/JVI.70.7.4269-4274.1996]
27 **Watanabe T**, Sorensen EM, Naito A, Schott M, Kim S, Ahlquist P. Involvement of host cellular multivesicular body functions in hepatitis B virus budding. *Proc Natl Acad Sci USA* 2007; **104**: 10205-10210 [PMID: 17551004 DOI: 10.1073/pnas.0704000104]
28 **Allweiss L**, Volz T, Giersch K, Kah J, Raffa G, Petersen J, Lohse AW, Beninati C, Pollicino T, Urban S, Lütgehetmann M, Dandri M. Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA in vivo. *Gut* 2018; **67**: 542-552 [PMID: 28428345 DOI: 10.1136/gutjnl-2016-312162]
29 **Herrscher C**, Roingeard P, Blanchard E. Hepatitis B Virus Entry into Cells. *Cells* 2020; **9**: 1486 [PMID: 32570893 DOI: 10.3390/cells9061486]
30 **Wi J**, Jeong MS, Hong HJ. Construction and Characterization of an Anti-Hepatitis B Virus preS1 Humanized Antibody that Binds to the Essential Receptor Binding Site. *J Microbiol Biotechnol* 2017; **27**: 1336-1344 [PMID: 28478661 DOI: 10.4014/jmb.1703.03066]
31 **Kim SH**, Shin YW, Hong KW, Chang KH, Ryoo KH, Paik SH, Kim JM, Brotman B, Pfahler W, Prince AM. Neutralization of hepatitis B virus (HBV) by human monoclonal antibody against HBV

surface antigen (HBsAg) in chimpanzees. *Antiviral Res* 2008; **79**: 188-191 [PMID: 18479762 DOI: 10.1016/j.antiviral.2008.03.006]

32 **Krepstakies M**, Lucifora J, Nagel CH, Zeisel MB, Holstermann B, Hohenberg H, Kowalski I, Gutsmann T, Baumert TF, Brandenburg K, Hauber J, Protzer U. A new class of synthetic peptide inhibitors blocks attachment and entry of human pathogenic viruses. *J Infect Dis* 2012; **205**: 1654-1664 [PMID: 22457281 DOI: 10.1093/infdis/jis273]

33 **Petcu DJ**, Aldrich CE, Coates L, Taylor JM, Mason WS. Suramin inhibits *in vitro* infection by duck hepatitis B virus, Rous sarcoma virus, and hepatitis delta virus. *Virology* 1988; **167**: 385-392 [PMID: 2462306]

34 **Xia Y**, Cheng X, Blossey CK, Wisskirchen K, Esser K, Protzer U. Secreted Interferon-Inducible Factors Restrict Hepatitis B and C Virus Entry In Vitro. *J Immunol Res* 2017; **2017**: 4828936 [PMID: 28367455 DOI: 10.1155/2017/4828936]

35 **Tsukuda S**, Watashi K, Hojima T, Isogawa M, Iwamoto M, Omagari K, Suzuki R, Aizaki H, Kojima S, Sugiyama M, Saito A, Tanaka Y, Mizokami M, Sureau C, Wakita T. A new class of hepatitis B and D virus entry inhibitors, proanthocyanidin and its analogs, that directly act on the viral large surface proteins. *Hepatology* 2017; **65**: 1104-1116 [PMID: 27863453 DOI: 10.1002/hep.28952]

36 **Yan H**, Peng B, Liu Y, Xu G, He W, Ren B, Jing Z, Sui J, Li W. Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide. *J Virol* 2014; **88**: 3273-3284 [PMID: 24390325 DOI: 10.1128/JVI.03478-13]

37 **Lucifora J**, Esser K, Protzer U. Ezetimibe blocks hepatitis B virus infection after virus uptake into hepatocytes. *Antiviral Res* 2013; **97**: 195-197 [PMID: 23266293 DOI: 10.1016/j.antiviral.2012.12.008]

38 **Ko C**, Park WJ, Park S, Kim S, Windisch MP, Ryu WS. The FDA-approved drug irbesartan inhibits HBV-infection in HepG2 cells stably expressing sodium taurocholate co-transporting polypeptide. *Antivir Ther* 2015; **20**: 835-842 [PMID: 25929767 DOI: 10.3851/IMP2965]

39 **Watashi K**, Sluder A, Daito T, Matsunaga S, Ryo A, Nagamori S, Iwamoto M, Nakajima S, Tsukuda S, Borroto-Esoda K, Sugiyama M, Tanaka Y, Kanai Y, Kusuhara H, Mizokami M, Wakita T. Cyclosporin A and its analogs inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane transporter, sodium taurocholate cotransporting polypeptide (NTCP). *Hepatology* 2014; **59**: 1726-1737 [PMID: 24375637 DOI: 10.1002/hep.26982]

40 **Shimura S**, Watashi K, Fukano K, Peel M, Sluder A, Kawai F, Iwamoto M, Tsukuda S, Takeuchi JS, Miyake T, Sugiyama M, Ogasawara Y, Park SY, Tanaka Y, Kusuhara H, Mizokami M, Sureau C, Wakita T. Cyclosporin derivatives inhibit hepatitis B virus entry without interfering with NTCP transporter activity. *J Hepatol* 2017; **66**: 685-692 [PMID: 27890789 DOI: 10.1016/j.jhep.2016.11.009]

41 **Wedemeyer H**, Schöneweis K, Bogomolov PO, Chulanov V, Stepanova T, Viacheslav M, Allweiss L, Dandri M, Ciesek S, Dittmer U, Haefeli W-E, Alexandrov A, Urban S. 48 wk of high dose (10 mg) bulevirtide as monotherapy or with peginterferon alfa-2a in patients with chronic HBV/HDV coinfection. *J Hepatol* 2020; **73** Suppl 1: S52-S53 [DOI: 10.1016/S0168-8278(20)30651-6]

42 **Lucifora J**, Protzer U. Attacking hepatitis B virus cccDNA--The holy grail to hepatitis B cure. *J Hepatol* 2016; **64**: S41-S48 [PMID: 27084036 DOI: 10.1016/j.jhep.2016.02.009]

43 **Lucifora J**, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, Sprinzl MF, Koppensteiner H, Makowska Z, Volz T, Remouchamps C, Chou WM, Thasler WE, Hüser N, Durantel D, Liang TJ, Münk C, Heim MH, Browning JL, Dejardin E, Dandri M, Schindler M, Heikenwalder M, Protzer U. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science* 2014; **343**: 1221-1228 [PMID: 24557838 DOI: 10.1126/science.1243462]

44 **Bockmann JH**, Stadler D, Xia Y, Ko C, Wettengel JM, Schulze Zur Wiesch J, Dandri M, Protzer U. Comparative Analysis of the Antiviral Effects Mediated by Type I and III Interferons in Hepatitis B Virus-Infected Hepatocytes. *J Infect Dis* 2019; **220**: 567-577 [PMID: 30923817 DOI: 10.1093/infdis/jiz143]

45 **Xia Y**, Stadler D, Lucifora J, Reisinger F, Webb D, Hösel M, Michler T, Wisskirchen K, Cheng X, Zhang K, Chou WM, Wettengel JM, Malo A, Bohne F, Hoffmann D, Eyer F, Thimme R, Falk CS, Thasler WE, Heikenwalder M, Protzer U. Interferon-γ and Tumor Necrosis Factor-α Produced by T Cells Reduce the HBV Persistence Form, cccDNA, Without Cytolysis. *Gastroenterology* 2016; **150**: 194-205 [PMID: 26416327 DOI: 10.1053/j.gastro.2015.09.026]

46 **Wang L**, Zhu Q, Zeng J, Yan Z, Feng A, Young J, Gao L. PS-074-A first-in-class orally available HBV cccDNA destabilizer ccc_R08 achieved sustainable HBsAg and HBV DNA suppression in the HBV circle mouse model through elimination of cccDNA-like molecules in the mouse liver. *J Hepatol* 2019; **70** Suppl 1: e48 [DOI: 10.1016/S0618-8278(19)30086-6]

47 **Cradick TJ**, Keck K, Bradshaw S, Jamieson AC, McCaffrey AP. Zinc-finger nucleases as a novel therapeutic strategy for targeting hepatitis B virus DNAs. *Mol Ther* 2010; **18**: 947-954 [PMID: 20160705 DOI: 10.1038/mt.2010.20]

48 **Ramanan V**, Shlomai A, Cox DB, Schwartz RE, Michailidis E, Bhatta A, Scott DA, Zhang F, Rice CM, Bhatia SN. CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Sci Rep* 2015; **5**: 10833 [PMID: 26035283 DOI: 10.1038/srep10833]

49 **Chen J**, Zhang W, Lin J, Wang F, Wu M, Chen C, Zheng Y, Peng X, Li J, Yuan Z. An efficient antiviral strategy for targeting hepatitis B virus genome using transcription activator-like effector

nucleases. *Mol Ther* 2014; **22**: 303-311 [PMID: 24025750 DOI: 10.1038/mt.2013.212]
50 **Seeger C**, Sohn JA. Complete Spectrum of CRISPR/Cas9-induced Mutations on HBV cccDNA. *Mol Ther* 2016; **24**: 1258-1266 [PMID: 27203444 DOI: 10.1038/mt.2016.94]
51 **Martinez MG**, Inchauspe A, Delberghe E, Chapus F, Neveu G, Alam A, Carter K, Testoni B, Zoulim F. Targeting hepatitis B virus with CRISPR/Cas9 approach. *J Hepatol* 2020; **73** Suppl 1: S841-S842 [DOI: 10.1016/S0168-8278(20)32126-7]
52 **Murai K**, Kodama T, Hikita H, Shimoda A, Fukuaka M, Fukutomi K, Tahata Y, Makino Y, Yamada R, Sakamori R, Tatsumi T, Takehara T. Novel Anti-HBV Therapies Using CRISPR/Cas9 Targeting HBV Genome Strongly Suppress HBV. *Hepatology* 2020; **72** Suppl 1: 61A-62A [DOI: 10.1002/hep.31578]
53 **Belloni L**, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, Petersen J, Raimondo G, Dandri M, Levrero M. IFN-α inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. *J Clin Invest* 2012; **122**: 529-537 [PMID: 22251702 DOI: 10.1172/JCI58847]
54 **Cougot D**, Wu Y, Cairo S, Caramel J, Renard CA, Lévy L, Buendia MA, Neuveut C. The hepatitis B virus X protein functionally interacts with CREB-binding protein/p300 in the regulation of CREB-mediated transcription. *J Biol Chem* 2007; **282**: 4277-4287 [PMID: 17158882 DOI: 10.1074/jbc.M606774200]
55 **Gilmore S**, Tam D, Dick R, Appleby T, Birkus G, Willkom M, Delaney WE, Notte GT, Feierbach B. Antiviral activity of GS-5801, a liver-targeted prodrug of a lysine demethylase 5 inhibitor, in a hepatitis B virus primary human hepatocyte infection model. *J Hepatol* 2017; **66** Suppl 1: S690-S691 [DOI: 10.1016/S0168-8278(17)31855-X]
56 **Erken R**, Stelma F, Roy E, Sampson D, Radreau P, André P, Vonderscher J, Meldrum E, Sousa CM, Jacob E, Schreuder T, Pierrillas P, Laveille C, Scalfaro P, Reesink H. First clinical evaluation in chronic hepatitis B patients of the synthetic farnesoid X receptor agonist EYP001. *J Hepatol* 2018; **68** Suppl 1: S488-S489 [DOI: 10.1016/S0168-8278(18)31226-1]
57 **Decorsière A**, Mueller H, van Breugel PC, Abdul F, Gerossier L, Beran RK, Livingston CM, Niu C, Fletcher SP, Hantz O, Strubin M. Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. *Nature* 2016; **531**: 386-389 [PMID: 26983541 DOI: 10.1038/nature17170]
58 **Sekiba K**, Otsuka M, Ohno M, Yamagami M, Kishikawa T, Suzuki T, Ishibashi R, Seimiya T, Tanaka E, Koike K. Inhibition of HBV Transcription From cccDNA With Nitazoxanide by Targeting the HBx-DDB1 Interaction. *Cell Mol Gastroenterol Hepatol* 2019; **7**: 297-312 [PMID: 30704981 DOI: 10.1016/j.jcmgh.2018.10.010]
59 **Rossignol JF**, Bréchot C. A Pilot Clinical Trial of Nitazoxanide in the Treatment of Chronic Hepatitis B. *Hepatol Commun* 2019; **3**: 744-747 [PMID: 31168509 DOI: 10.1002/hep4.1339]
60 **Sekiba K**, Otsuka M, Ohno M, Yamagami M, Kishikawa T, Seimiya T, Suzuki T, Tanaka E, Ishibashi R, Funato K, Koike K. Pevonedistat, a Neuronal Precursor Cell-Expressed Developmentally Down-Regulated Protein 8-Activating Enzyme Inhibitor, Is a Potent Inhibitor of Hepatitis B Virus. *Hepatology* 2019; **69**: 1903-1915 [PMID: 30586159 DOI: 10.1002/hep.30491]
61 **Yu HB**, Jiang H, Cheng ST, Hu ZW, Ren JH, Chen J. AGK2, A SIRT2 Inhibitor, Inhibits Hepatitis B Virus Replication *In Vitro* And *In Vivo*. *Int J Med Sci* 2018; **15**: 1356-1364 [PMID: 30275764 DOI: 10.7150/ijms.26125]
62 **Gallay P**, Ure D, Bobardt M, Chatterji U, Ou J, Trepanier D, Foster R. The cyclophilin inhibitor CRV431 inhibits liver HBV DNA and HBsAg in transgenic mice. *PLoS One* 2019; **14**: e0217433 [PMID: 31181107 DOI: 10.1371/journal.pone.0217433]
63 **Rawal PR**, Tripathi DM, Sarin SK, Nain V, Kaur S. Targeting HBx by CRISPR/Cas9 system effectively reduced the EMT and stemness characteristics of HCC cells in vitro. *Hepatology* 2020; **72** Suppl 1: 61A
64 **Chery J**. RNA therapeutics: RNAi and antisense mechanisms and clinical applications. *Postdoc J* 2016; **4**: 35-50 [PMID: 27570789 DOI: 10.14304/surya.jpr.v4n7.5]
65 **Yuen MF**, Schiefke I, Yoon JH, Ahn SH, Heo J, Kim JH, Lik Yuen Chan H, Yoon KT, Klinker H, Manns M, Petersen J, Schluep T, Hamilton J, Given BD, Ferrari C, Lai CL, Locarnini SA, Gish RG. RNA Interference Therapy With ARC-520 Results in Prolonged Hepatitis B Surface Antigen Response in Patients With Chronic Hepatitis B Infection. *Hepatology* 2020; **72**: 19-31 [PMID: 31654573 DOI: 10.1002/hep.31008]
66 **Gane E**, Locarnini S, Lim TH, Strasser S, Sievert W, Cheng W, Thompson A, Given B, Schluep T, Hamilton J, Biermer M, Kalmeijer R, Beumont-Mauviel M, Lenz O, Cloherty G, Ka-Ho Wong D, Schwabe C, Jackson K, Ferrari C, Lai CL, Gish RG, Yuen MF. Short-term treatment with RNA interference therapy, JNJ-3989, results in sustained hepatitis B surface antigen supression in patients with chronic hepatitis B receiving nucleos(t)ide analogue treatment. *J Hepatol* 2020; **73** Suppl 1: S20 [DOI: 10.1016/S0168-8278(20)30597-3]
67 **Gane E**, Lim YS, Tangkijvanich P, O'Beirne J, Lim TH, Bakardjiev A, Ding X, Connolly L, Huang S, Kim J, Pang P, Yuen MF. Preliminary safety and antiviral activity of VIR-2218, an X-targeting HBV RNAi therapeutic, in chronic hepatitis B patients. *J Hepatol* 2020; **73** Suppl 1: S50-S51 [DOI: 10.1016/S0168-8278(20)30647-4]
68 **Streinu-Cercel A**, Gane E, Cheng W, Sievert W, Roberts S, Ahn SH, Kim YJ, Agarwal K, Niforos D, Symonds B, Mendez P. A phase 2a study evaluating the multi-dose activity of ARB-1467 in HBeAg positive and negative virally suppressed subjects with hepatitis B. *J Hepatol* 2017; **66** Suppl 1: S688-S689 [DOI: 10.1016/S0168-8278(17)31850-0]

69 **Yuen MF**, Berliba E, Kim YJ, Holmes JA, Lim Y-S, Strasser SI, Schwabe C, Jucov A, Lee ACH, Thi EP, Harasym T, Pamulapati GR, Wattamwar P, Kunta J, Sofia M, Sevinsky H, Gray K, Eley T, Picchio G, Sims KD, Gane E. Safety and Pharmacodynamics of the GalNAc-siRNA AB-729 in subjects with Chronic Hepatitis B infection. *Hepatology* 2020; **72** Suppl 1: 62A-63A

70 **Yuen MF**, Lim TH, Kim W, Tongkijvonich P, Yoon JH, Sievert W, Sukeepoisornjoroen W, Thompson A, Schwabe C, Brown B, Achnecl H, Gane E. HBV RNAi inhibitor RG6346 in Phase 1b-2a trial was safe, well-tolerated, and resulted in substantial and durable reductions in serum HBsAg levels. Late breaking abstract to the AASLD's The Liver Meeting® Digital Experience™ 2020; American Association for the Study of Liver Diseases, 2020: 14

71 **Han K**, Cremer J, Elston R, Oliver S, Baptiste-Brown S, Chen S, Gardiner D, Davies M, Saunders J, Hamatake R, Losos J, Leivers M, Hood S, van der Berg F, Paff M, Ritter JM, Theodore D. A Randomized, Double-Blind, Placebo-Controlled, First-Time-in-Human Study to Assess the Safety, Tolerability, and Pharmacokinetics of Single and Multiple Ascending Doses of GSK3389404 in Healthy Subjects. *Clin Pharmacol Drug Dev* 2019; **8**: 790-801 [PMID: 30861337 DOI: 10.1002/cpdd.670]

72 **Yuen MF**, Heo J, Jang JW, Yoon JH, Kweon YO, Park SJ, Bennett CF, Kwoh J. AS067-Hepatitis B virus (HBV) surface antigen (HBsAg) inhibition with isis 505358 in chronic hepatitis B (CHB) patients on stable nucleos (t)ide analogue (NA) regimen and in NA -naive CHB patients: phase 2a, randomized, double-blind, placebo-controlled study. *J Hepatol* 2020; **73** Suppl 1: S49-S50 [DOI: 10.1016/S0168-8278(20)30646-2]

73 **You S**, Yates P, Elston R, Tao Y, Paff M, Theodore D. Short-term therapy with GSK3228836 in chronic hepatitis B (CHB) patients results in reductions in HBcrAg and HBV RNA: Phase 2a, randomized, double-blind, placebo-controlled study. Late breaking abstract to the AASLD's The Liver Meeting® Digital Experience™ 2020; American Association for the Study of Liver Diseases, 2020: 52

74 **Hong J**, Tan H, Lin T-I, Kang H, Nie Y, Bhattacharya A, Rajendra P, Blatt LM, Symons JA, Beigelman LN. Combination of Antisense Oligonucleotides (ASOS) ALG-020572 and ALG-020576 against Hepatitis B Virus (HBV) improves activity and can be combined with other anti-HBV agents. *Hepatology* 2020; **72** Suppl 1: 63A

75 **Yuen MF**, Gane E, Kim DJ, Chan H, Surujbally B, Pavlonic V, Triyatni M, Grippo J, Kim HJ, Leerapun A, Lim TH, Lim YS, Tanwandee T, Kim W, Cheng W, HuTH, Wat C. RO7062931 antisense oligonucleotide phase 1 study demonstrates target engagement in patients with chronic hepatitis B on established nucleos(t)ide therapy. *J Hepatol* 2020; **73** Suppl 1: S51 [DOI: 10.1016/S0168-8278(20)30648-6]

76 **Cortese MF**, Garcia-Garcia S, Casillas R, Lopez-Martinez R, Ruiz BP, Sopena S, Tabernero D, Ferrer-Costa RM, Barciela MR, Buti Ferret MA, Rodriguez-Frías F. Gene Silencing by GAPMERS: A New Therapeutic Tool in HBV Infection. *Hepatology* 2020; **72** Suppl 1: 504A-505A [DOI: 10.1002/hep.31579]

77 **Mueller H**, Wildum S, Luangsay S, Walther J, Lopez A, Tropberger P, Ottaviani G, Lu W, Parrott NJ, Zhang JD, Schmucki R, Racek T, Hoflack JC, Kueng E, Point F, Zhou X, Steiner G, Lütgehetmann M, Rapp G, Volz T, Dandri M, Yang S, Young JAT, Javanbakht H. A novel orally available small molecule that inhibits hepatitis B virus expression. *J Hepatol* 2018; **68**: 412-420 [PMID: 29079285 DOI: 10.1016/j.jhep.2017.10.014]

78 **Zhou T**, Block T, Liu F, Kondratowicz AS, Sun L, Rawat S, Branson J, Guo F, Steuer HM, Liang H, Bailey L, Moore C, Wang X, Cuconatti A, Gao M, Lee ACH, Harasym T, Chiu T, Gotchev D, Dorsey B, Rijnbrand R, Sofia MJ. HBsAg mRNA degradation induced by a dihydroquinolizinone compound depends on the HBV posttranscriptional regulatory element. *Antiviral Res* 2018; **149**: 191-201 [PMID: 29133129 DOI: 10.1016/j.antiviral.2017.11.009]

79 **Zlotnick A**, Venkatakrishnan B, Tan Z, Lewellyn E, Turner W, Francis S. Core protein: A pleiotropic keystone in the HBV lifecycle. *Antiviral Res* 2015; **121**: 82-93 [PMID: 26129969 DOI: 10.1016/j.antiviral.2015.06.020]

80 **Zhang X**, Cheng J, Ma J, Hu Z, Wu S, Hwang N, Kulp J, Du Y, Guo JT, Chang J. Discovery of Novel Hepatitis B Virus Nucleocapsid Assembly Inhibitors. *ACS Infect Dis* 2019; **5**: 759-768 [PMID: 30525438 DOI: 10.1021/acsinfecdis.8b00269]

81 **Guo F**, Zhao Q, Sheraz M, Cheng J, Qi Y, Su Q, Cuconati A, Wei L, Du Y, Li W, Chang J, Guo JT. HBV core protein allosteric modulators differentially alter cccDNA biosynthesis from de novo infection and intracellular amplification pathways. *PLoS Pathog* 2017; **13**: e1006658 [PMID: 28945802 DOI: 10.1371/journal.ppat.1006658]

82 **Gane E**, Yuen MF, Bo Q, Schwabe C, Tanwandee T, Das S, Jin Y, Gao L, Zhou X, Wang Y, Feng S, Meinel D, Zhu M. FRI-219-RO7049389, a core protein allosteric modulator, demonstrates robust decline in HBV DNA and HBV RNA in chronic HBV infected patients. *J Hepatol* 2019; **70** Suppl 1: e491 [DOI: 10.1016/S0618-8278(19)30964-8]

83 **Zhao N**, Jia B, Zhao H, Xu J, Sheng X, Luo L, Huang Z, Wang X, Ren Q, Zhang Y, Zhao X, Cui Y. A First-in-Human Trial of GLS4, a Novel Inhibitor of Hepatitis B Virus Capsid Assembly, following Single- and Multiple-Ascending-Oral-Dose Studies with or without Ritonavir in Healthy Adult Volunteers. *Antimicrob Agents Chemother* 2019; **64**: e01686-19 [PMID: 31636065 DOI: 10.1128/AAC.01686-19]

84 **Tai Z**, Tian Q, Zhao X, Xie J, Lu Y, Tan Y, Zhao W, Ma X, Yuan X, Song H, Xue T, Wang J. Discovery of KL060332, a potential best-in-class capsid inhibitor. *Hepatology* 2020; **72** Suppl 1:

504A

85 **Yuen MF**, Gane EJ, Kim DJ, Weilert F, Yuen Chan HL, Lalezari J, Hwang SG, Nguyen T, Flores O, Hartman G, Liaw S, Lenz O, Kakuda TN, Talloen W, Schwabe C, Klumpp K, Brown N. Antiviral Activity, Safety, and Pharmacokinetics of Capsid Assembly Modulator NVR 3-778 in Patients with Chronic HBV Infection. *Gastroenterology* 2019; **156**: 1392-1403. e7 [PMID: 30625297 DOI: 10.1053/j.gastro.2018.12.023]

86 **Berke JM**, Dehertogh P, Vergauwen K, Mostmans W, Vandyck K, Raboisson P, Pauwels F. Antiviral Properties and Mechanism of Action Studies of the Hepatitis B Virus Capsid Assembly Modulator JNJ-56136379. *Antimicrob Agents Chemother* 2020; **64**: e02439-19 [PMID: 32094138 DOI: 10.1128/AAC.02439-19]

87 **Janssen H**, Hou J, Asselah T, Chan H, Zoulim F, Tanaka Y, Janczewska E, Nahass R, Bourgeois S, Buti M, Lampertico P, Lenz O, Verbinnen T, Vandenbossche JJ, Talloen W, Biermer M, Kalmeijer R, Beumont-Mauviel M, Shukla U. LBP12-Efficacy and safety results of the phase 2 JNJ-56136379 JADE study in patients with chronic hepatitis B: Interim week 24 data. *J Hepatol* 2020; **73** Suppl 1: S129-S130 [DOI: 10.1016/S0168-8278(20)30773-X]

88 **Fung S**, Sulkowski M, Lalezari J, Schiff ER, Dieterich D, Hassanein T, Kwo P, Elkhashab M, Nahass R, Ayoub W, Han SH, Bonacini M, Alves K, Zayed Hany, Huang Q, Colonno R, Knox S, Ramji A, Bennett M, Gane E, Ravendhran N, Park J, Jacobson I, Bae H, Chan S, Hann HW, Ma X, Nguyen T, Yuen MF. Antiviral activity and safety of the hepatitis B core inhibitor ABIH0731 administered with a nucleos(t)ide reverse transcriptase inhibitor in patients with HBeAg-negative chronic hepatitis B infection. *J Hepatol* 2020; **73** Suppl 1: S51-S52 [DOI: 10.1016/S0168-8278(20)30649-8]

89 **Yuen MF**, Agarwal K, Ma X, Nguyen T, Schiff ER, Hann HW, Dieterich D, Nahass R, Park J, Chan S, Han HS, Gane E, Bennett M, Alves K, Zayed H, Huang Q, Colonno R, Knox S, Stamm L, Bonacini M, Jacobson I, Ayoub W, Weilert F, Ravendhran N, Ramji A, Kwo P, Elkhashab M, Hassanein T, Bae H, Lalezari J, Fung S, Sulkowski M. Antiviral activity and safety of the hepatitis B core inhibitor ABIH0731 administered with a nucleos(t)ide reverse transcriptase inhibitor in patients with HBeAg-positive chronic hepatitis B infection in a long-term extension study. *J Hepatol* 2020; **73** Suppl 1: S140 [DOI: 10.1016/S0168-8278(20)30790-X]

90 **Agarwal K**, Niu J, Ding Y, Gane E, Nguyen T, Alves K, Evanchick M, Zayed H, Huang Q, Knox S, Stamm L, Colonno R, Hassanein T, Kim DJ, Lim YS, Yuen MF. Antiviral activity, pharmacokinetics and safety of the secondgeneration hepatitis B core inhibitor ABI-H2158 in Phase 1b study of patients with HBeAg-positive chronic hepatitis B infection. *J Hepatol* 2020; **73** Suppl 1: S125 [DOI: 10.1016/S0168-8278(20)30767-4]

91 **Ganem D**, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118-1129 [PMID: 15014185 DOI: 10.1056/NEJMra031087]

92 **Real CI**, Werner M, Paul A, Gerken G, Schlaak JF, Vaillant A, Broering R. Nucleic acid-based polymers effective against hepatitis B Virus infection in patients don't harbor immunostimulatory properties in primary isolated liver cells. *Sci Rep* 2017; **7**: 43838 [PMID: 28272460 DOI: 10.1038/srep43838]

93 **Bazinet M**, Anderson M, Pântea V, Placinta G, Moscalu I, Cebotarescu V, Cojuhari L, Jimbei P, Iarovoi L, Smesnoi V, Musteata T, Jucov A, Gersch J, Holzmayer V, Kuhns M, Cloherty G, Vaillant A. Analysis of HbsAg isoforms during and after NAP-based combination therapy in the REP 301, REP 301-LTF and REP 401 studies. *Hepatology* 2020; **72** Suppl 1: 500A-501A

94 **Nie Y**, Tan H, Kao C, Ren S, Pandey R, Chanda S, Blatt L, Beigelman LN, Symons JA, Hong J. S-Antigen Traffic-Inhibiting Oligonucleotide Polymers (STOPs) Effectively Inhibit Hepatitis B Surface Antigen (HbsAg) Secretion in Both Hepatitis B Virus (HBV) Cell Lines and HBV Infected Cells. *Hepatology* 2020; **72** Suppl 1: 502A-503A

95 **Bazinet M**, Pântea V, Placinta G, Moscalu I, Cebotarescu V, Cojuhari L, Jimbei P, Iarovoi L, Smesnoi V, Musteata T, Jucov A, Dittmer U, Krawczyk A, Vaillant A. Safety and Efficacy of 48 Weeks REP 2139 or REP 2165, Tenofovir Disoproxil, and Pegylated Interferon Alfa-2a in Patients with Chronic HBV Infection Naïve to Nucleos(t)ide Therapy. *Gastroenterology* 2020; **158**: 2180-2194 [PMID: 32147484 DOI: 10.1053/j.gastro.2020.02.058]

96 **Gohil V**, Misner D, Chanda S, Zhang Q, Liu J, Hong J, Pandey R, Rajwanshi VK, Williams C, Yogaratnam J, Fry J, Smith DB, Symons JA, Blatt LM, Beigelman LN, Lin TI. The S-Antigen Transport-Inhibiting Oligonucleotide Polymer (STOPS™) ALG-010133 Demonstrates a Favorable Preclinical Profile for the Treatment of Chronic Hepatitis B. *Hepatology* 2020; **72** Suppl 1: 508A

97 **Thimme R**, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH, Chisari FV. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* 2003; **77**: 68-76 [PMID: 12477811 DOI: 10.1128/jvi.77.1.68-76.2003]

98 **Asabe S**, Wieland SF, Chattopadhyay PK, Roederer M, Engle RE, Purcell RH, Chisari FV. The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. *J Virol* 2009; **83**: 9652-9662 [PMID: 19625407 DOI: 10.1128/JVI.00867-09]

99 **Paul S**, Dickstein A, Saxena A, Terrin N, Viveiros K, Balk EM, Wong JB. Role of surface antibody in hepatitis B reactivation in patients with resolved infection and hematologic malignancy: A meta-analysis. *Hepatology* 2017; **66**: 379-388 [PMID: 28128861 DOI: 10.1002/hep.29082]

100 **Liaw YF**, Chen YC, Sheen IS, Chien RN, Yeh CT, Chu CM. Impact of acute hepatitis C virus superinfection in patients with chronic hepatitis B virus infection. *Gastroenterology* 2004; **126**: 1024-1029 [PMID: 15057742 DOI: 10.1053/j.gastro.2004.01.011]

101 **Zhang Y**, Liang C. Innate recognition of microbial-derived signals in immunity and inflammation. *Sci China Life Sci* 2016; **59**: 1210-1217 [PMID: 27888386 DOI: 10.1007/s11427-016-0325-6]

102 **Moresco EM**, LaVine D, Beutler B. Toll-like receptors. *Curr Biol* 2011; **21**: R488-R493 [PMID: 21741580 DOI: 10.1016/j.cub.2011.05.039]

103 **Williams JB**, Hüppner A, Mulrooney-Cousins PM, Michalak TI. Differential Expression of Woodchuck Toll-Like Receptors 1-10 in Distinct Forms of Infection and Stages of Hepatitis in Experimental Hepatitis B Virus Infection. *Front Microbiol* 2018; **9**: 3007 [PMID: 30581424 DOI: 10.3389/fmicb.2018.03007]

104 **Chen Z**, Cheng Y, Xu Y, Liao J, Zhang X, Hu Y, Zhang Q, Wang J, Zhang Z, Shen F, Yuan Z. Expression profiles and function of Toll-like receptors 2 and 4 in peripheral blood mononuclear cells of chronic hepatitis B patients. *Clin Immunol* 2008; **128**: 400-408 [PMID: 18565796 DOI: 10.1016/j.clim.2008.04.006]

105 **Riordan SM**, Skinner N, Kurtovic J, Locarnini S, Visvanathan K. Reduced expression of toll-like receptor 2 on peripheral monocytes in patients with chronic hepatitis B. *Clin Vaccine Immunol* 2006; **13**: 972-974 [PMID: 16894001 DOI: 10.1128/CVI.00396-05]

106 **Vincent IE**, Zannetti C, Lucifora J, Norder H, Protzer U, Hainaut P, Zoulim F, Tommasino M, Trépo C, Hasan U, Chemin I. Hepatitis B virus impairs TLR9 expression and function in plasmacytoid dendritic cells. *PLoS One* 2011; **6**: e26315 [PMID: 22046272 DOI: 10.1371/journal.pone.0026315]

107 **Wu J**, Lu M, Meng Z, Trippler M, Broering R, Szczeponek A, Krux F, Dittmer U, Roggendorf M, Gerken G, Schlaak JF. Toll-like receptor-mediated control of HBV replication by nonparenchymal liver cells in mice. *Hepatology* 2007; **46**: 1769-1778 [PMID: 17929296 DOI: 10.1002/hep.21897]

108 **Zhang X**, Ma Z, Liu H, Liu J, Meng Z, Broering R, Yang D, Schlaak JF, Roggendorf M, Lu M. Role of Toll-like receptor 2 in the immune response against hepadnaviral infection. *J Hepatol* 2012; **57**: 522-528 [PMID: 22617154 DOI: 10.1016/j.jhep.2012.05.004]

109 **Lanford RE**, Guerra B, Chavez D, Giavedoni L, Hodara VL, Brasky KM, Fosdick A, Frey CR, Zheng J, Wolfgang G, Halcomb RL, Tumas DB. GS-9620, an oral agonist of Toll-like receptor-7, induces prolonged suppression of hepatitis B virus in chronically infected chimpanzees. *Gastroenterology* 2013; **144**: 1508-1517, 1517.e1-1517. 10 [PMID: 23415804 DOI: 10.1053/j.gastro.2013.02.003]

110 **Isogawa M**, Robek MD, Furuichi Y, Chisari FV. Toll-like receptor signaling inhibits hepatitis B virus replication in vivo. *J Virol* 2005; **79**: 7269-7272 [PMID: 15890966 DOI: 10.1128/JVI.79.11.7269-7272.2005]

111 **Menne S**, Tumas DB, Liu KH, Thampi L, AlDeghaither D, Baldwin BH, Bellezza CA, Cote PJ, Zheng J, Halcomb R, Fosdick A, Fletcher SP, Daffis S, Li L, Yue P, Wolfgang GH, Tennant BC. Sustained efficacy and seroconversion with the Toll-like receptor 7 agonist GS-9620 in the Woodchuck model of chronic hepatitis B. *J Hepatol* 2015; **62**: 1237-1245 [PMID: 25559326 DOI: 10.1016/j.jhep.2014.12.026]

112 **Janssen HLA**, Brunetto MR, Kim YJ, Ferrari C, Massetto B, Nguyen AH, Joshi A, Woo J, Lau AH, Gaggar A, Subramanian GM, Yoshida EM, Ahn SH, Tsai NCS, Fung S, Gane EJ. Safety, efficacy and pharmacodynamics of vesatolimod (GS-9620) in virally suppressed patients with chronic hepatitis B. *J Hepatol* 2018; **68**: 431-440 [PMID: 29104121 DOI: 10.1016/j.jhep.2017.10.027]

113 **Boni C**, Vecchi A, Rossi M, Laccabue D, Giuberti T, Alfieri A, Lampertico P, Grossi G, Facchetti F, Brunetto MR, Coco B, Cavallone D, Mangia A, Santoro R, Piazzolla V, Lau A, Gaggar A, Subramanian GM, Ferrari C. TLR7 Agonist Increases Responses of Hepatitis B Virus-Specific T Cells and Natural Killer Cells in Patients With Chronic Hepatitis B Treated With Nucleos(T)Ide Analogues. *Gastroenterology* 2018; **154**: 1764-1777. e7 [PMID: 29378197 DOI: 10.1053/j.gastro.2018.01.030]

114 **Bertoletti A**, Le Bert N. Fine-Tuning TLR-7-Based Therapy for Functional HBV Cure. *Hepatol Commun* 2019; **3**: 1289-1292 [PMID: 31592063 DOI: 10.1002/hep4.1420]

115 **Korolowizc KE**, Li B, Huang X, Yon C, Rodrigo E, Corpuz M, Plouffe DM, Kallakury BV, Suresh M, Wu TY, Miller AT, Menne S. Liver-Targeted Toll-Like Receptor 7 Agonist Combined With Entecavir Promotes a Functional Cure in the Woodchuck Model of Hepatitis B Virus. *Hepatol Commun* 2019; **3**: 1296-1310 [PMID: 31592075 DOI: 10.1002/hep4.1397]

116 **Luk A**, Jiang Q, Glavini K, Triyatni M, Zhao N, Racek T, Zhu Y, Grippo JF. A Single and Multiple Ascending Dose Study of Toll-Like Receptor 7 Agonist (RO7020531) in Chinese Healthy Volunteers. *Clin Transl Sci* 2020; **13**: 985-993 [PMID: 32268000 DOI: 10.1111/cts.12791]

117 **Wu L**, Niu J, Gane EJ, Slaets L, Creus AD, Ding Y, Schwabe C, Goeyvaerts N, Xu Z, Huo D, Remoortere PV, Schwertschlag U, Vandenbossche JJ. Population pharmacokinetic/pharmacodynamic (PK/PD) models of JNJ-64794964, a toll-like receptor (TLR)-7 agonist in healthy adult subjects. *Hepatology* 2020; **72** Suppl 1: 506A

118 **Hu Y**, Tang L, Zhu Z, Meng H, Chen T, Zhao S, Jin Z, Wang Z, Jin G. A novel TLR7 agonist as adjuvant to stimulate high quality HBsAg-specific immune responses in an HBV mouse model. *J Transl Med* 2020; **18**: 112 [PMID: 32131853 DOI: 10.1186/s12967-020-02275-2]

119 **Gorden KB**, Gorski KS, Gibson SJ, Kedl RM, Kieper WC, Qiu X, Tomai MA, Alkan SS, Vasilakos JP. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J Immunol* 2005; **174**: 1259-1268 [PMID: 15661881 DOI: 10.4049/jimmunol.174.3.1259]

120 **Daffis S**, Balsitis S, Chamberlain J, Zheng J, Santos R, Rowe W, Ramakrishnan D, Pattabiraman D, Spurlock S, Chu R, Kang D, Mish M, Ramirez R, Li L, Li B, Ma S, Hung M, Voitenleitner C, Yon

C, Suresh M, Menne S, Cote P, Delaney WE 4th, Mackman R, Fletcher SP. Toll-Like Receptor 8 Agonist GS-9688 Induces Sustained Efficacy in the Woodchuck Model of Chronic Hepatitis B. *Hepatology* 2021; **73**: 53-67 [PMID: 32246499 DOI: 10.1002/hep.31255]

121 **Mackman RL**, Mish M, Chin G, Perry JK, Appleby T, Aktoudianakis V, Metobo S, Pyun P, Niu C, Daffis S, Yu H, Zheng J, Villasenor AG, Zablocki J, Chamberlain J, Jin H, Lee G, Suekawa-Pirrone K, Santos R, Delaney WE 4th, Fletcher SP. Discovery of GS-9688 (Selgantolimod) as a Potent and Selective Oral Toll-Like Receptor 8 Agonist for the Treatment of Chronic Hepatitis B. *J Med Chem* 2020; **63**: 10188-10203 [PMID: 32407112 DOI: 10.1021/acs.jmedchem.0c00100]

122 **Janssen HL**, Lim YS, Kim HJ, Tseng CH, Coffin CS, Elkhashab M, Anh SH, Nguyen AH, Chen D, Wallin JJ, Tan SK, Yang JC, Gaggar A, Brainard DM, Fung S, Kim YJ, Kao JH, Chuang WL. Safety and efficacy of 24 wk treatment with oral TLR8 agonist, Selgantolimod, in viremic adult patients with chronic Hepatitis B: a phase 2 study. *Hepatology* 2020; **72** Suppl 1: 506A-507A

123 **Baum P**, Odegard V. ASGR1-TLR8, an ASGR1-directed TLR8 Immunotac(TM) therapeutic, is a potent myeloid cell agonist with liver-localized activity for the treatment of chronic HBV. *Hepatology* 2020; **72** Suppl 1: 503A

124 **Rehwinkel J**, Gack MU. RIG-I-like receptors: their regulation and roles in RNA sensing. *Nat Rev Immunol* 2020; **20**: 537-551 [PMID: 32203325 DOI: 10.1038/s41577-020-0288-3]

125 **Motta V**, Soares F, Sun T, Philpott DJ. NOD-like receptors: versatile cytosolic sentinels. *Physiol Rev* 2015; **95**: 149-178 [PMID: 25540141 DOI: 10.1152/physrev.00009.2014]

126 **Hou Z**, Zhang J, Han Q, Su C, Qu J, Xu D, Zhang C, Tian Z. Hepatitis B virus inhibits intrinsic RIG-I and RIG-G immune signaling *via* inducing miR146a. *Sci Rep* 2016; **6**: 26150 [PMID: 27210312 DOI: 10.1038/srep26150]

127 **Kanneganti TD**. Central roles of NLRs and inflammasomes in viral infection. *Nat Rev Immunol* 2010; **10**: 688-698 [PMID: 20847744 DOI: 10.1038/nri2851]

128 **Korolowicz KE**, Iyer RP, Czerwinski S, Suresh M, Yang J, Padmanabhan S, Sheri A, Pandey RK, Skell J, Marquis JK, Kallakury BV, Tucker RD, Menne S. Antiviral Efficacy and Host Innate Immunity Associated with SB 9200 Treatment in the Woodchuck Model of Chronic Hepatitis B. *PLoS One* 2016; **11**: e0161313 [PMID: 27552102 DOI: 10.1371/journal.pone.0161313]

129 **Yuen R**, Chen C-Y, Liu C-J, Jeng RW-J, Elkhashab M, Coffin C, Kim W, Greenbloom S, Ramji A, Lim YS, Kim YJ, Fung S, Kim DJ, Jang J, Lee KS, Afdhal N, Lyer R, Macfarlane C, Jackson aka, Locarnini S, Chan H. Ascending dose cohort study of inarigivir-A novel RIG I agonist in chronic HBV patients: Final results of the ACHIEVE trial. *J Hepatol* 2019; **70** Suppl 1: e47-e48 [DOI: 10.1016/S0618-8278(19)30084-2]

130 **Tao J**, Zhou X, Jiang Z. cGAS-cGAMP-STING: The three musketeers of cytosolic DNA sensing and signaling. *IUBMB Life* 2016; **68**: 858-870 [PMID: 27706894 DOI: 10.1002/iub.1566]

131 **Ito H**, Kanbe A, Hara A, Ishikawa T. Induction of humoral and cellular immune response to HBV vaccine can be up-regulated by STING ligand. *Virology* 2019; **531**: 233-239 [PMID: 30928701 DOI: 10.1016/j.virol.2019.03.013]

132 **Guo F**, Han Y, Zhao X, Wang J, Liu F, Xu C, Wei L, Jiang JD, Block TM, Guo JT, Chang J. STING agonists induce an innate antiviral immune response against hepatitis B virus. *Antimicrob Agents Chemother* 2015; **59**: 1273-1281 [PMID: 25512416 DOI: 10.1128/AAC.04321-14]

133 **Zhu J**, Paul WE. Heterogeneity and plasticity of T helper cells. *Cell Res* 2010; **20**: 4-12 [PMID: 20010916 DOI: 10.1038/cr.2009.138]

134 **Sakaguchi S**, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008; **133**: 775-787 [PMID: 18510923 DOI: 10.1016/j.cell.2008.05.009]

135 **Ye B**, Liu X, Li X, Kong H, Tian L, Chen Y. T-cell exhaustion in chronic hepatitis B infection: current knowledge and clinical significance. *Cell Death Dis* 2015; **6**: e1694 [PMID: 25789969 DOI: 10.1038/cddis.2015.42]

136 **Dong Y**, Li X, Zhang L, Zhu Q, Chen C, Bao J, Chen Y. $CD4^+$ T cell exhaustion revealed by high PD-1 and LAG-3 expression and the loss of helper T cell function in chronic hepatitis B. *BMC Immunol* 2019; **20**: 27 [PMID: 31390978 DOI: 10.1186/s12865-019-0309-9]

137 **McLane LM**, Abdel-Hakeem MS, Wherry EJ. CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. *Annu Rev Immunol* 2019; **37**: 457-495 [PMID: 30676822 DOI: 10.1146/annurev-immunol-041015-055318]

138 **Lopes AR**, Kellam P, Das A, Dunn C, Kwan A, Turner J, Peppa D, Gilson RJ, Gehring A, Bertoletti A, Maini MK. Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection. *J Clin Invest* 2008; **118**: 1835-1845 [PMID: 18398508 DOI: 10.1172/JCI33402]

139 **Peppa D**, Gill US, Reynolds G, Easom NJ, Pallett LJ, Schurich A, Micco L, Nebbia G, Singh HD, Adams DH, Kennedy PT, Maini MK. Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. *J Exp Med* 2013; **210**: 99-114 [PMID: 23254287 DOI: 10.1084/jem.20121172]

140 **Thaventhiran T**, Sethu S, Yeang H, Al-Huseini L, Hamdam J, Sathish J. T cell co-inhibitory receptors-functions and signalling mechanisms. *J Clin Cell Immunol* 2012; **S12**: 004 [DOI: 10.4172/2155-9899.S12-004]

141 **Lau GK**, Lok AS, Liang RH, Lai CL, Chiu EK, Lau YL, Lam SK. Clearance of hepatitis B surface antigen after bone marrow transplantation: role of adoptive immunity transfer. *Hepatology* 1997; **25**: 1497-1501 [PMID: 9185774 DOI: 10.1002/hep.510250631]

142 **Bohne F**, Chmielewski M, Ebert G, Wiegmann K, Kürschner T, Schulze A, Urban S, Krönke M, Abken H, Protzer U. T cells redirected against hepatitis B virus surface proteins eliminate infected

hepatocytes. *Gastroenterology* 2008; **134**: 239-247 [PMID: 18166356 DOI: 10.1053/j.gastro.2007.11.002]

143 **Gehring AJ**, Xue SA, Ho ZZ, Teoh D, Ruedl C, Chia A, Koh S, Lim SG, Maini MK, Stauss H, Bertoletti A. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. *J Hepatol* 2011; **55**: 103-110 [PMID: 21145860 DOI: 10.1016/j.jhep.2010.10.025]

144 **Tan AT**, Schreiber S. Adoptive T-cell therapy for HBV-associated HCC and HBV infection. *Antiviral Res* 2020; **176**: 104748 [PMID: 32087191 DOI: 10.1016/j.antiviral.2020.104748]

145 **Festag MM**, Festag J, Fräßle SP, Asen T, Sacherl J, Schreiber S, Mück-Häusl MA, Busch DH, Wisskirchen K, Protzer U. Evaluation of a Fully Human, Hepatitis B Virus-Specific Chimeric Antigen Receptor in an Immunocompetent Mouse Model. *Mol Ther* 2019; **27**: 947-959 [PMID: 30852138 DOI: 10.1016/j.ymthe.2019.02.001]

146 **Wisskirchen K**, Kah J, Malo A, Asen T, Volz T, Allweiss L, Wettengel JM, Lütgehetmann M, Urban S, Bauer T, Dandri M, Protzer U. T cell receptor grafting allows virological control of Hepatitis B virus infection. *J Clin Invest* 2019; **129**: 2932-2945 [PMID: 31039136 DOI: 10.1172/JCI120228]

147 **Morgan RA**, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, Dudley ME, Feldman SA, Yang JC, Sherry RM, Phan GQ, Hughes MS, Kammula US, Miller AD, Hessman CJ, Stewart AA, Restifo NP, Quezado MM, Alimchandani M, Rosenberg AZ, Nath A, Wang T, Bielekova B, Wuest SC, Akula N, McMahon FJ, Wilde S, Mosetter B, Schendel DJ, Laurencot CM, Rosenberg SA. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother* 2013; **36**: 133-151 [PMID: 23377668 DOI: 10.1097/CJI.0b013e3182829903]

148 **Johnson LA**, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CC, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM, Rosenberg SA. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 2009; **114**: 535-546 [PMID: 19451549 DOI: 10.1182/blood-2009-03-211714]

149 **Bertoletti A**, Tan AT. Challenges of CAR- and TCR-T cell-based therapy for chronic infections. *J Exp Med* 2020; **217** [PMID: 32163104 DOI: 10.1084/jem.20191663]

150 **Fergusson JR**, Wallace Z, Connolly MM, Woon AP, Suckling RJ, Hine DW, Barber C, Bunjobpol W, Choi BS, Crespillo S, Dembek M, Dieckmann N, Donoso J, Godinho LF, Grant T, Howe D, McCully ML, Perot C, Sarkar A, Seifert FU, Singh PK, Stegmann KA, Turner B, Verma A, Walker A, Leonard S, Maini MK, Wiederhold K, Dorrell L, Simmons R, Knox A. Immune-Mobilizing Monoclonal T Cell Receptors Mediate Specific and Rapid Elimination of Hepatitis B-Infected Cells. *Hepatology* 2020; **72**: 1528-1540 [PMID: 32770836 DOI: 10.1002/hep.31503]

151 **Leonard S**, Paterson R, Godinho L, Howe D, Monteiro M, Hague RM, Atkin K, Sarkar A, Suckling R, Bunjobpol W, Kay D, Grant T, Perot C, Knox A. Novel HLA-E specific ImmTAVR molecules for the treatment of Hepatitis B. *Hepatology* 2020; **72** Suppl 1: 59A-60A

152 **Fuertes Marraco SA**, Neubert NJ, Verdeil G, Speiser DE. Inhibitory Receptors Beyond T Cell Exhaustion. *Front Immunol* 2015; **6**: 310 [PMID: 26167163 DOI: 10.3389/fimmu.2015.00310]

153 **Johnson DB**, Peng C, Sosman JA. Nivolumab in melanoma: latest evidence and clinical potential. *Ther Adv Med Oncol* 2015; **7**: 97-106 [PMID: 25755682 DOI: 10.1177/1758834014567469]

154 **Sundar R**, Cho BC, Brahmer JR, Soo RA. Nivolumab in NSCLC: latest evidence and clinical potential. *Ther Adv Med Oncol* 2015; **7**: 85-96 [PMID: 25755681 DOI: 10.1177/1758834014567470]

155 **Gane E**, Verdon DJ, Brooks AE, Gaggar A, Nguyen AH, Subramanian GM, Schwabe C, Dunbar PR. Anti-PD-1 blockade with nivolumab with and without therapeutic vaccination for virally suppressed chronic hepatitis B: A pilot study. *J Hepatol* 2019; **71**: 900-907 [PMID: 31306680 DOI: 10.1016/j.jhep.2019.06.028]

156 **Zhang F**, Wei H, Wang X, Bai Y, Wang P, Wu J, Jiang X, Wang Y, Cai H, Xu T, Zhou A. Structural basis of a novel PD-L1 nanobody for immune checkpoint blockade. *Cell Discov* 2017; **3**: 17004 [PMID: 28280600 DOI: 10.1038/celldisc.2017.4]

157 **Yu X**, Zheng Y, Mao R, Su Z, Zhang J. BTLA/HVEM Signaling: Milestones in Research and Role in Chronic Hepatitis B Virus Infection. *Front Immunol* 2019; **10**: 617 [PMID: 30984188 DOI: 10.3389/fimmu.2019.00617]

158 **Hasanpourghadi M**, Luber A, Magowan C, Zhou X, Ertl H. Glycoprotein D, a checkpoint inhibitor of early T cell activation, broadens HBV T cell antigen recognition and produces sustained antiviral declines in preclinical studiesa. *Hepatology* 2020; **72** Suppl 1: 505A

159 **Zhang XY**, Huang KY, Zhu W, Zhang Ge, Chen JH, Huang X, Liu HY, Zhai YF, Yang DJ, Hou JL. Targeting inhibitor of apoptosis proteins (IAPs) enhances intrahepatic antiviral immunity to clear hepatitis B virus infection in vivo. *J Hepatol* 2020; **73** Suppl 1: S5-S6 [DOI: 10.1016/S0168-8278(20)30572-9]

160 **Stasi C**, Silvestri C, Voller F. Hepatitis B vaccination and immunotherapies: an update. *Clin Exp Vaccine Res* 2020; **9**: 1-7 [PMID: 32095435 DOI: 10.7774/cevr.2020.9.1.1]

161 **Vandepapelière P**, Lau GK, Leroux-Roels G, Horsmans Y, Gane E, Tawandee T, Merican MI, Win KM, Trepo C, Cooksley G, Wettendorff M, Ferrari C; Therapeutic HBV Vaccine Group of Investigators. Therapeutic vaccination of chronic hepatitis B patients with virus suppression by antiviral therapy: a randomized, controlled study of co-administration of HBsAg/AS02 candidate

vaccine and lamivudine. *Vaccine* 2007; **25**: 8585-8597 [PMID: 18031872 DOI: 10.1016/j.vaccine.2007.09.072]

162 **Pulendran B**, Ahmed R. Immunological mechanisms of vaccination. *Nat Immunol* 2011; **12**: 509-517 [PMID: 21739679 DOI: 10.1038/ni.2039]

163 **Kosinska AD**, Bauer T, Protzer U. Therapeutic vaccination for chronic hepatitis B. *Curr Opin Virol* 2017; **23**: 75-81 [PMID: 28453967 DOI: 10.1016/j.coviro.2017.03.011]

164 **Yoon SK**, Seo YB, Im SJ, Bae SH, Song MJ, You CR, Jang JW, Yang SH, Suh YS, Song JS, Kim BM, Kim CY, Jeong SH, Sung YC. Safety and immunogenicity of therapeutic DNA vaccine with antiviral drug in chronic HBV patients and its immunogenicity in mice. *Liver Int* 2015; **35**: 805-815 [PMID: 24620920 DOI: 10.1111/liv.12530]

165 **Shi J**, Ma Y, Zhu J, Chen Y, Sun Y, Yao Y, Yang Z, Xie J. A Review on Electroporation-Based Intracellular Delivery. *Molecules* 2018; **23** [PMID: 30469344 DOI: 10.3390/molecules23113044]

166 **Paulsen D**, Weber O, Ruebsamen-Schaeff H, Tennant BC, Menne S. AIC649 Induces a Bi-Phasic Treatment Response in the Woodchuck Model of Chronic Hepatitis B. *PLoS One* 2015; **10**: e0144383 [PMID: 26656974 DOI: 10.1371/journal.pone.0144383]

167 **Addy I**, Jambrecina A, Berg T, Boemmel F, Kropeit D, Vank C, Bigge A, Nedoschinsky K, Stobernack HP, Rangaraju M, Kummer S, Matschl U, Gehrimg A, Eberhard J, Altfeld M, Wiesch JSZ, Zimmermann H, Paulsen D. First in Human, single ascending dose clinical trial of AIC649 in patients with chronic hepatitis. *J Hepatol* 2019; **70** Suppl 1: e478-e479 [DOI: 10.1016/S0618-8278(19)30944-2]

168 **Zoulim F**, Fournier C, Habersetzer F, Sprinzl M, Pol S, Coffin CS, Leroy V, Ma M, Wedemeyer H, Lohse AW, Thimme R, Lugardon K, Martin P, Bastien B, Sansas B, Adda N, Halluard C, Bendjama K, Brandely M, Inchauspé G. Safety and immunogenicity of the therapeutic vaccine TG1050 in chronic hepatitis B patients: a phase 1b placebo-controlled trial. *Hum Vaccin Immunother* 2020; **16**: 388-399 [PMID: 31373537 DOI: 10.1080/21645515.2019.1651141]

169 **Vaccitech Ltd**. First participant dosed in Phase 1 clinical study with VTP-300 immunotherapeutic to treat chronically infected Hepatitis B patients. 2020 June 23. [cited 20 September 2020]. In: B3C newswire [Internet]. Available from: https://www.vaccitech.co.uk/first-participant-dosed-in-phase-1-clinical-study-with-vtp-300-immunotherapeutic-to-treat-chronically-infected-hepatitis-b-patients/

170 **Robinson HL**, Amara RR. T cell vaccines for microbial infections. *Nat Med* 2005; **11**: S25-S32 [PMID: 15812486 DOI: 10.1038/nm1212]

171 **Lim YS**, Mutimer D, Heo J, Tak WY, Rosenberg W, Jang BK, Kim YJ, Forton D, Tasker S, Georges B. A phase 1b evaluation of HepTcell HBV-specific immunotherapy in nuc-controlled, eAg negative chronic HBV infection. *J Hepatol* 2019; **70** Suppl 1: e50-e51 [DOI: 10.1016/S0618-8278(19)30090-8]

172 **Altimmune Inc**. Altimmune Announces IND Clearance for a Phase 2 Trial of HepTcell™ Immunotherapeutic for the Treatment of Chronic Hepatitis B. 2020 June 22. [cited 25 September 2020]. In: GlobeNewswire [Internet]. Available from: https://www.globenewswire.com/news-release/2020/06/22/2051121/0/en/Altimmune-Announces-IND-Clearance-for-a-Phase-2-Trial-of-HepTcell-Immunotherapeutic-for-the-Treatment-of-Chronic-Hepatitis-B.html

173 **Boni C**, Janssen HLA, Rossi M, Yoon SK, Vecchi A, Barili V, Yoshida EM, Trinh H, Rodell TC, Laccabue D, Alfieri A, Brillo F, Fisicaro P, Acerbi G, Pedrazzi G, Andreone P, Cursaro C, Margotti M, Santoro R, Piazzolla V, Brunetto MR, Coco B, Cavallone D, Zhao Y, Joshi A, Woo J, Lau AH, Gaggar A, Subramanian GM, Massetto B, Fung S, Ahn SH, Ma X, Mangia A, Ferrari C. Combined GS-4774 and Tenofovir Therapy Can Improve HBV-Specific T-Cell Responses in Patients With Chronic Hepatitis. *Gastroenterology* 2019; **157**: 227-241. e7 [PMID: 30930022 DOI: 10.1053/j.gastro.2019.03.044]

174 **Swisher JF**, Feldman GM. The many faces of FcγRI: implications for therapeutic antibody function. *Immunol Rev* 2015; **268**: 160-174 [PMID: 26497519 DOI: 10.1111/imr.12334]

175 **Wen YM**, Mu L, Shi Y. Immunoregulatory functions of immune complexes in vaccine and therapy. *EMBO Mol Med* 2016; **8**: 1120-1133 [PMID: 27572622 DOI: 10.15252/emmm.201606593]

176 **Mócsai A**, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol* 2010; **10**: 387-402 [PMID: 20467426 DOI: 10.1038/nri2765]

177 **Ma A**, Motyka B, Gutfreund K, Shi YE, George R. A dendritic cell receptor-targeted chimeric immunotherapeutic protein (C-HBV) for the treatment of chronic hepatitis B. *Hum Vaccin Immunother* 2020; **16**: 756-778 [PMID: 31687879 DOI: 10.1080/21645515.2019.1689080]

178 **Zhou C**, Li C, Gong GZ, Wang S, Zhang JM, Xu DZ, Guo LM, Ren H, Xu M, Xie Q, Pan C, Xu J, Hu Z, Geng S, Zhou X, Wang X, Mi H, Zhao G, Yu W, Wen YM, Huang L, Wang XY, Wang B. Analysis of immunological mechanisms exerted by HBsAg-HBIG therapeutic vaccine combined with Adefovir in chronic hepatitis B patients. *Hum Vaccin Immunother* 2017; **13**: 1989-1996 [PMID: 28665747 DOI: 10.1080/21645515.2017.1335840]

179 **Xu DZ**, Zhao K, Guo LM, Li LJ, Xie Q, Ren H, Zhang JM, Xu M, Wang HF, Huang WX, Bai XF, Niu JQ, Liu P, Chen XY, Shen XL, Yuan ZH, Wang XY, Wen YM. A randomized controlled phase IIb trial of antigen-antibody immunogenic complex therapeutic vaccine in chronic hepatitis B patients. *PLoS One* 2008; **3**: e2565 [PMID: 18596958 DOI: 10.1371/journal.pone.0002565]

180 **Lux A**, Yu X, Scanlan CN, Nimmerjahn F. Impact of immune complex size and glycosylation on IgG binding to human FcγRs. *J Immunol* 2013; **190**: 4315-4323 [PMID: 23509345 DOI: 10.4049/jimmunol.1200501]

181 **Halstead SB**, Mahalingam S, Marovich MA, Ubol S, Mosser DM. Intrinsic antibody-dependent

enhancement of microbial infection in macrophages: disease regulation by immune complexes. *Lancet Infect Dis* 2010; **10**: 712-722 [PMID: 20883967 DOI: 10.1016/S1473-3099(10)70166-3]

182 **Wang XY**, Wang B, Wen YM. From therapeutic antibodies to immune complex vaccines. *NPJ Vaccines* 2019; **4**: 2 [PMID: 30675393 DOI: 10.1038/s41541-018-0095-z]

183 **Al Mahtab M**, Akbar SMF, Aguilar JC, Guillen G, Penton E, Tuero A, Yoshida O, Hiasa Y, Onji M. Treatment of chronic hepatitis B naïve patients with a therapeutic vaccine containing HBs and HBc antigens (a randomized, open and treatment controlled phase III clinical trial). *PLoS One* 2018; **13**: e0201236 [PMID: 30133478 DOI: 10.1371/journal.pone.0201236]

184 **Yoshida O**, Imai Y, Shiraishi K, Tokumoto Y, Sanada T, Kohara-Tsukiyama K, Miyazaki T, Kamishita T, AI Mahtab M, Aguilar JC, Guillen Ge, Fazle Akbar SM, Kohara M, Hiasa Y. HBsAg reduction by nasal administration of a therapeutic vaccine containing HBsAg and HBcAg (Nasvac) in patients with chronic HBV infection: the result of 18 mo follow up. *Hepatology* 2020; **72** Suppl 1: 80

185 **Cargill T**, Narayan S Chinnakannan SK, Lee LN, Hutchings C, Klenerman P, Barnes E. Optimising delivery of therapeutic hepatitis B vaccines to induce resident memory T cells in the liver. *J Hepatol* 2020; **73** Suppl 1: S886 [DOI: 10.1016/S0168-8278(20)32209-1]

186 **Bayat B**, Lorin C, Vassilev V, Warter L. A new therapeutic candidate vaccine can overcome hepatitis B virus-induced immune tolerance in a mouse model of chronic infection. *J Hepatol* 2020; **73** Suppl 1: S571 [DOI: 10.1016/S0168-8278(20)31615-9]

187 **Dai S**, Wang H, Deng F. Advances and challenges in enveloped virus-like particle (VLP)-based vaccines. *J Immunol Sci* 2018; **2**: 36-41 [DOI: 10.29245/2578-3009/2018/2.1118]

188 **Bu W**, Joyce MG, Nguyen H, Banh DV, Aguilar F, Tariq Z, Yap ML, Tsujimura Y, Gillespie RA, Tsybovsky Y, Andrews SF, Narpala SR, McDermott AB, Rossmann MG, Yasutomi Y, Nabel GJ, Kanekiyo M, Cohen JI. Immunization with Components of the Viral Fusion Apparatus Elicits Antibodies That Neutralize Epstein-Barr Virus in B Cells and Epithelial Cells. *Immunity* 2019; **50**: 1305-1316. e6 [PMID: 30979688 DOI: 10.1016/j.immuni.2019.03.010]

189 **Kanekiyo M**, Joyce MG, Gillespie RA, Gallagher JR, Andrews SF, Yassine HM, Wheatley AK, Fisher BE, Ambrozak DR, Creanga A, Leung K, Yang ES, Boyoglu-Barnum S, Georgiev IS, Tsybovsky Y, Prabhakaran MS, Andersen H, Kong WP, Baxa U, Zephir KL, Ledgerwood JE, Koup RA, Kwong PD, Harris AK, McDermott AB, Mascola JR, Graham BS. Author Correction: Mosaic nanoparticle display of diverse influenza virus hemagglutinins elicits broad B cell responses. *Nat Immunol* 2019; **20**: 765 [PMID: 30980067 DOI: 10.1038/s41590-019-0395-0]

190 **Wang W**, Zhou X, Bian Y, Wang S, Chai Q, Guo Z, Wang Z, Zhu P, Peng H, Yan X, Li W, Fu YX, Zhu M. Dual-targeting nanoparticle vaccine elicits a therapeutic antibody response against chronic hepatitis B. *Nat Nanotechnol* 2020; **15**: 406-416 [PMID: 32123380 DOI: 10.1038/s41565-020-0648-y]

191 **Thi EP**, Dhillon AP, Ardzinski A, Bidirici-Ertekin L, Cobarrubias KD, Cuconati A, Kondratowicz AS, Kwak K, Li AHL, Miller A, Pasetka C, Pei L, Phelps JR, Snead NM, Wang X, Ye X, Sofia MJ, Lee ACH. ARB-1740, a RNA Interference Therapeutic for Chronic Hepatitis B Infection. *ACS Infect Dis* 2019; **5**: 725-737 [PMID: 30403127 DOI: 10.1021/acsinfecdis.8b00191]

192 **Mani N**, Cole AG, Phelps JR, Ardzinski A, Cobarrubias KD, Cuconati A, Dorsey BD, Evangelista E, Fan K, Guo F, Guo H, Guo JT, Harasym TO, Kadhim S, Kultgen SG, Lee ACH, Li AHL, Long Q, Majeski SA, Mao R, McClintock KD, Reid SP, Rijnbrand R, Snead NM, Micolochick Steuer HM, Stever K, Tang S, Wang X, Zhao Q, Sofia MJ. Preclinical Profile of AB-423, an Inhibitor of Hepatitis B Virus Pregenomic RNA Encapsidation. *Antimicrob Agents Chemother* 2018; **62**: e00082-18 [PMID: 29555628 DOI: 10.1128/AAC.00082-18]

193 **Zhang Q**, Jekle A, Serebryany V, Welch M, Liu J, Vendeville S, Debing Y, Buh Kum D, Ren S, Liu C, Deval J, Misner D, Chanda S, Mukherjee S, Raboisson P, Symons JA, Blatt LM, Biegelman LN, Smith DB. Best in class preclinical characteristics of ALG-000184, a prodrug of the capsid assembly modulator ALG-001075 for the treatment of chronic hepatitis B. *Hepatology* 2020; **72** Suppl 1: 503A

194 **Amblard F**, Boucle S, Bassit L, Cox B, Sari O, Tao S, Chen Z, Ozturk T, Verma K, Russell O, Rat V, Rocquigny HD, Fiquet O, Boussand M, Santo JD, Strick-Marchand H, Schinazi RF. Novel Hepatitis B Virus Capsid Assembly Modulator Induces Potent Antiviral Responses In Vitro and in Humanized Mice. *Antimicrob Agents Chemother* 2020; **64**: 01701-19 [PMID: 31712213 DOI: 10.1128/AAC.01701-19]

195 **Delaney WE 4th**, Edwards R, Colledge D, Shaw T, Furman P, Painter G, Locarnini S. Phenylpropenamide derivatives AT-61 and AT-130 inhibit replication of wild-type and lamivudine-resistant strains of hepatitis B virus in vitro. *Antimicrob Agents Chemother* 2002; **46**: 3057-3060 [PMID: 12183271 DOI: 10.1128/aac.46.9.3057-3060.2002]

196 **Chen W**, Liu F, Zhao Q, Ma X, Lu D, Li H, Zeng Y, Tong X, Zeng L, Liu J, Yang L, Zuo J, Hu Y. Discovery of Phthalazinone Derivatives as Novel Hepatitis B Virus Capsid Inhibitors. *J Med Chem* 2020; **63**: 8134-8145 [PMID: 32692159 DOI: 10.1021/acs.jmedchem.0c00346]

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MINIREVIEWS

Artificial intelligence in perioperative management of major gastrointestinal surgeries

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Abstract

Artificial intelligence (AI) demonstrated by machines is based on reinforcement learning and revolves around the usage of algorithms. The purpose of this review was to summarize concepts, the scope, applications, and limitations in major gastrointestinal surgery. This is a narrative review of the available literature on the key capabilities of AI to help anesthesiologists, surgeons, and other physicians to understand and critically evaluate ongoing and new AI applications in perioperative management. AI uses available databases called "big data" to formulate an algorithm. Analysis of other data based on these algorithms can help in early diagnosis, accurate risk assessment, intraoperative management, automated drug delivery, predicting anesthesia and surgical complications and postoperative outcomes and can thus lead to effective perioperative management as well as to reduce the cost of treatment. Perioperative physicians, anesthesiologists, and surgeons are well-positioned to help integrate AI into modern surgical practice. We all need to partner and collaborate with data scientists to collect and analyze data across all phases of perioperative care to provide clinical scenarios and context. Careful implementation and use of AI along with real-time human interpretation will revolutionize perioperative care, and is the way forward in future perioperative management of major surgery.

Key Words: Algorithms; Artificial intelligence; Big data; Data management; Machine learning; Perioperative care

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Core Tip: Artificial intelligence (AI) has revolutionized the way surgery and anesthesia are taught and practiced. Applications of AI in anesthesia are risk prediction, control of anesthesia by closed-loop anesthesia delivery systems, monitoring the depth of anesthesia, robotic intubation, monitoring cardiac output based on algorithms and ultrasound guidance. In surgery, AI focuses on generating evidence-based, real-time clinical decision support designed to optimize patient care and surgeon workflow. AI can be used to appropriately convey the results of prognosis and treatment algorithms to patients. Nevertheless, there is a lack of problem-solving by AI and a continuing dependence of human analysis.

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INTRODUCTION

Artificial Intelligence (AI) which means intelligence demonstrated by machines is based on reinforcement learning and revolves around the usage of algorithms. AI has already found an irreplaceable place in our lives with it use by smartphones, personal assistants like Alexa and many other applications. AI derives inspiration from the brain's complex neural network and uses artificial "neurons" that learn by comparing themselves to the desired output and "reinforce" connections that are useful, thus creating the basis of the artificial neural network approach. The healthcare field generates vast medical databases, also known as "big data" that include healthcare records, imaging, pathology, and surgical videos. Analysis of big data with the help of AI can help diagnose diseases early as well as predict outcomes better, which will eventually lead to effective and economical healthcare. Involvement of AI in perioperative management includes preoperative assessment with accurate risk predictions, intraoperative management includes intubating and operating robots, infusion devices, monitoring the depth of anesthesia (DOA), and early detection of anesthesia and surgical complications. The vast spectrum of AI and the ability of deep neural networks (DNN) to analyze intraoperative videos, the learning curve, and near misses may be reduced in minimally invasive surgery[1]. Postoperatively, AI has shown a role in reducing surgical site infections (SSI), intra-abdominal SSI after gastrointestinal (GI) surgeries[2,3], and identifying anastomotic leaks from electronic health records (EHRs)[4,5]. The applications of AI in the near future are expected to be significantly practice-changing, hence it becomes essential for surgeons and anesthesiologists to be updated with current applications and possible future uses.

BASICS OF AI IN A NUTSHELL

AI is a vast field in which machine learning (ML) forms a subfield (Figure 1). ML enables machines to learn from experience just as humans do. ML depends on the ability to perform tasks based on algorithms without actual programming and can be divided into supervised, unsupervised, and reinforcement learning[6]. Supervised learning enables the ML algorithm to achieve a predesigned outcome, unsupervised ML does not have prescribed output categories and can detect subtle patterns in large datasets that are imperceptible to human analysis. In supervised ML, datasets are divided into a training set and a test set to test the algorithm using the new dataset. Reinforcement learning is similar to conditioning and involves repeated trial and error situations that lead to a reward. DNN or deep learning involves multiple layers of the network that allow for the learning of more complex patterns (Figure 2). In DNN, to achieve the best association between the input and output layer, there are multiple intermediate hidden layers in that are fed data by their previous layers and thus influence the outcome. DNN can use EHR variables and accurately predict anastomotic leaks or mortality in postoperative patients[4,5]. Conventional neural

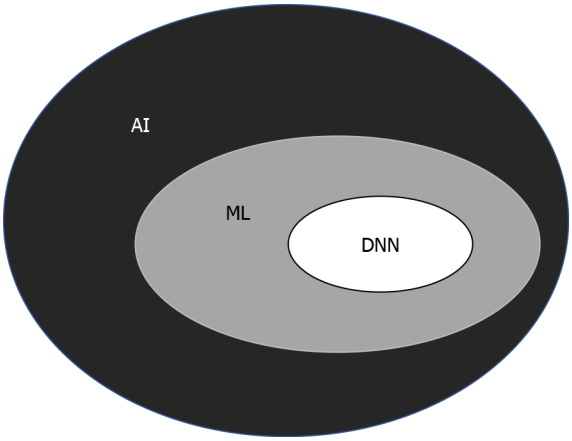


Figure 1 Machine learning is a subfield of artificial intelligence; deep neural networks in turn form a subfield of machine learning. AI: Artificial intelligence; DNN: Deep neural networks; ML: Machine learning.

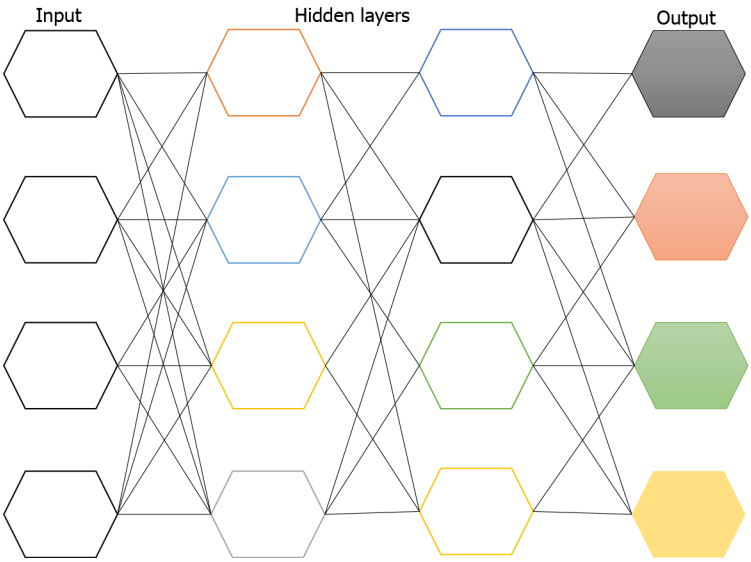


Figure 2 Deep neural networks have multiple intermediate layers that are hidden between the input and output layers.

networks, recurrent neural networks, and residual neural networks are currently commonly used architectures in deep learning. Techniques used by AI include computer vision (CV) and natural language processing (NLP). CV allows AI to identify objects through the processing of patterns and images; NLP allows machines to analyze human language beyond their machine language or code. CV and NLP have diverse applications in surgery and anesthesia.

PREOPERATIVE MANAGEMENT AND PLANNING

Preoperative planning of GI surgeries includes the combined use of pathological diagnosis, endoscopy, and imaging to identify patients at risk of complications, for early detection, and for timely treatment. Digital pathology with the transfer of slide images is in practice to report them remotely and can help obtain second opinions by remote centers with pathological dilemmas. Due to the sheer volume of specimen and ability to focus the microscope in an area of the field, even expert pathologists are bound to miss areas of interest. AI helps to analyze the whole field as well as to detect subtle changes[7,8]. AI algorithms can identify celiac disease[9], *Helicobacter pylori*[10], grade liver fibrosis[11], classify colonic polyps[12], grade dysplasia[13], predict 5-year overall survival in colorectal cancer from pathology[14], and predict microsatellite instability[15]. The drawbacks include large data storage and the need for an efficient network, computer, and equipment. ML learns from the data feed and because of

baseline inter-individual variability amongst pathologists, there is a grey zone regarding a 100% precision. Endoscopists have embraced AI and currently used it increase detection of polyps as well as to differentiate hyperplastic from adenomatous polyps based on endoscopic images[16,17]. However, most of the additional polyps detected by trained DNN are sub-centimetric adenomas[17]. It can also be used to differentiate malignant *vs* nonmalignant tissue, identify the depth of invasion, and margins of endoscopic resection[18,19]. An important feat achieved through AI is the evaluation of huge numbers of endoscopic images obtained by wireless capsule endoscopy[20] as well as an integrated ultrasound system in the capsule for evaluation as described by Sonopill[21]. Owing to the problem of organ deformation in the abdomen, integration of AI in imaging of GI pathologies has not been as fast as in breast or lung diseases. However, for a complex solid organ like the liver AI has expanded the scope of complex surgeries by three-dimensional (3D) visualization, virtual simulation surgery[22-24] and navigation-assisted surgery (see below). With that technology, it is possible to assess patients with large tumors initially considered inoperable[25]. The ability of 3D imaging to assess biliary anatomy and the liver remnant helps to predict the optimal surgical path to avoid residual tissue in complex hepatolithiasis[26-28]. 3D imaging has helped to better understand pancreatic vascular anatomy and determine the resectability of tumors[29-31]. Photoacoustic imaging combines optical imaging with high resolution ultrasound and large penetration depth. This has shown promise in Crohn's disease to assess intestinal hemoglobin levels and differentiate between active and inactive disease to avoid unnecessary invasive procedures[32].

The other aspect of perioperative planning is decision making and risk assessment. The surgical decision is not made only by the surgeon, a shared-decision includes patient involvement in the management, increases patient satisfaction, compliance and allows the patient to cope up with any unfortunate complications[33]. IBM Watson Oncology was built to help oncologists to keep up to date with current evidence and guide decision making. Despite the associated hype, it has not been able to deliver well for GI cancers[34,35]. Unfortunately, the available risk assessment models like major adverse cardiac events, the Revised Cardiac Risk Index, and Gupta Perioperative Risk for Myocardial Infarction or Cardiac Arrest underestimate the risk involved[36-38]. The American Society of Anesthesiologists (ASA) classification, which is used widely is also not devoid of subjective assessment errors[39]. AI and ML platforms that are fed data from vast databases of patients outcomes can identify and accurately predict risk based on the variables available EHRs. The MySurgeryRisk platform uses EHR data of 285 variables and has been shown to predict perioperative risk with better accuracy than clinical judgement[5]. It utilizes the big data from EHRs and obviates manual input of variables to give accurate mortality prediction. ML has been shown to accurately predict mortality, sepsis, and acute kidney injury using intraoperative data[40-43]. The Predictive OpTimization Trees in Emergency Surgery Risk (POTTER) calculator is an ML platform based on the American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP) database to calculate perioperative risk and mortality[44]. POTTER is accurate, user-friendly and can be integrated with EHR data to identify and prevent risks like SSI, sepsis, pneumonia, urinary tract infection, cardiac complications, and prolonged intensive care unit (ICU) stay in postoperative patients. Similarly, for the prevention of anesthesia-related complications, ML algorithms can interpret data from previous surgery that includes both patient risk factors and postoperative outcomes to recommend the anesthetic drugs to be used[45].

INTRAOPERATIVE MANAGEMENT

AI has led to several advancements for the induction and maintenance of anesthesia in the current era. Intubation robots like the Kepler intubation system, which uses a video laryngoscope and a robotic arm to place the endotracheal tube, has shown a 91% success rate[46]. Monitoring DOA requires the assessment of a multitude of parameters and is a complex task to be converted in real-time to an ML algorithm. AI utilizes the bispectral index (BIS), which is derived from electroencephalogram data, to maintain tight feedback and control of DOA[47]. McSleepy automated intravenous infusion machines use the BIS along with vital signs to maintain DOA by administering propofol, narcotics, and muscle relaxants[48]. The maintenance of DOA is a critical balance between infusion and assessment of parameters and under-dosing or excessive DOA may be caused by equipment imbalance, therefore automated

anesthesia is still in its infancy and not ready to be adopted in general practice. Closed-loop anesthesia delivery systems which are another automated delivery system based on BIS have been shown to be effective and efficient[49].

Apart from induction and maintenance, automated regional anesthetic blockade has been performed by the da Vinci® system, and the Magellan robot has been used to place peripheral nerve blocks[50,51]. Intraoperative intelligence can help to predict return of consciousness after general anesthesia, and neural networks can predict post-induction hypotension as well as the rate of recovery from neuromuscular blockade [47]. Hatib *et al*[52] have utilized arterial waveforms to formulate a model that was able to predict hypotension 15 min before its occurrence using waveform analysis. In an unblinded randomized clinical trial (HYPE trial), the use of a ML-derived early warning system compared resulted in less intraoperative hypotension with standard care[53]. Neural networks can assist in the identification of vertebral level and epidural landmarks for postoperative pain from ultrasound images[47]. In supra major abdominal surgeries like cytoreductive surgeries or hyperthermic intraperitoneal chemotherapy, it is recommended to use cardiac output monitoring. Currently, noninvasive arterial pressure-based cardiac output monitoring is used in a majority of cases[54]. A noise-robust bioimpedance approach for cardiac output monitoring using a regression support vector machine and DNN is the most accurate and robust method for monitoring cardiac output, but it has not been used in surgical patients[55].

Today's operation theater (OR) utilizes a multitude of anesthesia and surgical machines that provide a regular status report and adequate assistance. The heterogeneous sensors in the OR are known as "context-aware assistance" and help in the smooth functioning of the surgical procedures[56]. ML and data annotation can be used to identify the phases of surgery and apply them to identify any deviation or delay in surgical steps[57]. Surgical navigation technology or computer-assisted abdominal surgery use preoperative or intraoperative imaging to track surgical instruments and help to describe hidden surgical anatomy. These have been described in case reports in laparoscopic adrenalectomy[58], splenectomy[59], pancreaticoduodenectomy[60], and esophagectomy[61]. However, integrating this entire system in current practice is difficult because of the effort required in the setup and the problem of organ deformation in the abdomen. Liver surgery, with its complex biliary and vascular anatomy, makes a perfect match for the use of navigation-assisted procedures[62-65]. Prediction of the resectability of peritoneal carcinomatosis often requires a laparotomy to assess the burden of disease. ML like the random forest model have been shown to predict resectability with an accuracy of 97.82% in peritoneal carcinomatosis[66] and could help prevent unnecessary laparotomies and to improve the efficiency of the OR.

The da Vinci Surgical System® (Intuitive Surgical, Sunnyvale, CA, United States) is the most commonly used robotic platform and is used for a majority of pelvic surgeries, where it has shown superior postoperative outcomes[67]. Robotic surgery gives surgeons full control with robotic arms, but there is an increase in operative time and cost. A standalone robotic camera can reduce costs and has been evaluated for pancreatic procedures[68] and for laparoscopic cholecystectomy[69]. In laparoscopic cholecystectomy, there was an increase in operating time, which was possibly caused by a lack of real autonomy or reinforced learning and needs to be controlled by the surgeon either verbally or by manual interaction. The human-machine interface can be controlled by gaze gesture recognition or recognition of the surgeon's head movement to control the laparoscope or endoscope remotely[70-72]. As an alternative to automated anesthesia and robotic surgery, Jacob *et al*[73,74], designed Gestonurse, a robotic scrub nurse with a sensor that understands nonverbal hand gestures, can help to deliver surgical instruments to surgeons. The Smart tissue anastomosis robot is used for minimally invasive suturing and it has been shown in experimental models to have better accuracy and consistency than human surgeons[75,76]. Minimally invasive laparoscopic surgery is a continuously evolving process for surgeons, requires skill and has a learning curve. With AI it is possible to identify the real-time automatic surgical phase for real-time workflow recognition and teaching[77]. A DNN approach has shown an accuracy of 91.9% for the identification of surgical phases in 71 patients with laparoscopic sigmoidectomy[77]. With the help of AI and DNN, it will be possible to predict the occurrence of intrasurgical events and to avoid near-miss events in laparoscopic surgery[1].

POSTOPERATIVE MANAGEMENT

A smooth postoperative recovery for all patients is the goal. NLP allows ML algorithms to understand EHR data and to utilize it to predict outcomes[5,78]. ML algorithms have been shown to accurately predict superficial SSI, organ space SSI, sepsis, and bleeding requiring transfusion after liver, pancreatic, or colorectal surgery[79]. Statistically, it has shown to outperform the ASA and ACS-Surgical Risk Calculator. One of the most undesirable complications after major GI surgeries is an anastomotic leak. Various clinical signs and biochemical and radiological investigations are used to assess leaks. The use of NLP data from EHRs can predict anastomotic leaking after colorectal or bariatric surgery[5,80]. AI has been shown to predict the risk of postoperative pancreatic fistula (POPF) after pancreaticoduodenectomy by using prediction platform algorithms. A patient's predicted POPF risk could guide clinical management and prevent or mitigate untoward outcomes[81]. ML has been used in the postoperative period or in ICUs in patients with sepsis to predict urine output and fluid status after fluid administration and to help avoid fluid overload and oliguria[82].

As has been recently published, an ML algorithm performed by the DASH Analytics High-Definition Care Platform can use EHR data predict the risk of SSI and suggest ways to reduce the risk in real-time during the closure. The platform reduced SSIs by 74% in 3 years at the University of Iowa Hospitals and Clinics[83]. AI research has also focused on minimizing postoperative pain and maximizing patient comfort. Postoperative pain after abdominal surgeries was studied in a randomized controlled trial (RCT) involving 50 patients using the Nociception Level (NOL) index, a multiparameter AI-driven index designed to monitor nociception during surgery. That study found no differences in perioperative opioid (fentanyl and morphine) consumption, but there was a 1.6-point improvement in postoperative pain scores in the NOL-guided group[84] (Figure 3).

EDUCATION AND TRAINING

AI can help in the training of anesthesiologists by recreating realistic case scenarios not encountered in routine training, which makes them more prepared for possible situations in the OR. Virtual reality (VR) uses computer technology to simulate the real environment. Minimally invasive surgeries or liver surgeries that require a high level of skill or expertise, are performed better by surgeons who have been trained by VR[85,86]. Currently, there is no robust objective assessment tool to assess competency in advanced laparoscopic colorectal surgery. Observational clinical human reliability analysis and assessment of errors in laparoscopic videos may potentially be useful for specialist recertification and revalidation[87,88].

ROLE IN THE CORONAVIRUS DISEASE 2019 PANDEMIC

The coronavirus disease 2019 (COVID-19) pandemic has changed lives worldwide and has affected healthcare delivery to a great extent. Elective major surgeries were put on hold because of the sudden need of beds and the risk of postoperative infection with the virus, which could lead to adverse outcomes[89]. Healthcare workers were at high risk for infection along with the elderly and individuals with comorbidities. As of July 2020, more than 1800 healthcare workers from 64 countries had died of COVID-19[90]. Intubation generates aerosols that increase the risk of infection, and the use of intubation robots might reduce exposure of healthcare workers. The da Vinci® system, the most widely used surgical robot can reduce exposure allowing minimal contact with the patient. Robotic surgeries require fewer OR personnel than open surgeries, require less intrabdominal pressure than laparoscopic surgery, which reduces aerosol generation[91,92]. Robotic procedures are moving towards contactless surgeries with the help of magnetic navigation systems, which require an external magnet placed on the patient's body[93]. Delaying elective surgery has resulted in a patient backlog. Clearing the backlog requires the efficient use of OR time and resources. An ML algorithm with a custom Python script has been shown to optimize the efficiency of operating room booking times that resulted in a reduction in nursing overtime of 21%, which was equivalent to saving of half a million dollars[94]. Robots can be used to perform surgery on weekends and after hours under supervision that will help to clear the surgical backlog and mitigate surgeon fatigue[95]. Training programs have also

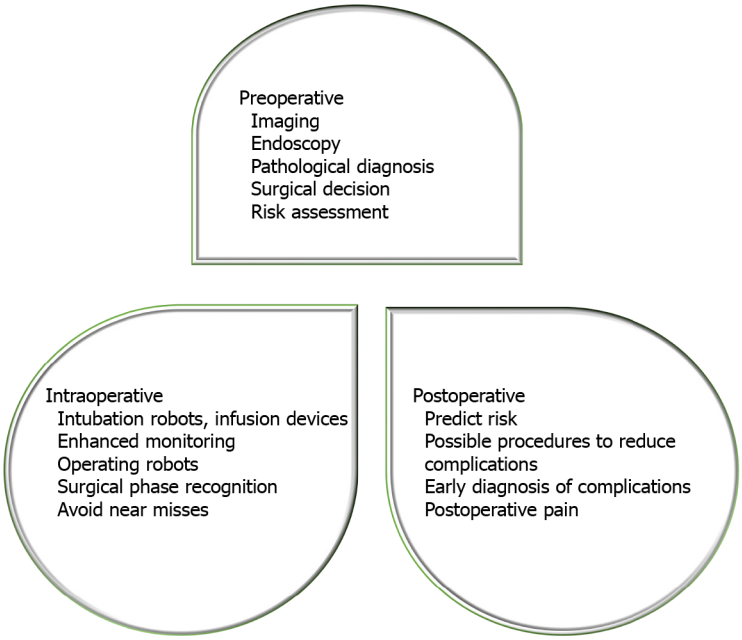


Figure 3 Comprehensive view of areas that reflect the benefits of artificial intelligence in management of major gastrointestinal surgery.

been transformed by the pandemic is the surgical. Conversion of hospitals to dedicated COVID-19 centers, halting of non-urgent surgeries, and reallocation of space to ICU services have drastically hampered the training of surgery residents[96]. VR can allow residents to engage in minimally invasive surgeries amongst others rather than experiencing a complete void in surgical training[85,86]. AI can help to resolve and provide alternative solutions to a few of the problems resulting from the pandemic.

LIMITATIONS

AI should not be looked upon as the answer to all problems. AI can help identify subtle differences in large datasets, but there are certain instances where traditional methods perform better. AI is as good as the data that is used to generate the answers. The results will depend on the questions asked, the variables labelled, and the datasets that are available. Any glitches in those components will result in an incorrect algorithm. Also, the generalizability of the algorithm is dependent on the data that is used to generate it. If the algorithm has been derived from data extracted from an Asian population, then it cannot be generalized and used to predict accurate answers for white Americans. Similarly, there unknown confounders might be present. For example, in one study, an algorithm was more likely to classify a skin lesion as malignant if an image had a ruler in it[97]. The practice of evidence-based medicine has been built upon strictly regulated RCTs. To adopt AI as standard, it will need to undergo vigorous RCTs to confirm their performance in a blinded fashion. Lack of transparency because of multiple layers in a neural network becomes problematic and may not be considered trustworthy or accepted in healthcare because wrong results can lead to devastating consequences[98].

LEGAL IMPLICATIONS

Ethicos is a Greek word meaning a way of conduct accepted in society. *Moralis* is a Latin word that denotes judgement of the appropriateness of a given action. Most of the time, ethics and morality are used interchangeably. The ethical and legal implications of AI are heavily debated. It has already been explained that if the algorithm is flawed, AI will generate inaccurate results. Those errors are different from ones that occurring because of network loss or computer malfunction. AI was initially used only to augment clinical decision making but with its current development and autonomy, flaws in the device that harm patients, resulting in medical negligence, require that

responsibility needs to be predetermined. The allocation of responsibility was traditionally to the surgeon, but with the self-learning capability of AI, it may not be possible for the surgeon to override certain procedures. Legal implications have still not reached a consensus regarding allocation. O'Sullivan *et al*[99] have classified the responsibility of AI and autonomous robotic surgery as (1) accountability; (2) liability; and (3) culpability. Accountability means the capacity of a system to explain its actions, liability is subject to action by the legal system, and culpability relates to punishment. Accountability can be determined by recording the actions, but the issues of liability and culpability require a consensus to adapt to the scenario of AI and robotics.

CONCLUSION

AI is emerging as a powerful tool in healthcare, with the ultimate aim of achieving better patient outcomes. However, we have not yet achieved that goal. Many aspects need to be refined or addressed, beginning with algorithm development and extending to legal and ethical issues. Future advances could definitely allow patients entering the clinic to be given accurate perioperative risks related to both anesthesia and surgery derived from previous surgical experience and patient histories. Intraoperatively along with automated anesthesia and robotic surgery, AI could help predict events like hypotension or delay in surgical steps or to avoid near misses. AI could identify the risk of postoperative complications like sepsis or renal failure as well as anastomotic leak to plan for early intervention. The use of AI in the OR is not intended to replace the surgeon or the anesthesiologist but to expand human capacity and capability through enhanced vision, dexterity, and complementary machine intelligence for improved surgical safety and outcomes.

REFERENCES

1 **Bonrath EM**, Gordon LE, Grantcharov TP. Characterising 'near miss' events in complex laparoscopic surgery through video analysis. *BMJ Qual Saf* 2015; **24**: 516-521 [PMID: 25947330 DOI: 10.1136/bmjqs-2014-003816]

2 **da Silva DA**, Ten Caten CS, Dos Santos RP, Fogliatto FS, Hsuan J. Predicting the occurrence of surgical site infections using text mining and machine learning. *PLoS One* 2019; **14**: e0226272 [PMID: 31834905 DOI: 10.1371/journal.pone.0226272]

3 **Chapman AB**, Mowery DL, Swords DS, Chapman WW, Bucher BT. Detecting Evidence of Intra-abdominal Surgical Site Infections from Radiology Reports Using Natural Language Processing. *AMIA Annu Symp Proc* 2017; **2017**: 515-524 [PMID: 29854116]

4 **Soguero-Ruiz C**, Hindberg K, Rojo-Alvarez JL, Skrovseth SO, Godtliebsen F, Mortensen K, Revhaug A, Lindsetmo RO, Augestad KM, Jenssen R. Support Vector Feature Selection for Early Detection of Anastomosis Leakage From Bag-of-Words in Electronic Health Records. *IEEE J Biomed Health Inform* 2016; **20**: 1404-1415 [PMID: 25312965 DOI: 10.1109/JBHI.2014.2361688]

5 **Brennan M**, Puri S, Ozrazgat-Baslanti T, Feng Z, Ruppert M, Hashemighouchani H, Momcilovic P, Li X, Wang DZ, Bihorac A. Comparing clinical judgment with the MySurgeryRisk algorithm for preoperative risk assessment: A pilot usability study. *Surgery* 2019; **165**: 1035-1045 [PMID: 30792011 DOI: 10.1016/j.surg.2019.01.002]

6 **Hashimoto DA**, Rosman G, Rus D, Meireles OR. Artificial Intelligence in Surgery: Promises and Perils. *Ann Surg* 2018; **268**: 70-76 [PMID: 29389679 DOI: 10.1097/SLA.0000000000002693]

7 **Komura D**, Ishikawa S. Machine learning approaches for pathologic diagnosis. *Virchows Arch* 2019; **475**: 131-138 [PMID: 31222375 DOI: 10.1007/s00428-019-02594-w]

8 **Nielsen JA**, Lager DJ, Lewin M, Weber JJ, Roberts CA. Incidence of diagnostic change in colorectal polyp specimens after deeper sectioning at 2 different laboratories staffed by the same pathologists. *Am J Clin Pathol* 2013; **140**: 231-237 [PMID: 23897260 DOI: 10.1309/AJCPPXU66QZAZNYN]

9 **Wei JW**, Wei JW, Jackson CR, Ren B, Suriawinata AA, Hassanpour S. Automated Detection of Celiac Disease on Duodenal Biopsy Slides: A Deep Learning Approach. *J Pathol Inform* 2019; **10**: 7 [PMID: 30984467 DOI: 10.4103/jpi.jpi_87_18]

10 **Martin DR**, Hanson JA, Gullapalli RR, Schultz FA, Sethi A, Clark DP. A Deep Learning Convolutional Neural Network Can Recognize Common Patterns of Injury in Gastric Pathology. *Arch Pathol Lab Med* 2020; **144**: 370-378 [PMID: 31246112 DOI: 10.5858/arpa.2019-0004-OA]

11 **Yu Y**, Wang J, Ng CW, Ma Y, Mo S, Fong ELS, Xing J, Song Z, Xie Y, Si K, Wee A, Welsch RE, So PTC, Yu H. Deep learning enables automated scoring of liver fibrosis stages. *Sci Rep* 2018; **8**: 16016 [PMID: 30375454 DOI: 10.1038/s41598-018-34300-2]

12 **Korbar B**, Olofson AM, Miraflor AP, Nicka CM, Suriawinata MA, Torresani L, Suriawinata AA, Hassanpour S. Deep Learning for Classification of Colorectal Polyps on Whole-slide Images. *J Pathol*

Inform 2017; **8**: 30 [PMID: 28828201 DOI: 10.4103/jpi.jpi_34_17]
13 **Tomita N**, Abdollahi B, Wei J, Ren B, Suriawinata A, Hassanpour S. Attention-Based Deep Neural Networks for Detection of Cancerous and Precancerous Esophagus Tissue on Histopathological Slides. *JAMA Netw Open* 2019; **2**: e1914645 [PMID: 31693124 DOI: 10.1001/jamanetworkopen.2019.14645]
14 **Bychkov D**, Linder N, Turkki R, Nordling S, Kovanen PE, Verrill C, Walliander M, Lundin M, Haglund C, Lundin J. Deep learning based tissue analysis predicts outcome in colorectal cancer. *Sci Rep* 2018; **8**: 3395 [PMID: 29467373 DOI: 10.1038/s41598-018-21758-3]
15 **Kather JN**, Pearson AT, Halama N, Jäger D, Krause J, Loosen SH, Marx A, Boor P, Tacke F, Neumann UP, Grabsch HI, Yoshikawa T, Brenner H, Chang-Claude J, Hoffmeister M, Trautwein C, Luedde T. Deep learning can predict microsatellite instability directly from histology in gastrointestinal cancer. *Nat Med* 2019; **25**: 1054-1056 [PMID: 31160815 DOI: 10.1038/s41591-019-0462-y]
16 **Byrne MF**, Chapados N, Soudan F, Oertel C, Linares Pérez M, Kelly R, Iqbal N, Chandelier F, Rex DK. Real-time differentiation of adenomatous and hyperplastic diminutive colorectal polyps during analysis of unaltered videos of standard colonoscopy using a deep learning model. *Gut* 2019; **68**: 94-100 [PMID: 29066576 DOI: 10.1136/gutjnl-2017-314547]
17 **Urban G**, Tripathi P, Alkayali T, Mittal M, Jalali F, Karnes W, Baldi P. Deep Learning Localizes and Identifies Polyps in Real Time With 96% Accuracy in Screening Colonoscopy. *Gastroenterology* 2018; **155**: 1069-1078. e8 [PMID: 29928897 DOI: 10.1053/j.gastro.2018.06.037]
18 **Tokai Y**, Yoshio T, Aoyama K, Horie Y, Yoshimizu S, Horiuchi Y, Ishiyama A, Tsuchida T, Hirasawa T, Sakakibara Y, Yamada T, Yamaguchi S, Fujisaki J, Tada T. Application of artificial intelligence using convolutional neural networks in determining the invasion depth of esophageal squamous cell carcinoma. *Esophagus* 2020; **17**: 250-256 [PMID: 31980977 DOI: 10.1007/s10388-020-00716-x]
19 **Ichimasa K**, Kudo SE, Mori Y, Misawa M, Matsudaira S, Kouyama Y, Baba T, Hidaka E, Wakamura K, Hayashi T, Kudo T, Ishigaki T, Yagawa Y, Nakamura H, Takeda K, Haji A, Hamatani S, Mori K, Ishida F, Miyachi H. Artificial intelligence may help in predicting the need for additional surgery after endoscopic resection of T1 colorectal cancer. *Endoscopy* 2018; **50**: 230-240 [PMID: 29272905 DOI: 10.1055/s-0043-122385]
20 **Aoki T**, Yamada A, Aoyama K, Saito H, Tsuboi A, Nakada A, Niikura R, Fujishiro M, Oka S, Ishihara S, Matsuda T, Tanaka S, Koike K, Tada T. Automatic detection of erosions and ulcerations in wireless capsule endoscopy images based on a deep convolutional neural network. *Gastrointest Endosc* 2019; **89**: 357-363. e2 [PMID: 30670179 DOI: 10.1016/j.gie.2018.10.027]
21 **Cummins G**, Lay H, Cox B, Seetohul V, Qiu Y, Stewart F, Faerber J, Mitrakos V, Al Rawhani M, Beeley J, Watson DE. Sonopill: A platform for gastrointestinal disease diagnosis and therapeutics. Proceedings of the 6th Joint Workshop on New Technologies for Computer/Robot Assisted Surgery; Pisa, Italy. 2016: 12-14
22 **Mise Y**, Hasegawa K, Satou S, Shindoh J, Miki K, Akamatsu N, Arita J, Kaneko J, Sakamoto Y, Kokudo N. How Has Virtual Hepatectomy Changed the Practice of Liver Surgery? *Ann Surg* 2018; **268**: 127-133 [PMID: 28288065 DOI: 10.1097/SLA.0000000000002213]
23 **Fang CH**, Tao HS, Yang J, Fang ZS, Cai W, Liu J, Fan YF. Impact of three-dimensional reconstruction technique in the operation planning of centrally located hepatocellular carcinoma. *J Am Coll Surg* 2015; **220**: 28-37 [PMID: 25456781 DOI: 10.1016/j.jamcollsurg.2014.09.023]
24 **Fang CH**, Liu J, Fan YF, Yang J, Xiang N, Zeng N. Outcomes of hepatectomy for hepatolithiasis based on 3-dimensional reconstruction technique. *J Am Coll Surg* 2013; **217**: 280-288 [PMID: 23870220 DOI: 10.1016/j.jamcollsurg.2013.03.017]
25 **Fang C**, Zhang P, Qi X. Digital and intelligent liver surgery in the new era: Prospects and dilemmas. *EBioMedicine* 2019; **41**: 693-701 [PMID: 30773479 DOI: 10.1016/j.ebiom.2019.02.017]
26 **Xiang N**, Fang C. [Application of hepatic segment resection combined with rigid choledochoscope in the treatment of complex hepatolithiasis guided by three-dimensional visualization technology]. *Zhonghua Waike Zazhi* 2015; **53**: 335-339 [PMID: 26082246]
27 **Xie A**, Fang C, Huang Y, Fan Y, Pan J, Peng F. Application of three-dimensional reconstruction and visible simulation technique in reoperation of hepatolithiasis. *J Gastroenterol Hepatol* 2013; **28**: 248-254 [PMID: 23190368 DOI: 10.1111/jgh.12066]
28 **Li G**, Fang CH, Fan YF, Wu TC, Zhong SZ. A comparative study of the diagnostic accuracy of the medical image three-dimensional visualization system, MRCP, CT and US in hepatolithiasis. *Hepatogastroenterology* 2014; **61**: 1901-1907 [PMID: 25713886]
29 **Fang CH**, Kong D, Wang X, Wang H, Xiang N, Fan Y, Yang J, Zhong SZ. Three-dimensional reconstruction of the peripancreatic vascular system based on computed tomographic angiography images and its clinical application in the surgical management of pancreatic tumors. *Pancreas* 2014; **43**: 389-395 [PMID: 24622068 DOI: 10.1097/MPA.0000000000000035]
30 **Fang CH**, Zhu W, Wang H, Xiang N, Fan Y, Yang J, Wang X, Zhong Sz. A new approach for evaluating the resectability of pancreatic and periampullary neoplasms. *Pancreatology* 2012; **12**: 364-371 [PMID: 22898639 DOI: 10.1016/j.pan.2012.05.006]
31 **Miyamoto R**, Oshiro Y, Nakayama K, Kohno K, Hashimoto S, Fukunaga K, Oda T, Ohkohchi N. Three-dimensional simulation of pancreatic surgery showing the size and location of the main pancreatic duct. *Surg Today* 2017; **47**: 357-364 [PMID: 27368278 DOI: 10.1007/s00595-016-1377-6]
32 **Knieling F**, Neufert C, Hartmann A, Claussen J, Urich A, Egger C, Vetter M, Fischer S, Pfeifer L,

Hagel A, Kielisch C, Görtz RS, Wildner D, Engel M, Röther J, Uter W, Siebler J, Atreya R, Rascher W, Strobel D, Neurath MF, Waldner MJ. Multispectral Optoacoustic Tomography for Assessment of Crohn's Disease Activity. *N Engl J Med* 2017; **376**: 1292-1294 [PMID: 28355498 DOI: 10.1056/NEJMc1612455]

33 **Loftus TJ**, Tighe PJ, Filiberto AC, Efron PA, Brakenridge SC, Mohr AM, Rashidi P, Upchurch GR Jr, Bihorac A. Artificial Intelligence and Surgical Decision-making. *JAMA Surg* 2020; **155**: 148-158 [PMID: 31825465 DOI: 10.1001/jamasurg.2019.4917]

34 **Somashekhar S**, Sepúlveda M, Norden AD, Rauthan A, Arun K, Patil P, Ethadka RY, Kumar RC. Early experience with IBM Watson for Oncology (WFO) cognitive computing system for lung and colorectal cancer treatment. *J Clin Onco* 2017; **35**: 8527 [DOI: 10.1200/JCO.2017.35.15_suppl.8527]

35 **Tian Y**, Liu X, Wang Z, Cao S, Liu Z, Ji Q, Li Z, Sun Y, Zhou X, Wang D, Zhou Y. Concordance Between Watson for Oncology and a Multidisciplinary Clinical Decision-Making Team for Gastric Cancer and the Prognostic Implications: Retrospective Study. *J Med Internet Res* 2020; **22**: e14122 [PMID: 32130123 DOI: 10.2196/14122]

36 **Lee TH**, Marcantonio ER, Mangione CM, Thomas EJ, Polanczyk CA, Cook EF, Sugarbaker DJ, Donaldson MC, Poss R, Ho KK, Ludwig LE, Pedan A, Goldman L. Derivation and prospective validation of a simple index for prediction of cardiac risk of major noncardiac surgery. *Circulation* 1999; **100**: 1043-1049 [PMID: 10477528 DOI: 10.1161/01.cir.100.10.1043]

37 **Gupta PK**, Gupta H, Sundaram A, Kaushik M, Fang X, Miller WJ, Esterbrooks DJ, Hunter CB, Pipinos II, Johanning JM, Lynch TG, Forse RA, Mohiuddin SM, Mooss AN. Development and validation of a risk calculator for prediction of cardiac risk after surgery. *Circulation* 2011; **124**: 381-387 [PMID: 21730309 DOI: 10.1161/CIRCULATIONAHA.110.015701]

38 **Wijeysundera DN**, Pearse RM, Shulman MA, Abbott TEF, Torres E, Ambosta A, Croal BL, Granton JT, Thorpe KE, Grocott MPW, Farrington C, Myles PS, Cuthbertson BH; METS study investigators. Assessment of functional capacity before major non-cardiac surgery: an international, prospective cohort study. *Lancet* 2018; **391**: 2631-2640 [PMID: 30070222 DOI: 10.1016/S0140-6736(18)31131-0]

39 **Wolters U**, Wolf T, Stützer H, Schröder T. ASA classification and perioperative variables as predictors of postoperative outcome. *Br J Anaesth* 1996; **77**: 217-222 [PMID: 8881629 DOI: 10.1093/bja/77.2.217]

40 **Adhikari L**, Ozrazgat-Baslanti T, Ruppert M, Madushani RWMA, Paliwal S, Hashemighouchani H, Zheng F, Tao M, Lopes JM, Li X, Rashidi P, Bihorac A. Improved predictive models for acute kidney injury with IDEA: Intraoperative Data Embedded Analytics. *PLoS One* 2019; **14**: e0214904 [PMID: 30947282 DOI: 10.1371/journal.pone.0214904]

41 **Henry KE**, Hager DN, Pronovost PJ, Saria S. A targeted real-time early warning score (TREWScore) for septic shock. *Sci Transl Med* 2015; **7**: 299ra122 [PMID: 26246167 DOI: 10.1126/scitranslmed.aab3719]

42 **Delahanty RJ**, Kaufman D, Jones SS. Development and Evaluation of an Automated Machine Learning Algorithm for In-Hospital Mortality Risk Adjustment Among Critical Care Patients. *Crit Care Med* 2018; **46**: e481-e488 [PMID: 29419557 DOI: 10.1097/CCM.0000000000003011]

43 **Koyner JL**, Carey KA, Edelson DP, Churpek MM. The Development of a Machine Learning Inpatient Acute Kidney Injury Prediction Model. *Crit Care Med* 2018; **46**: 1070-1077 [PMID: 29596073 DOI: 10.1097/CCM.0000000000003123]

44 **Bertsimas D**, Dunn J, Velmahos GC, Kaafarani HMA. Surgical Risk Is Not Linear: Derivation and Validation of a Novel, User-friendly, and Machine-learning-based Predictive OpTimal Trees in Emergency Surgery Risk (POTTER) Calculator. *Ann Surg* 2018; **268**: 574-583 [PMID: 30124479 DOI: 10.1097/SLA.0000000000002956]

45 **Karpagavalli S**, Jamuna KS, and Vijaya MS. Machine learning approach for pre-operative anaesthetic risk prediction. *Int J Rece Trends Engineering* 2009; **1**: 19-22

46 **Hemmerling TM**, Taddei R, Wehbe M, Zaouter C, Cyr S, Morse J. First robotic tracheal intubations in humans using the Kepler intubation system. *Br J Anaesth* 2012; **108**: 1011-1016 [PMID: 22457483 DOI: 10.1093/bja/aes034]

47 **Hashimoto DA**, Witkowski E, Gao L, Meireles O, Rosman G. Artificial Intelligence in Anesthesiology: Current Techniques, Clinical Applications, and Limitations. *Anesthesiology* 2020; **132**: 379-394 [PMID: 31939856 DOI: 10.1097/ALN.0000000000002960]

48 **Hughes DJ**. Automated anesthesia and natural Orifice transuluminal endoscopic surgery. *Surg Technol* 2011

49 **Puri GD**, Kumar B, Aveek J. Closed-loop anaesthesia delivery system (CLADS) using bispectral index: a performance assessment study. *Anaesth Intensive Care* 2007; **35**: 357-362 [PMID: 17591128 DOI: 10.1177/0310057X0703500306]

50 **Tighe PJ**, Badiyan SJ, Luria I, Boezaart AP, Parekattil S. Technical communication: robot-assisted regional anesthesia: a simulated demonstration. *Anesth Analg* 2010; **111**: 813-816 [PMID: 20581161 DOI: 10.1213/ANE.0b013e3181e66386]

51 **Wehbe M**, Giacalone M, Hemmerling TM. Robotics and regional anesthesia. *Curr Opin Anaesthesiol* 2014; **27**: 544-548 [PMID: 25115767 DOI: 10.1097/ACO.0000000000000117]

52 **Hatib F**, Jian Z, Buddi S, Lee C, Settels J, Sibert K, Rinehart J, Cannesson M. Machine-learning Algorithm to Predict Hypotension Based on High-fidelity Arterial Pressure Waveform Analysis. *Anesthesiology* 2018; **129**: 663-674 [PMID: 29894315 DOI: 10.1097/ALN.0000000000002300]

53 **Wijnberge M**, Geerts BF, Hol L, Lemmers N, Mulder MP, Berge P, Schenk J, Terwindt LE,

Hollmann MW, Vlaar AP, Veelo DP. Effect of a Machine Learning-Derived Early Warning System for Intraoperative Hypotension *vs* Standard Care on Depth and Duration of Intraoperative Hypotension During Elective Noncardiac Surgery: The HYPE Randomized Clinical Trial. *JAMA* 2020; **323**: 1052-1060 [PMID: 32065827 DOI: 10.1001/jama.2020.0592]

54 **Solanki SL**, Mukherjee S, Agarwal V, Thota RS, Balakrishnan K, Shah SB, Desai N, Garg R, Ambulkar RP, Bhorkar NM, Patro V, Sinukumar S, Venketeswaran MV, Joshi MP, Chikkalingegowda RH, Gottumukkala V, Owusu-Agyemang P, Saklani AP, Mehta SS, Seshadri RA, Bell JC, Bhatnagar S, Divatia JV. Society of Onco-Anaesthesia and Perioperative Care consensus guidelines for perioperative management of patients for cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (CRS-HIPEC). *Indian J Anaesth* 2019; **63**: 972-987 [PMID: 31879421 DOI: 10.4103/ija.IJA_765_19]

55 **Murphy EK**, Amoh J, Arshad SH, Halter RJ, Odame K. Noise-robust bioimpedance approach for cardiac output measurement. *Physiol Meas* 2019; **40**: 074004 [PMID: 30840932 DOI: 10.1088/1361-6579/ab0d45]

56 **Vercauteren T**, Unberath M, Padoy N, Navab N. CAI4CAI: The Rise of Contextual Artificial Intelligence in Computer Assisted Interventions. *Proc IEEE Inst Electr Electron Eng* 2020; **108**: 198-214 [PMID: 31920208 DOI: 10.1109/JPROC.2019.2946993]

57 **Bodenstedt S**, Wagner M, Müller-Stich BP, Weitz J, Speidel S. Artificial Intelligence-Assisted Surgery: Potential and Challenges. *Visc Med* 2020; **36**: 450-455 [PMID: 33447600 DOI: 10.1159/000511351]

58 **Marescaux J**, Rubino F, Arenas M, Mutter D, Soler L. Augmented-reality-assisted laparoscopic adrenalectomy. *JAMA* 2004; **292**: 2214-2215 [PMID: 15536106 DOI: 10.1001/jama.292.18.2214-c]

59 **Ieiri S**, Uemura M, Konishi K, Souzaki R, Nagao Y, Tsutsumi N, Akahoshi T, Ohuchida K, Ohdaira T, Tomikawa M, Tanoue K, Hashizume M, Taguchi T. Augmented reality navigation system for laparoscopic splenectomy in children based on preoperative CT image using optical tracking device. *Pediatr Surg Int* 2012; **28**: 341-346 [PMID: 22130783 DOI: 10.1007/s00383-011-3034-x]

60 **Onda S**, Okamoto T, Kanehira M, Suzuki F, Ito R, Fujioka S, Suzuki N, Hattori A, Yanaga K. Identification of inferior pancreaticoduodenal artery during pancreaticoduodenectomy using augmented reality-based navigation system. *J Hepatobiliary Pancreat Sci* 2014; **21**: 281-287 [PMID: 23970384 DOI: 10.1002/jhbp.25]

61 **Kenngott HG**, Neuhaus J, Müller-Stich BP, Wolf I, Vetter M, Meinzer HP, Köninger J, Büchler MW, Gutt CN. Development of a navigation system for minimally invasive esophagectomy. *Surg Endosc* 2008; **22**: 1858-1865 [PMID: 18157716 DOI: 10.1007/s00464-007-9723-9]

62 **Okamoto T**, Onda S, Matsumoto M, Gocho T, Futagawa Y, Fujioka S, Yanaga K, Suzuki N, Hattori A. Utility of augmented reality system in hepatobiliary surgery. *J Hepatobiliary Pancreat Sci* 2013; **20**: 249-253 [PMID: 22399157 DOI: 10.1007/s00534-012-0504-z]

63 **Kingham TP**, Scherer MA, Neese BW, Clements LW, Stefansic JD, Jarnagin WR. Image-guided liver surgery: intraoperative projection of computed tomography images utilizing tracked ultrasound. *HPB (Oxford)* 2012; **14**: 594-603 [PMID: 22882196 DOI: 10.1111/j.1477-2574.2012.00487.x]

64 **Peterhans M**, vom Berg A, Dagon B, Inderbitzin D, Baur C, Candinas D, Weber S. A navigation system for open liver surgery: design, workflow and first clinical applications. *Int J Med Robot* 2011; **7**: 7-16 [PMID: 21341357 DOI: 10.1002/rcs.360]

65 **Nozaki T**, Fujiuchi Y, Komiya A, Fuse H. Efficacy of DynaCT for surgical navigation during complex laparoscopic surgery: an initial experience. *Surg Endosc* 2013; **27**: 903-909 [PMID: 23052511 DOI: 10.1007/s00464-012-2531-x]

66 **Maubert A**, Birtwisle L, Bernard JL, Benizri E, Bereder JM. Can machine learning predict resecability of a peritoneal carcinomatosis? *Surg Oncol* 2019; **29**: 120-125 [PMID: 31196475 DOI: 10.1016/j.suronc.2019.04.008]

67 **Moran PS**, O'Neill M, Teljeur C, Flattery M, Murphy LA, Smyth G, Ryan M. Robot-assisted radical prostatectomy compared with open and laparoscopic approaches: a systematic review and meta-analysis. *Int J Urol* 2013; **20**: 312-321 [PMID: 23311943 DOI: 10.1111/iju.12070]

68 **Gumbs AA**, Croner R, Rodriguez A, Zuker N, Perrakis A, Gayet B. 200 consecutive laparoscopic pancreatic resections performed with a robotically controlled laparoscope holder. *Surg Endosc* 2013; **27**: 3781-3791 [PMID: 23644837 DOI: 10.1007/s00464-013-2969-5]

69 **Gillen S**, Pletzer B, Heiligensetzer A, Wolf P, Kleeff J, Feussner H, Fürst A. Solo-surgical laparoscopic cholecystectomy with a joystick-guided camera device: a case-control study. *Surg Endosc* 2014; **28**: 164-170 [PMID: 23990155 DOI: 10.1007/s00464-013-3142-x]

70 **Fujii K**, Gras G, Salerno A, Yang GZ. Gaze gesture based human robot interaction for laparoscopic surgery. *Med Image Anal* 2018; **44**: 196-214 [PMID: 29277075 DOI: 10.1016/j.media.2017.11.011]

71 **Nishikawa A**, Hosoi T, Koara K, Negoro D, Hikita A, Asano S, Kakutani H, Miyazaki F, Sekimoto M, Yasui M, Miyake Y. FAce MOUSe: A novel human-machine interface for controlling the position of a laparoscope. *IEEE Trans Rob Autom* 2003; **19**: 825-841 [DOI: 10.1109/TRA.2003.817093]

72 **Hong N**, Kim M, Lee C, Kim S. Head-mounted interface for intuitive vision control and continuous surgical operation in a surgical robot system. *Med Biol Eng Comput* 2019; **57**: 601-614 [PMID: 30280331 DOI: 10.1007/s11517-018-1902-4]

73 **Jacob M**, Li YT, Akingba G, Wachs JP. Gestonurse: a robotic surgical nurse for handling surgical instruments in the operating room. *J Robot Surg* 2012; **6**: 53-63 [PMID: 27637980 DOI: 10.1007/s11701-011-0325-0]

74 **Jacob MG**, Li YT, Akingba GA, Wachs JP. Collaboration with a robotic scrub nurse. *Commun ACM*

2013; **56**: 68-75 [DOI: 10.1145/2447976.2447993]
75 **Leonard S**, Wu KL, Kim Y, Krieger A, Kim PC. Smart tissue anastomosis robot (STAR): a vision-guided robotics system for laparoscopic suturing. *IEEE Trans Biomed Eng* 2014; **61**: 1305-1317 [PMID: 24658254 DOI: 10.1109/TBME.2014.2302385]
76 **Shademan A**, Decker RS, Opfermann JD, Leonard S, Krieger A, Kim PC. Supervised autonomous robotic soft tissue surgery. *Sci Transl Med* 2016; **8**: 337ra64 [PMID: 27147588 DOI: 10.1126/scitranslmed.aad9398]
77 **Kitaguchi D**, Takeshita N, Matsuzaki H, Takano H, Owada Y, Enomoto T, Oda T, Miura H, Yamanashi T, Watanabe M, Sato D, Sugomori Y, Hara S, Ito M. Real-time automatic surgical phase recognition in laparoscopic sigmoidectomy using the convolutional neural network-based deep learning approach. *Surg Endosc* 2020; **34**: 4924-4931 [PMID: 31797047 DOI: 10.1007/s00464-019-07281-0]
78 **Murff HJ**, FitzHenry F, Matheny ME, Gentry N, Kotter KL, Crimin K, Dittus RS, Rosen AK, Elkin PL, Brown SH, Speroff T. Automated identification of postoperative complications within an electronic medical record using natural language processing. *JAMA* 2011; **306**: 848-855 [PMID: 21862746 DOI: 10.1001/jama.2011.1204]
79 **Merath K**, Hyer JM, Mehta R, Farooq A, Bagante F, Sahara K, Tsilimigras DI, Beal E, Paredes AZ, Wu L, Ejaz A, Pawlik TM. Use of Machine Learning for Prediction of Patient Risk of Postoperative Complications After Liver, Pancreatic, and Colorectal Surgery. *J Gastrointest Surg* 2020; **24**: 1843-1851 [PMID: 31385172 DOI: 10.1007/s11605-019-04338-2]
80 **Nudel J**, Bishara AM, de Geus SWL, Patil P, Srinivasan J, Hess DT, Woodson J. Development and validation of machine learning models to predict gastrointestinal leak and venous thromboembolism after weight loss surgery: an analysis of the MBSAQIP database. *Surg Endosc* 2021; **35**: 182-191 [PMID: 31953733 DOI: 10.1007/s00464-020-07378-x]
81 **Han IW**, Cho K, Ryu Y, Shin SH, Heo JS, Choi DW, Chung MJ, Kwon OC, Cho BH. Risk prediction platform for pancreatic fistula after pancreatoduodenectomy using artificial intelligence. *World J Gastroenterol* 2020; **26**: 4453-4464 [PMID: 32874057 DOI: 10.3748/wjg.v26.i30.4453]
82 **Lin PC**, Huang HC, Komorowski M, Lin WK, Chang CM, Chen KT, Li YC, Lin MC. A machine learning approach for predicting urine output after fluid administration. *Comput Methods Programs Biomed* 2019; **177**: 155-159 [PMID: 31319943 DOI: 10.1016/j.cmpb.2019.05.009]
83 **Cromwell JW**. DASH Analytics High Definition Care Platform Chosen for CDC Epicenter Trial in Surgical Site Infection. [cited 14 August 2020]. In: Businesswire [Internet]. Available from: https://www.businesswire.com/news/home/20180814005047/en/DASH-Analytics-High-Definition-Care-Platform-Chosen-for-CDC-Epicenter-Trial-in-Surgical-Site-Infection
84 **Meijer F**, Honing M, Roor T, Toet S, Calis P, Olofsen E, Martini C, van Velzen M, Aarts L, Niesters M, Boon M, Dahan A. Reduced postoperative pain using Nociception Level-guided fentanyl dosing during sevoflurane anaesthesia: a randomised controlled trial. *Br J Anaesth* 2020; **125**: 1070-1078 [PMID: 32950246 DOI: 10.1016/j.bja.2020.07.057]
85 **Zendejas B**, Cook DA, Bingener J, Huebner M, Dunn WF, Sarr MG, Farley DR. Simulation-based mastery learning improves patient outcomes in laparoscopic inguinal hernia repair: a randomized controlled trial. *Ann Surg* 2011; **254**: 502-9; discussion 509 [PMID: 21865947 DOI: 10.1097/SLA.0b013e31822c6994]
86 **Müller-Stich BP**, Löb N, Wald D, Bruckner T, Meinzer HP, Kadmon M, Büchler MW, Fischer L. Regular three-dimensional presentations improve in the identification of surgical liver anatomy - a randomized study. *BMC Med Educ* 2013; **13**: 131 [PMID: 24066729 DOI: 10.1186/1472-6920-13-131]
87 **Miskovic D**, Ni M, Wyles SM, Parvaiz A, Hanna GB. Observational clinical human reliability analysis (OCHRA) for competency assessment in laparoscopic colorectal surgery at the specialist level. *Surg Endosc* 2012; **26**: 796-803 [PMID: 22042584 DOI: 10.1007/s00464-011-1955-z]
88 **Foster JD**, Miskovic D, Allison AS, Conti JA, Ockrim J, Cooper EJ, Hanna GB, Francis NK. Application of objective clinical human reliability analysis (OCHRA) in assessment of technical performance in laparoscopic rectal cancer surgery. *Tech Coloproctol* 2016; **20**: 361-367 [PMID: 27154295 DOI: 10.1007/s10151-016-1444-4]
89 **Solanki SL**, Thota RS, Garg R, Pingle AA, Goswami J, Ranganath N, Mukherjee S, Gupta S, Patkar S, Chikkalingegowda RH, Jindal T, Ray MD, Upadhye SM, Divatia JV. Society of Onco-Anesthesia and Perioperative Care (SOAPC) advisory regarding perioperative management of onco-surgeries during COVID-19 pandemic. *Indian J Anaesth* 2020; **64**: S97-S102 [PMID: 32773846 DOI: 10.4103/ija.IJA_447_20]
90 **Medscape**. In Memoriam: healthcare workers who have died of COVID-19. [cited 1 April 2020]. In: Medscape [Internet]. Available from: https://www.medscape.com/viewarticle/927976
91 **Zheng MH**, Boni L, Fingerhut A. Minimally Invasive Surgery and the Novel Coronavirus Outbreak: Lessons Learned in China and Italy. *Ann Surg* 2020; **272**: e5-e6 [PMID: 32221118 DOI: 10.1097/SLA.0000000000003924]
92 **Angioli R**, Terranova C, Plotti F, Cafà EV, Gennari P, Ricciardi R, Aloisi A, Miranda A, Montera R, De Cicco Nardone C. Influence of pneumoperitoneum pressure on surgical field during robotic and laparoscopic surgery: a comparative study. *Arch Gynecol Obstet* 2015; **291**: 865-868 [PMID: 25260990 DOI: 10.1007/s00404-014-3494-z]
93 **Petruska AJ**, Ruetz F, Hong A, Regli L, Sürücü O, Zemmar A, Nelson BJ. Magnetic needle guidance for neurosurgery: Initial design and proof of concept. *IEEE* 2016; 4392-4397 [DOI:

10.1109/ICRA.2016.7487638]

94 **Rozario N**, Rozario D. Can machine learning optimize the efficiency of the operating room in the era of COVID-19? *Can J Surg* 2020; **63**: E527-E529 [PMID: 33180692 DOI: 10.1503/cjs.016520]

95 **Zemmar A**, Lozano AM, Nelson BJ. The rise of robots in surgical environments during COVID-19. *Nat Mach Intell* 2020; **2**: 566-572 [DOI: 10.1038/s42256-020-00238-2]

96 **Bernardi L**, Germani P, Del Zotto G, Scotton G, de Manzini N. Impact of COVID-19 pandemic on general surgery training program: An Italian experience. *Am J Surg* 2020; **220**: 1361-1363 [PMID: 32564874 DOI: 10.1016/j.amjsurg.2020.06.010]

97 **Esteva A**, Kuprel B, Novoa RA, Ko J, Swetter SM, Blau HM, Thrun S. Dermatologist-level classification of skin cancer with deep neural networks. *Nature* 2017; **542**: 115-118 [PMID: 28117445 DOI: 10.1038/nature21056]

98 **Kelly CJ**, Karthikesalingam A, Suleyman M, Corrado G, King D. Key challenges for delivering clinical impact with artificial intelligence. *BMC Med* 2019; **17**: 195 [PMID: 31665002 DOI: 10.1186/s12916-019-1426-2]

99 **O'Sullivan S**, Nevejans N, Allen C, Blyth A, Leonard S, Pagallo U, Holzinger K, Holzinger A, Sajid MI, Ashrafian H. Legal, regulatory, and ethical frameworks for development of standards in artificial intelligence (AI) and autonomous robotic surgery. *Int J Med Robot* 2019; **15**: e1968 [PMID: 30397993 DOI: 10.1002/rcs.1968]

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MINIREVIEWS

Direct antiviral agents in hepatitis C virus related liver disease: Don't count the chickens before they're hatched

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Abstract

Since molecules with direct-acting antiviral (DAA) became available, the landscape of the treatment of hepatitis C virus (HCV) infection has completely changed. The new drugs are extremely effective in eradicating infection, and treatment is very well tolerated with a duration of 8-12 wk. This review aims to report the outstanding clinical benefits of DAA and to highlight their critical disadvantages, identifying some clinically relevant hot topics. First, do the rates of virological response remain as high when patients with more advanced cirrhosis are considered? Large studies have shown slightly lower but still satisfactory rates of response in these patients. Nevertheless, modified schedules with an extended treatment duration and use of ribavirin may be necessary. Second, does the treatment of HCV infection affect the risk of occurrence and recurrence of liver cancer? Incidence is reduced after viral eradication but remains high enough to warrant periodic surveillance for an early diagnosis. In contrast, the risk of recurrence seems to be unaffected by viral clearance; however, DAA treatment improves survival because of the reduced risk of progression of liver disease. Third, can HCV treatment also have favorable effects on major comorbidities? HCV eradication is associated with a reduced incidence of diabetes, an improvement in glycemic control and a decreased risk of cardiovascular events; nevertheless, a risk of hypoglycemia during DAA treatment has been reported. Finally, is it safe to treat patients with HCV/ hepatitis B virus (HBV) coinfection? In this setting, HCV is usually the main driver of viral activity, while HBV replication is suppressed. Because various studies have described HBV reactivation after HCV clearance, a baseline evaluation for HBV coinfection and a

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Core Tip: The treatment of hepatitis C virus (HCV) infection has changed since direct-acting antivirals (DAAs) became available. DAA use has been extended to patients with advanced cirrhosis and severe comorbidities. Consequently, this review addresses the following questions: What benefits can be expected from eradicating HCV in patients who already have advanced liver disease? Are DAAs associated with an appreciable benefit in liver cancer? Can HCV treatment have favorable effects on the major concomitant disorders such as diabetes or cardiovascular disease? Is there any cause for concern when using DAAs in HCV patients with a concomitant hepatitis B virus infection?

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, with more than 70 million people infected all over the world[1]. HCV is responsible for many cases of hepatitis, liver cirrhosis, liver cancer (hepatocellular carcinoma, HCC), transplantation (orthotopic liver transplantation, OLT) and liver-related deaths. Initially, all the available treatments were interferon (IFN)-based. However, they were unsatisfactory mainly because the rates of viral response were between 30% and 50%, and their efficacy was strongly dependent on viral genotype and the degree of liver fibrosis. Furthermore, IFN-based treatments had a long duration of 24-48 wk and marked adverse effects limited their use in patients with advanced liver disease and major comorbidities.

More recently, IFN-free treatments with direct-acting antiviral (DAAs) drugs were registered. Their availability has substantially changed the HCV-infection setting. Indeed, DAAs are able to obtain rates of sustained virological response (SVR) higher than 95%, independently of virus genotype or degree of liver damage. They have short duration schedules of 8 wk to 12 wk and are extremely well tolerated[2-4]. More recently, the advent of pangenotypic DAAs has further extended and simplified their use[2,5].

The effects of viral clearance in patients with chronic hepatitis are well known. SVR is generally associated with a biochemical response (normalization of liver enzymes), improvement of liver necroinflammation and reduction in fibrosis progression or regression of fibrosis[6,7].

Due to their efficacy in advanced fibrosis and high tolerability, DAA use has been extended to patients with advanced and even decompensated cirrhosis and patients with severe comorbidities, raising new questions. What benefits can be expected from eradicating HCV in patients who already have advanced liver disease? Is DAA-induced SVR associated with an appreciable benefit in liver cancer? Can HCV treatment have favorable effects on major concomitant disorders such as diabetes or cardiovascular disease? Is there any cause for concern when using DAAs in HCV patients with a concomitant hepatitis B virus (HBV) infection?

WHAT BENEFITS CAN BE EXPECTED FROM ERADICATING HCV IN PATIENTS WHO ALREADY HAVE ADVANCED LIVER DISEASE?

The new-generation DAA regimens sofosbuvir/velpatasvir and glecaprevir/pibrentasvir have shown rates of SVR higher than 95% in patients with cirrhosis, independent of genotype. No concerns about their safety have been described[2,5,8]. Notably, these results have been confirmed in real-world studies conducted in large and heterogeneous populations, including patients with intravenous drug use, patients with advanced chronic kidney disease or those aged over 70, who are usually excluded from registrational trials[9,10].

Previous studies demonstrated the favorable effects of viral eradication on the histopathological features of cirrhosis, showing that SVR is associated with a histological decrease in fibrosis or cirrhosis regression[11]. More recently, various studies have shown the clinical advantages of treating HCV infection in patients with cirrhosis. One study analyzed a large French cohort of 1323 patients with biopsy-proven cirrhosis, showing that SVR was significantly associated with reductions in the risk of decompensation and of both overall and liver-related mortality[12]. The association between SVR and reduction in mortality for all causes was confirmed by another French study including 9895 cirrhotic patients followed up for more than 33 mo[6].

Another large cohort study from the United States evaluated the impact of DAA-induced SVR on all-cause mortality in 15059 HCV patients with advanced liver disease. SVR was independently associated with a reduced risk of death compared to no SVR (hazard ratio: 0.26; 95% confidence interval: 0.22-0.31; $P < 0.001$)[13].

Studies on hemodynamics have examined the relationship between SVR and portal hypertension. A reduction in the hepatic venous pressure gradient (HVPG) has been reported after SVR with DAA, leading to a reduced risk of hepatic decompensation[14].

Are such relevant results confirmed when patients with more advanced/decompensated cirrhosis are considered?

DAAs have been used in patients with Child-Pugh B and C cirrhosis who were unsuitable for treatment with IFN-based regimens. Although such patients remain harder to treat and protease inhibitors are contraindicated in this setting, various studies have reported SVR rates higher than 90% after adding ribavirin or prolonging treatment to 24 wk[15-17].

Even in patients with advanced cirrhosis, virological response is associated with a clinical benefit. Model for end-stage liver disease score and liver function improve in one third to one half of treated patients. Furthermore, treatment is well tolerated with no safety concerns[18].

Hepatic venous pressure gradient reduction after SVR has been observed even in Child-Pugh class B cirrhosis[19], but the benefits for portal pressure can be reduced in patients with more advanced liver cirrhosis. In a multicenter prospective study, although a significant reduction in hepatic vein pressure gradient was observed after SVR, this reduction was only marginal in patients who had higher, clinically significant portal hypertension at baseline[20].

Strong evidence of the clinical impact of DAAs in these patients can be found in the setting of OLT. HCV-related liver disease has been the most common indication for OLT in Western Europe for the last 20 years. DAA introduction has significantly contributed to change the OLT scenario, greatly decreasing the burden of HCV disease at waiting list registration. Data from an Italian cohort showed that after the extended approval of DAA therapies was granted in Italy in 2014, waiting list registrations for OLT due to HCV-related disease decreased from 43.3% of all waiting list registrations in the pre-DAA period to 37.2%[21].

Data from the European liver transplantation cohort showed that treatment with DAAs allowed as many as one third of patients already on waiting list to be delisted because of clinical improvement, with a very low risk of a subsequent need for relisting[22].

High rates of SVR have also been demonstrated in patients treated after transplantation[23]. A study from Spain conducted in 112 OLT recipients showed SVR to be associated with liver fibrosis regression and a significant improvement in liver function and survival[7].

In conclusion, DAA treatment allows high rates of SVR even in patients with advanced cirrhosis. These patients remain difficult to treat, are not suitable for protease inhibitors and may require treatment prolongation and ribavirin use. SVR is associated with an improvement in liver function and a reduction in portal pressure in a large proportion of patients. DAA availability has reduced the burden of HCV-

related disease in the liver transplantation setting (Table 1).

IS DAA-INDUCED SVR ASSOCIATED WITH AN APPRECIABLE BENEFIT IN HCC?

A number of studies have evaluated the role of DAA-induced SVR in both the occurrence and recurrence of HCC. Overall, SVR obtained with DAAs is associated with a 70% incidence reduction[24].

A retrospective cohort study on a large population from the United States evaluated the annual rates of HCC in HCV patients treated with DAA, comparing those who achieved SVR to those who did not. The study included 22500 patients treated with DAA; about one third of them had cirrhosis. SVR was achieved in 19518 but not in 2982. The highest risk of HCC was reported in cirrhotic patients. Even though SVR significantly reduced the risk of HCC, the absolute risk of HCC remained high in patients with cirrhosis despite SVR. Thus, patients with cirrhosis should be kept under long-term surveillance for HCC[25].

Another real-world study from Italy, including a cohort of more than 2000 cirrhotic patients, showed a significant reduction in HCC risk after achieving SVR in patients with Child-Pugh class A and class B disease. Nevertheless, the mean time lapse between exposure to DAAs and HCC occurrence was 9.8 mo, with no significant difference between patients with SVR and those without; this observation would suggest that the high incidence of HCC described in the first year after DAA might be largely related to the presence of previously unrecognized HCC cases. Thus, previously unrecognized HCC could largely contribute to an overestimation of the early risk of HCC after DAAs[24,26]. In this study, albumin levels and platelet counts, associated with liver function and portal hypertension, respectively, were identified as significant predictors of HCC occurrence.

More recently, similar results have been confirmed in long-term follow-up studies. A retrospective study from East Asia, involving a large cohort of 5814 HCV patients treated with DAA and followed up for a median of 2.9 years, reported a significant reduction in HCC risk in SVR patients compared with those without SVR[27].

Another retrospective study was recently conducted in 129 Veterans Health Administration hospitals. Results confirmed that in cirrhotic patients successfully treated with DAA, the occurrence of HCC was reduced but remained high enough to warrant long-term HCC surveillance. Interestingly, the risk of HCC remained stable over the 3 year observation period[28].

Several studies have evaluated the risk of HCC recurrence in DAA-treated HCV patients on HCC remission at DAA initiation. Some early studies raised concerns about an increased risk of HCC recurrence in patients treated with DAAs compared with those receiving IFN. Furthermore, other studies hypothesized that the rapid clearance of HCV during DAAs could decrease the immune surveillance of small HCC clones, increasing the risk of early HCC recurrence[29].

A meta-analysis published in 2017 compared the risk of HCC recurrence in patients treated with DAA or IFN-based treatments, finding no difference between the two groups. In particular, DAA treatment was not associated with a higher HCC recurrence[30]. More recently, a retrospective, population-based cohort study including 17836 patients confirmed that neither the HCC incidence rate nor HCC-free survival were significantly different in the DAA group compared to the IFN group[31].

A large retrospective cohort study from the United States and Canada found no significant differences in either overall or early HCC recurrence between patients who received DAAs and patients not receiving therapy after a complete response to HCC treatment[32]. Even if the risk of HCC recurrence appears not to be affected by curative DAA treatment, a reduction in the risk of decompensation of cirrhosis has been observed in these patients, and overall survival appears to be improved in those achieving SVR[33].

A retrospective cohort study from the United States and Canada, including 797 patients with previous HCV-related HCC who had achieved a complete HCC response at treatment initiation, confirmed that SVR after DAA treatment was associated with a significant reduction in the overall risk of death[34]. A history of previous HCC recurrence and previous main tumor size appear to be related with the risk of recurrence[35].

In conclusion, the available data show that HCC incidence is reduced in DAA-treated patients. The risk of HCC recurrence appears unaffected, but overall survival can be increased in these patients because of the reduced risk of decompensation. The

Table 1 Benefits and pitfalls of sustained virological response using direct-acting antivirals in different hepatitis C virus scenarios

Setting	Benefits	Pitfalls
Advanced liver disease	SVR rates higher than 95%; Reduced risk of decompensation and deathHVPG reduction	Nothing to report
Decompensated cirrhosis	Liver function improvement in one third to one half of patients; Treatment well-tolerated	Slightly lower SVR rates; Need for ribavirin or treatment elongation to 24 wk
HCC occurrence	Reduced occurrence of HCC	Still relevant risk of HCC; Need for periodic surveillance for early diagnosis
HCC recurrence	Improved survival due to reduced risk of decompensation	Risk of HCC recurrence unaffected by SVR; Need for periodic surveillance for early diagnosis
Diabetes	Reduced incidence of diabetes; Improvement of glycemic control	Risk of hypoglycemia in patients receiving antidiabetic medications; Adjustments of antidiabetic medication may be necessary
Cardiovascular diseases	Decreased risk of cardiovascular events; Reduction in cardiovascular deaths	Nothing to report
HBV coinfection	As for general HCV population	Risk for HBV reactivation; Pre-DAA screening for HBV indicated; Need for HBV treatment or on-treatment HBV monitoring as requested

DAA: Direct-acting antiviral; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HVPG: Hepatic venous pressure gradient; SVR: Sustained virological response.

risk of both the incidence and recurrence of DAA remains high enough to warrant long-term surveillance after treatment, even in patients with SVR (Table 1).

CAN HCV TREATMENT HAVE FAVORABLE EFFECTS ON MAJOR CONCOMITANT DISORDERS SUCH AS DIABETES OR CARDIOVASCULAR DISEASE?

An interesting, albeit unsolved issue regarding the association between HCV infection and glucose abnormalities [insulin resistance (IR) and type 2 diabetes mellitus (T2DM)]. Even though the mechanisms that lead to the development of IR and T2DM in predisposed HCV-infected patients are not fully understood, HCV seems to play a direct role by interfering with glucose metabolism. As indicated by many studies, HCV-infected subjects have a significantly higher risk of T2DM than noninfected controls[36,37], HCV-cleared patients[38] and HBV-infected patients[39]. Moreover, some data suggest that the higher risk might be independent of body mass index and family history[40], strengthening the hypothesis of HCV as a direct cause of T2DM.

Although it is quite clear that HCV infection increases the risk of developing T2DM, the data regarding whether the stage of liver disease could affect the development of T2DM are ambiguous. Indeed, some studies show that HCV-related cirrhosis (and in particular decompensated cirrhosis) is associated with a higher risk of DM than in non-HCV cirrhosis, HCV-cleared patients and chronic HCV-infections[38]. Due to this close association between HCV infection and the development of T2DM, many studies have evaluated whether glucose abnormalities could on the contrary affect the natural course of HCV infection.

To our knowledge, DM and IR are associated with a worse outcome in patients with chronic hepatitis C, with an increased risk of developing liver cirrhosis and decompensation over time[41]. In particular, the effects of fasting glucose levels seem to be increased in genotype 1 and genotype 2 patients[42].

In a cohort study, baseline diabetes was independently associated with a higher prevalence of ascites, bacterial infections and encephalopathy detected at inclusion and was independently associated with the development of ascites, renal dysfunction, bacterial infections and HCC during follow-up, showing T2DM as an independent prognostic factor in patients with chronic hepatitis C and cirrhosis. Transplantation-free survival was also shortened, independent of the baseline model for end-stage liver disease score[43]. Furthermore, some data suggest an important role for new-onset diabetes in chronic hepatitis C patients, which seems to lead to a significantly higher cumulative incidence of HCC[37,44].

The effects of HCV eradication on glycometabolic control have been widely investigated. Although SVR after IFN/ribavirin therapies was already known to be

associated with a significantly reduced risk of developing T2DM after long-term follow-up[45-48], data collected in the DAA era suggest a great benefit of antiviral treatment in HCV patients who already have glucose abnormalities. Many studies have shown a significant improvement in insulin sensitivity[49], hemoglobin A1c and fasting glucose plasma levels in patients who achieved SVR after DAA therapy[50-53]. A prospective case-control study showed IR improvements in 76.5% of patients who achieved SVR and a normalized IR in about 41% of them, with these data being confirmed 3 mo after treatment withdrawal[54]. Moreover, patients with SVR also required a lower dose of antidiabetic medications than those without[51]. Interestingly, these metabolic changes seem to be body mass index-independent[55]. Nevertheless, more studies are needed to clarify the long-term effects after DAA therapy.

All these observations lead to another question: Does HCV eradication affect diabetes mellitus-related long-term complications? The clinical impact of successful antiviral therapy on the long-term outcomes of T2DM in HCV patients remains largely unknown, mainly because of the lack of specific prospective studies. However, based on the above-mentioned data, it is reasonable to presume that HCV eradication could also reduce long-term metabolic-related complications. A Taiwanese population-based cohort study found that better renal and cardiovascular outcomes were obtained in the group treated with the antiviral IFN/ribavirin regimen than in the untreated cohort[56]. Another study showed a decreased incidence of acute coronary syndrome, end-stage renal disease, ischemic stroke and retinopathy after HCV eradication, both with IFN and with DAA treatment, regardless of cirrhosis[57]. Whether the reduction in cardiovascular events observed in diabetic patients after HCV eradication is due to a beneficial effect on T2DM itself or to the disappearance of systemic chronic inflammation remains to be clarified.

Although HCV eradication has important benefits for glycometabolic control, some negative aspects of DAA treatment in diabetic patients must be considered. In fact, a Health Canada review has pointed out a link between DAA use and the risk of dysglycemia (including hypo/hyperglycemia and new-onset diabetes). The evidence was stronger concerning the risk of hypoglycemia in patients with an increased insulin sensitivity and a decreased need for antidiabetic medications[58]. Thus, patients with diabetes who start an anti-HCV therapy with DAAs should be closely monitored for changes in glucose levels, particularly in the first 3 mo of treatment, and adjustments to their diabetic medication or doses may be necessary.

Independent of the association between HCV and diabetes and diabetes-related consequences, HCV infection seems to be directly associated with an increased risk of cardiovascular disease. Emerging data show that HCV infection might increase the risk of cardiovascular disease by 20%[59]. HCV RNA has been found in the carotid plaques of infected subjects[60]. Thus, HCV could cause atherosclerosis through colonization and replication within the arterial wall[61], but other mechanisms may be involved, such as oxidative stress and the imbalanced secretion of inflammatory molecules[62-65], including C-reactive protein, soluble adhesion molecules and soluble E-selectin[66].

Does SVR have a favorable impact on major adverse cardiovascular events? It has been demonstrated that HCV clearance improves carotid atherosclerosis. Indeed, an intima-media thickness ≥ 1 mm has been associated with a higher risk of cardiovascular events[67,68], but when evaluated at 9-12 mo after the end of DAA-therapy, intima-media thickness and carotid thickening appeared significantly decreased compared to baseline.

An analysis of a large cohort of HCV-infected veterans evaluated the impact of HCV treatment on cardiovascular events in a population free of any cardiovascular disease at baseline. The study included 4436 patients treated with a pegylated interferon–ribavirin regimen, 12667 patients treated with a DAA regimen and 17103 HCV-infected matched controls. Results showed that HCV treatment both with IFN-based regimens and with DAAs was associated with a significantly decreased incidence of all cardiovascular events: Acute myocardial infarction, unstable angina, congestive heart failure, peripheral vascular disease, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting and stroke. The greatest benefits were observed in the DAA-treated group. Patients with advanced liver fibrosis at baseline had a higher incidence of cardiovascular events than those with minimal or no fibrosis. Nevertheless, a marked reduction in cardiovascular disease incidence was observed in treated patients independently of the degree of liver fibrosis, and patients with more advanced fibrosis received the greatest advantage[69].

A reduction in cardiovascular deaths after viral eradication has been observed in cirrhotic patients[70]. Moreover, a recent evaluation of the Italian Rete Sicilia Selezione Terapia-HCV cohort confirmed that DAA-induced SVR is independently associated with a reduction in the risk of both hepatic and cardiovascular mortality[71,72]. This observation has been further confirmed in other studies[73].

In conclusion, DAA treatment in HCV patients with glucose abnormalities is associated with an improvement in glucose control and a reduced need for antidiabetic medications. Furthermore, HCV eradication in diabetic patients seems to reduce the incidence of long-term vascular complications. However, glucose levels in HCV diabetic patients undergoing DAA treatment should be closely monitored because of the risk of hypoglycemia, and diabetic medications may need some adjustment.

DAA treatment is associated with a significantly decreased incidence of all cardiovascular events independent of the degree of liver fibrosis and is also associated with a reduction in the risk of both hepatic and cardiovascular mortality (Table 1).

IS THERE ANY CAUSE FOR CONCERN WHEN USING DAAs IN HCV PATIENTS WITH A CONCOMITANT HBV INFECTION?

In patients with a HCV-HBV coinfection, HCV is usually the main driver of chronic inflammation and viral activity, while HBV DNA levels are generally low or undetectable[1]. In these patients, HBV flares have been described after HCV suppression both by IFN-based treatments and by DAAs[74,75].

In 2016, the Food and Drugs Administration issued an alert about the risk of HBV reactivation in patients treated with DAAs. Notably, in some cases HBV reactivation resulted in severe liver disorders or death. The full data were subsequently published. They referred to 29 cases of HBV reactivation in HCV-HBV patients after treatment with DAAs. The median time lapse between DAA initiation and HBV reactivation was 46 d. Before DAA initiation, 13 patients were hepatitis B surface antigen (HBsAg) positive, 4 patients were HBsAg negative, and 12 patients did not have HBsAg reported. Two cases resulted in death and one case in liver transplantation[76,77].

Since then, various reports have been published on HBV reactivation in HCV-HBV patients treated with DAAs, both in HBsAg positive and in HBsAg negative/anti-hepatitis B core (HBc) positive patients[75,78,79]. The risk of HBV reactivation is lower in HBsAg negative/anti-HBc positive than in HBsAg-positive patients. An asymptomatic increase in HBV DNA level is generally observed, although clinically relevant events are infrequent[80].

On the basis of all these data, the European Association for the Study of the Liver guidelines state that patients undergoing DAA treatment for hepatitis C should be tested for HBs antigen, anti-HBc antibodies and anti-HBs antibodies. If HBsAg is present, then therapy with anti-HBV nucleoside/nucleotide analogue is indicated. In HBsAg negative/anti-HBc positive patients, monitoring for alanine transaminase levels, HBsAg and HBV DNA would be warranted during and after anti-HCV therapy[1].

In conclusion, in patients with HCV/HBV coinfection there is a risk of HBV reactivation after HCV clearance. However, the risk is low, particularly in HBsAg-negative/anti-HBc-positive patients. It is generally associated with an asymptomatic increase in HBV DNA levels, but threatening clinical events have been described. Patients undergoing DAA treatment should be tested for HBV infection (Table 1).

CONCLUSION

DAAs are extremely effective in eradicating infection. Furthermore, treatments are very well-tolerated, and their duration is reduced. Most of the information has been obtained from studies including patients with chronic hepatitis or early cirrhosis, whereas large studies in patients with advanced cirrhosis have shown slightly lower but still quite satisfactory response rates, although modified treatment schedules may be necessary.

Despite these enthusiastic results, some important points must be kept in mind. First, although the incidence of liver cancer is reduced after viral eradication, it remains high enough to warrant the continuation of periodic surveillance for an early diagnosis. The risk of recurrence seems to be unaffected, even if survival improves in treated patients because of the reduced risk of liver disease progression.

Furthermore, HCV eradication is associated with a reduced diabetes incidence and improved glycemic control, but there are concerns about the risk of hypoglycemia after virus clearance.

In patients with HCV/HBV coinfection, HBV reactivation after HCV clearance has been described, sometimes associated with clinically relevant events. Baseline evaluation for HBV coinfection is therefore mandatory before treatment.

REFERENCES

1 **European Association for the Study of the Liver**. EASL recommendations on treatment of hepatitis C: Final update of the series☆. *J Hepatol* 2020; **73**: 1170-1218 [PMID: 32956768 DOI: 10.1016/j.jhep.2020.08.018]

2 **Feld JJ**, Jacobson IM, Hézode C, Asselah T, Ruane PJ, Gruener N, Abergel A, Mangia A, Lai CL, Chan HL, Mazzotta F, Moreno C, Yoshida E, Shafran SD, Towner WJ, Tran TT, McNally J, Osinusi A, Svarovskaia E, Zhu Y, Brainard DM, McHutchison JG, Agarwal K, Zeuzem S; ASTRAL-1 Investigators. Sofosbuvir and Velpatasvir for HCV Genotype 1, 2, 4, 5, and 6 Infection. *N Engl J Med* 2015; **373**: 2599-2607 [PMID: 26571066 DOI: 10.1056/NEJMoa1512610]

3 **Foster GR**, Afdhal N, Roberts SK, Bräu N, Gane EJ, Pianko S, Lawitz E, Thompson A, Shiffman ML, Cooper C, Towner WJ, Conway B, Ruane P, Bourlière M, Asselah T, Berg T, Zeuzem S, Rosenberg W, Agarwal K, Stedman CA, Mo H, Dvory-Sobol H, Han L, Wang J, McNally J, Osinusi A, Brainard DM, McHutchison JG, Mazzotta F, Tran TT, Gordon SC, Patel K, Reau N, Mangia A, Sulkowski M; ASTRAL-2 Investigators; ASTRAL-3 Investigators. Sofosbuvir and Velpatasvir for HCV Genotype 2 and 3 Infection. *N Engl J Med* 2015; **373**: 2608-2617 [PMID: 26575258 DOI: 10.1056/NEJMoa1512612]

4 **Foster GR**, Gane E, Asatryan A, Asselah T, Ruane PJ, PolS, Poordad F, Stedman CA, Dore G, Roberts SK, Kaita K, Vierling J, Vargas HE, Kort J, Lin C-W, Liu R, NgT, Mensa F. ENDURANCE-3: safety and efficacy of glecaprevir/pibrentasvir compared to sofosbuvir plus daclatasvir in treatment-naïve HCV genotype 3-infected patients without cirrhosis. *J Hepatol* 2017; **66**: S33 [DOI: 10.1016/S0168-8278(17)30326-4]

5 **Forns X**, Lee SS, Valdes J, Lens S, Ghalib R, Aguilar H, Felizarta F, Hassanein T, Hinrichsen H, Rincon D, Morillas R, Zeuzem S, Horsmans Y, Nelson DR, Yu Y, Krishnan P, Lin CW, Kort JJ, Mensa FJ. Glecaprevir plus pibrentasvir for chronic hepatitis C virus genotype 1, 2, 4, 5, or 6 infection in adults with compensated cirrhosis (EXPEDITION-1): a single-arm, open-label, multicentre phase 3 trial. *Lancet Infect Dis* 2017; **17**: 1062-1068 [PMID: 28818546 DOI: 10.1016/S1473-3099(17)30496-6]

6 **Carrat F**, Fontaine H, Dorival C, Simony M, Diallo A, Hezode C, De Ledinghen V, Larrey D, Haour G, Bronowicki JP, Zoulim F, Asselah T, Marcellin P, Thabut D, Leroy V, Tran A, Habersetzer F, Samuel D, Guyader D, Chazouilleres O, Mathurin P, Metivier S, Alric L, Riachi G, Gournay J, Abergel A, Cales P, Ganne N, Loustaud-Ratti V, D'Alteroche L, Causse X, Geist C, Minello A, Rosa I, Gelu-Simeon M, Portal I, Raffi F, Bourliere M, Pol S; French ANRS CO22 Hepather cohort. Clinical outcomes in patients with chronic hepatitis C after direct-acting antiviral treatment: a prospective cohort study. *Lancet* 2019; **393**: 1453-1464 [PMID: 30765123 DOI: 10.1016/S0140-6736(18)32111-1]

7 **Mauro E**, Crespo G, Montironi C, Londoño MC, Hernández-Gea V, Ruiz P, Sastre L, Lombardo J, Mariño Z, Díaz A, Colmenero J, Rimola A, Garcia-Pagán JC, Brunet M, Forns X, Navasa M. Portal pressure and liver stiffness measurements in the prediction of fibrosis regression after sustained virological response in recurrent hepatitis C. *Hepatology* 2018; **67**: 1683-1694 [PMID: 28960366 DOI: 10.1002/hep.29557]

8 **Wyles D**, Poordad F, Wang S, Alric L, Felizarta F, Kwo PY, Maliakkal B, Agarwal K, Hassanein T, Weilert F, Lee SS, Kort J, Lovell SS, Liu R, Lin CW, Pilot-Matias T, Krishnan P, Mensa FJ. Glecaprevir/pibrentasvir for hepatitis C virus genotype 3 patients with cirrhosis and/or prior treatment experience: A partially randomized phase 3 clinical trial. *Hepatology* 2018; **67**: 514-523 [PMID: 28926120 DOI: 10.1002/hep.29541]

9 **Mangia A**, Milligan S, Khalili M, Fagiuoli S, Shafran S, Carrat F, Ouzan D, Papatheodoridis G, Ramji A, Borgia S, Wedemeyer H, Piazzolla V, Hernández F, Wick N, Fishbein D, Lampertico P, Doucette K, Mertens M, Vanstraelen K, Turnés J. GS-03-Global real world evidence of sofosbuvir/velpatasvir as a simple, effective regimen for the treatment of chronic hepatitis C patients: Integrated analysis of 12 clinical practice cohorts". *J Hepatol* 2019; **70**: e2-e3 [DOI: 10.1016/S0618-8278(19)30003-9]

10 **Lampertico P**, Carrión JA, Curry M, Turnes J, Cornberg M, Negro F, Brown A, Persico M, Wick N, Porcalla A, Pangerl A, Crown E, Larsen L, Yu Y, Wedemeyer H. Real-world effectiveness and safety of glecaprevir/pibrentasvir for the treatment of patients with chronic HCV infection: A meta-analysis. *J Hepatol* 2020; **72**: 1112-1121 [PMID: 32061651 DOI: 10.1016/j.jhep.2020.01.025]

11 **D'Ambrosio R**, Aghemo A, Rumi MG, Ronchi G, Donato MF, Paradis V, Colombo M, Bedossa P. A morphometric and immunohistochemical study to assess the benefit of a sustained virological response in hepatitis C virus patients with cirrhosis. *Hepatology* 2012; **56**: 532-543 [PMID: 22271347 DOI: 10.1002/hep.25606]

12 **Nahon P**, Bourcier V, Layese R, Audureau E, Cagnot C, Marcellin P, Guyader D, Fontaine H, Larrey D, De Lédinghen V, Ouzan D, Zoulim F, Roulot D, Tran A, Bronowicki JP, Zarski JP, Leroy V, Riachi G, Calès P, Péron JM, Alric L, Bourlière M, Mathurin P, Dharancy S, Blanc JF, Abergel A, Serfaty L, Mallat A, Grangé JD, Attali P, Bacq Y, Wartelle C, Dao T, Benhamou Y, Pilette C, Silvain C, Christidis C, Capron D, Bernard-Chabert B, Zucman D, Di Martino V, Thibaut V, Salmon D, Ziol M, Sutton A, Pol S, Roudot-Thoraval F; ANRS CO12 CirVir Group. Eradication of Hepatitis C Virus Infection in Patients With Cirrhosis Reduces Risk of Liver and Non-Liver Complications. *Gastroenterology* 2017; **152**: 142-156. e2 [PMID: 27641509 DOI: 10.1053/j.gastro.2016.09.009]

13 **Backus LI**, Belperio PS, Shahoumian TA, Mole LA. Impact of Sustained Virologic Response with Direct-Acting Antiviral Treatment on Mortality in Patients with Advanced Liver Disease. *Hepatology* 2019; **69**: 487-497 [PMID: 28749564 DOI: 10.1002/hep.29408]

14 **Mandorfer M**, Hernández-Gea V, García-Pagán JC, Reiberger T. Noninvasive Diagnostics for Portal Hypertension: A Comprehensive Review. *Semin Liver Dis* 2020; **40**: 240-255 [PMID: 32557480 DOI: 10.1055/s-0040-1708806]

15 **Curry MP**, O'Leary JG, Bzowej N, Muir AJ, Korenblat KM, Fenkel JM, Reddy KR, Lawitz E, Flamm SL, Schiano T, Teperman L, Fontana R, Schiff E, Fried M, Doehle B, An D, McNally J, Osinusi A, Brainard DM, McHutchison JG, Brown RS Jr, Charlton M; ASTRAL-4 Investigators. Sofosbuvir and Velpatasvir for HCV in Patients with Decompensated Cirrhosis. *N Engl J Med* 2015; **373**: 2618-2628 [PMID: 26569658 DOI: 10.1056/NEJMoa1512614]

16 **Manns M**, Samuel D, Gane EJ, Mutimer D, McCaughan G, Buti M, Prieto M, Calleja JL, Peck-Radosavljevic M, Müllhaupt B, Agarwal K, Angus P, Yoshida EM, Colombo M, Rizzetto M, Dvory-Sobol H, Denning J, Arterburn S, Pang PS, Brainard D, McHutchison JG, Dufour JF, Van Vlierberghe H, van Hoek B, Forns X; SOLAR-2 investigators. Ledipasvir and sofosbuvir plus ribavirin in patients with genotype 1 or 4 hepatitis C virus infection and advanced liver disease: a multicentre, open-label, randomised, phase 2 trial. *Lancet Infect Dis* 2016; **16**: 685-697 [PMID: 26907736 DOI: 10.1016/S1473-3099(16)00052-9]

17 **Lu M**, Wu KH, Li J, Moorman AC, Spradling PR, Teshale EH, Boscarino JA, Daida YG, Schmidt MA, Rupp LB, Zhang T, Trudeau S, Gordon SC. Adjuvant ribavirin and longer direct-acting antiviral treatment duration improve sustained virological response among hepatitis C patients at risk of treatment failure. *J Viral Hepat* 2019; **26**: 1210-1217 [PMID: 31197910 DOI: 10.1111/jvh.13162]

18 **Foster GR**, Irving WL, Cheung MC, Walker AJ, Hudson BE, Verma S, McLauchlan J, Mutimer DJ, Brown A, Gelson WT, MacDonald DC, Agarwal K; HCV Research, UK. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. *J Hepatol* 2016; **64**: 1224-1231 [PMID: 26829205 DOI: 10.1016/j.jhep.2016.01.029]

19 **Afdhal N**, Everson GT, Calleja JL, McCaughan GW, Bosch J, Brainard DM, McHutchison JG, De-Oertel S, An D, Charlton M, Reddy KR, Asselah T, Gane E, Curry MP, Forns X. Effect of viral suppression on hepatic venous pressure gradient in hepatitis C with cirrhosis and portal hypertension. *J Viral Hepat* 2017; **24**: 823-831 [PMID: 28295923 DOI: 10.1111/jvh.12706]

20 **Lens S**, Alvarado-Tapias E, Mariño Z, Londoño MC, LLop E, Martinez J, Fortea JI, Ibañez L, Ariza X, Baiges A, Gallego A, Bañares R, Puente A, Albillos A, Calleja JL, Torras X, Hernández-Gea V, Bosch J, Villanueva C, Forns X, García-Pagán JC. Effects of All-Oral Anti-Viral Therapy on HVPG and Systemic Hemodynamics in Patients With Hepatitis C Virus-Associated Cirrhosis. *Gastroenterology* 2017; **153**: 1273-1283. e1 [PMID: 28734831 DOI: 10.1053/j.gastro.2017.07.016]

21 **Ferrarese A**, Germani G, Gambato M, Russo FP, Senzolo M, Zanetto A, Shalaby S, Cillo U, Zanus G, Angeli P, Burra P. Hepatitis C virus related cirrhosis decreased as indication to liver transplantation since the introduction of direct-acting antivirals: A single-center study. *World J Gastroenterol* 2018; **24**: 4403-4411 [PMID: 30344424 DOI: 10.3748/wjg.v24.i38.4403]

22 **Perricone G**, Duvoux C, Berenguer M, Cortesi PA, Vinaixa C, Facchetti R, Mazzarelli C, Rockenschaub SR, Martini S, Morelli C, Monico S, Volpes R, Pageaux GP, Fagiuoli S, Belli LS; European Liver and Intestine Transplant Association (ELITA). Delisting HCV-infected liver transplant candidates who improved after viral eradication: Outcome 2 years after delisting. *Liver Int* 2018; **38**: 2170-2177 [PMID: 29750389 DOI: 10.1111/Liv.13878]

23 **Poordad F**, Schiff ER, Vierling JM, Landis C, Fontana RJ, Yang R, McPhee F, Hughes EA, Noviello S, Swenson ES. Daclatasvir with sofosbuvir and ribavirin for hepatitis C virus infection with advanced cirrhosis or post-liver transplantation recurrence. *Hepatology* 2016; **63**: 1493-1505 [PMID: 26754432 DOI: 10.1002/hep.28446]

24 **Singal AG**, Lim JK, Kanwal F. AGA Clinical Practice Update on Interaction Between Oral Direct-Acting Antivirals for Chronic Hepatitis C Infection and Hepatocellular Carcinoma: Expert Review. *Gastroenterology* 2019; **156**: 2149-2157 [PMID: 30878469 DOI: 10.1053/j.gastro.2019.02.046]

25 **Kanwal F**, Kramer J, Asch SM, Chayanupatkul M, Cao Y, El-Serag HB. Risk of Hepatocellular Cancer in HCV Patients Treated With Direct-Acting Antiviral Agents. *Gastroenterology* 2017; **153**: 996-1005. e1 [PMID: 28642197 DOI: 10.1053/j.gastro.2017.06.012]

26 **Calvaruso V**, Cabibbo G, Cacciola I, Petta S, Madonia S, Bellia A, Tinè F, Distefano M, Licata A, Giannitrapani L, Prestileo T, Mazzola G, Di Rosolini MA, Larocca L, Bertino G, Digiacomo A, Benanti F, Guarneri L, Averna A, Iacobello C, Magro A, Scalisi I, Cartabellotta F, Savalli F, Barbara M, Davì A, Russello M, Scifo G, Squadrito G, Cammà C, Raimondo G, Craxì A, Di Marco V; Rete Sicilia Selezione Terapia–HCV (RESIST-HCV). Incidence of Hepatocellular Carcinoma in Patients With HCV-Associated Cirrhosis Treated With Direct-Acting Antiviral Agents. *Gastroenterology* 2018; **155**: 411-421. e4 [PMID: 29655836 DOI: 10.1053/j.gastro.2018.04.008]

27 **Tanaka Y**, Ogawa E, Huang CF, Toyoda H, Jun DW, Tseng CH, Hsu YC, Enomoto M, Takahashi H, Furusyo N, Yeh ML, Iio E, Yasuda S, Lam CP, Lee DH, Haga H, Yoon EL, Ahn SB, Wong G, Nakamuta M, Nomura H, Tsai PC, Jung JH, Song DS, Dang H, Maeda M, Henry L, Cheung R, Yuen MF, Ueno Y, Eguchi Y, Tamori A, Yu ML, Hayashi J, Nguyen MH; REAL-C Investigators. HCC risk post-SVR with DAAs in East Asians: findings from the REAL-C cohort. *Hepatol Int* 2020; **14**: 1023-1033 [PMID: 33277685 DOI: 10.1007/s12072-020-10105-2]

28 **Kanwal F**, Kramer JR, Asch SM, Cao Y, Li L, El-Serag HB. Long-Term Risk of Hepatocellular Carcinoma in HCV Patients Treated With Direct Acting Antiviral Agents. *Hepatology* 2020; **71**: 44-55 [PMID: 31222774 DOI: 10.1002/hep.30823]

29 **Reig M**, Mariño Z, Perelló C, Iñarrairaegui M, Ribeiro A, Lens S, Díaz A, Vilana R, Darnell A, Varela M, Sangro B, Calleja JL, Forns X, Bruix J. Unexpected high rate of early tumor recurrence in patients with HCV-related HCC undergoing interferon-free therapy. *J Hepatol* 2016; **65**: 719-726 [PMID: 27084592 DOI: 10.1016/j.jhep.2016.04.008]

30 **Waziry R**, Hajarizadeh B, Grebely J, Amin J, Law M, Danta M, George J, Dore GJ. Hepatocellular carcinoma risk following direct-acting antiviral HCV therapy: A systematic review, meta-analyses, and meta-regression. *J Hepatol* 2017; **67**: 1204-1212 [PMID: 28802876 DOI: 10.1016/j.jhep.2017.07.025]

31 **Li DK**, Ren Y, Fierer DS, Rutledge S, Shaikh OS, Lo Re V 3rd, Simon T, Abou-Samra AB, Chung RT, Butt AA. The short-term incidence of hepatocellular carcinoma is not increased after hepatitis C treatment with direct-acting antivirals: An ERCHIVES study. *Hepatology* 2018; **67**: 2244-2253 [PMID: 29205416 DOI: 10.1002/hep.29707]

32 **Singal AG**, Rich NE, Mehta N, Branch A, Pillai A, Hoteit M, Volk M, Odewole M, Scaglione S, Guy J, Said A, Feld JJ, John BV, Frenette C, Mantry P, Rangnekar AS, Oloruntoba O, Leise M, Jou JH, Bhamidimarri KR, Kulik L, Tran T, Samant H, Dhanasekaran R, Duarte-Rojo A, Salgia R, Eswaran S, Jalal P, Flores A, Satapathy SK, Wong R, Huang A, Misra S, Schwartz M, Mitrani R, Nakka S, Noureddine W, Ho C, Konjeti VR, Dao A, Nelson K, Delarosa K, Rahim U, Mavuram M, Xie JJ, Murphy CC, Parikh ND. Direct-Acting Antiviral Therapy Not Associated With Recurrence of Hepatocellular Carcinoma in a Multicenter North American Cohort Study. *Gastroenterology* 2019; **156**: 1683-1692. e1 [PMID: 30660729 DOI: 10.1053/j.gastro.2019.01.027]

33 **Cabibbo G**, Celsa C, Calvaruso V, Petta S, Cacciola I, Cannavò MR, Madonia S, Rossi M, Magro B, Rini F, Distefano M, Larocca L, Prestileo T, Malizia G, Bertino G, Benanti F, Licata A, Scalisi I, Mazzola G, Di Rosolini MA, Alaimo G, Averna A, Cartabellotta F, Alessi N, Guastella S, Russello M, Scifo G, Squadrito G, Raimondo G, Trevisani F, Craxì A, Di Marco V, Cammà C; Rete Sicilia Selezione Terapia – HCV (RESIST-HCV) and Italian Liver Cancer (ITA. LI.CA.) Group. Direct-acting antivirals after successful treatment of early hepatocellular carcinoma improve survival in HCV-cirrhotic patients. *J Hepatol* 2019; **71**: 265-273 [PMID: 30959157 DOI: 10.1016/j.jhep.2019.03.027]

34 **Singal AG**, Rich NE, Mehta N, Branch AD, Pillai A, Hoteit M, Volk M, Odewole M, Scaglione S, Guy J, Said A, Feld JJ, John BV, Frenette C, Mantry P, Rangnekar AS, Oloruntoba O, Leise M, Jou JH, Bhamidimarri KR, Kulik L, Ioannou GN, Huang A, Tran T, Samant H, Dhanasekaran R, Duarte-Rojo A, Salgia R, Eswaran S, Jalal P, Flores A, Satapathy SK, Kagan S, Gopal P, Wong R, Parikh ND, Murphy CC. Direct-Acting Antiviral Therapy for Hepatitis C Virus Infection Is Associated With Increased Survival in Patients With a History of Hepatocellular Carcinoma. *Gastroenterology* 2019; **157**: 1253-1263. e2 [PMID: 31374215 DOI: 10.1053/j.gastro.2019.07.040]

35 **Cabibbo G**, Petta S, Calvaruso V, Cacciola I, Cannavò MR, Madonia S, Distefano M, Larocca L, Prestileo T, Tinè F, Bertino G, Giannitrapani L, Benanti F, Licata A, Scalisi I, Mazzola G, Cartabellotta F, Alessi N, Barbàra M, Russello M, Scifo G, Squadrito G, Raimondo G, Craxì A, Di Marco V, Cammà C; Rete Sicilia Selezione Terapia - HCV (RESIST-HCV). Is early recurrence of hepatocellular carcinoma in HCV cirrhotic patients affected by treatment with direct-acting antivirals? *Aliment Pharmacol Ther* 2017; **46**: 688-695 [PMID: 28791711 DOI: 10.1111/apt.14256]

36 **White DL**, Ratziu V, El-Serag HB. Hepatitis C infection and risk of diabetes: a systematic review and meta-analysis. *J Hepatol* 2008; **49**: 831-844 [PMID: 18814931 DOI: 10.1016/j.jhep.2008.08.006]

37 **Desbois AC**, Cacoub P. Diabetes mellitus, insulin resistance and hepatitis C virus infection: A contemporary review. *World J Gastroenterol* 2017; **23**: 1697-1711 [PMID: 28321170 DOI: 10.3748/wjg.v23.i9.1697]

38 **Chen Y**, Ji H, Shao J, Jia Y, Bao Q, Zhu J, Zhang L, Shen Y. Different Hepatitis C Virus Infection Statuses Show a Significant Risk of Developing Type 2 Diabetes Mellitus: A Network Meta-Analysis. *Dig Dis Sci* 2020; **65**: 1940-1950 [PMID: 31758432 DOI: 10.1007/s10620-019-05918-7]

39 **Huang JF**, Dai CY, Hwang SJ, Ho CK, Hsiao PJ, Hsieh MY, Lee LP, Lin ZY, Chen SC, Wang LY, Shin SJ, Chang WY, Chuang WL, Yu ML. Hepatitis C viremia increases the association with type 2 diabetes mellitus in a hepatitis B and C endemic area: an epidemiological link with virological implication. *Am J Gastroenterol* 2007; **102**: 1237-1243 [PMID: 17531012 DOI: 10.1111/j.1572-0241.2007.01181.x]

40 **Visnegarwala F**, Chen L, Raghavan S, Tedaldi E. Prevalence of diabetes mellitus and dyslipidemia among antiretroviral naive patients co-infected with hepatitis C virus (HCV) and HIV-1 compared to patients without co-infection. *J Infect* 2005; **50**: 331-337 [PMID: 15845431 DOI: 10.1016/j.jinf.2004.06.001]

41 **Huang YW**, Yang SS, Fu SC, Wang TC, Hsu CK, Chen DS, Hu JT, Kao JH. Increased risk of cirrhosis and its decompensation in chronic hepatitis C patients with new-onset diabetes: a nationwide

cohort study. *Hepatology* 2014; **60**: 807-814 [PMID: 24919583 DOI: 10.1002/hep.27212]

42 **Hsu CS**, Liu CH, Liu CJ, Hsu SJ, Chen CL, Hwang JJ, Lai MY, Chen PJ, Chen DS, Kao JH. Association of metabolic profiles with hepatic fibrosis in chronic hepatitis C patients with genotype 1 or 2 infection. *J Gastroenterol Hepatol* 2010; **25**: 970-977 [PMID: 20546452 DOI: 10.1111/j.1440-1746.2009.06186.x]

43 **Elkrief L**, Chouinard P, Bendersky N, Hajage D, Larroque B, Babany G, Kutala B, Francoz C, Boyer N, Moreau R, Durand F, Marcellin P, Rautou PE, Valla D. Diabetes mellitus is an independent prognostic factor for major liver-related outcomes in patients with cirrhosis and chronic hepatitis C. *Hepatology* 2014; **60**: 823-831 [PMID: 24841704 DOI: 10.1002/hep.27228]

44 **Huang YW**, Wang TC, Yang SS, Lin SY, Fu SC, Hu JT, Liu CJ, Kao JH, Chen DS. Increased risk of hepatocellular carcinoma in chronic hepatitis C patients with new onset diabetes: a nation-wide cohort study. *Aliment Pharmacol Ther* 2015; **42**: 902-911 [PMID: 26211742 DOI: 10.1111/apt.13341]

45 **Arase Y**, Suzuki F, Suzuki Y, Akuta N, Kobayashi M, Kawamura Y, Yatsuji H, Sezaki H, Hosaka T, Hirakawa M, Ikeda K, Kumada H. Sustained virological response reduces incidence of onset of type 2 diabetes in chronic hepatitis C. *Hepatology* 2009; **49**: 739-744 [PMID: 19127513 DOI: 10.1002/hep.22703]

46 **Simó R**, Lecube A, Genescà J, Esteban JI, Hernández C. Sustained virological response correlates with reduction in the incidence of glucose abnormalities in patients with chronic hepatitis C virus infection. *Diabetes Care* 2006; **29**: 2462-2466 [PMID: 17065685 DOI: 10.2337/dc06-0456]

47 **Aghemo A**, Prati GM, Rumi MG, Soffredini R, D'Ambrosio R, Orsi E, De Nicola S, Degasperi E, Grancini V, Colombo M. Sustained virological response prevents the development of insulin resistance in patients with chronic hepatitis C. *Hepatology* 2012; **56**: 1681-1687 [PMID: 22619107 DOI: 10.1002/hep.25867]

48 **Romero-Gómez M**, Fernández-Rodríguez CM, Andrade RJ, Diago M, Alonso S, Planas R, Solá R, Pons JA, Salmerón J, Barcena R, Perez R, Carmona I, Durán S. Effect of sustained virological response to treatment on the incidence of abnormal glucose values in chronic hepatitis C. *J Hepatol* 2008; **48**: 721-727 [PMID: 18308416 DOI: 10.1016/j.jhep.2007.11.022]

49 **Alsebaey A**, Elhelbawy M, Abdel-Razek W, Hashim M, Elshenawy H, Waked I. HCV treatment with direct acting antivirals improves the insulin sensitivity. *Expert Rev Anti Infect Ther* 2019; **17**: 749-754 [PMID: 31393188 DOI: 10.1080/14787210.2019.1653184]

50 **Ribaldone DG**, Sacco M, Saracco GM. The Effect of Viral Clearance Achieved by Direct-Acting Antiviral Agents on Hepatitis C Virus Positive Patients with Type 2 Diabetes Mellitus: A Word of Caution after the Initial Enthusiasm. *J Clin Med* 2020; **9** [PMID: 32092892 DOI: 10.3390/jcm9020563]

51 **Carnovale C**, Pozzi M, Dassano A, D'Addio F, Gentili M, Magni C, Clementi E, Radice S, Fiorina P. The impact of a successful treatment of hepatitis C virus on glyco-metabolic control in diabetic patients: a systematic review and meta-analysis. *Acta Diabetol* 2019; **56**: 341-354 [PMID: 30478781 DOI: 10.1007/s00592-018-1257-1]

52 **Hum J**, Jou JH, Green PK, Berry K, Lundblad J, Hettinger BD, Chang M, Ioannou GN. Improvement in Glycemic Control of Type 2 Diabetes After Successful Treatment of Hepatitis C Virus. *Diabetes Care* 2017; **40**: 1173-1180 [PMID: 28659309 DOI: 10.2337/dc17-0485]

53 **Ciancio A**, Bosio R, Bo S, Pellegrini M, Sacco M, Vogliotti E, Fassio G, Bianco Mauthe Degerfeld AGF, Gallo M, Giordanino C, Terzi di Bergamo L, Ribaldone D, Bugianesi E, Smedile A, Rizzetto M, Saracco GM. Significant improvement of glycemic control in diabetic patients with HCV infection responding to direct-acting antiviral agents. *J Med Virol* 2018; **90**: 320-327 [PMID: 28960353 DOI: 10.1002/jmv.24954]

54 **Adinolfi LE**, Nevola R, Guerrera B, D'Alterio G, Marrone A, Giordano M, Rinaldi L. Hepatitis C virus clearance by direct-acting antiviral treatments and impact on insulin resistance in chronic hepatitis C patients. *J Gastroenterol Hepatol* 2018; **33**: 1379-1382 [PMID: 29228501 DOI: 10.1111/jgh.14067]

55 **Weidner P**, Boettche D, Zimmerer T, Burgermeister E, Teufel A, Ebert MPA, Antoni C. Impact of direct acting antiviral (DAA) treatment on glucose metabolism and reduction of pre-diabetes in patients with chronic hepatitis C. *J Gastrointestin Liver Dis* 2018; **27**: 281-289 [PMID: 30240472 DOI: 10.15403/jgld.2014.1121.273.daa]

56 **Hsu YC**, Lin JT, Ho HJ, Kao YH, Huang YT, Hsiao NW, Wu MS, Liu YY, Wu CY. Antiviral treatment for hepatitis C virus infection is associated with improved renal and cardiovascular outcomes in diabetic patients. *Hepatology* 2014; **59**: 1293-1302 [PMID: 24122848 DOI: 10.1002/hep.26892]

57 **Li J**, Gordon SC, Rupp LB, Zhang T, Trudeau S, Holmberg SD, Moorman AC, Spradling PR, Teshale EH, Boscarino JA, Schmidt MA, Daida YG, Lu M; CHeCS Investigators. Sustained virological response to hepatitis C treatment decreases the incidence of complications associated with type 2 diabetes. *Aliment Pharmacol Ther* 2019; **49**: 599-608 [PMID: 30650468 DOI: 10.1111/apt.15102]

58 Summary Safety Review - Direct-acting antivirals (DAAs) - Health Canada. 2020 Feb 17 [cited 27 December 2020]. In: Government of Canada [Internet]. Available from: https://hpr-rps.hres.ca/reg-content/summary-safety-review-detail.php?linkID=SSR00234

59 **Negro F**. Natural History of Hepatic and Extrahepatic Hepatitis C Virus Diseases and Impact of Interferon-Free HCV Therapy. *Cold Spring Harb Perspect Med* 2020; **10**: a036921 [PMID: 31636094 DOI: 10.1101/cshperspect.a036921]

60 **Boddi M**, Abbate R, Chellini B, Giusti B, Giannini C, Pratesi G, Rossi L, Pratesi C, Gensini GF, Paperetti L, Zignego AL. Hepatitis C virus RNA localization in human carotid plaques. *J Clin Virol* 2010; **47**: 72-75 [PMID: 19896417 DOI: 10.1016/j.jcv.2009.10.005]

61 **Petta S**, Amato M, Cabibi D, Cammà C, Di Marco V, Giordano C, Galluzzo A, Craxì A. Visceral adiposity index is associated with histological findings and high viral load in patients with chronic hepatitis C due to genotype 1. *Hepatology* 2010; **52**: 1543-1552 [PMID: 20799355 DOI: 10.1002/hep.23859]

62 **Rehermann B**. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. *Nat Med* 2013; **19**: 859-868 [PMID: 23836236 DOI: 10.1038/nm.3251]

63 **Petit JM**, Minello A, Jooste V, Bour JB, Galland F, Duvillard L, Verges B, Olsson NO, Gambert P, Hillon P. Decreased plasma adiponectin concentrations are closely related to steatosis in hepatitis C virus-infected patients. *J Clin Endocrinol Metab* 2005; **90**: 2240-2243 [PMID: 15644404 DOI: 10.1210/jc.2004-1266]

64 **Cua IH**, Hui JM, Bandara P, Kench JG, Farrell GC, McCaughan GW, George J. Insulin resistance and liver injury in hepatitis C is not associated with virus-specific changes in adipocytokines. *Hepatology* 2007; **46**: 66-73 [PMID: 17596870 DOI: 10.1002/hep.21703]

65 **González-Reimers E**, Quintero-Platt G, Martín-González C, Pérez-Hernández O, Romero-Acevedo L, Santolaria-Fernández F. Thrombin activation and liver inflammation in advanced hepatitis C virus infection. *World J Gastroenterol* 2016; **22**: 4427-4437 [PMID: 27182154 DOI: 10.3748/wjg.v22.i18.4427]

66 **Butt AA**, Xiaoqiang W, Budoff M, Leaf D, Kuller LH, Justice AC. Hepatitis C virus infection and the risk of coronary disease. *Clin Infect Dis* 2009; **49**: 225-232 [PMID: 19508169 DOI: 10.1086/599371]

67 **Howard G**, Sharrett AR, Heiss G, Evans GW, Chambless LE, Riley WA, Burke GL. Carotid artery intimal-medial thickness distribution in general populations as evaluated by B-mode ultrasound. ARIC Investigators. *Stroke* 1993; **24**: 1297-1304 [PMID: 8362421 DOI: 10.1161/01.str.24.9.1297]

68 **Chambless LE**, Heiss G, Folsom AR, Rosamond W, Szklo M, Sharrett AR, Clegg LX. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the Atherosclerosis Risk in Communities (ARIC) Study, 1987-1993. *Am J Epidemiol* 1997; **146**: 483-494 [PMID: 9290509 DOI: 10.1093/oxfordjournals.aje.a009302]

69 **Butt AA**, Yan P, Shuaib A, Abou-Samra AB, Shaikh OS, Freiberg MS. Direct-Acting Antiviral Therapy for HCV Infection Is Associated With a Reduced Risk of Cardiovascular Disease Events. *Gastroenterology* 2019; **156**: 987-996. e8 [PMID: 30445009 DOI: 10.1053/j.gastro.2018.11.022]

70 **Magro B**, CalvarusoV, Petta S, Cacciola I, Cabibbo G, Cartabellotta F, Di Rosolini A, Davì A, Cannavò M, Russello M, Di Stefano M, Scifo G, Di Lorenzo F, Prestileo T, LaRocca L, Montineri A, Fuduli G, Digiacomo A, Cannizzaro M, Madonia S, Licata A, Malizia G, Alaimo G, Bertino G, Cacopardo B, Iacobello C, Averna A, Guarneri L, Scalisi I, Mazzola G, Mondello L, Portelli V, Squadrito G, Cammà C, Raimondo G, Craxì A, Di Marco V. Liver and cardiovascular mortality after DAAS: data from the RESIST-HCV cohort. *Dig Liver Dis* 2019; **51**: e75-e76 [DOI: 10.1016/S1590-8658(19)30134-3]

71 **Calvaruso V**, Craxì A. Hepatic benefits of HCV cure. *J Hepatol* 2020; **73**: 1548-1556 [PMID: 32777323 DOI: 10.1016/j.jhep.2020.08.006]

72 **Calvaruso V**, PettaS, Cacciola I, Cabibbo G, Cartabellotta F, Di Rosolini A, Davì A, Cannavò M, Russello M, DistefanoM, Scifo G, Di Lorenzo F, Prestileo T, La Rocca L, Montineri A, Fiduli G, Digiacomo A, Cannizzaro M, Madonia S, Licata A, Malizia G, Alaimo G, Bertino G, Cacopardo B, Iacobello C, Averna A, Guarneri L, Scalisi I, Mazzola G, Mondello L, Portelli V, SquadritoG, Cammà C, Raimondo G, Craxì A, Di Marco V. Disease outcomes after DAA-induced SVR: data from the resist-HCV cohort. *J Hepatol* 2018; **68**: S83 [DOI: 10.1016/S0168-8278(18)30385-4]

73 **Cacoub P**, Nahon P, Layese R, Blaise L, Desbois AC, Bourcier V, Cagnot C, Marcellin P, Guyader D, Pol S, Larrey D, De Lédinghen V, Ouzan D, Zoulim F, Roulot D, Tran A, Bronowicki JP, Zarski JP, Riachi G, Calès P, Péron JM, Alric L, Bourlière M, Mathurin P, Blanc JF, Abergel A, Serfaty L, Mallat A, Grangé JD, Attali P, Bacq Y, Wartelle C, Dao T, Thabut D, Pilette C, Silvain C, Christidis C, Capron D, Thiefin G, Zucman D, Di Martino V, Bagnis CI, Ziol M, Sutton A, Letouze E, Roudot-Thoraval F, Audureau E; ANRS CO12 CirVir group. Prognostic value of viral eradication for major adverse cardiovascular events in hepatitis C cirrhotic patients. *Am Heart J* 2018; **198**: 4-17 [PMID: 29653647 DOI: 10.1016/j.ahj.2017.10.024]

74 **Potthoff A**, Berg T, Wedemeyer H; HEP-NET B/C Coinfection Study Group. Late hepatitis B virus relapse in patients co-infected with hepatitis B virus and hepatitis C virus after antiviral treatment with pegylated interferon-a2b and ribavirin. *Scand J Gastroenterol* 2009; **44**: 1487-1490 [PMID: 19900055 DOI: 10.3109/00365520903329585]

75 **Wang C**, Ji D, Chen J, Shao Q, Li B, Liu J, Wu V, Wong A, Wang Y, Zhang X, Lu L, Wong C, Tsang S, Zhang Z, Sun J, Hou J, Chen G, Lau G. Hepatitis due to Reactivation of Hepatitis B Virus in Endemic Areas Among Patients With Hepatitis C Treated With Direct-acting Antiviral Agents. *Clin Gastroenterol Hepatol* 2017; **15**: 132-136 [PMID: 27392759 DOI: 10.1016/j.cgh.2016.06.023]

76 **Bersoff-Matcha SJ**, Cao K, Jason M, Ajao A, Jones SC, Meyer T, Brinker A. Hepatitis B Virus Reactivation Associated With Direct-Acting Antiviral Therapy for Chronic Hepatitis C Virus: A Review of Cases Reported to the U.S. Food and Drug Administration Adverse Event Reporting System. *Ann Intern Med* 2017; **166**: 792-798 [PMID: 28437794 DOI: 10.7326/M17-0377]

77 **Pockros PJ**. Black Box Warning for Possible HBV Reactivation During DAA Therapy for Chronic HCV Infection. *Gastroenterol Hepatol (N Y)* 2017; **13**: 536-540 [PMID: 29038644]

78 **Madonia S**, Orlando E, Madonia G, Cannizzaro M. HCV/HBV coinfection: The dark side of DAAs treatment? *Liver Int* 2017; **37**: 1086-1087 [PMID: 27966812 DOI: 10.1111/Liv.13342]

79 **Ozaras R**, Mete B, Tabak F. Occult Hepatitis B and Risk of Reactivation After Hepatitis C Treatment With Direct-Acting Antivirals. *Clin Gastroenterol Hepatol* 2017; **15**: 605 [PMID: 27923721 DOI: 10.1016/j.cgh.2016.11.030]

80 **Liu CJ**, Chuang WL, Sheen IS, Wang HY, Chen CY, Tseng KC, Chang TT, Massetto B, Yang JC, Yun C, Knox SJ, Osinusi A, Camus G, Jiang D, Brainard DM, McHutchison JG, Hu TH, Hsu YC, Lo GH, Chu CJ, Chen JJ, Peng CY, Chien RN, Chen PJ. Efficacy of Ledipasvir and Sofosbuvir Treatment of HCV Infection in Patients Coinfected With HBV. *Gastroenterology* 2018; **154**: 989-997 [PMID: 29174546 DOI: 10.1053/j.gastro.2017.11.011]

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MINIREVIEWS

Rethinking the Barcelona clinic liver cancer guidelines: Intermediate stage and Child-Pugh B patients are suitable for surgery?

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Abstract

According to Barcelona Clinic Liver Cancer recommendations, intermediate stage hepatocellular carcinomas (stage B) are excluded from liver resection and are referred to palliative treatment. Moreover, Child-Pugh B patients are not usually candidates for liver resection. However, many hepatobiliary centers in the world manage patients with intermediate stage hepatocellular carcinoma or Child-Pugh B cirrhosis with liver resection, maintaining that hepatic resection is not contraindicated in selected patients with non–early-stage hepatocellular carcinoma and without normal liver function. Several studies demonstrate that resection provides the best survival benefit for selected patients in very early/early and even in intermediate stages of Barcelona Clinic Liver Cancer classification, and this treatment gives good results in the setting of multinodular, large tumors in patients with portal hypertension and/or Child-Pugh B cirrhosis. In this review we explore this controversial topic, and we show through the literature analysis how liver resection may improve the short- and long-term survival rate of carefully selected Barcelona Clinic Liver Cancer B and Child-Pugh B hepatocellular carcinoma patients. However, other large clinical studies are needed to clarify which patients with intermediate stage hepatocellular carcinoma are most likely to benefit from liver resection.

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Core Tip: According to Barcelona Clinic Liver Cancer recommendations, intermediate stage hepatocellular carcinomas are excluded from surgery. Also, Child-Pugh B patients with hepatocellular carcinoma are not usually candidates for liver resection. Nevertheless, several recent studies demonstrated that surgical resection can provide good survival benefit for patients with large and multinodular (diameter > 5 cm, number > 3) hepatocellular carcinoma or Child-Pugh B cirrhosis. In this review we discuss that liver resection may improve the short- and long-term survival of selected patients who have a stage B hepatocellular carcinoma or underlying Child-Pugh B cirrhosis.

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INTRODUCTION

The most common primary liver tumor is hepatocellular carcinoma (HCC), which is the third leading cause of cancer mortality worldwide[1]. The incidence of HCC is increasing in Western countries due to the effects of nonalcoholic fatty liver disease[2-5]. Literature data show how surgery is the therapeutic approach for HCC associated with the most favorable results[6]. The surgical treatments available for patients with HCC are liver resection (LR) or liver transplantation (LT)[2,4,7]. LT is theoretically considered the best treatment of HCC because in addition to treating the neoplasm it also corrects the underlying liver cirrhosis, minimizing the tumor recurrence[2,4,8]. Given the limited number of liver grafts, it is applied only in a small group of patients who meet the Milan criteria[9]. LR can be applied much more widely than LT, but it is usually performed on an unhealthy liver[2,3,7]. LR is a nonprojectable therapeutic option that includes the risk of cancer progression pending a suitable organ[10]. Nevertheless, LR is associated with considerable postoperative morbidity and mortality, and the early and late recurrence rates after this surgical approach remain high[11,12].

The American Association for the Study of Liver Diseases, the European Association for Study of the Liver, and the European Organization for Research and Treatment of Cancer have endorsed the Barcelona Clinic Liver Cancer (BCLC) staging and management system for treating HCC[13-16]. These Western guidelines limit LR to ideal patients, including those in BCLC stage 0 and a subset of patients in BCLC stage A, *i.e.* those with a single tumor without invasion of the main branches of the hepatic or portal vein, preoperative hepatic venous gradient < 10 mmHg, normal bilirubin values, Child-Pugh class A liver function, and good general condition (Figure 1). These stringent surgical indications combine low postoperative mortality rates and optimal long-term results with 5-year survival rates of up to 60%[3,5,17,18].

BCLC stage B include patients with Child-Pugh A and B scores with asymptomatic multicentric tumors (> 3) without vascular invasion or extrahepatic spread. For this group of patients, the BCLC staging system indicates transarterial chemoembolization (TACE) as the main treatment option[13]. TACE seems to provide a better 2-year survival rate than supportive treatment with an average life expectancy of 18-27 mo[19,20]. However, it is unclear whether TACE offers an effective benefit to long-term survival in these patients. BCLC stage B is a heterogeneous category that includes cases that vary widely in size of tumor, number of lesions, and liver function[19,21]. Due to this heterogeneity, TACE may not be the optimal therapy for all patients with stage B HCC[22,23]. Considering the poor prognosis associated with a nonresected HCC, several centers have moved away from these guidelines, reporting acceptable

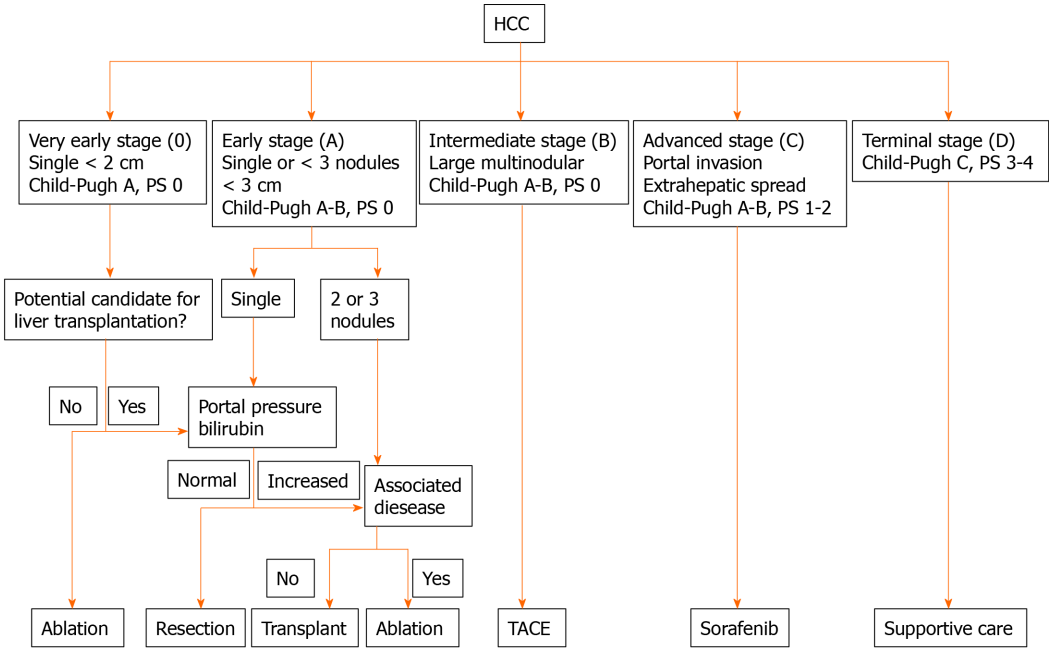


Figure 1 Child-Pugh class A liver function and good general condition. HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization.

short-term survival (mortality from 0% to 5%) and long-term survival rates (ranging from 40% to 60% at 5 years) in BCLC stage B patients treated with LR[24-27]. These recent studies have verified that surgical resection can be safely performed on a subset of patients presenting BCLC stage B HCC, resulting in a long-term survival rate longer than patients that underwent TACE[28,29]. In addition, TACE can only be performed in cases of preserved liver function, and consequently it is not indicated in patients with impaired hepatic functional reserve for the high risk of postoperative liver failure[19,20,30].

In the past decades the presence of underlying cirrhosis was noted to be a negative prognostic factor for HCC recurrence in patients treated with surgery[31,32]. The 5-year survival rates after LR for HCC were different between cirrhotic and noncirrhotic patients (55% *vs* 80%); moreover the survival in Child-Pugh B cirrhosis was poorer than in Child-Pugh A cirrhosis (55% *vs* 28%)[31]. The primary reason of this marked survival difference is currently considered the multicentric carcinogenesis associated with the chronic liver disease. Consequently, in the clinical setting, surgical resection in Child-Pugh B patients tends to be avoided due to the high incidence of postoperative complications and discouraging long-term results[27,33,34]. Minimally invasive techniques have recently been applied to liver surgery with important benefits for patients. In particular, laparoscopic techniques seem to be associated with a lower rate of liver failure in the postoperative period, allowing LR to be performed with satisfactory results even in patients with Child-Pugh B or C cirrhosis[35].

The purpose of this minireview is to illustrate the possible surgical treatment of HCC in intermediate stage of BCLC classification and in Child-Pugh B patients.

LIVER RESECTION IN BCLC-B STAGE PATIENTS

About 20% of patients with HCC are classified as BCLC-B (intermediate stage). These patients present a survival of 50% at 2-year follow-up[2,5,7]. According to Western international recommendations, patients with intermediate HCC are considered unresectable, and TACE is recommended as a standard treatment[14,15]. The cumulative survival time of patients with BCLC-B HCC has been reported to vary from 24 to 60 mo (with overall 5-year survival rate ranging from 0% to 10%) for the best responders to TACE[36,37]. Advances in surgical technique and perioperative care expanded the indications for LR, and consequently surgery has recently been proposed for stage B patients[26,29]. Several studies have shown that LR for an intermediate stage HCC is associated with a promising 5-year survival rate ranging from 25% to 60% with median survival time varying from 20 to 60 mo[38-41]. Moreover, data from other groups show that LR offers satisfactory results for patients

selected at intermediate and advanced stages of the BCLC classification (stage B and C), in the context of large or multinodular tumors[24,42,43].

In 2013, Torzilli *et al*[44] promoted a multicenter East–West observational study on LR in HCC, questioning the European Association for Study of the Liver-American Association for the Study of Liver Diseases guidelines. This study found that the patients who underwent resection for BCLC-B, defined as "single tumor with a diameter greater than 5 cm; two to three tumors with at least one greater than 3 cm in diameter; more than three tumors with any diameter" were characterized by 1-, 3-, and 5-year survival rates of 88%, 71%, and 57%, respectively. These figures were higher than those expected after TACE. Recently, a systematic review about the role of resective surgery in large (> 5 cm) and multinodular HCC found that LR was associated with a 5-year survival of 42% in Asian studies and 32% in non-Asian studies[45]. Chang *et al*[46] analyzed a large cohort grouped according to the tumor size and found that patients with large (between 5 and 10 cm) and huge (> 10 cm) HCCs were associated with 1-year survival of 82% and 68% respectively, and 5-year survival of 50% and 35%, respectively, after curative LR. Similar survival rates about huge (> 10 cm) HCCs were also recorded by other authors[42,47-49]. To date, patients with a single HCC larger than 5 cm are in a "grey area." Initially, large (HCCs > 5 cm) were categorized as BCLC stage B. Currently, some Western experts and Eastern guidelines propose to classify these patients as initial stage, beyond the size[6,50,51].

Another systematic review excluded all those patients in the so-called "grey area" of intermediate BCLC stage with a single large lesion (> 5 cm). Median survival of patients resected for multinodular HCCs in BCLC stage B was 37 mo with a 5-year survival rate of 35%[22]. The 5-year survival was higher for patients with a number of carcinomas less than four *vs* four or more (49% *vs* 23%). In fact, a substantial percentage of patients with multiple HCCs in the early stage and exceeding the BCLC criteria for surgery, survived longer than expected after curative resection[52-55].

A large meta-analysis, based on high-quality studies, suggested that LR may increase the overall survival in patients with intermediate and advanced liver cancer in comparison to TACE[56]. In a recent study, Tada *et al*[57] concluded that LR is superior to TACE in BCLC-B patients with HCC with three nodules or less in Child-Pugh A cirrhosis. The superiority of surgery over TACE in class B BCLC patients was confirmed in four meta-analyses[58-61], one clinical trial[28], and one large observational study[62].

Not all BCLC-B HCC patients are good candidates for surgery. LR is even considered a futile procedure for patients with unfavorable short and mid-term outcomes, such as 90-d postoperative death or early tumor recurrence (within 1 year)[63]. Xu *et al*[64] proposed a discriminant criterion (tumor size < 11 cm or number of lesions < 4) for selecting stage B patients with the aim of receiving curative surgery. Moreover, Wada *et al*[65] identified three types of HCC based on the number and the diameter on imaging: type 1, up to 3 lesions < 5 cm; type 2, up to 3 lesions ≥ 5 cm or 4 tumors of any size; and type 3, > 4 tumors. These authors demonstrated that type 1 patients were associated with better survival than type 3. Intuitively the prognosis of type 2 patients was intermediate between type 1 and type 3 patients.

According to these large and high-quality studies, the Japanese Society of Hepatology, the Asian Pacific Association for the Study of the Liver, and the Chinese Liver Cancer Staging System recently proposed treatment algorithms in which patients with intermediated stage HCC are potential candidates for hepatectomy[8,50,66].

LIVER RESECTION IN CHILD-PUGH B CIRRHOSIS PATIENTS

According to BCLC guidelines, the ideal candidate for LR is the early-stage cirrhotic patient with a single small size lesion[13-15]. On the contrary, it is generally accepted that early stage HCC in Child-Pugh C cirrhosis patients without significant associated diseases should be listed for LT, according to the inclusion criteria[9,67]. Moreover, the indications for TACE should be carefully evaluated in patients with impaired hepatic functional reserve for the high risk of liver failure[19,30]. Furthermore, definitive treatment indications for patients, presenting with HCC with underlying Child-Pugh B cirrhosis, are lacking, and consequently therapeutic allocation remains controversial [27,31,34,68]. In the clinical setting, the indication for LR is dictated by the severity of the hepatic dysfunction and the resection volume that should be performed on the basis of the size and number of HCCs.

In the past decades the presence of cirrhosis in HCC patients undergoing LR was associated with a higher rate of perioperative complications and reduced 5-year

survival rate compared to patients treated with LT; this prognostic difference was more evident in Child-Pugh class B patients[31]. Nevertheless, as a result of recent interest in expanding eligibility for LR, many surgeons believe that well-selected Child-Pugh B patients should not be excluded a priori because good short and long-term survival rates could probably be obtained[31,33,69]. In accordance with this approach, consensus-based HCC treatment guidelines of the Japan Society of Hepatology consider LR for a wide range of HCC patients, including those with Child–Pugh B liver function, multiple tumors (regardless of size), or minimal portal invasion[66]. However, it must be highlighted that surgical resection in Child-Pugh B cirrhosis is associated with considerable postoperative morbidity and mortality, and surgical indications should be carefully weighed. In a recent study the 90-d mortality in Child-Pugh B patients was 10% in cases of major hepatectomies and 3% in segmentectomies[70]. However, it should be remembered that the frequency of adverse events in hepatic surgery still remains significant[71]. A systemic review reported a postoperative complication rate ranging from 27% to 32% and mortality rate from 2.7% to 7.3% after LR among Child-Pugh A and B patients[45].

In a cohort of 137 cases of Child-Pugh B cirrhosis with primary HCC, Harimoto *et al*[72] demonstrated that the overall survival rate in patients that underwent LR was not different from the rate in living donor LT. The main difference between these two types of surgical treatment consisted in the higher incidence of HCC recurrence in patients undergoing LR[31,72,73]. In this context, LR for HCC in Child B patients can be performed with acceptable postoperative morbidity and mortality with a 5-year overall survival of 50%-70%[72,73].

Laparoscopic liver surgery has recently been associated with a lower rate of hepatic failure, ascites production, and overall postoperative complications. This is mainly due to the conservation of extrahepatic collaterals and reduced liver mobilization compared to the open approach[74-76]. Therefore, the development of the minimally invasive laparoscopic approach, aiming to minimize surgical stress and improve patient recovery, allows the subsequent application of these techniques in advanced cirrhotic patients[35,77]. Laparoscopic indications for LR was recently expanded to include major hepatectomies[78]. In the English literature the laparoscopic approach to HCC is well described for Child-Pugh A cirrhosis[74,76,79]. Recently, a single institution series reported LRs in Child-Pugh B and C cirrhotic patients with acceptable results. To date, these data are scarce and come mainly from Asian studies[80-83]. Brytska *et al*[81] performed a subgroup analysis in advanced cirrhotic patients undergoing laparoscopic resection for HCC, including 13 Child-Pugh B and 3 Child-Pugh C patients. Laparoscopic LRs included ten tumorectomies, two segmentectomies, three left lateral sectionectomy, and one right anterior sectionectomy. The authors reported an overall complication rate of 19%, with a 12% occurrence of major postoperative complications (pleural effusion and variceal bleeding), without 90-d mortality. They reported an overall survival of about 85% and a disease-free survival of 42% at the 5-year follow-up.

CONCLUSION

Currently, TACE and sorafenib are indicated for intermediate and advanced HCCs; surgery is not recommended for HCC in BCLC stage B or associated with Child-Pugh B cirrhosis because of unproven survival benefit.

With the advancement of surgical techniques and the consequent reduction of postoperative complications, many experiences have been accumulated to support the advantages of surgery in treating intermediate HCC patients[26,28,29,62]. Recent systematic reviews show how LR is associated to better long-term survival than TACE in patients with stage B HCC. The 5-year survival rate associated with LR can be up to 35%[22,23]. Indeed, in the past decades, several studies have highlighted that LRs performed on multinodular HCC offer satisfactory long-term results, associated with acceptable perioperative morbidity and mortality[52,84,85]. Moreover, the Japanese and Chinese guidelines recommend surgery as a treatment modality for a wide spectrum of HCC patients, including those with Child–Pugh B liver function, multiple tumors, or minimal portal invasion[50,66]. Based on encouraging data, many hepato-biliary centers around the world manage patients with intermediate stage liver cancer with LR, claiming that surgical resection is not contraindicated in selected patients with non-early-stage liver cancer[17,29,38,40,41].

Hence, the restriction of surgical resection to a small subset of patients, as suggested by the Barcelona approach, can be rethought for various reasons. First, the safety of

extensive hepatic surgery improved significantly, making LR a feasible option even for patients with suboptimal liver function or poor performance status[35,70]. Second, surgery does not counterpose TACE. The latter can be used as an additional procedure to decrease tumor volume and give chance for surgery in selected BCLC stage B[86-89]. Finally, the accuracy of the current diagnostic exams may be limited in particular cases of macronodular cirrhosis, leading to possible erroneous classifications with serious consequences on the therapeutic objectives (curative *vs* palliative). Preoperative case selection is essential to reduce surgery risks, possibly identifying the candidate who could profit rather than be harmed by LR.

In 2012, Bolondi *et al*[21] proposed a classification of BCLC B stage into four substages to better identify the prognosis of this heterogeneous class of patients and at the same time suggested the therapeutic options for each category. In this context, a multicenter observational study highlighted how surgical resection is associated with better results in B1/B2 stage than B3/B4[29], according to the subclassification proposed by Bolondi *et al*[21]. Moreover we believe that the type of resection must be balanced with the underlying liver function to direct the patient towards the safest effective oncological treatment. Indeed, the removal of the tumor and preservation of the liver parenchyma are equally important, as both contribute to determining patient survival. In this perspective the Kinki criteria simplify the Bolondi substaging system[90]. Based on Child-Pugh score (5-7 or 8-9) and "up-to-7" rule (the sum of the number of nodules plus the diameter of the largest lesion), BCLC stage B HCCs are subdivided into three subgroups (B1, B2, B3). B1 class is characterized by relatively preserved liver function (Child-Pugh A 5-6 or Child-Pugh B7) and tumor burden within "up-to-7" criterion. According to these authors only patients with B1 HCC can benefit from LR[90].

LR in Child-Pugh B cirrhosis has been reported as an alternative treatment to LT, with promising short and long-term outcomes[72,73]. Moreover laparoscopic LR can be safely performed in selected Child-Pugh B patients with acceptable perioperative outcomes, although to date long-term results are not available[80-83]. Further studies are needed to clarify which Child-Pugh B patients with HCC are suitable candidates for laparoscopic LR, hoping to optimize the chances of long-term survival[35]. To date minimally invasive surgery could be proposed in patients with a satisfactory functional reserve of the liver and less aggressive tumors[74]. Nevertheless, it should be noted that data are scanty and come mainly from single institution case series. Moreover, LR in Child-Pugh B patients is characterized by significant 90-d mortality and morbidity rates, and consequently a tailored therapeutic approach could reduce the frequency of adverse events, offering good long-term results[63,54,70].

In our opinion additional radiological and laboratory data (*e.g.*, tumor location and distribution, serological markers), predictive of radical resection, should be incorporated into the staging system to select patients who may benefit from surgery. In this perspective, the Japanese Society of Hepatology indicated contrast-enhanced magnetic resonance imaging the pivotal exam in the preoperative work-up[66].

According to the most recent experiences, LR may improve the survival of Child-Pugh B and BCLC B carcinoma when patients are carefully selected and surgery is performed in specialized centers. Moreover, the mini-invasive approach allows pushing up the limits of LR in this stage of the disease[75,82]. Consequently, indications for resection could be expanded to include patients from therapies associated with satisfactory results. Nevertheless, randomized controlled trials must be planned to clarify which patients presenting with HCC beyond the classical indications are most likely to benefit from LR. In conclusion, these findings support the future expansion of indications for surgical resection in Western therapeutic guidelines.

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REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Balogh J**, Victor D 3rd, Asham EH, Burroughs SG, Boktour M, Saharia A, Li X, Ghobrial RM,

Monsour HP Jr. Hepatocellular carcinoma: a review. *J Hepatocell Carcinoma* 2016; **3**: 41-53 [PMID: 27785449 DOI: 10.2147/JHC.S61146]
3 **Villanueva A**. Hepatocellular Carcinoma. *N Engl J Med* 2019; **380**: 1450-1462 [PMID: 30970190 DOI: 10.1056/NEJMra1713263]
4 **Dhanasekaran R**, Limaye A, Cabrera R. Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis, and therapeutics. *Hepat Med* 2012; **4**: 19-37 [PMID: 24367230 DOI: 10.2147/HMER.S16316]
5 **Fujiwara N**, Friedman SL, Goossens N, Hoshida Y. Risk factors and prevention of hepatocellular carcinoma in the era of precision medicine. *J Hepatol* 2018; **68**: 526-549 [PMID: 28989095 DOI: 10.1016/j.jhep.2017.09.016]
6 **Surveillance group**. ; Diagnosis group; Staging group; Surgery group; Local ablation group; TACE/TARE/HAI group; Target therapy/systemic therapy group; Radiotherapy group; Prevention group; Drafting group. Management consensus guideline for hepatocellular carcinoma: 2016 updated by the Taiwan Liver Cancer Association and the Gastroenterological Society of Taiwan. *J Formos Med Assoc* 2018; **117**: 381-403 [PMID: 29074347 DOI: 10.1016/j.jfma.2017.09.007]
7 **Llovet JM**, Fuster J, Bruix J; Barcelona-Clínic Liver Cancer Group. The Barcelona approach: diagnosis, staging, and treatment of hepatocellular carcinoma. *Liver Transpl* 2004; **10**: S115-S120 [PMID: 14762851 DOI: 10.1002/Lt.20034]
8 **Omata M**, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK, Sarin SK. Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. *Hepatol Int* 2017; **11**: 317-370 [PMID: 28620797 DOI: 10.1007/s12072-017-9799-9]
9 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699 [PMID: 8594428 DOI: 10.1056/NEJM199603143341104]
10 **Arii S**, Sata M, Sakamoto M, Shimada M, Kumada T, Shiina S, Yamashita T, Kokudo N, Tanaka M, Takayama T, Kudo M. Management of hepatocellular carcinoma: Report of Consensus Meeting in the 45th Annual Meeting of the Japan Society of Hepatology (2009). *Hepatol Res* 2010; **40**: 667-685 [PMID: 20633193 DOI: 10.1111/j.1872-034X.2010.00673.x]
11 **Imamura H**, Matsuyama Y, Tanaka E, Ohkubo T, Hasegawa K, Miyagawa S, Sugawara Y, Minagawa M, Takayama T, Kawasaki S, Makuuchi M. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. *J Hepatol* 2003; **38**: 200-207 [PMID: 12547409 DOI: 10.1016/s0168-8278(02)00360-4]
12 **Famularo S**, Donadon M, Cipriani F, Ardito F, Carissimi F, Perri P, Iaria M, Dominioni T, Zanello M, Conci S, Molfino S, LaBarba G, Ferrari C, Germani P, Patauner S, Pinotti E, Lodo E, Garatti M, Sciannamea I, Troci A, Conticchio M, Floridi A, Chiarelli M, Fumagalli L, Memeo R, Crespi M, Antonucci A, Zimmitti G, Zanus G, Zago M, Frena A, Tarchi P, Griseri G, Ercolani G, Baiocchi GL, Ruzzenente A, Jovine E, Maestri M, DallaValle R, Grazi GL, Giuliante F, Aldrighetti L, Torzilli G, Romano F; HE. RC.O.LE.S. Group. Hepatocellular carcinoma surgical and oncological trends in a national multicentric population: the HERCOLES experience. *Updates Surg* 2020; **72**: 399-411 [PMID: 32170630 DOI: 10.1007/s13304-020-00733-6]
13 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J; EASL Panel of Experts on HCC. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430 [PMID: 11592607 DOI: 10.1016/s0168-8278(01)00130-1]
14 **Heimbach JK**, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, Zhu AX, Murad MH, Marrero JA. AASLD guidelines for the treatment of hepatocellular carcinoma. *Hepatology* 2018; **67**: 358-380 [PMID: 28130846 DOI: 10.1002/hep.29086]
15 **European Association for the Study of the Liver**. European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]
16 **European Association for Study of Liver**. European Organisation for Research and Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *Eur J Cancer* 2012; **48**: 599-641 [PMID: 22424278 DOI: 10.1016/j.ejca.2011.12.021]
17 **Lim KC**, Chow PK, Allen JC, Siddiqui FJ, Chan ES, Tan SB. Systematic review of outcomes of liver resection for early hepatocellular carcinoma within the Milan criteria. *Br J Surg* 2012; **99**: 1622-1629 [PMID: 23023956 DOI: 10.1002/bjs.8915]
18 **Garancini M**, Nespoli S, Romano F, Uggeri F, Degrate L, Okolicsanyi S, Gianotti L. Surgical management of hepatocellular carcinoma within and beyond BCLC indications in a middle volume center. *J Visc Surg* 2018; **155**: 275-282 [PMID: 29606603 DOI: 10.1016/j.jviscsurg.2017.12.001]
19 **Raoul JL**, Sangro B, Forner A, Mazzaferro V, Piscaglia F, Bolondi L, Lencioni R. Evolving strategies for the management of intermediate-stage hepatocellular carcinoma: available evidence and expert opinion on the use of transarterial chemoembolization. *Cancer Treat Rev* 2011; **37**: 212-220 [PMID: 20724077 DOI: 10.1016/j.ctrv.2010.07.006]
20 **Park JW**, Amarapurkar D, Chao Y, Chen PJ, Geschwind JF, Goh KL, Han KH, Kudo M, Lee HC, Lee RC, Lesmana LA, Lim HY, Paik SW, Poon RT, Tan CK, Tanwandee T, Teng G, Cheng AL. Consensus recommendations and review by an International Expert Panel on Interventions in

Hepatocellular Carcinoma (EPOIHCC). *Liver Int* 2013; **33**: 327-337 [PMID: 23331661 DOI: 10.1111/liv.12083]

21 **Bolondi L**, Burroughs A, Dufour JF, Galle PR, Mazzaferro V, Piscaglia F, Raoul JL, Sangro B. Heterogeneity of patients with intermediate (BCLC B) Hepatocellular Carcinoma: proposal for a subclassification to facilitate treatment decisions. *Semin Liver Dis* 2012; **32**: 348-359 [PMID: 23397536 DOI: 10.1055/s-0032-1329906]

22 **Glantzounis GK**, Paliouras A, Stylianidi MC, Milionis H, Tzimas P, Roukos D, Pentheroudakis G, Felekouras E. The role of liver resection in the management of intermediate and advanced stage hepatocellular carcinoma. A systematic review. *Eur J Surg Oncol* 2018; **44**: 195-208 [PMID: 29258719 DOI: 10.1016/j.ejso.2017.11.022]

23 **Koh YX**, Tan HL, Lye WK, Kam JH, Chiow AKH, Tan SS, Choo SP, Chung AYF, Goh BKP. Systematic review of the outcomes of surgical resection for intermediate and advanced Barcelona Clinic Liver Cancer stage hepatocellular carcinoma: A critical appraisal of the evidence. *World J Hepatol* 2018; **10**: 433-447 [PMID: 29988922 DOI: 10.4254/wjh.v10.i6.433]

24 **Chang WT**, Kao WY, Chau GY, Su CW, Lei HJ, Wu JC, Hsia CY, Lui WY, King KL, Lee SD. Hepatic resection can provide long-term survival of patients with non-early-stage hepatocellular carcinoma: extending the indication for resection? *Surgery* 2012; **152**: 809-820 [PMID: 22766361 DOI: 10.1016/j.surg.2012.03.024]

25 **Ciria R**, López-Cillero P, Gallardo AB, Cabrera J, Pleguezuelo M, Ayllón MD, Luque A, Zurera L, Espejo JJ, Rodríguez-Perálvarez M, Montero JL, de la Mata M, Briceño J. Optimizing the management of patients with BCLC stage-B hepatocellular carcinoma: Modern surgical resection as a feasible alternative to transarterial chemoemolization. *Eur J Surg Oncol* 2015; **41**: 1153-1161 [PMID: 26118317 DOI: 10.1016/j.ejso.2015.05.023]

26 **Kim H**, Ahn SW, Hong SK, Yoon KC, Kim HS, Choi YR, Lee HW, Yi NJ, Lee KW, Suh KS; Korean Liver Cancer Association. Survival benefit of liver resection for Barcelona Clinic Liver Cancer stage B hepatocellular carcinoma. *Br J Surg* 2017; **104**: 1045-1052 [PMID: 28480964 DOI: 10.1002/bjs.10541]

27 **Bell R**, Pandanaboyana S, Lodge JPA, Prasad KR, Jones R, Hidalgo E. Primary liver resection for patients with cirrhosis and hepatocellular carcinoma: the role of surgery in BCLC early (A) and intermediate stages (B). *Langenbecks Arch Surg* 2017; **402**: 575-583 [PMID: 27456677 DOI: 10.1007/s00423-016-1475-3]

28 **Yin L**, Li H, Li AJ, Lau WY, Pan ZY, Lai EC, Wu MC, Zhou WP. Partial hepatectomy vs. transcatheter arterial chemoembolization for resectable multiple hepatocellular carcinoma beyond Milan Criteria: a RCT. *J Hepatol* 2014; **61**: 82-88 [PMID: 24650695 DOI: 10.1016/j.jhep.2014.03.012]

29 **Kariyama K**, Nouso K, Wakuta A, Oonishi A, Toyoda H, Tada T, Hiraoka A, Tsuji K, Itobayashi E, Ishikawa T, Takaguchi K, Tsutsui A, Shimada N, Kumada T. Treatment of Intermediate-Stage Hepatocellular Carcinoma in Japan: Position of Curative Therapies. *Liver Cancer* 2020; **9**: 41-49 [PMID: 32071908 DOI: 10.1159/000502479]

30 **Lencioni R**, de Baere T, Soulen MC, Rilling WS, Geschwind JF. Lipiodol transarterial chemoembolization for hepatocellular carcinoma: A systematic review of efficacy and safety data. *Hepatology* 2016; **64**: 106-116 [PMID: 26765068 DOI: 10.1002/hep.28453]

31 **Taura K**, Ikai I, Hatano E, Yasuchika K, Nakajima A, Tada M, Seo S, Machimoto T, Uemoto S. Influence of coexisting cirrhosis on outcomes after partial hepatic resection for hepatocellular carcinoma fulfilling the Milan criteria: an analysis of 293 patients. *Surgery* 2007; **142**: 685-694 [PMID: 17981188 DOI: 10.1016/j.surg.2007.05.009]

32 **Bilimoria MM**, Lauwers GY, Doherty DA, Nagorney DM, Belghiti J, Do KA, Regimbeau JM, Ellis LM, Curley SA, Ikai I, Yamaoka Y, Vauthey JN; International Cooperative Study Group on Hepatocellular Carcinoma. Underlying liver disease, not tumor factors, predicts long-term survival after resection of hepatocellular carcinoma. *Arch Surg* 2001; **136**: 528-535 [PMID: 11343543 DOI: 10.1001/archsurg.136.5.528]

33 **Poon RT**, Fan ST, Lo CM, Liu CL, Lam CM, Yuen WK, Yeung C, Wong J. Extended hepatic resection for hepatocellular carcinoma in patients with cirrhosis: is it justified? *Ann Surg* 2002; **236**: 602-611 [PMID: 12409666 DOI: 10.1097/00000658-200211000-00010]

34 **Choi SH**, Choi GH, Kim SU, Park JY, Joo DJ, Ju MK, Kim MS, Choi JS, Han KH, Kim SI. Role of surgical resection for multiple hepatocellular carcinomas. *World J Gastroenterol* 2013; **19**: 366-374 [PMID: 23372359 DOI: 10.3748/wjg.v19.i3.366]

35 **Vega EA**, Kutlu OC, Joechle K, De La Cruz N, Ko D, Conrad C. Preoperative Prognosticators of Safe Laparoscopic Hepatocellular Carcinoma Resection in Advanced Cirrhosis: a Propensity Score Matching Population-Based Analysis of 1799 Western Patients. *J Gastrointest Surg* 2019; **23**: 1157-1165 [PMID: 30820798 DOI: 10.1007/s11605-019-04139-7]

36 **Lin CT**, Hsu KF, Chen TW, Yu JC, Chan DC, Yu CY, Hsieh TY, Fan HL, Kuo SM, Chung KP, Hsieh CB. Comparing hepatic resection and transarterial chemoembolization for Barcelona Clinic Liver Cancer (BCLC) stage B hepatocellular carcinoma: change for treatment of choice? *World J Surg* 2010; **34**: 2155-2161 [PMID: 20407768 DOI: 10.1007/s00268-010-0598-x]

37 **Zhong JH**, Xiang BD, Gong WF, Ke Y, Mo QG, Ma L, Liu X, Li LQ. Comparison of long-term survival of patients with BCLC stage B hepatocellular carcinoma after liver resection or transarterial chemoembolization. *PLoS One* 2013; **8**: e68193 [PMID: 23874536 DOI: 10.1371/journal.pone.0068193]

38 **Delis SG**, Bakoyiannis A, Tassopoulos N, Athanassiou K, Kelekis D, Madariaga J, Dervenis C. Hepatic resection for hepatocellular carcinoma exceeding Milan criteria. *Surg Oncol* 2010; **19**: 200-207 [PMID: 19500972 DOI: 10.1016/j.suronc.2009.05.003]

39 **Kamiyama T**, Orimo T, Wakayama K, Shimada S, Nagatsu A, Yokoo H, Kamachi H, Yamashita K, Shimamura T, Taketomi A. Survival outcomes of hepatectomy for stage B Hepatocellular carcinoma in the BCLC classification. *World J Surg Oncol* 2017; **15**: 156 [PMID: 28830473 DOI: 10.1186/s12957-017-1229-x]

40 **Bhandare MS**, Patkar S, Shetty N, Polnaya A, Kulkarni S, Dusane RR, Shrikhande SV, Goel M. Liver resection for HCC outside the BCLC criteria. *Langenbecks Arch Surg* 2018; **403**: 37-44 [PMID: 29199380 DOI: 10.1007/s00423-017-1640-3]

41 **Wang H**, Qian YW, Wu MC, Cong WM. Liver Resection Is Justified in Patients with BCLC Intermediate Stage Hepatocellular Carcinoma without Microvascular Invasion. *J Gastrointest Surg* 2020; **24**: 2737-2747 [PMID: 31768830 DOI: 10.1007/s11605-019-04251-8]

42 **Furukawa K**, Shiba H, Horiuchi T, Shirai Y, Haruki K, Fujiwara Y, Sakamoto T, Gocho T, Yanaga K. Survival benefit of hepatic resection for hepatocellular carcinoma beyond the Barcelona Clinic Liver Cancer classification. *J Hepatobiliary Pancreat Sci* 2017; **24**: 199-205 [PMID: 28160422 DOI: 10.1002/jhbp.436]

43 **Zhong JH**, Ke Y, Gong WF, Xiang BD, Ma L, Ye XP, Peng T, Xie GS, Li LQ. Hepatic resection associated with good survival for selected patients with intermediate and advanced-stage hepatocellular carcinoma. *Ann Surg* 2014; **260**: 329-340 [PMID: 24096763 DOI: 10.1097/SLA.0000000000000236]

44 **Torzilli G**, Belghiti J, Kokudo N, Takayama T, Capussotti L, Nuzzo G, Vauthey JN, Choti MA, De Santibanes E, Donadon M, Morenghi E, Makuuchi M. A snapshot of the effective indications and results of surgery for hepatocellular carcinoma in tertiary referral centers: is it adherent to the EASL/AASLD recommendations? *Ann Surg* 2013; **257**: 929-937 [PMID: 23426336 DOI: 10.1097/SLA.0b013e31828329b8]

45 **Zhong JH**, Rodríguez AC, Ke Y, Wang YY, Wang L, Li LQ. Hepatic resection as a safe and effective treatment for hepatocellular carcinoma involving a single large tumor, multiple tumors, or macrovascular invasion. *Medicine (Baltimore)* 2015; **94**: e396 [PMID: 25621684 DOI: 10.1097/MD.0000000000000396]

46 **Chang YJ**, Chung KP, Chang YJ, Chen LJ. Long-term survival of patients undergoing liver resection for very large hepatocellular carcinomas. *Br J Surg* 2016; **103**: 1513-1520 [PMID: 27550624 DOI: 10.1002/bjs.10196]

47 **Tsoulfas G**, Mekras A, Agorastou P, Kiskinis D. Surgical treatment for large hepatocellular carcinoma: does size matter? *ANZ J Surg* 2012; **82**: 510-517 [PMID: 22548726 DOI: 10.1111/j.1445-2197.2012.06079.x]

48 **Lee SG**, Hwang S, Jung JP, Lee YJ, Kim KH, Ahn CS. Outcome of patients with huge hepatocellular carcinoma after primary resection and treatment of recurrent lesions. *Br J Surg* 2007; **94**: 320-326 [PMID: 17205495 DOI: 10.1002/bjs.5622]

49 **Ariizumi S**, Kotera Y, Takahashi Y, Katagiri S, Yamamoto M. Impact of hepatectomy for huge solitary hepatocellular carcinoma. *J Surg Oncol* 2013; **107**: 408-413 [PMID: 22903402 DOI: 10.1002/jso.23226]

50 **Xie DY**, Ren ZG, Zhou J, Fan J, Gao Q. 2019 Chinese clinical guidelines for the management of hepatocellular carcinoma: updates and insights. *Hepatobiliary Surg Nutr* 2020; **9**: 452-463 [PMID: 32832496 DOI: 10.21037/hbsn-20-480]

51 **Bruix J**, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **150**: 835-853 [PMID: 26795574 DOI: 10.1053/j.gastro.2015.12.041]

52 **Ho MC**, Huang GT, Tsang YM, Lee PH, Chen DS, Sheu JC, Chen CH. Liver resection improves the survival of patients with multiple hepatocellular carcinomas. *Ann Surg Oncol* 2009; **16**: 848-855 [PMID: 19159983 DOI: 10.1245/s10434-008-0282-7]

53 **Ishizawa T**, Hasegawa K, Aoki T, Takahashi M, Inoue Y, Sano K, Imamura H, Sugawara Y, Kokudo N, Makuuchi M. Neither multiple tumors nor portal hypertension are surgical contraindications for hepatocellular carcinoma. *Gastroenterology* 2008; **134**: 1908-1916 [PMID: 18549877 DOI: 10.1053/j.gastro.2008.02.091]

54 **Zhao WC**, Fan LF, Yang N, Zhang HB, Chen BD, Yang GS. Preoperative predictors of microvascular invasion in multinodular hepatocellular carcinoma. *Eur J Surg Oncol* 2013; **39**: 858-864 [PMID: 23669199 DOI: 10.1016/j.ejso.2013.04.003]

55 **Lee SY**, Ahn CS, Yoon YI, Lee SG, Hwang S, Kim KH, Moon DB, Ha TY, Song GW, Jung DH, Park GC. Long-term outcomes of liver resection for multiple hepatocellular carcinomas: Single-institution experience with 187 patients. *Ann Hepatobiliary Pancreat Surg* 2020; **24**: 437-444 [PMID: 33234746 DOI: 10.14701/ahbps.2020.24.4.437]

56 **Hyun MH**, Lee YS, Kim JH, Lee CU, Jung YK, Seo YS, Yim HJ, Yeon JE, Byun KS. Hepatic resection compared to chemoembolization in intermediate- to advanced-stage hepatocellular carcinoma: A meta-analysis of high-quality studies. *Hepatology* 2018; **68**: 977-993 [PMID: 29543988 DOI: 10.1002/hep.29883]

57 **Tada T**, Kumada T, Toyoda H, Tsuji K, Hiraoka A, Itobayashi E, Nouso K, Kariyama K, Ishikawa T, Hirooka M, Hiasa Y. Role of hepatic resection in patients with intermediate-stage hepatocellular carcinoma: A multicenter study from Japan. *Cancer Sci* 2017; **108**: 1414-1420 [PMID: 28406546

DOI: 10.1111/cas.13257]
58 **Liu W**, Zhou JG, Sun Y, Zhang L, Xing BC. Hepatic Resection Improved the Long-Term Survival of Patients with BCLC Stage B Hepatocellular Carcinoma in Asia: a Systematic Review and Meta-Analysis. *J Gastrointest Surg* 2015; **19**: 1271-1280 [PMID: 25943910 DOI: 10.1007/s11605-015-2811-6]
59 **Qi X**, Wang D, Su C, Li H, Guo X. Hepatic resection *vs* transarterial chemoembolization for the initial treatment of hepatocellular carcinoma: A systematic review and meta-analysis. *Oncotarget* 2015; **6**: 18715-18733 [PMID: 26243835 DOI: 10.18632/oncotarget.4134]
60 **Liang L**, Xing H, Zhang H, Zhong J, Li C, Lau WY, Wu M, Shen F, Yang T. Surgical resection *vs* transarterial chemoembolization for BCLC intermediate stage hepatocellular carcinoma: a systematic review and meta-analysis. *HPB (Oxford)* 2018; **20**: 110-119 [PMID: 29174493 DOI: 10.1016/j.hpb.2017.10.004]
61 **Labgaa I**, Taffé P, Martin D, Clerc D, Schwartz M, Kokudo N, Denys A, Halkic N, Demartines N, Melloul E. Comparison of Partial Hepatectomy and Transarterial Chemoembolization in Intermediate-Stage Hepatocellular Carcinoma: A Systematic Review and Meta-Analysis. *Liver Cancer* 2020; **9**: 138-147 [PMID: 32399428 DOI: 10.1159/000505093]
62 **Vitale A**, Burra P, Frigo AC, Trevisani F, Farinati F, Spolverato G, Volk M, Giannini EG, Ciccarese F, Piscaglia F, Rapaccini GL, Di Marco M, Caturelli E, Zoli M, Borzio F, Cabibbo G, Felder M, Gasbarrini A, Sacco R, Foschi FG, Missale G, Morisco F, Svegliati Baroni G, Virdone R, Cillo U; Italian Liver Cancer (ITA. LI.CA) group. Survival benefit of liver resection for patients with hepatocellular carcinoma across different Barcelona Clinic Liver Cancer stages: a multicentre study. *J Hepatol* 2015; **62**: 617-624 [PMID: 25450706 DOI: 10.1016/j.jhep.2014.10.037]
63 **Li C**, Shen JY, Zhang XY, Peng W, Wen TF, Yang JY, Yan LN. Predictors of Futile Liver Resection for Patients with Barcelona Clinic Liver Cancer Stage B/C Hepatocellular Carcinoma. *J Gastrointest Surg* 2018; **22**: 496-502 [PMID: 29119530 DOI: 10.1007/s11605-017-3632-6]
64 **Xu W**, Rao Q, An Y, Li M, Xu G, Sang X, Lu X, Zhang Z, Mao Y. Proposal for subclassification to select patients for hepatectomy with intermediate hepatocellular carcinoma and Child-Pugh A liver function: A double-center study from China. *Medicine (Baltimore)* 2018; **97**: e11800 [PMID: 30095644 DOI: 10.1097/MD.0000000000011800]
65 **Wada H**, Eguchi H, Noda T, Ogawa H, Yamada D, Tomimaru Y, Tomokuni A, Asaoka T, Kawamoto K, Gotoh K, Marubashi S, Umeshita K, Nagano H, Doki Y, Mori M. Selection criteria for hepatic resection in intermediate-stage (BCLC stage B) multiple hepatocellular carcinoma. *Surgery* 2016; **160**: 1227-1235 [PMID: 27395761 DOI: 10.1016/j.surg.2016.05.023]
66 **Kokudo N**, Takemura N, Hasegawa K, Takayama T, Kubo S, Shimada M, Nagano H, Hatano E, Izumi N, Kaneko S, Kudo M, Iijima H, Genda T, Tateishi R, Torimura T, Igaki H, Kobayashi S, Sakurai H, Murakami T, Watadani T, Matsuyama Y. Clinical practice guidelines for hepatocellular carcinoma: The Japan Society of Hepatology 2017 (4th JSH-HCC guidelines) 2019 update. *Hepatol Res* 2019; **49**: 1109-1113 [PMID: 31336394 DOI: 10.1111/hepr.13411]
67 **Yao FY**, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. *Hepatology* 2001; **33**: 1394-1403 [PMID: 11391528 DOI: 10.1053/jhep.2001.24563]
68 **Yeh CN**, Chen MF, Lee WC, Jeng LB. Prognostic factors of hepatic resection for hepatocellular carcinoma with cirrhosis: univariate and multivariate analysis. *J Surg Oncol* 2002; **81**: 195-202 [PMID: 12451624 DOI: 10.1002/jso.10178]
69 **Manzini G**, Henne-Bruns D, Porzsolt F, Kremer M. Is there a standard for surgical therapy of hepatocellular carcinoma in healthy and cirrhotic liver? *BMJ Open Gastroenterol* 2017; **4**: e000129 [PMID: 28405349 DOI: 10.1136/bmjgast-2016-000129]
70 **Berardi G**, Morise Z, Sposito C, Igarashi K, Panetta V, Simonelli I, Kim S, Goh BKP, Kubo S, Tanaka S, Takeda Y, Ettorre GM, Wilson GC, Cimino M, Chan CY, Torzilli G, Cheung TT, Kaneko H, Mazzaferro V, Geller DA, Han HS, Kanazawa A, Wakabayashi G, Troisi RI. Development of a nomogram to predict outcome after liver resection for hepatocellular carcinoma in Child-Pugh B cirrhosis. *J Hepatol* 2020; **72**: 75-84 [PMID: 31499131 DOI: 10.1016/j.jhep.2019.08.032]
71 **Giani A**, Cipriani F, Famularo S, Donadon M, Bernasconi DP, Ardito F, Fazio F, Nicolini D, Perri P, Giuffrida M, Pontarolo N, Zanello M, Lai Q, Conci S, Molfino S, Germani P, Pinotti E, Romano M, La Barba G, Ferrari C, Patauner S, Manzoni A, Sciannamea I, Fumagalli L, Troci A, Ferraro V, Floridi A, Memeo R, Crespi M, Chiarelli M, Antonucci A, Zimmitti G, Frena A, Percivale A, Ercolani G, Zanus G, Zago M, Tarchi P, Baiocchi GL, Ruzzenente A, Rossi M, Jovine E, Maestri M, Dalla Valle R, Grazi G, Vivarelli M, Ferrero A, Giuliante F, Torzilli G, Aldrighetti L, Gianotti L, Romano F, Ciulli C, Braga M, Ratti F, Costa G, Razionale F, Russolillo N, Marinelli L, De Peppo V, Cremaschi E, Calabrese F, Larghi Laureiro Z, Lazzari G, Cosola D, Montuori M, Salvador L, Cucchetti A, Franceschi A, Ciola M, Sega V, Calcagno P, Pennacchi L, Tedeschi M. Performance of Comprehensive Complication Index and Clavien-Dindo Complication Scoring System in Liver Surgery for Hepatocellular Carcinoma. *Cancers (Basel)* 2020; **12** [PMID: 33371419 DOI: 10.3390/cancers12123868]
72 **Harimoto N**, Yoshizumi T, Fujimoto Y, Motomura T, Mano Y, Toshima T, Itoh S, Harada N, Ikegami T, Uchiyama H, Soejima Y, Maehara Y. Surgery for Hepatocellular Carcinoma in Patients with Child-Pugh B Cirrhosis: Hepatic Resection Versus Living Donor Liver Transplantation. *World J Surg* 2018; **42**: 2606-2616 [PMID: 29372372]
73 **Harada N**, Shirabe K, Ikeda Y, Korenaga D, Takenaka K, Maehara Y. Surgical management of

hepatocellular carcinoma in Child-Pugh class B cirrhotic patients: hepatic resection and/or microwave coagulation therapy *vs* living donor liver transplantation. *Ann Transplant* 2012; **17**: 11-20 [PMID: 23274319 DOI: 10.12659/aot.883689]

74 **Ciria R**, Gomez-Luque I, Ocaña S, Cipriani F, Halls M, Briceño J, Okuda Y, Troisi R, Rotellar F, Soubrane O, Abu Hilal M. A Systematic Review and Meta-Analysis Comparing the Short- and Long-Term Outcomes for Laparoscopic and Open Liver Resections for Hepatocellular Carcinoma: Updated Results from the European Guidelines Meeting on Laparoscopic Liver Surgery, Southampton, UK, 2017. *Ann Surg Oncol* 2019; **26**: 252-263 [PMID: 30390167 DOI: 10.1245/s10434-018-6926-3]

75 **Morise Z**, Ciria R, Cherqui D, Chen KH, Belli G, Wakabayashi G. Can we expand the indications for laparoscopic liver resection? *J Hepatobiliary Pancreat Sci* 2015; **22**: 342-352 [PMID: 25663288 DOI: 10.1002/jhbp.215]

76 **Takahara T**, Wakabayashi G, Beppu T, Aihara A, Hasegawa K, Gotohda N, Hatano E, Tanahashi Y, Mizuguchi T, Kamiyama T, Ikeda T, Tanaka S, Taniai N, Baba H, Tanabe M, Kokudo N, Konishi M, Uemoto S, Sugioka A, Hirata K, Taketomi A, Maehara Y, Kubo S, Uchida E, Miyata H, Nakamura M, Kaneko H, Yamaue H, Miyazaki M, Takada T. Long-term and perioperative outcomes of laparoscopic *vs* open liver resection for hepatocellular carcinoma with propensity score matching: a multi-institutional Japanese study. *J Hepatobiliary Pancreat Sci* 2015; **22**: 721-727 [PMID: 26096910 DOI: 10.1002/jhbp.276]

77 **Di Sandro S**, Bagnardi V, Najjar M, Buscemi V, Lauterio A, De Carlis R, Danieli M, Pinotti E, Benuzzi L, De Carlis L. Minor laparoscopic liver resection for Hepatocellular Carcinoma is safer than minor open resection, especially for less compensated cirrhotic patients: Propensity score analysis. *Surg Oncol* 2018; **27**: 722-729 [PMID: 30449499 DOI: 10.1016/j.suronc.2018.10.001]

78 **Lin NC**, Nitta H, Wakabayashi G. Laparoscopic major hepatectomy: a systematic literature review and comparison of 3 techniques. *Ann Surg* 2013; **257**: 205-213 [PMID: 23263192 DOI: 10.1097/SLA.0b013e31827da7fe]

79 **Yoon YI**, Kim KH, Cho HD, Kwon JH, Jung DH, Park GC, Song GW, Ha TY, Lee SG. Long-term perioperative outcomes of pure laparoscopic liver resection *vs* open liver resection for hepatocellular carcinoma: a retrospective study. *Surg Endosc* 2020; **34**: 796-805 [PMID: 31161292 DOI: 10.1007/s00464-019-06831-w]

80 **Cai X**, Liang X, Yu T, Liang Y, Jing R, Jiang W, Li J, Ying H. Liver cirrhosis grading Child-Pugh class B: a Goliath to challenge in laparoscopic liver resection? *Hepatobiliary Surg Nutr* 2015; **4**: 391-397 [PMID: 26734623 DOI: 10.3978/j.issn.2304-3881.2015.09.02]

81 **Brytska N**, Han HS, Shehta A, Yoon YS, Cho JY, Choi Y. Laparoscopic liver resection for hepatitis B and C virus-related hepatocellular carcinoma in patients with Child B or C cirrhosis. *Hepatobiliary Surg Nutr* 2015; **4**: 373-378 [PMID: 26734621 DOI: 10.3978/j.issn.2304-3881.2015.04.06]

82 **Beard RE**, Wang Y, Khan S, Marsh JW, Tsung A, Geller DA. Laparoscopic liver resection for hepatocellular carcinoma in early and advanced cirrhosis. *HPB (Oxford)* 2018; **20**: 521-529 [PMID: 29317157 DOI: 10.1016/j.hpb.2017.11.011]

83 **Fuji H**, Seo S, Toda R, Yoh T, Ikeno Y, Fukumitsu K, Ishii T, Taura K, Hatano E, Kaido T, Uemoto S. Optimal introduction of laparoscopic liver resection for Child-Pugh B. *Asian J Endosc Surg* 2019; **12**: 287-293 [PMID: 30133178 DOI: 10.1111/ases.12634]

84 **Ruzzenente A**, Capra F, Pachera S, Iacono C, Piccirillo G, Lunardi M, Pistoso S, Valdegamberi A, D'Onofrio M, Guglielmi A. Is liver resection justified in advanced hepatocellular carcinoma? *J Gastrointest Surg* 2009; **13**: 1313-1320 [PMID: 19418103 DOI: 10.1007/s11605-009-0903-x]

85 **Fukami Y**, Kaneoka Y, Maeda A, Kumada T, Tanaka J, Akita T, Kubo S, Izumi N, Kadoya M, Sakamoto M, Nakashima O, Matsuyama Y, Kokudo T, Hasegawa K, Yamashita T, Kashiwabara K, Takayama T, Kokudo N, Kudo M; Liver Cancer Study Group of Japan. Liver Resection for Multiple Hepatocellular Carcinomas: A Japanese Nationwide Survey. *Ann Surg* 2020; **272**: 145-154 [PMID: 30672806 DOI: 10.1097/SLA.0000000000003192]

86 **Ronot M**, Cauchy F, Gregoli B, Breguet R, Allaham W, Paradis V, Soubrane O, Vilgrain V. Sequential transarterial chemoembolization and portal vein embolization before resection is a valid oncological strategy for unilobar hepatocellular carcinoma regardless of the tumor burden. *HPB (Oxford)* 2016; **18**: 684-690 [PMID: 27485063 DOI: 10.1016/j.hpb.2016.05.012]

87 **Tang YL**, Qi XS, Guo XZ. Hepatic Resection after Initial Transarterial Chemoembolization Versus Transarterial Chemoembolization Alone for the Treatment of Hepatocellular Carcinoma: A Meta-analysis of Observational Studies. *Asian Pac J Cancer Prev* 2015; **16**: 7871-7874 [PMID: 26625813 DOI: 10.7314/apjcp.2015.16.17.7871]

88 **Tustumi F**, Ernani L, Coelho FF, Bernardo WM, Junior SS, Kruger JAP, Fonseca GM, Jeismann VB, Cecconello I, Herman P. Preoperative strategies to improve resectability for hepatocellular carcinoma: a systematic review and meta-analysis. *HPB (Oxford)* 2018; **20**: 1109-1118 [PMID: 30057123 DOI: 10.1016/j.hpb.2018.06.1798]

89 **Qi X**, Liu L, Wang D, Li H, Su C, Guo X. Hepatic resection alone *vs* in combination with pre- and post-operative transarterial chemoembolization for the treatment of hepatocellular carcinoma: A systematic review and meta-analysis. *Oncotarget* 2015; **6**: 36838-36859 [PMID: 26451613 DOI: 10.18632/oncotarget.5426]

90 **Kudo M**, Arizumi T, Ueshima K, Sakurai T, Kitano M, Nishida N. Subclassification of BCLC B Stage Hepatocellular Carcinoma and Treatment Strategies: Proposal of Modified Bolondi's Subclassification (Kinki Criteria). *Dig Dis* 2015; **33**: 751-758 [PMID: 26488473 DOI: 10.1159/000439290]

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MINIREVIEWS

Potential risk factors for constipation in the community

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Abstract

Constipation is a common community health problem. There are many factors that are widely thought to be associated with constipation but real-world evidence of these associations is difficult to locate. These potential risk factors may be categorised as demographic, lifestyle and health-related factors. This review presents the available evidence for each factor by an assessment of quantitative data from cross-sectional studies of community-dwelling adults published over the last 30 years. It appears that there is evidence of an association between constipation and female gender, residential location, physical activity and some health-related factors such as self-rated health, some surgery, certain medical conditions and certain medications. The available evidence for most other factors is either conflicting or insufficient. Therefore, further research is necessary to determine if each factor is truly associated with constipation and whether it can be regarded as a potential risk factor. It is recommended that studies investigating a broad range of factors are conducted in populations in community settings. Multivariate analyses should be performed to account for all possible confounding factors. In this way, valuable evidence can be accumulated for a better understanding of potential risk factors for constipation in the community.

Key Words: Adults; Constipation; Epidemiology; Factors; Community

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Core Tip: Despite widespread beliefs that there are a number of potential risk factors for constipation in the community, this review highlights the paucity of real-world evidence for most factors. It is unclear whether most factors are associated with constipation because, apart from female gender, physical activity, residential location and some health-related factors, there is insufficient evidence or conflicting data available. Further research is required in community-dwelling adult populations to

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understand the importance of each potential risk factor in constipation. A broad range of factors should be investigated in same population samples using multivariate analysis to determine which factors are truly associated with constipation in the community.

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INTRODUCTION

One of the most common health problems faced by the community is constipation[1]. In general, constipation can be defined as a lack of satisfactory defecation[1] which incorporates various symptoms and may be either chronic or sporadic. Chronic constipation is usually defined by a set of clinical symptoms known as the Rome criteria[2]; the Rome criteria have been revised several times following their introduction as Rome I criteria in 1994. Any constipation may be defined as constipation which includes both chronic and sporadic constipation[3]; this condition is generally self-reported in epidemiological studies.

A number of factors are widely considered to be associated with constipation however real-world evidence of these associations appears to be sparse[4,5]. It is widely accepted that prime risk factors for constipation in the community include low exercise levels, low fibre intake and inadequate fluid intake[6]. However, these risk factors have been challenged in the past due to a paucity of clinical evidence[4,5]. In addition to these factors, there are other determinants of health which are reported in the literature to be associated with constipation. These include lifestyle and demographic factors[7]. Furthermore, medications and medical conditions are well established as two major secondary causes of constipation[8,9].

Since there appears to be questionable real-world evidence for these various risk factors despite their wide acceptance in the community, this literature review seeks to assess each potential risk factor by reviewing evidence from population-based studies of community-dwelling adult populations. The specific aims of this review were to identify demographic, lifestyle and health-related factors reported to be associated with constipation and evaluate the evidence for each factor.

SEARCH STRATEGY

A search of relevant published literature was performed using the Ovid interface to MEDLINE and Embase electronic databases. In addition, some articles were located in PubMed. The "ancestry approach"[10] was also used to locate pertinent studies by searching references of selected articles.

The search was filtered to include only English language articles and population-based studies. Index search terms in various combinations were applied using the three main Boolean operators – AND, OR and NOT, and included: "constipation", "adults", "gastrointestinal disorders", "prevalence", "epidemiology", "factors" and "risk factors". Articles meeting the inclusion criteria were classified into chronic constipation and any constipation according to the definition of constipation used in the study. Because of the large number of articles published on these topics, the search was limited to articles which were published between 1989 and 2019.

Retrieved articles that were eligible for inclusion included peer-reviewed research articles, as well as systematic reviews, describing epidemiological studies in community settings. Articles reporting constipation relating to irritable bowel syndrome, opioid-induced constipation in cancer, faecal incontinence, bowel cleansing, constipation in infants and children, constipation in palliative care patients, constipation in hospital in-patients and constipation in residential care facilities were deemed inappropriate for this review and were excluded from the final literature review sample. Articles describing specific subpopulations such as those relating to university students, older adult populations (50 years of age and older), female

populations and male populations were excluded. Since the focus for this review was population-based studies, surveys where the sample size was fewer than 100 participants were also excluded.

Articles meeting the preliminary inclusion criteria were further screened. Articles were excluded when found to report results from employee groups[11-14], patient groups[15-19], and medical database records[20,21] as these were deemed not to be community settings. Self-reported constipation incorporates many and varied symptoms[22,23] and chronic constipation, as defined by Rome criteria, incorporates all possible symptoms of chronic constipation[2]; therefore, articles reporting only one symptom such as stool consistency or bowel motion frequency rather than constipation per se were not included in the final sample[24-31]. Also excluded were articles where the adult population sample was found to include participants below the age of 15 years[32-35].

In some articles there were sufficient data available to enable calculation of results for potential risk factors even though these results were not published; these included calculations of prevalence percentages and gender ratios. Any calculated data are clearly marked as such in the tables. However, in some epidemiological studies of general populations, the results reporting potential risk factors have been reported by gender only and it was not possible to amalgamate the data for inclusion of several factors in this review.

RESULTS

A final sample comprising 53 articles was selected for inclusion in this literature review. Of these, 9 were systematic reviews and 44 were quantitative epidemiological studies of community-based general adult populations. Three systematic reviews were international[7,36,37], two related to specific regions – North America[38] and Europe plus Oceania[39], and two related to specific countries – Iran[40] and China[41]. Another two reviews related to specific factors – co-morbidities and haemorrhoids[42,43]. The epidemiological studies were all cross-sectional surveys of adults residing in the community.

Factors potentially associated with constipation which emanated from our review included demographic factors (age, gender, income, education, work status and geography), lifestyle factors and behaviours (physical activity, smoking, and fibre, fluid, alcohol and coffee intakes) and numerous health-related factors (including medical conditions and medications). Each of these factors are discussed in turn.

DEMOGRAPHIC AND SOCIOECONOMIC FACTORS

The following section describes demographic and socioeconomic factors potentially associated with constipation in adults in community settings.

Age

For both chronic constipation and any constipation, it seems that there is not a clear association with age since conflicting results have been reported (Table 1)[44-63]. There may be a higher prevalence of constipation in older age groups as reported in some literature reviews and epidemiological studies. However, other studies reported either no such association or a higher prevalence of constipation in younger age groups.

For chronic constipation, one systematic review[36] found no significant differences in prevalence between younger and older age groups whereas reviews of Chinese[41] and Iranian[40] studies indicated an increased prevalence with age. Six epidemiological studies indicated higher prevalence of chronic constipation in younger age groups[44-49], whilst higher prevalence in older age groups was only reported in four studies[50-53]. Furthermore, in establishing an association between chronic constipation and age groups, four other studies did not demonstrate any trends[54-57].

For any constipation (chronic and sporadic), one systematic review[7] found an increased prevalence of any constipation after the age of 60 years with the largest increase in prevalence experienced after 70 years. Trends of increasing prevalence with increasing age were observed in three reviews[37,40,41], but one review of North American studies[38] concluded that the relationship between age and any constipation could not be established. An increase in any constipation with increasing

Table 1 Age and constipation

Ref.	Location	Sample size	Definition of constipation	Age range (yr)	Prevalence (%)	Odds ratio (95%CI)	*P* value
Harari *et al*[62], 1989	United States	42375	Self-report (12 mo)	< 40; 40-49; 50-59; 60-69; 70-79; > 80	2.6; 2.6; 2.9; 4.1; 5.5; 10.0	1.00; 1.00 (0.84, 1.18); 1.11 (0.92, 1.33); 1.60 (1.36, 1.86); 2.11 (1.80, 2.46); 3.80 (3.22, 4.49)	NR
Drossman *et al*[44], 1993	United States	5430	Rome I	15-34; 35-49; > 45	3.8; 3.6; 3.5	NR	NR
Talley *et al*[49], 1993	United States	690	Rome I	30-39; 60-64	25; 15	NR	< 0.05
Pare *et al*[45], 2001	Canada	1149	Self-report (3 mo)	18-34; 35-49; 50-64; > 65	26.4; 28.4; 26.3; 27.4	NR	NR
Pare *et al*[45], 2001	Canada	1149	Rome I	18-34; 35-49; 50-64; > 65	20.3; 14.5; 15.1; 15.4	NR	NR
Pare *et al*[45], 2001	Canada	1149	Rome II	18-34; 35-49; 50-64; > 65	16.1; 12.9; 14.8; 16.7	NR	NR
Choung *et al*[63], 2006	United States	3022	Self-report (12 mo)	< 50; > 50	1.32[1]; 7.87[1]	NR	NR
Wald *et al*[61], 2008	United States	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60	13.4[1]; 19.1[1] 17.5[1]; 19.9[1]	NR	NR
Chang *et al*[46], 2007	United States	523	Rome III	< 50; > 50	18.2; 17.3	1.0; 0.94 (0.60, 1.48)	NR
Meinds *et al*[47], 2017	Netherlands	1259	Rome III	18-34; 35-46; 47-55; 56-64; 65-85	36.3; 26.6; 19.0; 19.2; 19.8	NR	< 0.001
Garrigues *et al*[60], 2004	Spain	349	Self-report (12 mo)	18-30; 31-50; 51-65	29.2; 29.2; 30.7	NR	NS
Fosnes *et al*[54], 2011	Norway	4622	Rome II	NR	NR	1.01 (1.003, 1.02)	0.005
Rey *et al*[55], 2014	Spain	1500	Rome III	18-40; 41-65; > 65	19; 19; 20	NR	
Ebling *et al*[50], 2014	Croatia	658	Rome III	20-34; 35-49; 50-69	16.2; 22.7; 26.2	NR	0.035; 0.182
Papatheodoridis *et al*[51], 2010	Greece	1000	Rome III or self-report (12 mo)	15-29; 30-44; 45-59; 60-64	12; 16; 18; 25	0.422 (0.226, 0.788); 0.721 (0.397, 1.310); 0.670 (0.362, 1.241); 1.0	0.007; 0.283; 0.203; 0.010
Wald *et al*[61], 2008	United Kingdom	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60	5.9[1]; 7.7[1]; 8.1[1]; 9.1[1]	NR	NR
Wald *et al*[61], 2008	France	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60	12.2[1]; 12.4[1]; 9.4[1]; 22.0[1]	NR	NR
Wald *et al*[61], 2008	Germany	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60	1.5[1]; 4.3[1]; 5.5[1]; 8.7[1]	NR	NR
Wald *et al*[61], 2008	Italy	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60	5.8[1]; 6.9[1]; 8.9[1] 10.6[1]	NR	NR
Wald *et al*[59], 2010	China	2100	Self-report (12 mo)	< 29; 30-44; 45-59	12.7[1]; 16.0[1]; 18.6[1]	NR	NR
Wald *et al*[59], 2010	Indonesia	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60;	11.6[1]; 13.5[1]; 15.3[1]; 9.6[1]	NR	NR
Wald *et al*[59], 2010	South Korea	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60;	18.1[1]; 15.6[1]; 16.9[1]; 14.1[1]	NR	NR
Jun *et al*[58], 2006	South Korea	1029	Self-report (3 mo)	15-19; 20-29; 30-39; 40-49; 50-59; > 60	22; 22; 15; 15; 14; 12	NR	0.003
Cheng *et al*[57], 2003	Hong Kong	3282	Rome II	< 30; 30-39; 40-49; 50-59; > 59	14.5; 13.6; 11.8; 13.7; 14.9	NR	NR
Lu *et al*[56], 2006	Taiwan	2018	Rome II	20-29; 30-39; 40-49; 50-59; 60-69; 70-79	12.2; 7.9; 7.4; 7.1; 10.4; 11.9	NR	0.04
Sorouri *et al*[53],	Iran	18180	Rome III	< 40; 40-60; >	1.4; 4.7; 4.9	1.01 (1, 1.01)	< 0.05

2010				60			
Moezi *et al*[52], 2018	Iran	9264	Rome IV	40-59; > 60	6.9[1]; 11.9[1]	1.55 (1.31, 1.83)	< 0.001
Wald *et al*[59], 2010	Argentina	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60	11.6[1]; 12.3[1]; 17.1[1]; 17.3[1]	NR	NR
Wald *et al*[59], 2010	Colombia	2000	Self-report; (12 mo)	< 29; 30-44; 45-59; > 60	19.6[1]; 25.6[1]; 25.3[1]; 25.5[1]	NR	NR
Wald *et al*[59], 2010	Brazil	2000	Self-report (12 mo)	< 29; 30-44; 45-59; > 60	13.9[1]; 17.3[1]; 19.4[1]; 19.0[1]	NR	NR
Howell *et al*[48], 2006	Sydney	1673	Rome II	25-34; 35-44; 45-54; 55-64	37.8; 27.7; 27.4; 27.6	NR	0.03

[1]Calculated from data published. NR: Not reported; NS: Not significant.

age has been reported in epidemiological studies from various countries[58-61]; however, the prevalence of any constipation decreased with increasing age in studies elsewhere[58,59] (Table 1). In other epidemiological studies, no clear association of age and any constipation was seen[45,59-61].

Female gender

The prevalence of constipation is consistently higher in females compared to males in all systematic reviews and almost all epidemiological studies included in this review (Table 2)[22,23,45-81]. Most studies reporting gender differences have used female/male (F/M) ratios to express the result with only a few studies reporting odds ratios. Based on these data, it would appear that females are approximately twice as likely as males to report chronic constipation and more than twice as likely to report any constipation.

For chronic constipation, systematic reviews have reported mean F/M ratios of 1.4[41], 1.89[36] and 1.75[38,39]. Similar ratios are also seen in most epidemiological studies where F/M ratios have ranged from 1 to 10 in 24 studies conducted in various countries (Table 2). Only one study has shown a greater prevalence in males where the F/M ratio was 0.84[50]. Odds ratios for chronic constipation in females were reported as 2.22 in a global systematic review[36] and ranged from 1.0 to 4.8 in epidemiological studies[46,47,51,53,54,60,64-66].

For any constipation, systematic reviews have reported mean F/M ratios ranging from 2.1. to 2.65[7,38,39]. In 26 epidemiological studies, F/M ratios have ranged from 1.10 to 6.75 across 17 countries (Table 2). In 8 of these studies the difference between genders was reported to be statistically significant ($P < 0.05$). Odds ratios for any constipation in females ranged from 2.0 to 3.8 in systematic reviews[37,38] and epidemiological studies[59-61,67].

Income level

It is not clear from this literature review whether constipation and income are associated. The association of income level and constipation appears to vary by country but even within one country conflicting results have been reported (Table 3). In many countries, an inverse (negative) relationship has been found between constipation and income, with a higher prevalence of constipation with lower incomes.

For chronic constipation, a Canadian study showed evidence of an inverse relationship[45] but this was not the case in a United States study[68]. In Iran and Brazil, there was significantly higher prevalence in those with lower income[52,66] but there was no inverse relationship in South Korea[58] and Hong Kong[57]. An inverse relationship was also reported in an Australian study[82].

For any constipation, an inverse relationship was seen in the United Kingdom, Germany, Brazil, Colombia and China, but no such trend was evident in France, Italy, South Korea and Indonesia[59,61,75]. North American studies indicate that the prevalence of any constipation increases as income decreases[38,61].

Educational level

The association of educational level and constipation is not clear with studies in various countries showing mixed results (Table 4).

In studies of chronic constipation, there was evidence of an inverse relationship, *i.e.*, higher prevalence of constipation in those with lower levels of education, in United States, Chinese, Croatian and Iranian studies[41,49,50,52,53,83] but in other studies,

Table 2 Gender and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Prev. males (%)	Prev. females (%)	F/M ratio	Odds ratio (95%CI)	*P* value
Everhart *et al*[67], 1989	United States	11204	25-74	Self-report (NTP)	3.6	11.4	3.17[1]	3.8 (2.6, 5.6)	NR
Talley *et al*[64], 1991	United States	835	30-64	Rome I	13.9	20.8	1.50[1]	1.6 (1.1, 2.3)	NR
Talley *et al*[49], 1993	United States	690	30-64	Self-reportRome I	2.7; 18.3	7.3; 20.1	2.70[1]; 1.01[1]	NR	NR
Drossman *et al*[44], 1993	United States	5430	> 15	Rome I	2.4	4.8	2.00[1]	1.99 (1.5, 2.7)	NR
Stewart *et al*[68], 1999	United States	10018	> 18	Rome II	12.0	16.0	1.33[1]	NR	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Self-report (3 mo)	18.4	35.4	1.92[1]	NR	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Rome I	12.0	21.0	1.75[1]	NR	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Rome II	8.3	21.1	2.54[1]	NR	NR
Choung *et al*[63], 2006	United States	2718	20-95	Self-report (12 mo)	2.76[1]	6.44[1]	2.33[1]	NR	NR
Chang *et al*[46], 2007	United States	523	30-64	Rome III	17.8	17.8	1.00	1.0 (0.64, 1.57)	NR
Wald *et al*[61], 2008	United States	2000	> 15	Self-report (12 mo)	13.6	21.4	1.57	NR	NR
Choung *et al*[65], 2012	United States	2853	> 20	Rome II	20.1[1]	27.7[1]	1.38[1]	1.6 (1.3, 1.9)	< 0.01
Choung *et al*[69], 2016	United States	2327	> 25	Rome III (mod)	5.1	8.7	1.71[1]	NR	NR
Meinds *et al*[47], 2017	Netherlands	1259	> 18	Rome III	18.8	29.3	1.56[1]	1.8 (1.4, 2.3)	< 0.001
Van Kerkhoven *et al*[70], 2008	Netherlands	1616	> 18	Self-report (4 wk)	7	18	2.57[1]	NR	< 0.01
Garrigues *et al*[60], 2004	Spain	349	18-65	Self-report (12 mo)	18.0	40.1	2.23[1]	2.9 (1.68, 4.98)	0.0001
Garrigues *et al*[60], 2004	Spain	349	18-65	Rome II	5.5	22.0	4.0[1]	4.58 (1.98, 10.60)	0.0004
Walter *et al*[22], 2002	Sweden	1610	31-76	Self-report (NTP)	8.3	19.8	2.39[1]	NR	< 0.0001
Haug *et al*[71], 2002	Norway	62651	> 20	Self-report (12 mo)	1.5	5.7	3.8[1]	NR	< 0.05
Fosnes *et al*[54], 2011	Norway	4622	31-76	Rome II	6.7[1]	19.3[1]	2.88[1]	3.24 (2.61, 4.02)	< 0.001
Gaburri *et al*[72], 1989	Italy	519	NR	Self-report (3 yr)	1.2[1]	8.1[1]	6.75[1]	NR	NR
Heaton *et al*[73], 1993	United Kingdom	1892	26-69	Self-report (NTP)	14.7	31.1	2.12[1]	NR	< 0.001
Wald *et al*[61], 2008	United Kingdom	2000	> 15	Self-report (12 mo)	4.2	10.9	2.60	NR	NR
Wald *et al*[61], 2008	Germany	2000	> 15	Self-report (12 mo)	3.0	7.5	2.5	NR	NR
Wald *et al*[61], 2008	Italy	2000	> 15	Self-report (12 mo)	4.9	10.8	2.2	NR	NR
Wald *et al*[61], 2008	France	2000	> 15	Self-report (12 mo)	8.6	18.9	2.2	NR	NR
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	10.6	27.6	2.7	NR	NR
Esteban y Peña *et al*[74], 2014	Spain	7341	> 16	Self-report (NTP)	1.9	5.9	3.1[1]	NR	< 0.001
Enck *et al*[75], 2016	Germany	15002	> 18	Self-report (12 mo)	NR	NR	2.3	NR	NR
Ebling *et al*[50], 2014	Croatia	658	20-70	Rome III	24.3	20.3	0.84[1]	NR	0.2260.126
Papatheodoridis *et al*[51], 2010	Greece	1000	15-64	Rome III or self-report (12 mo)	11	21	1.91[1]	2.10 (1.41, 3.12)	< 0.001
Wald *et al*[61], 2008	Brazil	2000	> 15	Self-report	8.5	24.2	2.85	NR	NR
Wald *et al*[59], 2010	Argentina	2000	> 15	Self-report	7.9	20.2	2.56	NR	NR

Wald *et al*[59], 2010	Colombia	2000	> 15	Self-report	14.7	28.3	1.93	NR	NR
Schmidt *et al*[66], 2016	Brazil	2162	> 18	Rome III	5.3	21.9	4.13[1]	4.3 (3.1, 6.1)	NR
Ho *et al*[76], 1998	Singapore	706	21-95	Rome II	2.8	5.6	2.0[1]	NR	NR
Chen *et al*[77], 2000	Singapore	271	> 16	Rome II	3.6	11.3	3.14[1]	NR	< 0.05
Cheng *et al*[57], 2003	Hong Kong	3282	18-80	Rome II	13.9	14.5	1.04[1]	NR	NR
Lu *et al*[56], 2006	Taiwan	2018	> 20	Rome II	7.0[1]	10.6[1]	1.51[1]	NR	< 0.001
Chang *et al*[78], 2012	Taiwan	4275	> 19	Rome III	2.8	6.2	2.21[1]	NR	0.001
Jun *et al*[58], 2006	South Korea	1029	> 15	Self-report (3 mo)	10.4	22.8	2.19[1]	NR	< 0.001
Jeong *et al*[79], 2008	South Korea	1417	18-69	Rome II	0.5	5.0	10.0[1]	NR	< 0.05
Wald *et al*[61], 2008	South Korea	2000	> 15	Self-report (12 mo)	10.7	22.7	2.12	NR	NR
Tamura *et al*[23], 2016	Japan	5155	20-79	Self-report (NTP)	19.1	37.5	1.96[1]	NR	< 0.001
Wald *et al*[59], 2010	Indonesia	2000	> 15	Self-report (12 mo)	10.7	15.1	1.41	NR	NR
Wald *et al*[59], 2010	China	2100	15-60	Self-report (12 mo)	10.8	19.7	1.82	NR	NR
Sorouri *et al*[53], 2010	Iran	18180	NR	Rome III	1.2	3.7	3.08[1]	1.83 (1.44, 2.32)	< 0.01
Moezi *et al*[52], 2018	Iran	9264	40-75	Rome IV	6.7	9.3	1.44	NR	< 0.001
Talley *et al*[80], 1998	Australia	730	> 18	BSQ	6.3	21.1	3.35	NR	NR
Howell *et al*[48], 2006	Australia	1673	25-64	Rome II	25.1	36.0	1.43	NR	NR
Koloski *et al*[81], 2015	Australia	3260	> 18	Rome III	3.25[1]	8.95[1]	2.75[1]	NR	NR

[1]Calculated from published data. Prev.: Prevalence; F/M: Female/Male; Mod: Modified; NR: Not reported; NS: Not significant; NTP: No time period specified; BSQ: Bowel symptom questionnaire (similar to Rome criteria).

there was no clear evidence of this or any other trend[45,46,51,55,57,65,68].

Most North American studies[38,45,61,67] have shown an inverse relationship between prevalence of any constipation and years of education. In studies of any constipation in other countries, both trends were observed; in the United Kingdom, France, Germany, Italy and South Korea[61,75], there was an inverse relationship between any constipation and educational level but the opposite was found in Brazil, China and Indonesia[59,61]. No trends were found in Spain, Argentina and Colombia[59,60].

Residential region within countries

The prevalence of constipation appears to vary by residential region within some countries (Table 5). Significant differences in the prevalence of any constipation have been observed in China between different regions and between rural and urban locations, with a significantly higher prevalence in rural areas[41]. However, in Croatia there was a significantly higher prevalence of chronic constipation in urban populations[50]. Regional differences have also been reported for any constipation in Canada and Greece[45,51] and in Spain for chronic constipation[55].

Other demographic and socioeconomic factors

The association of work or employment status and constipation is not clear with studies in various countries showing mixed results (Table 6). In Germany, there appears to be an increased prevalence of any constipation in those not working[75] but in North America chronic constipation seems to be more prevalent in those working[45,49].

Similarly, the association of marital status and constipation is not clear but there may be a tendency for a lower prevalence of chronic constipation in those who are married (Table 6).

One United States study has shown a lower prevalence of chronic constipation in white participants compared to other ethnic groups[44]. Ethnicity differences have also been reported in China[41].

Table 3 Income level and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Income/year or week	Prevalence (%)	Odds ratio (95%CI)	*P* value
Stewart *et al*[68], 1999	United States	10018	> 18	Rome II	< $20000; $20-29900; $30-49900; > $50000	NR	1; 0.90; 1.10; 1.02	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Self-report (3 mo)	< $20000; $20-39900; $40-59900; $60-79900; > $80000	33.8; 23.7; 24.3; 28.0; 21.8	1.55; 1.01; 1.11; 1.28; 1	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Rome I	< $20000; $20-39900; $40-59900; $60-79900; > $80000	18.5; 16.3; 17.6; 13.1; 12.1	1.53; 1.35; 1.45; 1.08; 1	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Rome II	< $20000; $20-39900; $40-59900; $60-79900; > $80000	15.3; 14.3; 13.9; 14.5; 8.3	1.84; 1.72; 1.67; 1.75; 1	NR
Wald *et al*[61], 2008	United States	2000	> 15	Self-report (12 mo)	Low; Middle; High	20.9; 16.1; 16.8	NR	NR
Wald *et al*[61], 2008	United Kingdom	2000	> 15	Self-report (12 mo)	Low; Middle;High	8.8; 7.27.1	NR	NR
Wald *et al*[61], 2008	France	2000	> 15	Self-report (12 mo)	Low; Middle; High	14.9; 11.3; 15.8	NR	NR
Wald *et al*[61], 2008	Germany	2000	> 15	Self-report (12 mo)	Low; Middle; High	7.2; 3.6; 5.3	NR	NR
Enck *et al*[75], 2016	Germany	15002	> 18	Self-report (12 mo)	< 1000; 1000-1500; 1500-2000; 2000-2500; 2500-3000; 3000-4000; > 4000	19.0[1]; 18.8[1]; 14.6[1]; 13.6[1]; 13.3[1]; 11.3[1]; 11.5[1]	NR	< 0.001
Wald *et al*[61], 2008	Italy	2000	> 15	Self-report (12 mo)	Low; Middle; High	7.8; 7.0; 8.4	NR	NR
Wald *et al*[61], 2008	Brazil	2000	> 15	Self-report (12 mo)	Low; Middle; High	17.9; 15.8; 14.2	NR	NR
Schmidt *et al*[66], 2016	Brazil	2162	> 18	Rome III	2-15; 1.5-2; 1-1.5; 0.5-1; 0-0.5	11.1; 9.7; 13.8; 15.4; 21.8	1.0; 0.8 (0.5, 1.4); 1.3 (0.8, 2.1); 1.4 (0.9, 2.2); 1.9 (1.2, 3.0)	NR
Wald *et al*[61], 2008	South Korea	2000	> 15	Self-report (12 mo)	Low; Middle; High	17.1; 15.7; 17.1	NR	NR
Wald *et al*[59], 2010	Colombia	2000	> 15	Self-report (12 mo)	Low; Middle; High	23.9; 20.3; 14.8	NR	NR
Wald *et al*[59], 2010	China	2100	15-60	Self-report (12 mo)	Low; Middle; High	16.2; 15.5; 13.2	NR	NR
Wald *et al*[59], 2010	Indonesia	2000	> 15	Self-report (12 mo)	Low; Middle; High	13.1; 12.2; 14.0	NR	NR
Moezi *et al*[52], 2018	Iran	9264	40-75	Rome IV	Low; High	9.1; 6.5	NR	0.024
Cheng *et al*[57], 2003	Hong Kong	3282	18-80	Rome II	Nil; < 10000; 10000-19999; 20000-29999; 30000-39999; 40000-49999; > 50000	13.6; 14.8; 12.5; 12.6; 12.5; 12.5; 13.1	NR	NR
Jun *et al*[58], 2006	South Korea	1029	> 15	Rome II	< 1000; 1010-2000; 2010-3000; 3010-4000; > 4010	16; 15; 20; 26; 24	NR	0.044

Bytzer *et al*[82], 2001	Australia	8185	> 18	Rome II	5th quintile (lowest); 4th quintile; 3rd quintile; 2nd quintile; 1st quintile (highest)	10.2; 10.3; 9.6; 8.7; 6.3	NR	NR

[1]Calculated from data published.

Note: Income is stated in various currencies. NR: Not reported; NS: Not significant.

LIFESTYLE AND BEHAVIOURAL FACTORS

The following section describes lifestyle and behavioural factors potentially associated with constipation in adults in community settings.

Physical activity

There is limited evidence that low levels of physical activity and physical inactivity are associated with a high prevalence of constipation. Low levels of exercise/physical activity were significantly associated with increasing rates of both chronic[52,55] and any constipation[60,61] in studies conducted in various countries (Table 7).

Smoking

Conflicting data indicates that there is no clear association of smoking with the prevalence of chronic or any constipation (Table 8). United States and Iranian studies[52,83] have suggested that smoking may be a possible risk factor in chronic constipation. However other United States studies[46,65] and studies in Greece and Taiwan[51,56] found no significant differences in the prevalence of chronic constipation in smokers and non-smokers. Furthermore, one United States study[49] and one study in Norway[71] found that smoking was a negative risk factor for chronic and any constipation respectively.

Fibre

There is little evidence that low fibre intake is associated with a high prevalence of either chronic or any constipation (Table 9). In one Spanish study, both low and high fibre intakes were associated with increased prevalence of any constipation[60] and in another Spanish study there was no significant association with chronic constipation[55].

Fluid

There is little evidence that fluid intake is associated with the prevalence of chronic or any constipation (Table 10). The only evidence occurred in one Spanish study where chronic constipation was inversely related to fluid intake, defined as glasses of liquids consumed daily[55]. One United States study showed no association of coffee with chronic constipation[46].

Alcohol

There is limited evidence that alcohol consumption may be associated with a decreased prevalence of chronic constipation (Table 11).

Several studies have investigated the association of alcohol and chronic constipation. Increasing alcohol intake was a negative risk factor for chronic constipation in a United States study[49] and also in a Norwegian study[54]. A similar trend was observed in Taiwan[56] and other United States studies, but the opposite trend was found in Iran, but no relationships reached significance[46,52,56,83].

HEALTH-RELATED FACTORS

The following section describes health-related factors potentially associated with constipation in adults in community settings.

Self-rated health

Fair or poor self-rated health was significantly associated with an increased prevalence of chronic constipation in two European studies[50,75] (Table 12).

Table 4 Educational level and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Educational level	Prevalence (%)	Odds ratio (95%CI)	*P* value
Everhart *et al*[67], 1989	United States	11204	25-74	Self-report (NTP)	> 8 yr; 9-11 yr; > 12 yr	NR	2.78; 1.35; 1	NR
Talley *et al*[49], 1993	United States	690	30-64	Rome I	< HS; HS; > HS	23.3; 18.4; 18.0	NR	NR
Stewart *et al*[68], 1999	United States	10018	> 18	Rome II	< 12 yr; 12 yr; 13-15.9 yr; > 16 yr	NR	1; 1.49; 1.41; 1.39	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Self-report (3 mo)	Grade school; Some HS; HS; Diploma; Tech school; Some college; College; Grad school	28.8; 35.1; 24.6; 29.4; 20.5; 25.4; 31.5; 19.6	NR	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Rome I	Grade school; Some HS; HS; Diploma; Tech school; Some college; College; Grad school	14.3; 23.8; 18.8; 22.1; 15.0; 9.9; 17.2; 11.3	NR	NR
Pare *et al*[45], 2001	Canada	1149	> 18	Rome II	Grade school; Some HS; HS; Diploma; Tech school; Some college; College; Grad school	4.3; 21.7; 18.6; 18.4; 12.0; 8.6; 6.5; 10.0	NR	NR
Wald *et al*[61], 2008	United States	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	24.7; 18.9; 15.4	1.82 (1.16, 2.86)	< 0.01
Choung *et al*[83], 2007	United States	3022	20-95	BDQ	< HS; HS/College; > College	NR	1.3 (0.5, 3.1); 1.0; 0.8 (0.5, 3.1)	NS
Chang *et al*[46], 2007	United States	523	30-64	Rome III	< HS; HS/College; > College	16.7; 19.8; 14.8	0.81 (0.27, 2.46);1.00; 0.70 (0.43, 1.14)	NR
Choung *et al*[65], 2012	United States	2853	> 20	Rome II	< HS; HS/College; > College	7.0; 26.5; 20.3	NR	0.002
Wald *et al*[61], 2008	United Kingdom	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	9.4; 7.0; 7.3	NR	NR
Wald *et al*[61], 2008	France	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	17.6; 13.0; 12.7	1.48 (1.01, 2.15)	< 0.05
Wald *et al*[61], 2008	Germany	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	5.6; 5.5; 4.5	NR	NR
Enck *et al*[75], 2016	Germany		> 18	Self-report (12 mo)	< Secondary; Secondary; Tertiary	19.6; 15.3; 15.6	NR	< 0.001
Ebling *et al*[50], 2014	Croatia	658	20-69	Rome III	< Elementary; Elementary; High school; Bachelor; University	50.0; 25.6; 20.2; 23.0; 23.4	0.278; 0.229; 0.248; 0.383	0.065; 0.028; 0.060; 0.178
Wald *et al*[61], 2008	Italy	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	8.0; 8.1; 6.0	NR	NR
Papatheodoridis *et al*[51], 2010	Greece	1000	15-64	Rome III or self-report (12 mo)	Primary or less; Secondary; Higher	18; 14; 17	NR	0.31
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	Primary Secondary; University	19; 18; 20	NR	NR
Garrigues *et al*[60], 2004	Spain	349	18-65	Self-report (12 mo)	Basic; Primary Secondary or more	31.4; 26.4; 30.3	NR	NS
Wald *et al*[59], 2010	Argentina	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	15.1; 13.4; 14.8	NR	NR
Wald *et al*[59], 2010	Colombia	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	25.2; 21.0; 24.5	NR	NR
Wald *et al*[59], 2010	Brazil	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	17.0; 15.2; 26.5	0.58 (0.36, 0.92)	< 0.03
Wald *et al*[59],	China	2100	15-60	Self-report (12	< Secondary; Secondary;	10.2; 16.5; 14.4	NR	NR

2010				mo)	Tertiary			
Wald *et al*[59], 2010	Indonesia	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	10.6; 13.2; 19.0	NR	NR
Wald *et al*[59], 2010	Korea	2000	> 15	Self-report (12 mo)	< Secondary; Secondary; Tertiary	18.1; 16.7; 16.0	NR	NR
Cheng *et al*[57], 2003	Hong Kong	3282	18-80	Rome II	Nil; Primary; Junior HS; HS; Matriculation; University	14.5; 12.2; 14.8; 12.6; 14.0; 14.1	NR	NR
Sorouri *et al*[53], 2010	Iran	18180	NR	Rome III	Illiterate; < Diploma; HS diploma; University; > Masters	2.9; 2.1; 2.5; 2.1; 1.6	NR	NR
Moezi *et al*[52], 2018	Iran	9264	40-75	Rome IV	Illiterate; Other	9.0; 7.2	NR	0.002
Howell *et al*[48], 2006	Australia	1673	25-64	Rome II	Low; Low-mid; Mid-upper; High	30.6; 31.6; 38.9; 25.1	1.50 (0.97, 2.31); 1.49 (1.02, 2.18); 1.91 (1.33, 2.73)1	0.07; 0.04; 0.001

[1]Calculated from published data.

HS: High school; BDQ: Bowel disease questionnaire (similar to Rome criteria); NR: Not reported; NS: Not significant.

Table 5 Residential region and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Region of residence	Prevalence (%)	Odds ratio (95%CI)	*P* value
Pare *et al*[45], 2000	Canada	1149	> 18	Self-report (3 mo)	Atlantic; Quebec; Ontario; Prairies; British Columbia	26.7; 25.8; 26.7; 25.6; 32.3	NR	NR
Pare *et al*[45], 2000	Canada	1149	> 18	Rome I	Atlantic; Quebec; Ontario; Prairies; British Columbia	18.2; 22.1; 13.5; 16.4; 14.5	NR	NR
Pare *et al*[45], 2000	Canada	1149	> 18	Rome II	Atlantic; Quebec; Ontario; Prairies; British Columbia	15.9; 18.6; 13.7; 14.0; 11.9	NR	NR
Papatheodoridis *et al*[51], 2010	Greece	1000	15-64	Rome III or self-report (12 mo)	Athens; Thessaloniki; Other cities	13; 18; 19	0.581 (0.399, 0.844); 0.928 (0.539, 1.598);1.0	0.004; 0.787; 0.017
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	Mediterranean; Centre; Atlantic	21; 17; 15	NR	NR
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	Urban; Rural	20; 18	NR	NR
Ebling *et al*[50], 2014	Croatia	658	20-69	Rome III	Urban; Rural	22.2; 17.5	1.947	0.003
Chu *et al*[41], 2014	China		> 18	Rome II	Hong Kong; Mainland	14.0; 6.4	NR	< 0.001
Chu *et al*[41], 2014	China		> 18	Rome II	North; South	15.5; 3.3	NR	< 0.001
Chu *et al*[41], 2014	China		> 18	Rome II	East; Midwest	4.0; 11.0	NR	< 0.001
Chu *et al*[41], 2014	China		> 18	Rome II	Urban; Rural	6.7; 7.2	NR	< 0.001

[1]Calculated from data published. NR: Not reported.

Medical conditions

Various medical conditions have been reported to be potentially associated with constipation in epidemiological studies (Table 13).

Gastrointestinal disorders: In a review of co-morbidities[42], dyspepsia, heartburn, gastroesophageal reflux disease (GORD) and nausea/vomiting were commonly associated with chronic constipation. The association of GORD with constipation has been reported in several epidemiological studies[41,52,65]. Other gastrointestinal disorders including colorectal cancer and diverticulitis have also been associated with any constipation[43] and chronic constipation[65,69].

Anorectal disorders, particularly haemorrhoids, are frequently associated with constipation. A review of 7 studies conducted up to 2009[43] found a significant association between any constipation and haemorrhoids. Haemorrhoids also have

Table 6 Other demographic/socioeconomic factors and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Factor	Variable	Prevalence (%)	Odds ratio (95%CI)	*P* value
Talley *et al*[49], 1993	United States	690	30-64	Self-report; Rome I	Marital status	Married; Not married	17.8; 22.1	NR	NR
Chang *et al*[46], 2007	United States	523	30-64	Rome III	Marital status	Married; Single; Other	17.2; 23.1; 19.1	1.0; 1.27 (0.46, 3.48); 1.07 (0.53, 2.16)	NS
Choung *et al*[83], 2007	United States	3022	20-95	BDQ	Marital status	Married; Not married	16; 22	0.8 (0.5, 1.2); 1.0	NS
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	Marital status	Married; Single; Other	20; 16; 22	NR	NR
Ebling *et al*[50], 2014	Croatia	658	20-69	Rome III	Marital status; House; hold size	Divorced; Large size	35.7; NR	2.91; 1.19	0.039; 0.01
Sorouri *et al*[53], 2010	Iran	18180	NR	Rome III	Marital status	Married; Single; Widowed; Divorced	3.5; 0.7; 8.8; 12.5	NR	NR
Moezi *et al*[52], 2018	Iran	9264	40-75	Rome IV	Marital status	Divorced or Widowed	12.9	NR	< 0.001
Pare *et al*[45], 2000	Canada	1149	> 18	Self-report (3 mo)	Work status	Employed; Unemployed; Retired	27.2; 23.4; 25.5	NR	NR
Pare *et al*[45], 2000	Canada	1149	> 18	Rome I	Work status	Employed; Unemployed; Retired	16.3; 9.2; 15.0	NR	NR
Pare *et al*[45], 2000	Canada	1149	> 18	Rome II	Work status	Employed; Unemployed; Retired	14.1; 5.7; 16.2	NR	NR
Talley *et al*[49], 1993	United States	690	30-64	Rome I	Work status	Employed; Unemployed	20.1; 12.0	NR	NR
Enck *et al*[75], 2016	Germany	15002	> 18	Self-report (12 mo)	Work status	Full-time; Part-time; Unemployed	10.8[1]; 13.3[1]; 18.3[1]	NR	< 0.001
Drossman *et al*[44], 1993	United States	5430	> 15	Rome I	Ethnicity	White; Other	NR	0.54 (0.3, 0.9)	NR

[1]Calculated from data published.

NR: Not reported; NS: Not significant; BDQ: Bowel disease questionnaire (similar to Rome criteria).

been found to be commonly associated with chronic constipation[41,66]. Other anorectal disorders found to be associated with any constipation include fistulas, anal fissures and rectal prolapse[43,66].

Depression, anxiety and insomnia: There is evidence that depression and anxiety are associated with chronic and any constipation, and limited evidence for insomnia.

In a review of comorbidities, depression was the most commonly reported psychiatric condition associated with chronic constipation, occurring in 15% to 29% of chronic constipation patients[42]. In other studies not included in the review, depression and anxiety were found to be significantly associated with both chronic or any constipation[52,57,71,84]; one of these studies also found insomnia to be significantly associated with chronic constipation[52].

Neurological diseases: Odds ratios for chronic constipation in multiple sclerosis have been reported to be 5.5[69] and 2.41[54] in two studies; chronic constipation in Parkinson's disease had an odds ratio of 6.5[69].

Obesity: There is conflicting evidence of any association of obesity or body weight with chronic or any constipation.

In a review of comorbidities, chronic constipation was frequently associated with obesity (20% to 37% of chronic constipation patients) and being overweight (17% to 40% of chronic constipation patients)[42]. However, other epidemiological studies

Table 7 Physical activity and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Physical activity status	Prevalence (%)	Odds ratio (95%CI)	*P* value
Garrigues *et al*[60], 2004	Spain	349	18-65	Self-report (12 mo)	Never; Sometimes; Habitually	23.2; 10.9; 7.4	1.00; 0.43 (0.20, 0.89); 0.31 (0.11, 0.87)	0.02; 0.03
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	Regular sport; Long walks; Short walks; No regular walk	14; 16; 24; 30	1.00; 0.97 (0.66, 1.43); 1.52 (1.06, 2.19); 2.04 (1.23, 3.39)	< 0.01
Wald *et al*[61], 2008	United States, United Kingdom, France, Germany, Italy, Brazil and South Korea	14000	> 15	Self-report (12 mo)	Active; Reduced activity	NR	1.00; 1.23 (1.07, 1.40)	< 0.05
Moezi *et al*[52], 2018	Iran	9264	40-75	Rome IV	Low; Medium; High	10.9[1]; 7.8[1]; 5.6[1]	1.00; 0.74 (0.62, 0.89); 0.56 (0.46, 0.68)	< 0.001

[1]Calculated from data published. NR: Not reported.

Table 8 Smoking and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Smoking status	Prevalence (%)	Odds ratio (95%CI)	*P* value
Choung *et al*[65], 2012	United States	2853	> 20	Rome II	Current smoker	26.3	NR	NS
Choung *et al*[83], 2007	United States	7805	20-95	BDQ	Non-smoker; Smoker	16; 20	1.00; 1.40 (0.7, 2.7)	NS
Talley *et al*[49], 1993	United States	690	30-64	Rome I	No cigarettes; > 15/d	20.9; 12.3	4.7 (1.6, 13.7)	< 0.05
Chang *et al*[46], 2007	United States	523	30-64	Rome III	Never; Current; Past; Ever	18.9; 17.3; 17.0; 17.1	1.0; 0.90 (0.47, 1.73); 0.88 (0.53, 1.45); 0.89 (0.57, 1.39)	NS
Haug *et al*[71], 2002	Norway	62651	> 20	Self-report (12 mo)	Non-smoker; Smoker	NR; NR	1.00; 0.83	NR
Papatheodoridis *et al*[51], 2010	Greece	1000	15-64	Rome III or self-report (12 mo)	Active; Inactive	16; 16	NR	0.98
Lu *et al*[56], 2006	Taiwan	2018	> 20	Rome II	Non-smoker; Smoker	8.8[1]; 6.5[1]	NR	NS

[1]Calculated from data published.
NR: Not reported; NS: Not significant; BDQ: Bowel disease questionnaire (similar to Rome criteria).

have found no clear association between body mass index (BMI) and chronic or self-reported constipation[46,50-52,55,65,85].

Other medical conditions: In a review of comorbidities, diabetes was found to be frequently associated with chronic constipation (4.7% to 11.8% of chronic constipation patients) in a comorbidity review[42]. Some, but not all, epidemiological studies have also reported this association[56,69,86].

For chronic constipation, constipation with cardiovascular disease had an odds ratio of 1.5 in one study[69]. In the same study, the odds ratio for constipation in angina was 1.4 and in another study it was 1.86[54]. Stroke was associated with chronic constipation in Brazilian study[66]. Musculoskeletal complaints were found to be associated with chronic constipation[54], including back or joint pain[52]. Urinary tract disorders have also been reported to be associated with constipation[43].

Table 9 Fibre and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Dietary intake of fibre	Prevalence (%)	Odds ratio (95%CI)	*P* value
Garrigues *et al*[60], 2004	Spain	349	18-65	Self-report (12 mo)	Low fibre; Medium fibre; High fibre	19.2; 10.9; 20.9	1.00; 0.38 (0.15, 0.96); 1.05 (0.35, 3.17)	0.04; 0.93
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	1st quintile; 2nd quintile; 3rd quintile; 4th quintile; 5th quintile	23; 18; 17; 18; 20	NR	NS

NR: Not reported; NS: Not significant.

Table 10 Fluid and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Fluid intake per day	Prevalence (%)	Odds ratio (95%CI)	*P* value
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	7 glasses or less; 8-9 glasses; 10-11 glasses; 12-14 glasses; 15 glasses or more	21; 20; 19; 18; 16	NR	< 0.01
Chang *et al*[46], 2007	United States	523	30-64	Rome III	No coffee; Coffee	18.7; 17.8	1.00; 0.94 (0.5, 1.77)	NS

NR: Not reported; NS: Not significant.

Surgery

Recent surgery is well-known to be a risk factor for constipation; this may be associated with medications including general anaesthetics and opioid analgesics as well as being sedentary following surgery. However, the long-term effects of different surgical procedures may contribute to chronic constipation. Gynaecological, abdominal and anorectal surgery were significantly associated with an increased risk of chronic constipation in four epidemiological studies[53,66,69,83] but cholecystectomy and appendectomy were not significant in others[56,65] (Table 14).

Medications

Constipation is a common side effect of many drug classes[87,88] but this is not always evident in population-based studies because few have reported concomitant drug use.

The number of medications used may be associated with chronic or any constipation. In a Norwegian study[54], the use of one or more medications was found to be associated with chronic constipation (Table 15). Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs), particularly ibuprofen, were significantly associated with chronic constipation in this and other studies[46,49,54] (Table 16). Other classes of drugs including digoxin, glyceryl trinitrate, atorvastatin, furosemide and levothyroxine have also been found to be significantly associated with chronic constipation[54].

DISCUSSION

This is the first comprehensive review of epidemiological studies of community populations to present a detailed assessment of real-world evidence relating to all possible potential risk factors for defined adult constipation. This review of studies spanning 30 years of research identified many factors considered to be potentially associated with constipation in community-dwelling adults. These studies frequently refer to these factors as risk factors or potential risk factors for constipation. A risk factor is any factor which is proven to cause an increased prevalence of a disease but in cross-sectional studies only associations can be identified[89]. There are two issues to consider in determining the association of any factor with the prevalence of

Table 11 Alcohol and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Weekly alcohol consumption	Prevalence (%)	Odds ratio (95%CI)	*P* value
Choung *et al*[83], 2007	United States	7805	20-95	BDQ	No alcohol; Alcohol	16; 17	1; 1.1 (0.7, 1.7)	NS
Talley *et al*[49], 1993	United States	690	30-64	Rome I	No alcohol; > 7 drinks	19.7; 9.4[1]	3.6 (1.2, 10.4)	< 0.05
Chang *et al*[46], 2007	United States	523	30-64	Rome III	0 drinks; 1-2; 3-6; > 7; Any alcohol	19.3; 19.2; 14.1; 14.9; 16.3	1.0; 0.99 (0.55, 1.79); 0.69 (0.34, 1.39); 0.73 (0.35, 1.53); 0.82 (0.52, 1.29)	NS
Fosnes *et al*[54], 2011	Norway	4622	31-76	Rome II	> Once; < Once	10.6[1]; 14.2[1]	0.94 (0.89, 0.99)	0.024
Choung *et al*[65], 2012	United States	2853	> 20	Rome II	1-6 drinks; 7+ drinks	25.3[1]; 23.1[1]	NR	NS
Moezi *et al*[52], 2018	Iran	9264	40-75	Rome IV	No alcohol; Alcohol	7.9[1]; 9.7[1]	NR	NS
Lu *et al*[56], 2006	Taiwan	2018	> 20	Rome II	No alcohol; Alcohol	8.9[1]; 7.4[1]	NR	NS

[1]Calculated from data published.
NR: Not reported; NS: Not significant; BDQ: Bowel disease questionnaire (similar to Rome criteria).

Table 12 Self-rated health and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Self-rated health	Prevalence (%)	Odds ratio (95%CI)	*P* value
Ebling *et al*[50], 2014	Croatia	658	20-69	Rome III	Lower	NR	0.628	< 0.001
Enck *et al*[75], 2016	Germany	15002	> 18	Self-report (12 mo)	Very good; Good; Satisfactory; Less good; Bad	9.1[1]; 12.2[1]; 18.4[1]; 22.1[1]; 28.2[1]	NR	< 0.001

[1]Calculated from data published. NR: Not reported.

constipation in population-based studies. Firstly, the studies investigating factors associated with constipation have been cross-sectional which precludes any links to causality[89]. Factors identified as being associated with constipation could be potential risk factors, or they could be caused by constipation or both. For example, factors such as poor self-rated health, haemorrhoids and depression may be either risk factors for constipation or these factors could be resulting from constipation itself. In the case of haemorrhoids, it is hypothesized that the straining of constipation leads to the development of haemorrhoids[43]. Secondly, published cross-sectional studies investigating factors associated with constipation have tended to focus on a small number of factors of interest, ignoring other factors which may be confounding variables. For example, drugs used by participants may cause constipation as a side effect and this may influence the results obtained. If a wide range of factors is not studied, the possibility of confounding bias exists and diminishes the value of the results[90]. In the period of this review, there has been no comprehensive epidemiological study which has investigated a wide range of associated factors in the same population sample. In addition, many early studies have assessed factors only on univariate analysis, not multivariate, and therefore have not taken all confounding variables into account when determining which factors are associated with constipation.

Table 13 Medical conditions and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Condition	Prevalence (%)	Odds ratio (95%CI)	*P* value
Schmidt *et al*[66], 2016	Brazil	2162	> 18	Rome III	Fistula; Haemorrhoids; Anal fissures; Nervous disease; Stroke	54.5; 29.3; 35.8; 24.5; 48.3	3.8 (1.5, 9.7); 1.9 (1.3, 2.7); 2.2 (1.3, 3.6); 1.6 (1.1, 2.1); 5.3 (2.3, 12.1)	NR
Cheng *et al*[57], 2003	Hong Kong	3282	18-80	Rome II	Anxiety; Depression	NR	NR	< 0.0001; < 0.0001
Koloski *et al*[84], 2002	Australia	2910	> 18	Rome I	Anxiety; Depression	5.6; 4.2	NR	< 0.05; < 0.05
Bytzer *et al*[86], 1989	Australia	8185	> 18	BDQ	Control; Diabetes mellitus	9.2; 11.4	1.00; 1.54	NR
Choung *et al*[69], 2016	United States	2327	> 25	Rome III	Rectal cancer; Neurological dis; Parkinson's dis; Multiple sclerosis; Metabolic dis; Cardiovascular dis; Angina; Psychiatric disorder	NR	4.7 (1.0, 22.2); 1.5 (1.1, 1.9); 6.5 (2.9, 14.4); 5.5 (1.9, 15.8); 1.4 (1.1, 1.9); 1.5 (1.1, 1.9); 1.4 (1.1, 1.9); 1.3 (1.0, 1.7)	NR
Choung *et al*[65], 2012	United States	2853	> 20	Rome II	Dyspepsia; GORD	46.9; 34.3	NR; NR	< 0.01; < 0.01
Enck *et al*[75], 2016	Germany	15002	> 18	Self-report (12 mo)	Back pain; Circulation problem; Gynecological; Urological; Gastrointestinal	19.0[1]; 25.2[1]; 35.1[1]; 34.3[1]; 31.6[1]	NR	< 0.001 < 0.001; < 0.001 < 0.001; < 0.001
Ebling *et al*[50], 2014	Croatia	658	20-69	Rome III	BMI; Anemia	NR; 40.0	1.051; NR	0.777; < 0.01
Chang *et al*[46], 2007	United States	523	30-64	Rome III	BMI 1st Q; BMI 2nd Q; BMI 3rd Q; BMI 4th Q	19.4; 13.6; 18.0; 20.7	1.0; 0.65 (0.32, 1.32); 0.92 (0.46, 1.82); 1.07 (0.55, 2.10)	NS
Rey *et al*[55], 2014	Spain	1500	> 18	Rome III	Normal; Overweight; Obese	22; 15; 20	NR	NR
Papatheodoridis *et al*[51], 2010	Greece	1000	15-64	Rome III or self-report (12 mo)	Underweight; Normal weight; Overweight; Obese	18; 14; 17; 20	NR	0.21
Pourhoseingholi *et al*[85], 2008	Iran	2547	NR	Self-report (NTP)	BMI < 25; BMI 25-30; BMI > 30	40.4; 38.9; 40.7	NR	NS
Haug *et al*[71], 2002	Norway	60998	> 20	Self-report (12 mo)	Anxiety; Depression	NR; NR	1.86 (1.67, 2.07); 1.46 (1.30, 1.65)	NR
Fosnes *et al*[54], 2011	Norway	4622	31-76	Rome II	BMI; M/S complaints; Angina; MS	NR	0.95 (0.93, 0.97); 1.04 (1.002, 1.09); 1.86 (1.21, 2.85); 2.14 (1.03, 5.66)	< 0.001; 0.042; 0.004; 0.043
Lu *et al*[56], 2006	Taiwan	2018	> 20	Rome II	Diabetes; Hypertension	14.1[1];11.1[1]	NR	NS; NS
Moezi *et al*[52], 2018	Iran	9264	40-75	Rome IV	Insomnia; Anxiety; Depression; Back or joint pain; GORD	13.5[1]; 11.7[1]; 12.4[1]; 9.4[1]; 11.7[1]	1.62 (1.36, 1.93); 1.38 (1.15, 1.65); 1.22 (1.01, 1.48); 1.38 (1.14, 1.67); 1.51 (1.28, 1.78)	< 0.001; < 0.001; < 0.001; < 0.001; < 0.001

[1]Calculated from data published.

NR: Not reported; NS: Not significant; BDQ: Bowel disease questionnaire (similar to Rome criteria); BMI: Body mass index; M/S: Musculoskeletal; MS: multiple sclerosis; GORD: Gastroesophageal reflux disease.

In assessing the results of studies reviewed, it is clear that there is insufficient evidence for an association of most factors with constipation (Table 17). Regarding demographic factors, female gender is strongly associated with an increased prevalence of constipation; there are various possible explanations for this such as the influence of sex hormones[40]. There is no clear evidence that increasing age is associated with increased constipation. Contrary to widespread beliefs, many epidemiological studies show higher prevalence of constipation in younger age groups. Whilst increasing age effects may be explained by anatomical changes or medications[40],

Table 14 Surgery and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Surgery	Odds ratio (95%CI)	*P* value
Choung *et al*[83], 2007	United States	7805	20-95	BDQ	Abdominal	1.2 (0.7, 2.0)	NS
Choung *et al*[69], 2016	United States	2327	> 25	Rome III	Anorectal surgery; Hysterectomy	3.3 (1.2, 9.1); 1.5 (1.0, 2.2)	0.02; 0.033
Schmidt *et al*[66], 2016	Brazil	2162	> 18	Rome III	Anorectal surgery	5.3 (2.3, 12.1)	NR
Lu *et al*[56], 2006	Taiwan	2018	> 20	Rome II	Appendectomy; Cholecystectomy; Hysterectomy	NR	NS; NS; NS
Sorouri *et al*[53], 2010	Iran	18180	NR	Rome III	Abdominal surgery	0.66 (0.52, 0.83)	< 0.01

NR: Not reported; NS: Not significant; BDQ: Bowel disease questionnaire (similar to Rome criteria).

Table 15 Number of medications and constipation

Ref.	Location	Sample size	Age	Definition of constipation	Number of drugs taken	Odds ratio (95%CI)	*P* value
Fosnes *et al*[54], 2011	Norway	4622	31-76	Rome II	0; 1; 2-3; 4 or more	1; 1.34 (1.07, 1.69); 1.26 (0.99, 1.61); 1.21 (0.85, 1.71)	0.012; 0.062; 0.288

Table 16 Medications and constipation

Ref.	Location	Sample size	Age range (yr)	Definition of constipation	Medications	Prevalence (%)	Odds ratio (95%CI)	*P* value
Fosnes *et al*[54], 2011	Norway	4622	31-76	Rome II	Digoxin; GTN; Furosemide; Atorvastatin; Tibolone; Levothyroxine; Ibuprofen	NR	NR	0.025; 0.015; 0.001; 0.037; 0.038; < 0.001; 0.001
Talley *et al*[49], 1993	United States	690	30-64	Rome I	Aspirin > 7 tabs/wk	31.1	2.6 (1.2, 5.7)	< 0.05
Chang *et al*[46], 2007	United States	523	30-64	Rome III	Paracetamol; Aspirin; NSAIDs	25.3; 23.0; 26.6	1.50 (0.91, 2.47) 1.67 (1.04, 2.70) 1.80 (1.09, 2.98)	NS; < 0.05; < 0.05

GTN: Glyceryl trinitrate; NSAIDs: Non-steroidal anti-inflammatory drugs; NR: Not reported; NS: Not significant.

there is no obvious explanation for the high prevalence of constipation in younger adults. Geographic location within a country may be associated with constipation and there are indications that ethnicity may also be associated, however there are conflicting data regarding marital status. The data for socioeconomic factors such as income levels, educational levels and work status are conflicting and appear to vary by country. Similarly, the data for lifestyle factors is mixed. Whilst there is evidence that low physical activity levels are associated with constipation, there is only limited evidence for low fluid intakes and no evidence for low fibre intakes. The effects of smoking, alcohol, and coffee on constipation are unclear and could not be confirmed in this review. However, it is clear that some health-related factors are associated with constipation. This includes low self-rated health, some surgical procedures, some medications, and various medical conditions including depression, haemorrhoids, neurological diseases and some gastrointestinal, cardiovascular and musculoskeletal disorders.

Further research is required to comprehensively assess each of these factors. There are many variations and complexities involved in the epidemiological studies conducted to date. Firstly, differences in population samples, study designs, data

Table 17 Summary of evidence from population-based studies for factors potentially associated with constipation

Category	Factor	Association with constipation
Demographic	Age	Conflicting data, probably only > 70 yr
	Female gender	Associated
	Income level	Conflicting data, probably country specific
	Educational level	Conflicting data, probably country specific
	Residential location	Associated
	Work status	Conflicting data
	Marital status	Conflicting data
	Ethnicity	Possible association
Lifestyle	Physical activity	Associated
	Fibre	No evidence for low fibre
	Fluid	Possible association
	Smoking	Conflicting data
	Alcohol	No clear evidence
	Coffee	No association
Health-related	Self-rated health	Associated
	Obesity	Conflicting data
	Depression	Associated
	Anorectal	Associated – haemorrhoids and other conditions
	Gastrointestinal	Associated
	Neurological	Associated – MS, Parkinson's disease
	Endocrine	Associated - diabetes
	Cardiovascular	Associated
	Musculoskeletal	Associated
	Surgery	Associated – gynaecological, anorectal, abdominal
	No. of medications	Possible association
	NSAIDs	Associated
	Aspirin	Associated
	Other drugs	Possible association

NSAIDs: Non-steroidal anti-inflammatory drugs; MS: Multiple sclerosis.

collection methods and analyses may contribute to the different results obtained. Also, results may be affected by differences in the constipation definition – different criteria used to define chronic or any constipation[3]. Similarly, different criteria used to define comorbid conditions will affect results[42]. When considering comorbid conditions, it is possible that medications being used as treatment may be causing, wholly or in part, the constipation. This could certainly be the case in conditions such as depression, musculoskeletal disorders and cardiovascular diseases where constipation is a known side effect of many medications used for treatment[87,88]. Any increased prevalence of constipation with age may be more related to secondary causes such as comorbid conditions and medications[91]. Most constipation management protocols recommend increases in dietary fibre, fluid intake and physical activity[9,92,93]; there is the possibility that any studies showing high levels of these factors being associated with high constipation prevalence may be indicating that these are consequences of constipation management rather than risk factors for constipation.

This comprehensive and contemporary review of studies conducted in community settings extends earlier work that questioned the existence of real-world evidence for potential risk factors of constipation[4,5]. The strengths of this review are the restriction to population-based studies of community-dwelling adults and the restriction to studies where defined constipation (any or chronic), rather than stool characteristics, was assessed. Also this review was restricted to cross-sectional surveys of the community and excluded studies of convenience samples such as patient or employee groups which were considered not to reflect community settings. One limitation is the risk of bias because only articles published in English were reviewed which may have restricted studies of non-white populations. However, earlier reviews reported a lack of available data from developing countries[7,36].

CONCLUSION

Apart from female gender, residential location, physical activity and some health-related factors, it is unclear whether most other potential risk factors are associated with constipation because of insufficient evidence or conflicting data. In view of the complexities involved in previous research, it is essential that further research is conducted in community-dwelling adult populations to better understand the importance of each risk factor in constipation. It is recommended that a broad range of factors be investigated in same population samples using multivariate analysis to uncover which factors are associated with any constipation or chronic constipation in the community.

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REFERENCES

1 **Talley NJ**. Definitions, epidemiology, and impact of chronic constipation. *Rev Gastroenterol Disord* 2004; **4** Suppl 2: S3-S10 [PMID: 15184814]
2 **Drossman DA**. Functional Gastrointestinal Disorders: History, Pathophysiology, Clinical Features and Rome IV. *Gastroenterology* 2016 [PMID: 27144617 DOI: 10.1053/j.gastro.2016.02.032]
3 **Werth BL**. Epidemiology of constipation in adults: Why estimates of prevalence differ. *J Epidemiol Res* 2019; **5**: 37-49 [DOI: 10.5430/jer.v5n1p37]
4 **Müller-Lissner SA**, Kamm MA, Scarpignato C, Wald A. Myths and misconceptions about chronic constipation. *Am J Gastroenterol* 2005; **100**: 232-242 [PMID: 15654804 DOI: 10.1111/j.1572-0241.2005.40885.x]
5 **Leung FW**. Etiologic factors of chronic constipation: review of the scientific evidence. *Dig Dis Sci* 2007; **52**: 313-316 [PMID: 17219073 DOI: 10.1007/s10620-006-9298-7]
6 **Annells M**, Koch T. Constipation and the preached trio: diet, fluid intake, exercise. *Int J Nurs Stud* 2003; **40**: 843-852 [PMID: 14568365 DOI: 10.1016/s0020-7489(03)00075-0]
7 **Mugie SM**, Benninga MA, Di Lorenzo C. Epidemiology of constipation in children and adults: a systematic review. *Best Pract Res Clin Gastroenterol* 2011; **25**: 3-18 [PMID: 21382575 DOI: 10.1016/j.bpg.2010.12.010]
8 **Sbahi H**, Cash BD. Chronic Constipation: a Review of Current Literature. *Curr Gastroenterol Rep* 2015; **17**: 47 [PMID: 26449614 DOI: 10.1007/s11894-015-0471-z]
9 **Wald A**. Constipation: Advances in Diagnosis and Treatment. *JAMA* 2016; **315**: 185-191 [PMID: 26757467 DOI: 10.1001/jama.2015.16994]
10 **Morrison SM**, Symes L. An integrative review of expert nursing practice. *J Nurs Scholarsh* 2011; **43**: 163-170 [PMID: 21605320 DOI: 10.1111/j.1547-5069.2011.01398.x]
11 **Tuteja AK**, Talley NJ, Joos SK, Woehl JV, Hickam DH. Is constipation associated with decreased physical activity in normally active subjects? *Am J Gastroenterol* 2005; **100**: 124-129 [PMID: 15654791 DOI: 10.1111/j.1572-0241.2005.40516.x]
12 **Pamuk ON**, Pamuk GE, Celik AF. Revalidation of description of constipation in terms of recall bias and visual scale analog questionnaire. *J Gastroenterol Hepatol* 2003; **18**: 1417-1422 [PMID: 14675272 DOI: 10.1046/j.1440-1746.2003.03155.x]
13 **Santacruz CC**, López MO, García MV, Pérez CF, Miguel JC. Do bad habits bring a double constipation risk? *Turk J Gastroenterol* 2018; **29**: 580-587 [PMID: 30260781 DOI:

10.5152/tjg.2018.17533]
14 **Najafimehr H**, Yadegari H, Taherinejad H, Manhoie K, Rasooli SR, Moradi A, Akbariju MJ, Mohseni H, Ghadimi S, Mohaghegh Shalmani H. The effect of working in an auto factory on functional constipation and bowel habits. *Gastroenterol Hepatol Bed Bench* 2019; **12**: S101-S107 [PMID: 32099609]
15 **Herz MJ**, Kahan E, Zalevski S, Aframian R, Kuznitz D, Reichman S. Constipation: a different entity for patients and doctors. *Fam Pract* 1996; **13**: 156-159 [PMID: 8732327 DOI: 10.1093/fampra/13.2.156]
16 **Gage H**, Kaye J, Kimber A, Storey L, Egan M, Qiao Y, Trend P. Correlates of constipation in people with Parkinson's. *Parkinsonism Relat Disord* 2011; **17**: 106-111 [PMID: 21130017 DOI: 10.1016/j.parkreldis.2010.11.003]
17 **Ng KS**, Nassar N, Hamd K, Nagarajah A, Gladman MA. Prevalence of functional bowel disorders and faecal incontinence: an Australian primary care survey. *Colorectal Dis* 2015; **17**: 150-159 [PMID: 25359460 DOI: 10.1111/codi.12808]
18 **Ballou S**, Katon J, Singh P, Rangan V, Lee HN, McMahon C, Iturrino J, Lembo A, Nee J. Chronic Diarrhea and Constipation Are More Common in Depressed Individuals. *Clin Gastroenterol Hepatol* 2019; **17**: 2696-2703 [PMID: 30954714 DOI: 10.1016/j.cgh.2019.03.046]
19 **Talley NJ**, Boyce PM, Owen BK, Newman P, Paterson KJ. Initial validation of a bowel symptom questionnaire and measurement of chronic gastrointestinal symptoms in Australians. *Aust N Z J Med* 1995; **25**: 302-308 [PMID: 8540870 DOI: 10.1111/j.1445-5994.1995.tb01894.x]
20 **Talley NJ**, Jones M, Nuyts G, Dubois D. Risk factors for chronic constipation based on a general practice sample. *Am J Gastroenterol* 2003; **98**: 1107-1111 [PMID: 12809835 DOI: 10.1111/j.1572-0241.2003.07465.x]
21 **Arora G**, Mannalithara A, Mithal A, Triadafilopoulos G, Singh G. Concurrent conditions in patients with chronic constipation: a population-based study. *PLoS One* 2012; **7**: e42910 [PMID: 23071488 DOI: 10.1371/journal.pone.0042910]
22 **Walter S**, Hallböök O, Gotthard R, Bergmark M, Sjödahl R. A population-based study on bowel habits in a Swedish community: prevalence of faecal incontinence and constipation. *Scand J Gastroenterol* 2002; **37**: 911-916 [PMID: 12229965 DOI: 10.1080/003655202760230865]
23 **Tamura A**, Tomita T, Oshima T, Toyoshima F, Yamasaki T, Okugawa T, Kondo T, Kono T, Tozawa K, Ikehara H, Ohda Y, Fukui H, Watari J, Miwa H. Prevalence and Self-recognition of Chronic Constipation: Results of an Internet Survey. *J Neurogastroenterol Motil* 2016; **22**: 677-685 [PMID: 27426278 DOI: 10.5056/jnm15187]
24 **Siproudhis L**, Pigot F, Godeberge P, Damon H, Soudan D, Bigard MA. Defecation disorders: a French population survey. *Dis Colon Rectum* 2006; **49**: 219-227 [PMID: 16362804 DOI: 10.1007/s10350-005-0249-8]
25 **Bassotti G**, Bellini M, Pucciani F, Bocchini R, Bove A, Alduini P, Battaglia E, Bruzzi P; Italian Constipation Study Group. An extended assessment of bowel habits in a general population. *World J Gastroenterol* 2004; **10**: 713-716 [PMID: 14991944 DOI: 10.3748/wjg.v10.i5.713]
26 **Sanjoaquin MA**, Appleby PN, Spencer EA, Key TJ. Nutrition and lifestyle in relation to bowel movement frequency: a cross-sectional study of 20630 men and women in EPIC-Oxford. *Public Health Nutr* 2004; **7**: 77-83 [PMID: 14972075 DOI: 10.1079/phn2003522]
27 **Markland AD**, Palsson O, Goode PS, Burgio KL, Busby-Whitehead J, Whitehead WE. Association of low dietary intake of fiber and liquids with constipation: evidence from the National Health and Nutrition Examination Survey. *Am J Gastroenterol* 2013; **108**: 796-803 [PMID: 23567352 DOI: 10.1038/ajg.2013.73]
28 **Roberts MC**, Millikan RC, Galanko JA, Martin C, Sandler RS. Constipation, laxative use, and colon cancer in a North Carolina population. *Am J Gastroenterol* 2003; **98**: 857-864 [PMID: 12738468 DOI: 10.1111/j.1572-0241.2003.07386.x]
29 **Chinzon D**, Dias-Bastos TR, Medeiros da Silva A, Eisig JN, Latorre Mdo R. Epidemiology of constipation in São Paulo, Brazil: a population-based study. *Curr Med Res Opin* 2015; **31**: 57-64 [PMID: 25295483 DOI: 10.1185/03007995.2014.973485]
30 **Lam**, Kennedy, Chen, Lubowski, Talley. Prevalence of faecal incontinence: obstetric and constipation-related risk factors; a population-based study. *Colorectal Dis* 1999; **1**: 197-203 [PMID: 23577806 DOI: 10.1046/j.1463-1318.1999.00044.x]
31 **Shen L**, Huang C, Lu X, Xu X, Jiang Z, Zhu C. Lower dietary fibre intake, but not total water consumption, is associated with constipation: a population-based analysis. *J Hum Nutr Diet* 2019; **32**: 422-431 [PMID: 31087475 DOI: 10.1111/jhn.12589]
32 **Sandler RS**, Jordan MC, Shelton BJ. Demographic and dietary determinants of constipation in the US population. *Am J Public Health* 1990; **80**: 185-189 [PMID: 2297063 DOI: 10.2105/ajph.80.2.185]
33 **Adibi P**, Behzad E, Pirzadeh S, Mohseni M. Bowel habit reference values and abnormalities in young Iranian healthy adults. *Dig Dis Sci* 2007; **52**: 1810-1813 [PMID: 17410463 DOI: 10.1007/s10620-006-9509-2]
34 **Rajput M**, Saini SK. Prevalence of constipation among the general population: a community-based survey from India. *Gastroenterol Nurs* 2014; **37**: 425-429 [PMID: 25461464 DOI: 10.1097/SGA.0000000000000074]
35 **Tokuda Y**, Takahashi O, Ohde S, Shakudo M, Yanai H, Shimbo T, Fukuhara S, Hinohara S, Fukui T. Gastrointestinal symptoms in a Japanese population: a health diary study. *World J Gastroenterol* 2007; **13**: 572-578 [PMID: 17278223 DOI: 10.3748/wjg.v13.i4.572]

36 **Suares NC**, Ford AC. Prevalence of, and risk factors for, chronic idiopathic constipation in the community: systematic review and meta-analysis. *Am J Gastroenterol* 2011; **106**: 1582-91; quiz 1581, 1592 [PMID: 21606976 DOI: 10.1038/ajg.2011.164]

37 **Schmidt FM**, Santos VL. Prevalence of constipation in the general adult population: an integrative review. *J Wound Ostomy Continence Nurs* 2014; **41**: 70-6; quiz E1 [PMID: 24378694 DOI: 10.1097/01.WON.0000438019.21229.b7]

38 **Higgins PD**, Johanson JF. Epidemiology of constipation in North America: a systematic review. *Am J Gastroenterol* 2004; **99**: 750-759 [PMID: 15089911 DOI: 10.1111/j.1572-0241.2004.04114.x]

39 **Peppas G**, Alexiou VG, Mourtzoukou E, Falagas ME. Epidemiology of constipation in Europe and Oceania: a systematic review. *BMC Gastroenterol* 2008; **8**: 5 [PMID: 18269746 DOI: 10.1186/1471-230X-8-5]

40 **Iraji N**, Keshteli AH, Sadeghpour S, Daneshpajouhnejad P, Fazel M, Adibi P. Constipation in Iran: SEPAHAN Systematic Review No. 5. *Int J Prev Med* 2012; **3**: S34-S41 [PMID: 22826768]

41 **Chu H**, Zhong L, Li H, Zhang X, Zhang J, Hou X. Epidemiology characteristics of constipation for general population, pediatric population, and elderly population in china. *Gastroenterol Res Pract* 2014; **2014**: 532734 [PMID: 25386187 DOI: 10.1155/2014/532734]

42 **Nellesen D**, Chawla A, Oh DL, Weissman T, Lavins BJ, Murray CW. Comorbidities in patients with irritable bowel syndrome with constipation or chronic idiopathic constipation: a review of the literature from the past decade. *Postgrad Med* 2013; **125**: 40-50 [PMID: 23816770 DOI: 10.3810/pgm.2013.03.2640]

43 **Talley NJ**, Lasch KL, Baum CL. A gap in our understanding: chronic constipation and its comorbid conditions. *Clin Gastroenterol Hepatol* 2009; **7**: 9-19 [PMID: 18829389 DOI: 10.1016/j.cgh.2008.07.005]

44 **Drossman DA**, Li Z, Andruzzi E, Temple RD, Talley NJ, Thompson WG, Whitehead WE, Janssens J, Funch-Jensen P, Corazziari E. U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. *Dig Dis Sci* 1993; **38**: 1569-1580 [PMID: 8359066 DOI: 10.1007/BF01303162]

45 **Pare P**, Ferrazzi S, Thompson WG, Irvine EJ, Rance L. An epidemiological survey of constipation in canada: definitions, rates, demographics, and predictors of health care seeking. *Am J Gastroenterol* 2001; **96**: 3130-3137 [PMID: 11721760 DOI: 10.1111/j.1572-0241.2001.05259.x]

46 **Chang JY**, Locke GR, Schleck CD, Zinsmeister AR, Talley NJ. Risk factors for chronic constipation and a possible role of analgesics. *Neurogastroenterol Motil* 2007; **19**: 905-911 [PMID: 17988275 DOI: 10.1111/j.1365-2982.2007.00974.x]

47 **Meinds RJ**, van Meegdenburg MM, Trzpis M, Broens PM. On the prevalence of constipation and fecal incontinence, and their co-occurrence, in the Netherlands. *Int J Colorectal Dis* 2017; **32**: 475-483 [PMID: 27913883 DOI: 10.1007/s00384-016-2722-3]

48 **Howell SC**, Quine S, Talley NJ. Low social class is linked to upper gastrointestinal symptoms in an Australian sample of urban adults. *Scand J Gastroenterol* 2006; **41**: 657-666 [PMID: 16716963 DOI: 10.1080/00365520500442567]

49 **Talley NJ**, Weaver AL, Zinsmeister AR, Melton LJ 3rd. Functional constipation and outlet delay: a population-based study. *Gastroenterology* 1993; **105**: 781-790 [PMID: 8359649 DOI: 10.1016/0016-5085(93)90896-k]

50 **Ebling B**, Gulić S, Jurcić D, Martinac M, Gmajnić R, Bilić A, Pribić S, Levak MT. Demographic, anthropometric and socioeconomic characteristics of functional constipation in Eastern Croatia. *Coll Antropol* 2014; **38**: 539-546 [PMID: 25144985]

51 **Papatheodoridis GV**, Vlachogiannakos J, Karaitianos I, Karamanolis DG. A Greek survey of community prevalence and characteristics of constipation. *Eur J Gastroenterol Hepatol* 2010; **22**: 354-360 [PMID: 19474747 DOI: 10.1097/MEG.0b013e32832bfdf0]

52 **Moezi P**, Salehi A, Molavi H, Poustchi H, Gandomkar A, Imanieh MH, Malekzadeh R. Prevalence of Chronic Constipation and Its Associated Factors in Pars Cohort Study: A Study of 9000 Adults in Southern Iran. *Middle East J Dig Dis* 2018; **10**: 75-83 [PMID: 30013755 DOI: 10.15171/mejdd.2018.94]

53 **Sorouri M**, Pourhoseingholi MA, Vahedi M, Safaee A, Moghimi-Dehkordi B, Pourhoseingholi A, Habibi M, Zali MR. Functional bowel disorders in Iranian population using Rome III criteria. *Saudi J Gastroenterol* 2010; **16**: 154-160 [PMID: 20616409 DOI: 10.4103/1319-3767.65183]

54 **Fosnes GS**, Lydersen S, Farup PG. Constipation and diarrhoea - common adverse drug reactions? *BMC Clin Pharmacol* 2011; **11**: 2 [PMID: 21332973 DOI: 10.1186/1472-6904-11-2]

55 **Rey E**, Balboa A, Mearin F. Chronic constipation, irritable bowel syndrome with constipation and constipation with pain/discomfort: similarities and differences. *Am J Gastroenterol* 2014; **109**: 876-884 [PMID: 24589666 DOI: 10.1038/ajg.2014.18]

56 **Lu CL**, Chang FY, Chen CY, Luo JC, Lee SD. Significance of Rome II-defined functional constipation in Taiwan and comparison with constipation-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2006; **24**: 429-438 [PMID: 16842471 DOI: 10.1111/j.1365-2036.2006.02949.x]

57 **Cheng C**, Chan AO, Hui WM, Lam SK. Coping strategies, illness perception, anxiety and depression of patients with idiopathic constipation: a population-based study. *Aliment Pharmacol Ther* 2003; **18**: 319-326 [PMID: 12895216 DOI: 10.1046/j.1365-2036.2003.01663.x]

58 **Jun DW**, Park HY, Lee OY, Lee HL, Yoon BC, Choi HS, Hahm JS, Lee MH, Lee DH, Kee CS. A population-based study on bowel habits in a Korean community: prevalence of functional constipation

and self-reported constipation. *Dig Dis Sci* 2006; **51**: 1471-1477 [PMID: 16832618 DOI: 10.1007/s10620-006-9087-3]

59 **Wald A**, Mueller-Lissner S, Kamm MA, Hinkel U, Richter E, Schuijt C, Mandel KG. Survey of laxative use by adults with self-defined constipation in South America and Asia: a comparison of six countries. *Aliment Pharmacol Ther* 2010; **31**: 274-284 [PMID: 19832728 DOI: 10.1111/j.1365-2036.2009.04169.x]

60 **Garrigues V**, Gálvez C, Ortiz V, Ponce M, Nos P, Ponce J. Prevalence of constipation: agreement among several criteria and evaluation of the diagnostic accuracy of qualifying symptoms and self-reported definition in a population-based survey in Spain. *Am J Epidemiol* 2004; **159**: 520-526 [PMID: 14977649 DOI: 10.1093/aje/kwh072]

61 **Wald A**, Scarpignato C, Mueller-Lissner S, Kamm MA, Hinkel U, Helfrich I, Schuijt C, Mandel KG. A multinational survey of prevalence and patterns of laxative use among adults with self-defined constipation. *Aliment Pharmacol Ther* 2008; **28**: 917-930 [PMID: 18644012 DOI: 10.1111/j.1365-2036.2008.03806.x]

62 **Harari D**, Gurwitz JH, Avorn J, Bohn R, Minaker KL. Bowel habit in relation to age and gender. Findings from the National Health Interview Survey and clinical implications. *Arch Intern Med* 1996; **156**: 315-320 [PMID: 8572842]

63 **Choung RS**, Locke GR 3rd, Zinsmeister AR, Melton LJ 3rd, Talley NJ. Alternating bowel pattern: what do people mean? *Aliment Pharmacol Ther* 2006; **23**: 1749-1755 [PMID: 16817919 DOI: 10.1111/j.1365-2036.2006.02953.x]

64 **Talley NJ**, Zinsmeister AR, Van Dyke C, Melton LJ 3rd. Epidemiology of colonic symptoms and the irritable bowel syndrome. *Gastroenterology* 1991; **101**: 927-934 [PMID: 1889716 DOI: 10.1016/0016-5085(91)90717-y]

65 **Choung RS**, Locke GR 3rd, Rey E, Schleck CD, Baum C, Zinsmeister AR, Talley NJ. Factors associated with persistent and nonpersistent chronic constipation, over 20 years. *Clin Gastroenterol Hepatol* 2012; **10**: 494-500 [PMID: 22289877 DOI: 10.1016/j.cgh.2011.12.041]

66 **Schmidt FM**, de Gouveia Santos VL, de Cássia Domansky R, Neves JM. Constipation: Prevalence and Associated Factors in Adults Living in Londrina, Southern Brazil. *Gastroenterol Nurs* 2016; **39**: 204-211 [PMID: 27258460 DOI: 10.1097/SGA.0000000000000224]

67 **Everhart JE**, Go VL, Johannes RS, Fitzsimmons SC, Roth HP, White LR. A longitudinal survey of self-reported bowel habits in the United States. *Dig Dis Sci* 1989; **34**: 1153-1162 [PMID: 2787735 DOI: 10.1007/BF01537261]

68 **Stewart WF**, Liberman JN, Sandler RS, Woods MS, Stemhagen A, Chee E, Lipton RB, Farup CE. Epidemiology of constipation (EPOC) study in the United States: relation of clinical subtypes to sociodemographic features. *Am J Gastroenterol* 1999; **94**: 3530-3540 [PMID: 10606315 DOI: 10.1111/j.1572-0241.1999.01642.x]

69 **Choung RS**, Rey E, Richard Locke G 3rd, Schleck CD, Baum C, Zinsmeister AR, Talley NJ. Chronic constipation and co-morbidities: A prospective population-based nested case-control study. *United European Gastroenterol J* 2016; **4**: 142-151 [PMID: 26966534 DOI: 10.1177/2050640614558476]

70 **van Kerkhoven LA**, Eikendal T, Laheij RJ, van Oijen MG, Jansen JB. Gastrointestinal symptoms are still common in a general Western population. *Neth J Med* 2008; **66**: 18-22 [PMID: 18219063]

71 **Haug TT**, Mykletun A, Dahl AA. Are anxiety and depression related to gastrointestinal symptoms in the general population? *Scand J Gastroenterol* 2002; **37**: 294-298 [PMID: 11916191 DOI: 10.1080/003655202317284192]

72 **Gaburri M**, Bassotti G, Bacci G, Cinti A, Bosso R, Ceccarelli P, Paolocci N, Pelli MA, Morelli A. Functional gut disorders and health care seeking behavior in an Italian non-patient population. *Recenti Prog Med* 1989; **80**: 241-244 [PMID: 2762661]

73 **Heaton KW**, Cripps HA. Straining at stool and laxative taking in an English population. *Dig Dis Sci* 1993; **38**: 1004-1008 [PMID: 8508693 DOI: 10.1007/BF01295713]

74 **Esteban y Peña M**, García RJ, Olalla JM, Llanos EV, de Miguel AG, Cordero XF. Impact of the most frequent chronic health conditions on the quality of life among people aged > 15 years in Madrid. *Eur J Public Health* 2010; **20**: 78-84 [PMID: 19587225 DOI: 10.1093/eurpub/ckp098]

75 **Enck P**, Leinert J, Smid M, Köhler T, Schwille-Kiuntke J. Prevalence of constipation in the German population - a representative survey (GECCO). *United European Gastroenterol J* 2016; **4**: 429-437 [PMID: 27403310 DOI: 10.1177/2050640615603009]

76 **Ho KY**, Kang JY, Seow A. Prevalence of gastrointestinal symptoms in a multiracial Asian population, with particular reference to reflux-type symptoms. *Am J Gastroenterol* 1998; **93**: 1816-1822 [PMID: 9772037 DOI: 10.1111/j.1572-0241.1998.00526.x]

77 **Chen LY**, Ho KY, Phua KH. Normal bowel habits and prevalence of functional bowel disorders in Singaporean adults--findings from a community based study in Bishan. Community Medicine GI Study Group. *Singapore Med J* 2000; **41**: 255-258 [PMID: 11109339]

78 **Chang FY**, Chen PH, Wu TC, Pan WH, Chang HY, Wu SJ, Yeh NH, Tang RB, Wu L, James FE. Prevalence of functional gastrointestinal disorders in Taiwan: questionnaire-based survey for adults based on the Rome III criteria. *Asia Pac J Clin Nutr* 2012; **21**: 594-600 [PMID: 23017318]

79 **Jeong JJ**, Choi MG, Cho YS, Lee SG, Oh JH, Park JM, Cho YK, Lee IS, Kim SW, Han SW, Choi KY, Chung IS. Chronic gastrointestinal symptoms and quality of life in the Korean population. *World J Gastroenterol* 2008; **14**: 6388-6394 [PMID: 19009657 DOI: 10.3748/wjg.14.6388]

80 **Talley NJ**, Boyce P, Jones M. Identification of distinct upper and lower gastrointestinal symptom groupings in an urban population. *Gut* 1998; **42**: 690-695 [PMID: 9659166 DOI:

10.1136/gut.42.5.690]
81 **Koloski NA**, Jones M, Young M, Talley NJ. Differentiation of functional constipation and constipation predominant irritable bowel syndrome based on Rome III criteria: a population-based study. *Aliment Pharmacol Ther* 2015; **41**: 856-866 [PMID: 25736433 DOI: 10.1111/apt.13149]
82 **Bytzer P**, Howell S, Leemon M, Young LJ, Jones MP, Talley NJ. Low socioeconomic class is a risk factor for upper and lower gastrointestinal symptoms: a population based study in 15 000 Australian adults. *Gut* 2001; **49**: 66-72 [PMID: 11413112 DOI: 10.1136/gut.49.1.66]
83 **Choung RS**, Locke GR 3rd, Schleck CD, Zinsmeister AR, Talley NJ. Cumulative incidence of chronic constipation: a population-based study 1988-2003. *Aliment Pharmacol Ther* 2007; **26**: 1521-1528 [PMID: 17919271 DOI: 10.1111/j.1365-2036.2007.03540.x]
84 **Koloski NA**, Talley NJ, Boyce PM. Epidemiology and health care seeking in the functional GI disorders: a population-based study. *Am J Gastroenterol* 2002; **97**: 2290-2299 [PMID: 12358247 DOI: 10.1111/j.1572-0241.2002.05783.x]
85 **Pourhoseingholi MA**, Kaboli SA, Pourhoseingholi A, Moghimi-Dehkordi B, Safaee A, Mansoori BK, Habibi M, Zali MR. Obesity and functional constipation; a community-based study in Iran. *J Gastrointestin Liver Dis* 2009; **18**: 151-155 [PMID: 19565043]
86 **Bytzer P**, Talley NJ, Leemon M, Young LJ, Jones MP, Horowitz M. Prevalence of gastrointestinal symptoms associated with diabetes mellitus: a population-based survey of 15,000 adults. *Arch Intern Med* 2001; **161**: 1989-1996 [PMID: 11525701 DOI: 10.1001/archinte.161.16.1989]
87 **Branch RL**, Butt TF. Drug-induced constipation. *Adv Drug React Bull* 2009; **257**: 987-990 [DOI: 10.1097/FAD.0b013e32833080d1]
88 **Toney RC**, Wallace D, Sekhon S, Agrawal RM. Medication induced constipation and diarrhea. *Pract Gastroenterol* 2008; **32**: 12-28
89 **Harkness GA**. Epidemiology in Nursing Practice. St. Louis: Mosby-Year Book Inc., 1995: 78-83
90 **Hemkens LG**, Ewald H, Naudet F, Ladanie A, Shaw JG, Sajeev G, Ioannidis JPA. Interpretation of epidemiologic studies very often lacked adequate consideration of confounding. *J Clin Epidemiol* 2018; **93**: 94-102 [PMID: 28943377 DOI: 10.1016/j.jclinepi.2017.09.013]
91 **Gallagher P**, O'Mahony D. Constipation in old age. *Best Pract Res Clin Gastroenterol* 2009; **23**: 875-887 [PMID: 19942165 DOI: 10.1016/j.bpg.2009.09.001]
92 **Tack J**, Müller-Lissner S, Stanghellini V, Boeckxstaens G, Kamm MA, Simren M, Galmiche JP, Fried M. Diagnosis and treatment of chronic constipation--a European perspective. *Neurogastroenterol Motil* 2011; **23**: 697-710 [PMID: 21605282 DOI: 10.1111/j.1365-2982.2011.01709.x]
93 **Pare P**. The approach to diagnosis and treatment of chronic constipation: suggestions for a general practitioner. *Can J Gastroenterol* 2011; **25** Suppl B: 36B-40B [PMID: 22114756 DOI: 10.1155/2011/368189]

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MINIREVIEWS

Requirements for implementation of artificial intelligence in the practice of gastrointestinal pathology

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Abstract

Tremendous advances in artificial intelligence (AI) in medical image analysis have been achieved in recent years. The integration of AI is expected to cause a revolution in various areas of medicine, including gastrointestinal (GI) pathology. Currently, deep learning algorithms have shown promising benefits in areas of diagnostic histopathology, such as tumor identification, classification, prognosis prediction, and biomarker/genetic alteration prediction. While AI cannot substitute pathologists, carefully constructed AI applications may increase workforce productivity and diagnostic accuracy in pathology practice. Regardless of these promising advances, unlike the areas of radiology or cardiology imaging, no histopathology-based AI application has been approved by a regulatory authority or for public reimbursement. Thus, implying that there are still some obstacles to be overcome before AI applications can be safely and effectively implemented in real-life pathology practice. The challenges have been identified at different stages of the development process, such as needs identification, data curation, model development, validation, regulation, modification of daily workflow, and cost-effectiveness balance. The aim of this review is to present challenges in the process of AI development, validation, and regulation that should be overcome for its implementation in real-life GI pathology practice.

Key Words: Artificial intelligence; Deep learning; Digital image analysis; Digital pathology; Clinical implementation; Gastrointestinal cancer

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Core Tip: The advances in artificial intelligence (AI) will revolutionize medical practice, as well as other areas of medicine. Deep learning algorithms have shown promising benefits in various areas of diagnostic histopathology. Despite this, AI technology is not widely used as a medical device and is not approved by a regulatory authority. Thus, implying that certain improvements in the development process are still necessary for the implementation of AI in the real-life histopathology-practice. This paper aims to provide a review of recent AI developments in gastrointestinal pathology and the challenges in their implementation.

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INTRODUCTION

The integration of artificial intelligence (AI) will cause a revolution in various areas of medicine[1], including gastrointestinal (GI) pathology, in the next decade. Advances in slide scanner technology have made it possible to quickly digitalize histological slides at high resolution, which could be used in clinical practice, research, and education [2-4]. The drastic increase in computing capacity and improvement in information technology (IT) infrastructure has allowed rapid and efficient processing of large-sized data such as whole slide images (WSIs). In recent years, there has been an increase in computer applications utilizing AI to analyze images[5].

AI is an umbrella terminology for the different strategies a computer can employ to think and learn like a human. Pathological AI models have progressed from expert systems to conventional machine learning (ML) and deep learning (DL)[6]. Both expert systems and conventional ML use expert knowledge and expert-defined rules about objects. On the contrary, DL directly extracts features from the raw data and leverages multiple hidden layers of data for the output[7] (Figure 1). Compared to conventional ML, DL is simpler to conduct, performs with high-precision, and is cost-effective[5,8]. Its implementation enhances the reproducibility of the subjective visual assessment by human pathologists and integrates multiple parameters for precision medicine[9,10]. Currently, DL algorithms have shown promising benefits in different facets of diagnostic histopathology, such as tumor identification, classification, prognosis prediction, and biomarker/genetic alteration prediction[5,11]. In addition, various AI applications have been developed for GI pathology[12-14].

AI applications using DL algorithms have demonstrated various benefits in the field of GI pathology. Recent reviews (gastric and colorectal) provide an overview of the rapid and extensive progress in the field[5,11-14]. In 2017, the Philips IntelliSite (Philips Electronics, Amsterdam, The Netherlands) whole-slide scanner was approved by the Food and Drug Administration (FDA) in the United States. The implementation of AI in pathology is also promoted by various startups such as DeepLens[15] and PathAI[16]. Some institutions have agreed to digitize their pathology workflow[17,18]. Although these advances are promising, unlike in the field of radiology or cardiology imaging[19], no histopathology-related AI application has been approved by a regulatory authority or for public reimbursement. This indicates that there are still many obstacles to be resolved before the introduction of AI applications in real-life histopathology practice (Figure 2).

In this review, we aim to present and summarize challenges in the process of development, validation, and regulation that should be overcome for the implementation of AI in real-life GI pathology practice. The complete and comprehensive review of the literature on GI pathology-related AI applications is beyond the scope of this paper and is well described elsewhere[12-14]. Here, we focused on how we can adopt these recent advancements in our daily practice.

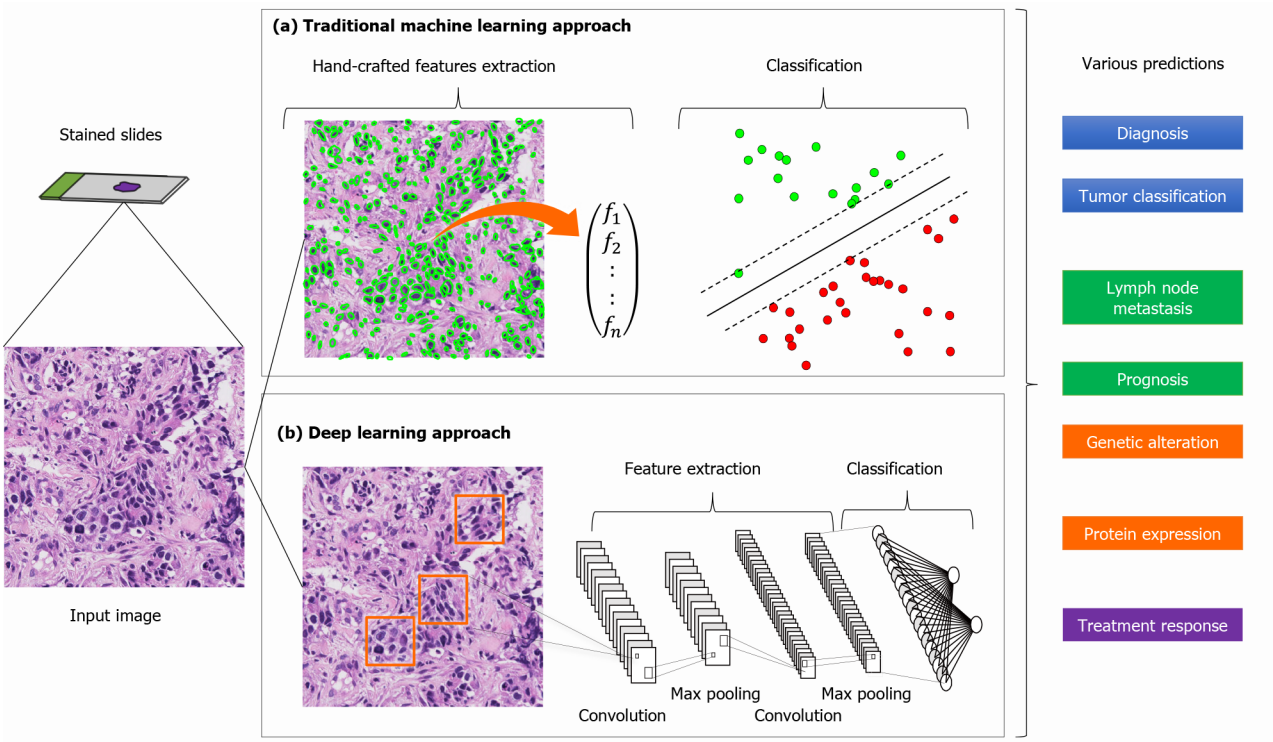


Figure 1 General workflow of construction of artificial intelligence model in pathology. Stained slides are converted to digital input images by a slide scanner. Both (a) hand-crafted feature engineering and (b) deep learning approach generate outputs of classification, which are applied to various clinically relevant predictions.

AI-APPLICATIONS IN GI PATHOLOGY

AI applications in tumor pathology, including GI cancers[4,5] have been developed for tumor diagnosis, subtyping, grading, staging, prognosis prediction, and identification of biomarkers and genetic alterations. In the current decade, the implementation of DL technologies has dramatically improved the accuracy of digital image analysis[5]. DL is one of the ML methods that are particularly effective for digital image analysis[6]. DL is based on the use of convolutional neural networks (CNNs), consisting of millions of artificial neurons, assembled in several layers that are capable of translating its input data (pixel value matrix for an image) into a more abstract representation (Figure 1). The various layers of mathematical computation are fed into a dataset of digitized images annotated with a specific label (*e.g.*, carcinoma or benign lesion); ultimately, the CNN learns how to categorize images according to their respective labels. They automatically identify the most distinctive and common characteristics of each type of object. CNNs outperform hand-crafted or conventional ML techniques (using support vector machines or random forests), by a substantial margin, in image classification[8,20]. In GI pathology, the prediction targets also include tumor classification, the clinical outcome of the patient, and genetic alterations within the tumor (Tables 1 and 2).

In addition, a variety of ML methods have been developed. The strengths and weaknesses of typical ML methods are summarized in Table 3. All of the current ML methods have their advantages and disadvantages, and it is necessary to select an appropriate method according to the purpose of image analysis. DL-based methods are most commonly used in current image analysis of GI pathology; however, they have limitations of requiring substantial data sets and insufficient interpretability. In the future, the development of new ML methods that can compensate for the disadvantages of current ML methods will further accelerate the development of AI-models.

Histopathological AI-applications in gastric cancer

Several attempts have been made to classify pathological images of gastric cancer using AI (Table 1). Before we go into details of AI research review, it should be noted that the comparison of performances should not rely only on accuracy; we should pay attention to the task difficulty in the research framework, *i.e.*, (1) dataset size (results

Table 1 Artificial intelligence applications in gastric cancer pathology

Ref.	Task	No. of cases/data set	Machine learning method	Performance
Bollschweiler *et al*[79]	Prognosis prediction	135 cases	ANN	Accuracy (93%)
Duraipandian *et al*[80]	Tumor classification	700 slides	GastricNet	Accuracy (100%)
Cosatto *et al*[65]	Tumor classification	> 12000 WSIs	MIL	AUC (0.96)
Sharma *et al*[21]	Tumor classification	454 cases	CNN	Accuracy (69% for cancer classification), accuracy (81% for necrosis detection)
Jiang *et al*[81]	Prognosis prediction	786 cases	SVM classifier	AUCs (up to 0.83)
Qu *et al*[82]	Tumor classification	9720 images	DL	AUCs (up to 0.97)
Yoshida *et al*[23]	Tumor classification	3062 gastric biopsy specimens	ML	Overall concordance rate (55.6%)
Kather *et al*[34]	Prediction of microsatellite instability	1147 cases (gastric and colorectal cancer)	Deep residual learning	AUC (0.81 for gastric cancer; 0.84 for colorectal cancer)
Garcia *et al*[30]	Tumor classification	3257 images	CNN	Accuracy (96.9%)
León *et al*[83]	Tumor classification	40 images	CNN	Accuracy (up to 89.7%)
Fu *et al*[32]	Prediction of genomic alterations, gene expression profiling, and immune infiltration	> 1000 cases (gastric, colorectal, esophageal, and liver cancers)	Neural networks.	AUC (0.9) for BRAF mutations prediction in thyroid cancers
Liang *et al*[84]	Tumor classification	1900 images	DL	Accuracy (91.1%)
Sun *et al*[85]	Tumor classification	500 images	DL	Accuracy (91.6%)
Tomita *et al*[24]	Tumor classification	502 cases (esophageal adenocarcinoma and Barret esophagus)	Attention-based deep learning	Accuracy (83%)
Wang *et al*[86]	Tumor classification	608 images	Recalibrated multi-instance deep learning	Accuracy (86.5%)
Iizuka *et al*[22]	Tumor classification	1746 biopsy WSIs	CNN, RNN	AUCs (up to 0.98), accuracy (95.6%)
Kather *et al*[33]	Prediction of genetic alterations and gene expression signatures	> 1000 cases (gastric, colorectal, and pancreatic cancer)	Neural networks	AUC (up to 0.8)

ANN: Artificial neural network; GastricNet: The deep learning framework; WSIs: Whole slide images; MIL: Multi-instance learning; AUC: Area under the curve; CNN: Convolutional neural networks; SVM: Support vector machine; DL: Deep learning; ML: Machine learning; RNN: Recurrent neural networks.

for small sample size are less reliable), (2) resolution of detection (tissue level or region level), (3) number of categories to be classified, (4) multi-site validation (sources of training and test dataset are from the same site or not), and (5) constraints on target lesion (*e.g.*, adenocarcinoma only, or any lesions except lymphoma). Sharma and colleagues documented the detection of gastric cancer in histopathological images using two DL-based methods: one analyzed the morphological features of the whole image, while the other investigated the focal features of the image independently. These models showed an average accuracy of up to 89.7%[21]. Iizuka *et al*[22] reported an AI algorithm, based on CNNs and recurrent neural networks, to classify gastric biopsy images into gastric adenocarcinoma, adenoma, and non-neoplastic tissue. Within three independent test datasets, the algorithm demonstrated an area under the curve (AUC) of 0.97 for the classification of gastric adenocarcinoma. Yoshida *et al*[23], using gastric biopsy specimens, contrasted the classification outcomes of experienced pathologists with those of the NEC Corporation-built ML-based program "e-Pathologist". While the total concordance rate between them was only 55.6 percent (1702/3062), the concordance rate was as high as 90.6 percent (1033/1140) for the biopsy specimens negative for a neoplastic lesion. Tomita *et al*[24] attempted to automate the identification of pre-neoplastic/neoplastic lesions in Barrett esophagus or gastric adenomas/adenocarcinomas.

The above tumor classification studies have shown that AI can be used for histopathological image analysis. However, other obstacles are hindering its use in real-life practice. For example, although the workload of pathologists can be

Table 2 Artificial intelligence applications in colorectal cancer pathology

Ref.	Task	No. of cases/data set	Machine learning method	Performance
Xu *et al*[38]	Tumor classification: 6 classes (NL/ADC/MC/SC/PC/CCTA)	717 patches	AlexNet	Accuracy (97.5%)
Awan *et al*[87]	Tumor classification: Normal/Low-grade cancer/High-grade cancer	454 cases	Neural networks	Accuracy (97%, for 2-class; 91%, for 3-class)
Haj-Hassan *et al*[37]	Tumor classification: 3 classes (NL/AD/ADC)	30 multispectral image patches	CNN	Accuracy (99.2%)
Kainz *et al*[88]	Tumor classification: Benign/Malignant	165 images	CNN (LeNet-5)	Accuracy (95%-98%)
Korbar *et al*[36]	Tumor classification: 6 classes (NL/HP/SSP/TSA/TA/TVA-VA)	697 cases	ResNet	Accuracy (93.0%)
Yoshida *et al*[35]	Tumor classification	1328 colorectal biopsy WSIs	ML	Accuracy (90.1%, adenoma)
Alom *et al*[89]	Tumor microenvironment analysis: Classification, Segmentation and Detection	21135 patches	DCRN/R2U-Net	Accuracy (91.1%, classification)
Bychkov *et al*[42]	Prediction of colorectal cancer outcome (5-yr disease-specific survival).	420 cases	Recurrent neural networks	HR of 2.3, AUC (0.69)
Weis *et al*[90]	Evaluation of tumor budding	401 cases	CNN	Correlation R (0.86)
Ponzio *et al*[91]	Tumor classification: 3 classes (NL/AD/ADC)	27 WSIs (13500 patches)	VGG16	Accuracy (96 %)
Kather *et al*[34]	Tumor classification: 2 classes (NL/Tumor)	94 WSIs	ResNet18	AUC (> 0.99)
Kather *et al*[34]	Prediction of microsatellite instability	360 TCGA- DX (93408 patches), 378 TCGA- KR (60894 patches)	ResNet18	AUC: TCGA-DX – (0.77, TCGA-DX; 0.84, TCGA-KR)
Kather *et al*[26]	Tumor microenvironment analysis: classification of 9 cell types	86 WSIs (100000)	VGG19	Accuracy (94%-99%)
Kather *et al*[26]	Prognosis predictions	1296 WSIs	VGG19	Accuracy (94%-99%)
Kather *et al*[26]	Prognosis prediction	934 cases	Deep learning (comparison of 5 networks)	HR for overall survival of 1.99 (training set) and 1.63 (test set)
Geessink *et al*[29]	Prognosis prediction, quantification of intratumoral stroma	129 cases	Neural networks	HRs of 2.04 for disease-free survival
Sena *et al*[40]	Tumor classification: 4 classes (NL/HP/AD/ADC)	393 WSIs (12,565 patches)	CNN	Accuracy (80%)
Shapcott *et al*[92]	Tumor microenvironment analysis: detection and classification	853 patches and 142 TCGA images	CNN with a grid-based attention network	Accuracy (84%, training set; 65%, test set)
Sirinukunwattana *et al*[31]	Prediction of consensus molecular subtypes of colorectal cancer	1206 cases	Neural networks with domain-adversarial learning	AUC (0.84 and 0.95 in the two validation sets)
Swiderska-Chadaj *et al*[93]	Tumor Microenvironment Analysis: Detection of immune cell, CD3+, CD8+	28 WSIs	FCN/LSM/U-Net	Sensitivity (74.0%)
Yoon *et al*[39]	Tumor classification: 2 classes (NL/Tumor)	57 WSIs (10280 patches)	VGG	Accuracy (93.5%)
Echle *et al*[46]	Prediction of microsatellite instability	8836 cases	ShuffleNet Deep learning	AUC (0.92 in development cohort; 0.96 in validation cohort)
Iizuka *et al*[22]	Tumor classification: 3 classes (NL/AD/ADC)	4036 WSIs	CNN/RNN	AUCs (0.96, ADC; 0.99, AD)
Skrede *et al*[28]	Prognosis predictions	2022 cases	Neural networks with multiple instance learning	HR (3.04 after adjusting for established prognostic markers)

NL: Normal mucosa; ADC: Adenocarcinoma; MC: Mucinous carcinoma; SC: Serrated carcinoma; PC: Papillary carcinoma; CCTA: Cribriform comedo-type adenocarcinoma; AD: Adenoma; CNN: Convolutional neural network; HP: Hyperplastic polyp; SSP: Sessile serrated polyp; TSA: Traditional serrated adenoma; TA: Tubular adenoma; TVA: Tubulovillous adenoma; VA: Villous adenoma; WSI: Whole slide images; ML: Machine learning; DCRN: Densely connected recurrent convolutional network; R2U-Net: Recurrent residual U-Net; HR: Hazard ratio; AUC: Area under the curve; TCGA: The Cancer

Genome Atlas; ResNet: Residual network; VGG: Visual geometry group; RNN: Recurrent neural network; FCN: Fully convolutional networks; LSM: Locality-sensitive method.

Table 3 Advantages and disadvantages of representative machine-learning methods in the development of artificial intelligence-models for gastrointestinal pathology

AI model	Advantages	Disadvantages
Conventional ML (supervised)	User can reflect domain knowledge to features	Requires hand-crafted features; Accuracy depends heavily on the quality of feature extraction
Conventional ML (unsupervised)	Executable without labels	Results are often unstable; Interpretability of the results
Deep neural networks (CNN)	Automatic feature extraction; High accuracy	Requires a large dataset; Low explainability (Black box)
Multi-instance learning	Executable without detailed labels	Requires a large dataset; High computational cost
Semantic segmentation (FCN, U-Net)	Pixel-level detection gives the position, size, and shape of the target	High labeling cost
Recurrent neural networks	Learn sequential data	High computational cost
Generative adversarial networks	Learn to synthesize new realistic data	Complexity and instability in training

AI: Artificial intelligence; ML: Machine learning; CNN: Convolutional neural network; FCN: Fully convolutional network

minimized, by defining cases for no further review by a pathologist, even in "negative" gastric biopsies, other findings, in addition to neoplastic lesions, such as Helicobacter pylori infection, need to be reviewed and recorded. Therefore, AI application cannot be functional until it sufficiently represents diagnostic procedures of real-life practice.

The prediction of prognosis from histopathological images of GI cancers is also an attractive area for AI application. Considering the many types of histopathological prognostic features of cancer, such as tumor differentiation or lymphovascular involvement, the unveiling of hidden morphological features may be expected from AI for better prediction of clinical outcomes from the histopathological images alone[25-27]. After ingesting a sufficient number of histopathological images from patients with known outcomes, AI may comprehensively predict the patient's future outcomes. Recently, an exponentially increasing number of studies conducted for major GI cancers have demonstrated the feasibility of this concept[26,28,29]. Additionally, according to a recent study, tumor-infiltrating lymphocytes were associated with the prognosis of patients with gastric cancer[30]. CNN model may detect tumor-infiltrating lymphocytes on histopathological specimens with an acceptable accuracy of 96.9%[30]. The development of DL models that incorporate clinical and multi-omics data is also a promising approach for predictive purposes[19]. Prognosis prediction by AI applications might be more accurate than that by the conventional pathological method; however, these AI-based predictions alone seem not to be accepted in clinical practice due to lack of interpretability. If doctors and patients cannot understand the reason for prediction, they will not recognize misprediction by AI. We cannot provide patients' care based on prediction as in "fortune-telling." Biological and clinical reasons for the prediction by AI application must be understood prior to its implementation into clinical practice.

Some researchers have also attempted to predict biomarker status from histopathological images alone using AI applications. Specimens of various GI cancers can be processed to identify molecular markers that may predict responses to targeted therapies. Research has shown that certain clinically relevant molecular alterations in GI cancers are associated with specific histopathological features detected on hematoxylin-eosin (HE) slides; there have been some successful attempts to adopt AI applications for HE sections as surrogate markers for these alterations[31-34].

Histopathological AI-applications in colorectal cancer

As in gastric cancer, various AI applications have recently been developed for colorectal cancer (Table 2). Regarding tumor classification, several AI algorithms have been trained to classify the dataset into two to six specific classes, such as normal, hyperplasia, adenoma, adenocarcinoma, and histological subtypes of polyps or

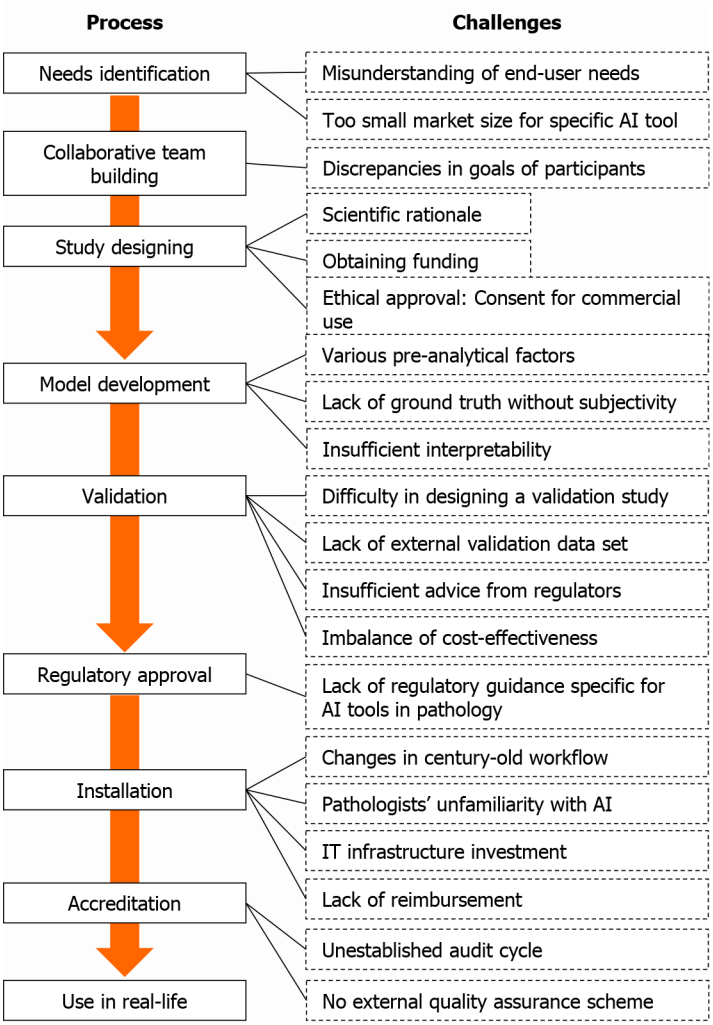


Figure 2 Challenges for implementation in the development process of an artificial intelligence application. The process of development and implementation of an artificial intelligence (AI) application is composed of multiple steps from needs identification to use in real-life (left). In each step, various challenges keep AI applications from being implemented into clinical practice (right). AI: Artificial intelligence; IT: Information technology.

adenocarcinomas[22,35-40]. Korbar *et al*[36] reported that the AI model, constructed using over 400 WSIs, could classify five types of colorectal polyps with an accuracy of 93%. Wei *et al*[41] demonstrated that the DL model, trained using WSIs, could classify colorectal polyps, even in datasets from the other hospitals, with reproducibility. Its accuracy was comparable to that of a local pathologist. While most researches exhibit promising performance, a precise comparison of performances among these AI applications is impossible and irrelevant; each model is derived from different datasets with different annotations and focuses on different tasks. To accurately compare the performance of AI models, it is necessary to have them perform a common task using a standardized dataset with standardized annotations.

Further, a few studies have predicted prognosis using pathological images for colorectal cancer[26,34,42]. Bychkov *et al*[42] used 420 tissue microarray-WSIs to predict the 5-year disease-specific survival of patients and obtained an AUC of 0.69. Kather *et al*[26] used more than 1000 histological images, collected from three institutions, to predict the prognosis of the patient; they observed accuracy of 99%. Another study, using the ResNet model for direct identification of microsatellite instability (MSI) on histological images, demonstrated an AUC of 0.77 for both FFPE and frozen specimens from The Cancer Genome Atlas (TCGA)[34]. The identification of colorectal cancer with MSI is crucial; these tumors are reportedly highly responsive to immunomodulating therapies[43,44]; moreover, the MSI could be a clue for the diagnosis of Lynch syndrome[45]. MSI is usually identified by polymerase chain reaction (PCR), but not all patients are screened for MSI in clinical practice. Echle *et al*[46] recently developed a DL model to detect colorectal cancer with MSI using more than 8800 images. The DL algorithm demonstrated an AUC of 0.96 in the multi-institutional validation cohort. Furthermore, the consensus molecular subtype of

colorectal cancer could be predicted from the images of colorectal surgical specimens using a CNN-based model[31]. Although prediction of molecular alterations by AI application might seem attractive, as clinically relevant biomarkers cannot be identified using HE stained slides and conventional PCR assay are both expensive and time-consuming, AI can neither achieve complete concordance with the gold standard test nor replace it. Thus, users must consider how to employ AI for predicting biomarkers with an appropriate, cost-effective balance in real-life practice.

A ROAD TO IMPLEMENTATION OF AI APPLICATIONS INTO REAL-LIFE PRACTICE

To achieve clinical implementation of the AI, several steps should be considered (Figure 2). Colling *et al*[47] presented an expected roadmap for the routine use of AI in pathology practice. They highlighted the main aspects of designing and applying AI in daily practice. The steps concerning design creation, ethics, financing, development, validation and regulation, implementation, and effect on the workforce were closely reviewed. For pathological image analysis, various problems exist in the execution of these steps, which would prevent the AI from being implemented in the clinical practice for GI cancers.

Identification of the true needs in daily practice

AI applications can either conduct routine tasks, usually performed by pathologists, or offer novel insights into diseases that are not possible by human pathologists[12]. The applications are needed to fill gaps and address unmet needs without impacting the daily workflow in the pathology department. The needs include mitosis detection, tumor-percentage calculation, lymph node metastasis, and other activities that are considered monotonous, repetitive, or vulnerable to higher interobserver variability.

The initial step in the development of the AI application is to recognize the true clinical need and define a possible solution. The novel AI applications can be developed by various stakeholders, including pathologists, physicians, computer scientists, engineers, IT companies, and drug companies. However, viewpoints between the professionals in academia and industry differ. For example, individuals in academia and businesses have different goals, such as grant funding, academic publications, and profitable commercial products.

Even if there is a problem that pathologists are eager to solve, the market size of the problem could be small. If the cost of developing an AI application to solve the problem cannot be recovered by the subsequent profit from the sale of the application, the company may not develop it. There is a wide range of classification tasks in diagnostic pathology, and it is difficult to secure an appropriate market for an AI application specializing only in a single task. For example, an AI algorithm can detect lymph node metastases in breast cancer as reliably as human pathologists[48,49]. Still, this tool has not been widely used or approved by the regulatory authorities. Although there could be many reasons, one is the imbalance between the overall cost of its implementation and the benefit of detecting only breast cancer lymph node metastases in real-life pathology practice.

Another significant concern is obtaining consent for the use of patient data in AI-model development[50]. Although the consent for research use could be obtained in most studies, patients might not consent to commercial use of their data required for product development, which could be an obstacle when developing products for clinical implementation. Therefore, consent should be obtained at the beginning of the research, conveying the possibility of its commercial use for product development; a framework for global data sharing should be developed.

For the development of AI algorithms, at least three parties need to collaborate, which include pathologists who know the true needs, academic professionals who can develop technology, and companies that will promote AI applications as products. In addition, to obtain a sufficiently sized market, it may be vital to develop global networks and online services using the cloud.

Development

After a concept of AI has been conceived and collaboratively established, the development of AI is carried out through the following steps: defining the output, designing the algorithm, collection of a pilot or larger follow-up sample, annotation and processing of data, and performing statistical analysis of the data.

High-quality data set curation is one of the major hurdles in the development of AI applications. Generally, CNNs require hundreds or thousands of data sets of pathological images to achieve significant performance and sufficient generalizability[51]. For rare tumors, researchers can obtain a very limited number of images; thus, it requires efficient data augmentation techniques and learning methods to resolve this issue. Conversely, in the case of transfer learning, small-scale datasets consisting of < 100 digital slides may suffice[52].

In addition, publicly available datasets should be developed for global data sharing. However, few such datasets are available in pathology, partly due to confidentiality, copyright, and financial problems[53]. Even under such circumstances, TCGA provides many WSIs and associated molecular data[54]. However, even TCGA data does not include sufficient numbers of cases for training AI applications for clinical implementation. Another potential source of datasets could be the public challenges provided for developing DL algorithms[55].

The development of AI applications with sufficient performance needs training on huge datasets demonstrating scanning[56] and staining protocol variability[56,57]. The major challenges for its implementation into practice are laboratory infrastructure and reproducibility and robustness of the AI model. Recently, automated methods for reducing blur in images have been developed. Automated algorithms (for example, HistoQC[58] and DeepFocus[59] can reportedly standardize the quality of WSIs; these AI applications automatically detects optimum quality regions and eliminates out-of-focus or artifact-related regions. Standardization of the color, displayed by histopathological slides, is important for the accuracy of AI; the color variations are often produced due to differences in batches or manufacturers of staining reagents, variations in the thickness of tissue sections, the difference in staining protocols, and disparity in scanning characteristics. These variations lead to inadequate classification by AI applications[56,60]. AI algorithms have been developed to standardize the data[61], including staining[62] and color characteristics[63].

After data set curation, the annotation of the dataset is required. Histopathological image annotation is not a simple task. The extent of annotation detail depends on the application of AI, which could vary from classification at the slide level to labeling at the pixel level. The annotation task, for many images, by human experts is time-consuming and tedious. In addition, variability in annotation performance, especially when the task is difficult, may affect the accuracy of the trained models. Moreover, for manufacturers, this task could be often expensive. Among GI pathologies, many lesions, such as intramucosal gastric carcinoma, do not have high interobserver reproducibility. When developing an AI application to assist pathologists in making a diagnosis, if the target disease shows significant interobserver variability, the correctness of the annotation of the dataset cannot be guaranteed, and the trained algorithm may not be able to reproduce performance in the dataset when used in other facilities, which may hinder its clinical implementation.

The problem of annotation in AI is an important research area. The majority of the AI models are trained using images of small tissue patches collected from WSIs. Since the patches, cropped from positive tissue, may not contain a tumor unless the tissue is filled with tumors, it is challenging to construct a high-accuracy model, particularly when pixel-level labeling is unavailable. To conduct patch-based training, without detailed annotation, multi-instance learning (MIL) algorithm can be used[64,65]. Cosatto *et al*[65] employed MIL for gastric cancer detection; they used over 12000 cases, 2/3rd for training and 1/3rd for the test, and achieved an AUC of 0.96. MIL is especially effective when there is a large dataset, and detailed annotations are impossible to obtain[51].

After the preparation of the annotated dataset, the model development process is usually composed of the following steps: preparation of the datasets for training, testing, and validation; selecting the ML framework, ML technique, and learning method. Once the learning process is completed, the output of the model is evaluated through performance metrics, and the hyperparameters are fine-tuned to improve performance. Considering the exponential increase in AI research for image analysis, this step does not seem to be a major obstacle to the implementation of AI in clinical practice.

Validation and regulation

As AI-based technologies grow increasingly, an evidence-based approach is required for their validation. Colling *et al*[47] presented summarized guidance by the current *in vitro* device regulation and their recommendations for the main components of validation. In laboratory medicine, apart from clinical evaluation, analytical validation should be considered[66]. The establishment of steps and criteria for the validation of

new tests against existing gold standards is essential. For image analysis validation, the technique is often compared with the "ground truth" (for example, comparing an AI-technology analyzing HER2 expression within the tumor to a detailed tumor assessment performed manually). It would be appropriate to compare the digital pathology technique with the performance of human pathologists. However, considering inter- and intra-observer variability in visual assessments of human pathologists, it is difficult to identify the ground truth; thus, it involves careful designing of the study and acceptance of the limitations of the present gold standard. Currently, most AI applications seem to have difficulty in establishing absolute ground truth. Therefore, the robustness and reproducibility of AI applications should be repeatedly validated in large and variable patient cohorts.

The relative lack of a validation cohort is an urgent issue in the development of AI-based applications. Histopathological slides, with detailed clinical data linked to them, cannot be often shared widely for reasons such as privacy protection. Annotations by pathologists, which are usually considered the "ground truth", are still controversial. Inter-observer variability and subjectivity in assessments by a pathologist indicate that a certain amount of uncertainty is inherent to ground truth. However, where the pathologist's assessment is the only available ground truth, it is important to enhance accuracy through validation as the next best measure. Efficient validation and testing require multicenter assessments involving multiple pathologists and datasets. If the AI application is intended to be used in real-life practice, it should be robust against pre-analytical variations within the target images, such as differences in staining conditions and WSI scanners, and its performance should be reproducible. With respect to this, a significant proportion of currently published AI research in GI cancers has not been externally validated.

Regulatory challenges

Appropriate regulations are required for the safe and effective use of AI in pathological practice. Unlike other laboratory tests, it is difficult to understand how predictions are made in AI applications; therefore, they are often viewed as black boxes. While various visualization techniques, including gradient saliency maps[67] and filter visualization methods, have been developed, it may not be possible for users to fully understand all the parameter changes causing erroneous performance or misprediction. Regulatory approval should be structured to minimize potential harm, define the risk-benefit balance, develop appropriate validation standards, and promote innovation[68].

Regulatory authorities, such as the FDA, the Centers for Medicare and Medicaid Services (CMS), and the European Union Conformité Européenne (EUCE) are not yet completely prepared for the implementation of AI applications in clinical medicine. As a result, AI-based devices are being controlled by prior and potentially obsolete guidelines for testing medical devices.

In the United States, the FDA is devising novel regulations for AI-based devices to make them safer and more effective[69]. CMS controls laboratory testing through the Clinical Laboratory Improvement Amendments (CLIA). CLIA stipulates that appropriate validation must be performed for all laboratory tests using human tissue before clinical implementation, regardless of their FDA approval. Currently, CLIA has no specific regulations for validating AI applications. The EUCE will replace the medical device directive in May 2021, and *in vitro* diagnostic medical device directives will be replaced by *in vitro* diagnostic regulation in May 2022[70]. Successful clinical implementation of AI-based applications will be assisted by the global market, and those clinically enforcing the applications will need to pay particular attention to the regulatory trends in their own country as well as in the US and EU. For AI applications to be approved by the FDA and EUCE, they should be established based on the updated details on FDA and EUCE regulations.

Implementation

Before implementing an AI application in real-life pathology practice, several obstacles must be addressed. Established business-use cases and a guarantee from pathologists for the use of the AI system should be accounted for before investing substantial time, energy, and funds on AI applications and required IT infrastructure.

The changes required for shifting daily workflow in the pathology department, from glass slides to WSIs, must be addressed. The department would require new digital pathology-related devices, a specific data management system, data storage facilities, and additional personnel to handle these changes. Simultaneously, an institutional IT infrastructure is required to enable users to operate through both on-site and cloud-based computing systems. Therefore, in the real-world, digital

pathology systems, requiring substantial investment, may hamper the implementation of these technologies[71]. Notably, augmented microscopy, connected directly to the cloud network service, might solve the issue of whole slide scanner installation. Chen and colleagues reported the augmented reality microscope, overlaying AI-based information onto the sample-view in real-time, may enable a seamless integration of AI into the routine workflow[72]. According to Hegde *et al*[73], the cloud-based AI application (SMILY, Similar image search for histopathology), developed by GOOGLE, irrespective of its annotation status, allows the search for morphologically similar features in a target image.

In addition, one must consider the relative inexperience of pathologists with AI-based technologies and acknowledge the range of issues the department would encounter prior to the implementation of AI. Second, a pathologist must buy-in to make significant improvements in a conventional century-old workflow. In view of the fact that progress does not happen immediately, the pathologist's management concerns should be dealt with separately from the technological hurdles. Initially, pathologists must commit to the installation of both digital pathology systems and AI applications to a pathology department. They have to understand the long-term risk-benefit balance of AI implementation. The present DL-based AI applications lack interpretability, which may contribute to patients' and clinicians' reluctance. Developing AI solutions that can be interpreted by end-users, thereby providing them with detailed descriptions of how their predictions are made, could be useful[74]. For lack of interpretability of DL model, various solutions, such as generating attention heat map[75], constructing interpretable model[76], creating external interpretive model[77], have been reported. However, this black box problem is not yet fully resolved.

On the downside, dependence on AI assistance for diagnoses can result in fewer opportunities for trainees to learn diagnostic skills. Although AI can be used as an auxiliary method to improve the quality and precision of clinical diagnoses, resident pathologists should be trained and encouraged to understand the utility, limitations, and pitfalls of AI application[78]. As molecular pathologists have become necessary, since the advent of genomic medicine, "computational pathologists"[47] will become necessary in the near future.

As with other clinical tests, ongoing post-marketing quality assurance is also essential for the safe and effective use of AI in clinical practice. Apart from laboratory testing processes, laboratory staff should understand the quality management system. As in conventional laboratory tests, a novel scheme of external quality assurance for AI applications in pathology should be urgently prepared for its implementation.

The use of AI applications in diagnostic practice poses complex new issues around the legal ramifications of signing a report prepared using AI by a pathologist. In order to incorporate their output into a pathological report, a pathologist should be confident in the performance of the algorithm; further, any algorithms used should be validated and regulated correctly. Although AI applications may not replace pathologists in view of this legal issue, they can be employed to support the pathologists in their clinical work. In particular, AI researchers are attempting to provide their predictions/results with confidence estimates and localize pathology-related features. This could help mitigate interpretability and confidence-building concerns.

CONCLUSION

The immense potential of AI in pathological practice can be harnessed by improving workflows, eliminating simple mistakes, increasing diagnostic reproducibility, and revealing predictions that are impossible with the use of conventional visual methods by human pathologists. The clinically implemented AI applications are expected to be user-friendly, explainable, robust, manageable, and cost-effective. Considering the current limited clinical awareness and uncertainty about how AI tools can be introduced into real-life practice, caution should be paid to their deployment. Eventually, AI applications may be implemented and used appropriately, provided they are supported by human pathologists, standardized usage recommendations, and harmonization of AI applications with present information systems.

AI can play a pivotal role in the practice of pathologists and the development of precision medicine for GI cancers. However, there are various barriers to its effective implementation. To overcome these barriers and implement AI at the practice level, it is necessary to work with a range of stakeholders, including pathologists, clinicians,

developers, regulators, and device vendors, to establish a strong network to grab true needs, expand the market, and use the application safely and efficiently.

REFERENCES

1 **Topol EJ**. High-performance medicine: the convergence of human and artificial intelligence. *Nat Med* 2019; **25**: 44-56 [PMID: 30617339 DOI: 10.1038/s41591-018-0300-7]

2 **Andras I**, Mazzone E, van Leeuwen FWB, De Naeyer G, van Oosterom MN, Beato S, Buckle T, O'Sullivan S, van Leeuwen PJ, Beulens A, Crisan N, D'Hondt F, Schatteman P, van Der Poel H, Dell'Oglio P, Mottrie A. Artificial intelligence and robotics: a combination that is changing the operating room. *World J Urol* 2020; **38**: 2359-2366 [PMID: 31776737 DOI: 10.1007/s00345-019-03037-6]

3 **Mukhopadhyay S**, Feldman MD, Abels E, Ashfaq R, Beltaifa S, Cacciabeve NG, Cathro HP, Cheng L, Cooper K, Dickey GE, Gill RM, Heaton RP Jr, Kerstens R, Lindberg GM, Malhotra RK, Mandell JW, Manlucu ED, Mills AM, Mills SE, Moskaluk CA, Nelis M, Patil DT, Przybycin CG, Reynolds JP, Rubin BP, Saboorian MH, Salicru M, Samols MA, Sturgis CD, Turner KO, Wick MR, Yoon JY, Zhao P, Taylor CR. Whole Slide Imaging Versus Microscopy for Primary Diagnosis in Surgical Pathology: A Multicenter Blinded Randomized Noninferiority Study of 1992 Cases (Pivotal Study). *Am J Surg Pathol* 2018; **42**: 39-52 [PMID: 28961557 DOI: 10.1097/PAS.0000000000000948]

4 **Niazi MKK**, Parwani AV, Gurcan MN. Digital pathology and artificial intelligence. *Lancet Oncol* 2019; **20**: e253-e261 [PMID: 31044723 DOI: 10.1016/S1470-2045(19)30154-8]

5 **Bera K**, Schalper KA, Rimm DL, Velcheti V, Madabhushi A. Artificial intelligence in digital pathology - new tools for diagnosis and precision oncology. *Nat Rev Clin Oncol* 2019; **16**: 703-715 [PMID: 31399699 DOI: 10.1038/s41571-019-0252-y]

6 **Rashidi HH**, Tran NK, Betts EV, Howell LP, Green R. Artificial Intelligence and Machine Learning in Pathology: The Present Landscape of Supervised Methods. *Acad Pathol* 2019; **6**: 2374289519873088 [PMID: 31523704 DOI: 10.1177/2374289519873088]

7 **LeCun Y**, Bengio Y, Hinton G. Deep learning. *Nature* 2015; **521**: 436-444 [PMID: 26017442 DOI: 10.1038/nature14539]

8 **Hinton GE**, Salakhutdinov RR. Reducing the dimensionality of data with neural networks. *Science* 2006; **313**: 504-507 [PMID: 16873662 DOI: 10.1126/science.1127647]

9 **Jain RK**, Mehta R, Dimitrov R, Larsson LG, Musto PM, Hodges KB, Ulbright TM, Hattab EM, Agaram N, Idrees MT, Badve S. Atypical ductal hyperplasia: interobserver and intraobserver variability. *Mod Pathol* 2011; **24**: 917-923 [PMID: 21532546 DOI: 10.1038/modpathol.2011.66]

10 **Elmore JG**, Longton GM, Carney PA, Geller BM, Onega T, Tosteson AN, Nelson HD, Pepe MS, Allison KH, Schnitt SJ, O'Malley FP, Weaver DL. Diagnostic concordance among pathologists interpreting breast biopsy specimens. *JAMA* 2015; **313**: 1122-1132 [PMID: 25781441 DOI: 10.1001/jama.2015.1405]

11 **Jiang Y**, Yang M, Wang S, Li X, Sun Y. Emerging role of deep learning-based artificial intelligence in tumor pathology. *Cancer Commun (Lond)* 2020; **40**: 154-166 [PMID: 32277744 DOI: 10.1002/cac2.12012]

12 **Calderaro J**, Kather JN. Artificial intelligence-based pathology for gastrointestinal and hepatobiliary cancers. *Gut* 2020 [PMID: 33214163 DOI: 10.1136/gutjnl-2020-322880]

13 **Niu PH**, Zhao LL, Wu HL, Zhao DB, Chen YT. Artificial intelligence in gastric cancer: Application and future perspectives. *World J Gastroenterol* 2020; **26**: 5408-5419 [PMID: 33024393 DOI: 10.3748/wjg.v26.i36.5408]

14 **Thakur N**, Yoon H, Chong Y. Current Trends of Artificial Intelligence for Colorectal Cancer Pathology Image Analysis: A Systematic Review. *Cancers (Basel)* 2020; **12** [PMID: 32668721 DOI: 10.3390/cancers12071884]

15 **Khan A**, Nawaz U, Ulhaq A, Robinson RW. Real-time plant health assessment *via* implementing cloud-based scalable transfer learning on AWS DeepLens. *PLoS One* 2020; **15**: e0243243 [PMID: 33332376 DOI: 10.1371/journal.pone.0243243]

16 **PathAI**. PathAI Present Machine Learning Models that Predict the Homologous Recombination Deficiency Status of Breast Cancer Biopsies at the 2020 SABCS. [cited 7 January 2021]. In: PathAI [Internet]. Available from: https://www.pathai.com/news/pathai-sabcs2020

17 **Pantanowitz L**, Sinard JH, Henricks WH, Fatheree LA, Carter AB, Contis L, Beckwith BA, Evans AJ, Lal A, Parwani AV; College of American Pathologists Pathology and Laboratory Quality Center. Validating whole slide imaging for diagnostic purposes in pathology: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch Pathol Lab Med* 2013; **137**: 1710-1722 [PMID: 23634907 DOI: 10.5858/arpa.2013-0093-CP]

18 **Cheng CL**, Azhar R, Sng SH, Chua YQ, Hwang JS, Chin JP, Seah WK, Loke JC, Ang RH, Tan PH. Enabling digital pathology in the diagnostic setting: navigating through the implementation journey in an academic medical centre. *J Clin Pathol* 2016; **69**: 784-792 [PMID: 26873939 DOI: 10.1136/jclinpath-2015-203600]

19 **Hamamoto R**, Suvarna K, Yamada M, Kobayashi K, Shinkai N, Miyake M, Takahashi M, Jinnai S, Shimoyama R, Sakai A, Takasawa K, Bolatkan A, Shozu K, Dozen A, Machino H, Takahashi S, Asada K, Komatsu M, Sese J, Kaneko S. Application of Artificial Intelligence Technology in

Oncology: Towards the Establishment of Precision Medicine. *Cancers (Basel)* 2020; **12** [PMID: 33256107 DOI: 10.3390/cancers12123532]

20 **de Groof AJ**, Struyvenberg MR, van der Putten J, van der Sommen F, Fockens KN, Curvers WL, Zinger S, Pouw RE, Coron E, Baldaque-Silva F, Pech O, Weusten B, Meining A, Neuhaus H, Bisschops R, Dent J, Schoon EJ, de With PH, Bergman JJ. Deep-Learning System Detects Neoplasia in Patients With Barrett's Esophagus With Higher Accuracy Than Endoscopists in a Multistep Training and Validation Study With Benchmarking. *Gastroenterology* 2020; **158**: 915-929. e4 [PMID: 31759929 DOI: 10.1053/j.gastro.2019.11.030]

21 **Sharma H**, Zerbe N, Klempert I, Hellwich O, Hufnagl P. Deep convolutional neural networks for automatic classification of gastric carcinoma using whole slide images in digital histopathology. *Comput Med Imaging Graph* 2017; **61**: 2-13 [PMID: 28676295 DOI: 10.1016/j.compmedimag.2017.06.001]

22 **Iizuka O**, Kanavati F, Kato K, Rambeau M, Arihiro K, Tsuneki M. Deep Learning Models for Histopathological Classification of Gastric and Colonic Epithelial Tumours. *Sci Rep* 2020; **10**: 1504 [PMID: 32001752 DOI: 10.1038/s41598-020-58467-9]

23 **Yoshida H**, Shimazu T, Kiyuna T, Marugame A, Yamashita Y, Cosatto E, Taniguchi H, Sekine S, Ochiai A. Automated histological classification of whole-slide images of gastric biopsy specimens. *Gastric Cancer* 2018; **21**: 249-257 [PMID: 28577229 DOI: 10.1007/s10120-017-0731-8]

24 **Tomita N**, Abdollahi B, Wei J, Ren B, Suriawinata A, Hassanpour S. Attention-Based Deep Neural Networks for Detection of Cancerous and Precancerous Esophagus Tissue on Histopathological Slides. *JAMA Netw Open* 2019; **2**: e1914645 [PMID: 31693124 DOI: 10.1001/jamanetworkopen.2019.14645]

25 **Courtiol P**, Maussion C, Moarii M, Pronier E, Pilcer S, Sefta M, Manceron P, Toldo S, Zaslavskiy M, Le Stang N, Girard N, Elemento O, Nicholson AG, Blay JY, Galateau-Sallé F, Wainrib G, Clozel T. Deep learning-based classification of mesothelioma improves prediction of patient outcome. *Nat Med* 2019; **25**: 1519-1525 [PMID: 31591589 DOI: 10.1038/s41591-019-0583-3]

26 **Kather JN**, Krisam J, Charoentong P, Luedde T, Herpel E, Weis CA, Gaiser T, Marx A, Valous NA, Ferber D, Jansen L, Reyes-Aldasoro CC, Zörnig I, Jäger D, Brenner H, Chang-Claude J, Hoffmeister M, Halama N. Predicting survival from colorectal cancer histology slides using deep learning: A retrospective multicenter study. *PLoS Med* 2019; **16**: e1002730 [PMID: 30677016 DOI: 10.1371/journal.pmed.1002730]

27 **Luo X**, Yin S, Yang L, Fujimoto J, Yang Y, Moran C, Kalhor N, Weissferdt A, Xie Y, Gazdar A, Minna J, Wistuba II, Mao Y, Xiao G. Development and Validation of a Pathology Image Analysis-based Predictive Model for Lung Adenocarcinoma Prognosis - A Multi-cohort Study. *Sci Rep* 2019; **9**: 6886 [PMID: 31053738 DOI: 10.1038/s41598-019-42845-z]

28 **Skrede OJ**, De Raedt S, Kleppe A, Hveem TS, Liestøl K, Maddison J, Askautrud HA, Pradhan M, Nesheim JA, Albregtsen F, Farstad IN, Domingo E, Church DN, Nesbakken A, Shepherd NA, Tomlinson I, Kerr R, Novelli M, Kerr DJ, Danielsen HE. Deep learning for prediction of colorectal cancer outcome: a discovery and validation study. *Lancet* 2020; **395**: 350-360 [PMID: 32007170 DOI: 10.1016/S0140-6736(19)32998-8]

29 **Geessink OGF**, Baidoshvili A, Klaase JM, Ehteshami Bejnordi B, Litjens GJS, van Pelt GW, Mesker WE, Nagtegaal ID, Ciompi F, van der Laak JAWM. Computer aided quantification of intratumoral stroma yields an independent prognosticator in rectal cancer. *Cell Oncol (Dordr)* 2019; **42**: 331-341 [PMID: 30825182 DOI: 10.1007/s13402-019-00429-z]

30 **García E**, Hermoza R, Beltran-Castanon C, Cano L, Castillo M, Castanneda C. Automatic Lymphocyte Detection on Gastric Cancer IHC Images Using Deep Learning. *IEEE* 2017; 200-204 [DOI: 10.1109/CBMS.2017.94]

31 **Sirinukunwattana K**, Domingo E, Richman SD, Redmond KL, Blake A, Verrill C, Leedham SJ, Chatzipli A, Hardy C, Whalley CM, Wu CH, Beggs AD, McDermott U, Dunne PD, Meade A, Walker SM, Murray GI, Samuel L, Seymour M, Tomlinson I, Quirke P, Maughan T, Rittscher J, Koelzer VH; S:CORT consortium. Image-based consensus molecular subtype (imCMS) classification of colorectal cancer using deep learning. *Gut* 2021; **70**: 544-554 [PMID: 32690604 DOI: 10.1136/gutjnl-2019-319866]

32 **Fu Y**, Jung AW, Torne RV, Gonzalez S, Vöhringer H, Shmatko A, Yates LR, Jimenez-Linan M, Moore L, Gerstung M. Pan-cancer computational histopathology reveals mutations, tumor composition and prognosis. *Nat Cancer* 2020; **1**: 800-810 [DOI: 10.1038/s43018-020-0085-8]

33 **Kather JN**, Heij LR, Grabsch HI, Loeffler C, Echle A, Muti HS, Krause J, Niehues JM, Sommer KAJ, Bankhead P, Kooreman LFS, Schulte JJ, Cipriani NA, Buelow RD, Boor P, Ortiz-Brüchle N, Hanby AM, Speirs V, Kochanny S, Patnaik A, Srisuwananukorn A, Brenner H, Hoffmeister M, van den Brandt PA, Jäger D, Trautwein C, Pearson AT, Luedde T. Pan-cancer image-based detection of clinically actionable genetic alterations. *Nat Cancer* 2020; **1**: 789-799 [DOI: 10.1038/s43018-020-0087-6]

34 **Kather JN**, Pearson AT, Halama N, Jäger D, Krause J, Loosen SH, Marx A, Boor P, Tacke F, Neumann UP, Grabsch HI, Yoshikawa T, Brenner H, Chang-Claude J, Hoffmeister M, Trautwein C, Luedde T. Deep learning can predict microsatellite instability directly from histology in gastrointestinal cancer. *Nat Med* 2019; **25**: 1054-1056 [PMID: 31160815 DOI: 10.1038/s41591-019-0462-y]

35 **Yoshida H**, Yamashita Y, Shimazu T, Cosatto E, Kiyuna T, Taniguchi H, Sekine S, Ochiai A. Automated histological classification of whole slide images of colorectal biopsy specimens.

Oncotarget 2017; **8**: 90719-90729 [PMID: 29207599 DOI: 10.18632/oncotarget.21819]
36 **Korbar B**, Olofson AM, Miraflor AP, Nicka CM, Suriawinata MA, Torresani L, Suriawinata AA, Hassanpour S. Deep Learning for Classification of Colorectal Polyps on Whole-slide Images. *J Pathol Inform* 2017; **8**: 30 [PMID: 28828201 DOI: 10.4103/jpi.jpi_34_17]
37 **Haj-Hassan H**, Chaddad A, Harkouss Y, Desrosiers C, Toews M, Tanougast C. Classifications of Multispectral Colorectal Cancer Tissues Using Convolution Neural Network. *J Pathol Inform* 2017; **8**: 1 [PMID: 28400990 DOI: 10.4103/jpi.jpi_47_16]
38 **Xu Y**, Jia Z, Wang LB, Ai Y, Zhang F, Lai M, Chang EI. Large scale tissue histopathology image classification, segmentation, and visualization *via* deep convolutional activation features. *BMC Bioinformatics* 2017; **18**: 281 [PMID: 28549410 DOI: 10.1186/s12859-017-1685-x]
39 **Yoon H**, Lee J, Oh JE, Kim HR, Lee S, Chang HJ, Sohn DK. Tumor Identification in Colorectal Histology Images Using a Convolutional Neural Network. *J Digit Imaging* 2019; **32**: 131-140 [PMID: 30066123 DOI: 10.1007/s10278-018-0112-9]
40 **Sena P**, Fioresi R, Faglioni F, Losi L, Faglioni G, Roncucci L. Deep learning techniques for detecting preneoplastic and neoplastic lesions in human colorectal histological images. *Oncol Lett* 2019; **18**: 6101-6107 [PMID: 31788084 DOI: 10.3892/ol.2019.10928]
41 **Wei JW**, Suriawinata AA, Vaickus LJ, Ren B, Liu X, Lisovsky M, Tomita N, Abdollahi B, Kim AS, Snover DC, Baron JA, Barry EL, Hassanpour S. Evaluation of a Deep Neural Network for Automated Classification of Colorectal Polyps on Histopathologic Slides. *JAMA Netw Open* 2020; **3**: e203398 [PMID: 32324237 DOI: 10.1001/jamanetworkopen.2020.3398]
42 **Bychkov D**, Linder N, Turkki R, Nordling S, Kovanen PE, Verrill C, Walliander M, Lundin M, Haglund C, Lundin J. Deep learning based tissue analysis predicts outcome in colorectal cancer. *Sci Rep* 2018; **8**: 3395 [PMID: 29467373 DOI: 10.1038/s41598-018-21758-3]
43 **Mandal R**, Samstein RM, Lee KW, Havel JJ, Wang H, Krishna C, Sabio EY, Makarov V, Kuo F, Blecua P, Ramaswamy AT, Durham JN, Bartlett B, Ma X, Srivastava R, Middha S, Zehir A, Hechtman JF, Morris LG, Weinhold N, Riaz N, Le DT, Diaz LA Jr, Chan TA. Genetic diversity of tumors with mismatch repair deficiency influences anti-PD-1 immunotherapy response. *Science* 2019; **364**: 485-491 [PMID: 31048490 DOI: 10.1126/science.aau0447]
44 **Le DT**, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Duffy SM, Goldberg RM, de la Chapelle A, Koshiji M, Bhaijee F, Huebner T, Hruban RH, Wood LD, Cuka N, Pardoll DM, Papadopoulos N, Kinzler KW, Zhou S, Cornish TC, Taube JM, Anders RA, Eshleman JR, Vogelstein B, Diaz LA Jr. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* 2015; **372**: 2509-2520 [PMID: 26028255 DOI: 10.1056/NEJMoa1500596]
45 **Lynch HT**, Snyder CL, Shaw TG, Heinen CD, Hitchins MP. Milestones of Lynch syndrome: 1895-2015. *Nat Rev Cancer* 2015; **15**: 181-194 [PMID: 25673086 DOI: 10.1038/nrc3878]
46 **Echle A**, Grabsch HI, Quirke P, van den Brandt PA, West NP, Hutchins GGA, Heij LR, Tan X, Richman SD, Krause J, Alwers E, Jenniskens J, Offermans K, Gray R, Brenner H, Chang-Claude J, Trautwein C, Pearson AT, Boor P, Luedde T, Gaisa NT, Hoffmeister M, Kather JN. Clinical-Grade Detection of Microsatellite Instability in Colorectal Tumors by Deep Learning. *Gastroenterology* 2020; **159**: 1406-1416. e11 [PMID: 32562722 DOI: 10.1053/j.gastro.2020.06.021]
47 **Colling R**, Pitman H, Oien K, Rajpoot N, Macklin P; CM-Path AI in Histopathology Working Group, Snead D, Sackville T, Verrill C. Artificial intelligence in digital pathology: a roadmap to routine use in clinical practice. *J Pathol* 2019; **249**: 143-150 [PMID: 31144302 DOI: 10.1002/path.5310]
48 **Steiner DF**, MacDonald R, Liu Y, Truszkowski P, Hipp JD, Gammage C, Thng F, Peng L, Stumpe MC. Impact of Deep Learning Assistance on the Histopathologic Review of Lymph Nodes for Metastatic Breast Cancer. *Am J Surg Pathol* 2018; **42**: 1636-1646 [PMID: 30312179 DOI: 10.1097/PAS.0000000000001151]
49 **Ehteshami Bejnordi B**, Veta M, Johannes van Diest P, van Ginneken B, Karssemeijer N, Litjens G, van der Laak JAWM; the CAMELYON16 Consortium, Hermsen M, Manson QF, Balkenhol M, Geessink O, Stathonikos N, van Dijk MC, Bult P, Beca F, Beck AH, Wang D, Khosla A, Gargeya R, Irshad H, Zhong A, Dou Q, Li Q, Chen H, Lin HJ, Heng PA, Haß C, Bruni E, Wong Q, Halici U, Öner MÜ, Cetin-Atalay R, Berseth M, Khvatkov V, Vylegzhanin A, Kraus O, Shaban M, Rajpoot N, Awan R, Sirinukunwattana K, Qaiser T, Tsang YW, Tellez D, Annuscheit J, Hufnagl P, Valkonen M, Kartasalo K, Latonen L, Ruusuvuori P, Liimatainen K, Albarqouni S, Mungal B, George A, Demirci S, Navab N, Watanabe S, Seno S, Takenaka Y, Matsuda H, Ahmady Phoulady H, Kovalev V, Kalinovsky A, Liauchuk V, Bueno G, Fernandez-Carrobles MM, Serrano I, Deniz O, Racoceanu D, Venâncio R. Diagnostic Assessment of Deep Learning Algorithms for Detection of Lymph Node Metastases in Women With Breast Cancer. *JAMA* 2017; **318**: 2199-2210 [PMID: 29234806 DOI: 10.1001/jama.2017.14585]
50 **Kotsenas AL**, Balthazar P, Andrews D, Geis JR, Cook TS. Rethinking Patient Consent in the Era of Artificial Intelligence and Big Data. *J Am Coll Radiol* 2021; **18**: 180-184 [PMID: 33413897 DOI: 10.1016/j.jacr.2020.09.022]
51 **Campanella G**, Hanna MG, Geneslaw L, Miraflor A, Werneck Krauss Silva V, Busam KJ, Brogi E, Reuter VE, Klimstra DS, Fuchs TJ. Clinical-grade computational pathology using weakly supervised deep learning on whole slide images. *Nat Med* 2019; **25**: 1301-1309 [PMID: 31308507 DOI: 10.1038/s41591-019-0508-1]
52 **Jones AD**, Graff JP, Darrow M, Borowsky A, Olson KA, Gandour-Edwards R, Datta Mitra A, Wei

D, Gao G, Durbin-Johnson B, Rashidi HH. Impact of pre-analytical variables on deep learning accuracy in histopathology. *Histopathology* 2019; **75**: 39-53 [PMID: 30801768 DOI: 10.1111/his.13844]

53 **Hipp JD**, Sica J, McKenna B, Monaco J, Madabhushi A, Cheng J, Balis UJ. The need for the pathology community to sponsor a whole slide imaging repository with technical guidance from the pathology informatics community. *J Pathol Inform* 2011; **2**: 31 [PMID: 21845229 DOI: 10.4103/2153-3539.83191]

54 **Cooper LA**, Demicco EG, Saltz JH, Powell RT, Rao A, Lazar AJ. PanCancer insights from The Cancer Genome Atlas: the pathologist's perspective. *J Pathol* 2018; **244**: 512-524 [PMID: 29288495 DOI: 10.1002/path.5028]

55 **Hartman DJ**, Van Der Laak JAWM, Gurcan MN, Pantanowitz L. Value of Public Challenges for the Development of Pathology Deep Learning Algorithms. *J Pathol Inform* 2020; **11**: 7 [PMID: 32318315 DOI: 10.4103/jpi.jpi_64_19]

56 **Yoshida H**, Yokota H, Singh R, Kiyuna T, Yamaguchi M, Kikuchi S, Yagi Y, Ochiai A. Meeting Report: The International Workshop on Harmonization and Standardization of Digital Pathology Image, Held on April 4, 2019 in Tokyo. *Pathobiology* 2019; **86**: 322-324 [PMID: 31707388 DOI: 10.1159/000502718]

57 **Inoue T**, Yagi Y. Color standardization and optimization in whole slide imaging. *Clin Diagn Pathol* 2020; **4** [PMID: 33088926 DOI: 10.15761/cdp.1000139]

58 **Janowczyk A**, Zuo R, Gilmore H, Feldman M, Madabhushi A. HistoQC: An Open-Source Quality Control Tool for Digital Pathology Slides. *JCO Clin Cancer Inform* 2019; **3**: 1-7 [PMID: 30990737 DOI: 10.1200/CCI.18.00157]

59 **Senaras C**, Niazi MKK, Lozanski G, Gurcan MN. DeepFocus: Detection of out-of-focus regions in whole slide digital images using deep learning. *PLoS One* 2018; **13**: e0205387 [PMID: 30359393 DOI: 10.1371/journal.pone.0205387]

60 **Komura D**, Ishikawa S. Machine Learning Methods for Histopathological Image Analysis. *Comput Struct Biotechnol J* 2018; **16**: 34-42 [PMID: 30275936 DOI: 10.1016/j.csbj.2018.01.001]

61 **Yagi Y**, Gilbertson JR. Digital imaging in pathology: the case for standardization. *J Telemed Telecare* 2005; **11**: 109-116 [PMID: 15901437 DOI: 10.1258/1357633053688705]

62 **Janowczyk A**, Basavanhally A, Madabhushi A. Stain Normalization using Sparse AutoEncoders (StaNoSA): Application to digital pathology. *Comput Med Imaging Graph* 2017; **57**: 50-61 [PMID: 27373749 DOI: 10.1016/j.compmedimag.2016.05.003]

63 **Vahadane A**, Peng T, Sethi A, Albarqouni S, Wang L, Baust M, Steiger K, Schlitter AM, Esposito I, Navab N. Structure-Preserving Color Normalization and Sparse Stain Separation for Histological Images. *IEEE Trans Med Imaging* 2016; **35**: 1962-1971 [PMID: 27164577 DOI: 10.1109/TMI.2016.2529665]

64 **Dietterich TG**, Lathrop RH, Lozano-Pérez T. Solving the multiple instance problem with axis-parallel rectangles. *Artificial Intelligence* 1997; **89**: 31-71 [DOI: 10.1016/S0004-3702(96)00034-3]

65 **Cosatto E**, Laquerre PF, Malon C, Graf HP, Saito A, Kiyuna T, Marugame A, Kamijo K. Automated gastric cancer diagnosis on H and E-stained sections; training a classifier on a large scale with multiple instance machine learning. Proceedings of SPIE - Progress in Biomedical Optics and Imaging, MI: 2013

66 **Mattocks CJ**, Morris MA, Matthijs G, Swinnen E, Corveleyn A, Dequeker E, Müller CR, Pratt V, Wallace A; EuroGentest Validation Group. A standardized framework for the validation and verification of clinical molecular genetic tests. *Eur J Hum Genet* 2010; **18**: 1276-1288 [PMID: 20664632 DOI: 10.1038/ejhg.2010.101]

67 **Pasa F**, Golkov V, Pfeiffer F, Cremers D, Pfeiffer D. Efficient Deep Network Architectures for Fast Chest X-Ray Tuberculosis Screening and Visualization. *Sci Rep* 2019; **9**: 6268 [PMID: 31000728 DOI: 10.1038/s41598-019-42557-4]

68 **Allen TC**. Regulating Artificial Intelligence for a Successful Pathology Future. *Arch Pathol Lab Med* 2019; **143**: 1175-1179 [PMID: 31268373 DOI: 10.5858/arpa.2019-0229-ED]

69 **U.S. Food and Drug Administration**. Proposed Regulatory Framework for Modifications to Artificial Intelligence/Machine Learning (AI/ML)-Based Software as a Medical Device (SaMD). [cited 7 January 2021]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/media/122535/download

70 **European Commission**. Medical Devices – Sector. [cited 7 January 2021]. In: European Commission [Internet]. Available from: https://ec.europa.eu/growth/sectors/medical-devices_en

71 **Retamero JA**, Aneiros-Fernandez J, Del Moral RG. Complete Digital Pathology for Routine Histopathology Diagnosis in a Multicenter Hospital Network. *Arch Pathol Lab Med* 2020; **144**: 221-228 [PMID: 31295015 DOI: 10.5858/arpa.2018-0541-OA]

72 **Chen PC**, Gadepalli K, MacDonald R, Liu Y, Kadowaki S, Nagpal K, Kohlberger T, Dean J, Corrado GS, Hipp JD, Mermel CH, Stumpe MC. An augmented reality microscope with real-time artificial intelligence integration for cancer diagnosis. *Nat Med* 2019; **25**: 1453-1457 [PMID: 31406351 DOI: 10.1038/s41591-019-0539-7]

73 **Hegde N**, Hipp JD, Liu Y, Emmert-Buck M, Reif E, Smilkov D, Terry M, Cai CJ, Amin MB, Mermel CH, Nelson PQ, Peng LH, Corrado GS, Stumpe MC. Similar image search for histopathology: SMILY. *NPJ Digit Med* 2019; **2**: 56 [PMID: 31304402 DOI: 10.1038/s41746-019-0131-z]

74 **Tosun AB**, Pullara F, Becich MJ, Taylor DL, Fine JL, Chennubhotla SC. Explainable AI (xAI) for Anatomic Pathology. *Adv Anat Pathol* 2020; **27**: 241-250 [PMID: 32541594 DOI:

10.1097/PAP.0000000000000264]
75 **Montavon G**, Samek W, Müller K-R. Methods for interpreting and understanding deep neural networks. *Digit Signal Process* 2018; **73**: 1-15 [DOI: 10.1016/j.dsp.2017.10.011]
76 **Yang JH**, Wright SN, Hamblin M, McCloskey D, Alcantar MA, Schrübbers L, Lopatkin AJ, Satish S, Nili A, Palsson BO, Walker GC, Collins JJ. A White-Box Machine Learning Approach for Revealing Antibiotic Mechanisms of Action. *Cell* 2019; **177**: 1649-1661. e9 [PMID: 31080069 DOI: 10.1016/j.cell.2019.04.016]
77 **Kuhn DR**, Kacker RN, Lei Y, Simos DE. Combinatorial Methods for Explainable AI. Proceedings of the 2020 IEEE International Conference on Software Testing, Verification and Validation Workshops (ICSTW); 2020 Oct 24-28. IEEE, 2020: 167-170
78 **Arora A**, Arora A. Pathology training in the age of artificial intelligence. *J Clin Pathol* 2021; **74**: 73-75 [PMID: 33020175 DOI: 10.1136/jclinpath-2020-207110]
79 **Bollschweiler EH**, Mönig SP, Hensler K, Baldus SE, Maruyama K, Hölscher AH. Artificial neural network for prediction of lymph node metastases in gastric cancer: a phase II diagnostic study. *Ann Surg Oncol* 2004; **11**: 506-511 [PMID: 15123460 DOI: 10.1245/ASO.2004.04.018]
80 **Duraipandian S**, Sylvest Bergholt M, Zheng W, Yu Ho K, Teh M, Guan Yeoh K, Bok Yan So J, Shabbir A, Huang Z. Real-time Raman spectroscopy for in vivo, online gastric cancer diagnosis during clinical endoscopic examination. *J Biomed Opt* 2012; **17**: 081418 [PMID: 23224179 DOI: 10.1117/1.JBO.17.8.081418]
81 **Jiang Y**, Xie J, Han Z, Liu W, Xi S, Huang L, Huang W, Lin T, Zhao L, Hu Y, Yu J, Zhang Q, Li T, Cai S, Li G. Immunomarker Support Vector Machine Classifier for Prediction of Gastric Cancer Survival and Adjuvant Chemotherapeutic Benefit. *Clin Cancer Res* 2018; **24**: 5574-5584 [PMID: 30042208 DOI: 10.1158/1078-0432.CCR-18-0848]
82 **Qu J**, Hiruta N, Terai K, Nosato H, Murakawa M, Sakanashi H. Gastric Pathology Image Classification Using Stepwise Fine-Tuning for Deep Neural Networks. *J Healthc Eng* 2018; **2018**: 8961781 [PMID: 30034677 DOI: 10.1155/2018/8961781]
83 **León F**, Gélvez M, Jaimes Z, Gelvez T, Arguello H. Supervised Classification of Histopathological Images Using Convolutional Neuronal Networks for Gastric Cancer Detection. 2019 XXII Symposium on Image, Signal Processing and Artificial Vision (STSIVA). IEEE, 2019: 1-5
84 **Liang Q**, Nan Y, Coppola G, Zou K, Sun W, Zhang D, Wang Y, Yu G. Weakly Supervised Biomedical Image Segmentation by Reiterative Learning. *IEEE J Biomed Health Inform* 2019; **23**: 1205-1214 [PMID: 29994489 DOI: 10.1109/JBHI.2018.2850040]
85 **Sun M**, Zhang G, Dang H, Qi X, Zhou X, Chang Q. Accurate Gastric Cancer Segmentation in Digital Pathology Images Using Deformable Convolution and Multi-Scale Embedding Networks. *IEEE Access* 2019; **7**: 75530-75541 [DOI: 10.1109/ACCESS.2019.2918800]
86 **Wang S**, Zhu Y, Yu L, Chen H, Lin H, Wan X, Fan X, Heng PA. RMDL: Recalibrated multi-instance deep learning for whole slide gastric image classification. *Med Image Anal* 2019; **58**: 101549 [PMID: 31499320 DOI: 10.1016/j.media.2019.101549]
87 **Awan R**, Sirinukunwattana K, Epstein D, Jefferyes S, Qidwai U, Aftab Z, Mujeeb I, Snead D, Rajpoot N. Glandular Morphometrics for Objective Grading of Colorectal Adenocarcinoma Histology Images. *Sci Rep* 2017; **7**: 16852 [PMID: 29203775 DOI: 10.1038/s41598-017-16516-w]
88 **Kainz P**, Pfeiffer M, Urschler M. Segmentation and classification of colon glands with deep convolutional neural networks and total variation regularization. *PeerJ* 2017; **5**: e3874 [PMID: 29018612 DOI: 10.7717/peerj.3874]
89 **Alom M**, Yakopcic C, Taha T, Asari V. Microscopic Nuclei Classification, Segmentation and Detection with improved Deep Convolutional Neural Network (DCNN) Approaches. 2018 Preprint. Available from: arXiv:1811.03447
90 **Weis CA**, Kather JN, Melchers S, Al-Ahmdi H, Pollheimer MJ, Langner C, Gaiser T. Automatic evaluation of tumor budding in immunohistochemically stained colorectal carcinomas and correlation to clinical outcome. *Diagn Pathol* 2018; **13**: 64 [PMID: 30153844 DOI: 10.1186/s13000-018-0739-3]
91 **Ponzio F**, Macii E, Ficarra E, Di Cataldo S. Colorectal Cancer Classification using Deep Convolutional Networks - An Experimental Study. Proceedings of the 11th International Joint Conference on Biomedical Engineering Systems and Technologies - Volume 2. Bioimaging, 2018: 58-66
92 **Shapcott M**, Hewitt KJ, Rajpoot N. Deep Learning With Sampling in Colon Cancer Histology. *Front Bioeng Biotechnol* 2019; **7**: 52 [PMID: 30972333 DOI: 10.3389/fbioe.2019.00052]
93 **Swiderska-Chadaj Z**, Pinckaers H, van Rijthoven M, Balkenhol M, Melnikova M, Geessink O, Manson Q, Sherman M, Polonia A, Parry J, Abubakar M, Litjens G, van der Laak J, Ciompi F. Learning to detect lymphocytes in immunohistochemistry with deep learning. *Med Image Anal* 2019; **58**: 101547 [PMID: 31476576 DOI: 10.1016/j.media.2019.101547]

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ORIGINAL ARTICLE

Basic Study

Fecal microbiota transplantation ameliorates experimental colitis *via* gut microbiota and T-cell modulation

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Abstract

BACKGROUND

Emerging evidence has demonstrated that fecal microbiota transplantation (FMT) has a promising therapeutic effect on mice with experimental colitis and patients with ulcerative colitis (UC), although the mechanism of FMT is unclear.

AIM

To evaluate the protective effect of FMT on UC and clarify its potential dependence on the gut microbiota, through association analysis of gut microbiota with colon transcriptome in mice.

METHODS

Dextran sodium sulfate (DSS)-induced experimental colitis was established and fecal microbiota was transplanted by gavage. Severity of colon inflammation was measured by body weight, disease activity index, colon length and histological score. Gut microbiota alteration was analyzed through 16S ribosomal ribonucleic acid sequencing. The differentially expressed genes (DEGs) in the colon were obtained by transcriptome sequencing. The activation status of colonic T lymphocytes in the lamina propria was evaluated by flow cytometry.

RESULTS

Compared with the DSS group, the weight loss, colon length shortening and inflammation were significantly alleviated in the FMT group. The scores of disease activity index and colon histology decreased obviously after FMT. FMT restored the balance of gut microbiota, especially by upregulating the relative abundance of *Lactobacillus* and downregulating the relative abundance of *Clostridium_sensu_stricto_1* and *Turicibacter*. In the transcriptomic analysis, 128 DEGs intersected after DSS treatment and FMT. Functional annotation analysis suggested that these DEGs were mainly involved in T-lymphocyte activation. In

ethics committee of the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University (Approval No: DW-P-2018-008-01).

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the DSS group, there was an increase in colonic T helper $CD4^+$ and T cytotoxic $CD8^+$ cells by flow cytometry. FMT selectively downregulated the ratio of colonic $CD4^+$ and $CD8^+$ T cells to maintain intestinal homeostasis. Furthermore, *Clostridium_sensu_stricto_1* was significantly related to inflammation-related genes including *REG3G*, *CCL8* and *IDO1*.

CONCLUSION

FMT ameliorated DSS-induced colitis in mice *via* regulating the gut microbiota and T-cell modulation.

Key Words: Fecal microbiota transplantation; Colitis; Gut microbiota; Transcriptome sequencing; T lymphocyte

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Core Tip: Previous studies shown that inflammatory bowel disease patients received satisfactory efficacy and safety after fecal microbiota transplantation (FMT) treatment. However, the mechanism of FMT remains unclear. Here, we set out animal experiments to explore the role of FMT in dextran sodium sulfate induced colitis in mice based on microbiome and transcriptome analysis.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a disease of global concern with a growing prevalence in young adults. The current treatments for IBD are not completely satisfactory. Modulating microbiota to correct dysbiosis is a new approach to treating IBD. Fecal microbiota transplantation (FMT) refers to infusion of a fecal suspension prepared from a healthy donor into the gut of a patient to restore a healthy composition and function of microbiota[1].

FMT has shown a valid therapeutic benefit after the onset of refractory recurrent *Clostridium difficile* infection by reintroducing a balanced microbiota to restore the gut microbial community. FMT has emerged as a highly effective treatment for refractory *Clostridium difficile* infection, achieving a cure rate of over 90%[2]. Reconstructing the gut microbiota through FMT also has benefits for patients with IBD[3,4]. The diversity of microbiota with FMT treatment increases to varying degrees among relieved patients. IBD patients show a decrease in overall biodiversity of the microbial ecosystem characterized by the loss of beneficial commensal microflora and the expansion of pathogenic bacteria[5]. In our previous studies and others, IBD patients received satisfactory efficacy and safety after FMT treatment[6-8]. However, the mechanism of FMT in the treatment of IBD is still unknown. In addition to reconstructing the gut microbiota, another role is that FMT activates a variety of anti-inflammatory mechanisms targeting mucosal immune cells[9]. Nevertheless, we only have a partial understanding of how FMT functionally modulates the intestinal immune system at present.

This study used wild-type mice induced by dextran sodium sulfate (DSS) as a model of colitis in order to identify the role of gut microbiota in mediating the effect of FMT on DSS-induced colitis. We evaluated the protective effect of FMT on mice with DSS-induced colitis and identified the related gut microbiota and intestinal mucosal immune signaling pathway. Our findings will provide a basis for explaining the mechanism of FMT in the treatment of IBD.

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MATERIALS AND METHODS

Animals

C57BL/10 mice (female; 7 wk to 8 wk of age; weighing 18-20 g; specific pathogen free grade) were purchased from the Model Animal Research Center of Nanjing University. All mice were reared in the experimental animal center of the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University in the specific pathogen free condition. Throughout the acclimatization and study periods, all animals were maintained on a 12 h light-dark cycle (21 ± 2 °C with a relatively constant humidity of 45% ± 10%) and had access to food and water *ad libitum*. Randomization was used to assign animals to the experimental groups. The animal experimental protocol was approved by experimental animal ethics committee of the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University, No: DW-P-2018-008-01.

DSS-induced colitis

To induce acute experimental colitis, mice were administered 2.5% (w/v) DSS (molecular weight, 36-50 kDa; MP Biomedicals, LLC, Irvine, CA, United States) in their drinking water and replaced it with normal water after 7 d. The mice in FMT group were fed with fecal microbiota from healthy mice from the 8th d, once every 2 d until the end of the experiment. The mice in the DSS group were fed normal saline as negative control at the same time. During intragastric administration, the procedures were strictly performed under sterile conditions. Animals were examined daily for change in net body weight and the disease activity index (DAI). The subsequent course of colitis development was evaluated daily by scoring *via* the DAI. DAI is a common index used to assess the severity of colitis combined with body weight loss, stool consistency and the presence of blood in the stool. The DAI score was calculated by grading on a 0-4 scale based on the following parameters: Change in bodyweight (1, 1%-5%; 2, 5%-10%; 3, 10%-15%; 4, ≥ 15%), stool consistency (0, normal; 2, loose stools; 4, diarrhea), and stool blood (0, no blood seen; 2, obvious blood with stool; 4, grossly bloody stool). At the end of the experiment, the mice were euthanized. The colon tissue was dissected; colons were measured for colon length, and tissues were examined for gross macroscopic appearance and stool consistency. Then, the colon was divided into different parts for different analysis and then fixed in 10% formalin for histological analysis or in ribonucleic acid (RNA) protective solution (Beyotime, Beijing, China) for transcriptomics analysis of colonic mucosa.

Feces preparation and transplantation

Feces were collected directly from healthy donor mice of the same strain. Fresh feces from 5 mice were pooled, mixed with sterile normal saline according to the concentration of 0.125 g/mL and homogenized immediately. The homogenate was centrifuged at 4 °C for 5 min at 1200 rpm, and the fecal sediment was used for transplantation. Gavage was carried out with a curved gavage needle appropriate for animal size. The volume of intragastric administration was 0.1 mL/10 g to each mouse, with care taken to avoid regurgitation. The two experimental groups stopped eating after 10:00 pm before transplantation, and FMT was always performed at 8:00 am.

Histopathology

The distal colon segments were placed in 10% neutral buffered formalin for 24 h, embedded in paraffin and cut into sections of 4 μm in thickness. The sections were then stained with hematoxylin and eosin (HE). HE-stained sections were examined for inflammation and tissue damage. Slices were evaluated by the experienced pathologist in a blinded manner, and histological score was assessed based on the following parameters according to previous research: Inflammation extent (0, none; 1, mild; 2, moderate; 3, severe), crypt aberrant (0, normal; 1, basal 1/3 damage; 2, basal 2/3 damage; 3, crypt lost and surface epithelium present; 4, crypt and surface epithelium lost), lymphocyte infiltration (0, 0%; 1, 10%; 2, 10%-25%; 3, 25%-50%; 4, > 50%) and colon wall aberrant (0, none; 1, mucosa; 2, submucosa; 3, transmural). The total histological score was the sum of all of the parameters evaluated[10].

Lamina propria mononuclear cells Isolation

To isolate lamina propria mononuclear cells, Peyer's Patches were firstly removed and then colonic tissue was incubated with 5 mmol/L ethylenediamine tetraacetic acid (Biosharp, Sakai, Japan) and 1 mmol/L DTT (Biofroxx, Einhausen, Germany) at 37 °C for 30 min, followed by digestion with collagenase IV (0.3 mg/mL, Sigma, St. Louis, MO, United States), DNase I (0.25 mg/mL, Biosharp) and Dispase II (3 mg/mL,

Shanghai YuanYe Biotechnology, Shanghai, China) (37 °C for 1 h). Colonic lamina propria lymphocytes were then separated with a Percoll gradient.

Flow cytometry analysis

For immunephenotyping, mononuclear cells isolated were stained with surface markers of APC-labeled anti-mouse CD4 (Biolegend, Cat. No. 100516, San Diego, CA, United States), FITC-labeled anti-mouse CD8a (Biolegend, Cat. No. 100706) for 30 min at 4 °C in the dark. Samples were passed on a FACSCanto II flow cytometer (BD). Data were analyzed using FlowJo v10 software (FlowJo, LLC, Ashland, OR, United States).

Fecal DNA extraction and 16S rRNA gene sequencing

Microbial community genomic deoxyribonucleic acid (DNA) was extracted from samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, United States) according to manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, United States). The hypervariable region V3-V4 of the bacterial 16S ribosomal RNA (rRNA) gene were amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (Applied Biosystems, Foster City, CA, United States). The polymerase chain reaction (PCR) amplification of 16S rRNA gene was performed as follows: Initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, single extension at 72 °C for 10 min, and end at 4 °C. The PCR mixtures contain 5 × TransStart FastPfu buffer 4 μL, 2.5 mmol/L dNTPs 2 μL, forward primer (5 μmol/L) 0.8 μL, reverse primer (5 μmol/L) 0.8 μL, TransStart FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng and finally ddH_2O up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, Madison, WI, United States). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, United States). The predominance of bacterial communities between groups were analyzed by linear discriminant analysis effect size method.

Transcriptome analysis

Total RNA from inflammatory colonic tissue was extracted. Samples about 1 cm long were for sequencing. The tissues from minimum and maximum histological score were removed. Then, we collected colon samples from four randomly chosen animals per group for sequencing, totaling 12 samples. A total amount of 2 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, United States) following manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Samples were then sequenced on an Illumina Hiseq 4000 platform, and paired-end 150 bp reads were generated. Next, we used Star and Cufflinks software to complete alignment and analyze transcripts and to make quantitative analysis of all genes. Differential genes were identified, and functional enrichment analysis of these differential genes was carried out to explore their functions. The primers were: Adapter, oligonucleotide sequences for TruSeq™ RNA and DNA Sample Prep Kits. RNA 5'Adapter (RA5), part # 15013205: 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG ACGCTCTTCCGATCT-3'; RNA3'Adapter (RA3), part # 15013207: 5'-GATCGGAA-GAGCACACGTCTGAACTCCAGTCAC (index) ATCTCGTATGCCGTCTTCTG CTTG-3'. Gene ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GO seq R packages based Wallenius non-central hyper-geometric distribution (Young *et al*[11]), which can adjust for gene length bias in DEGs. Differential expression analysis was performed using the DESeq R package (1.10.1). When there was no biological repetition, TMM was firstly used to standardize the readcount data, and then DEG seq was used for difference analysis, of which the threshold was q value < 0.005 and | log2FoldChange | > 1. For differential genes, if the log2Foldchange of the gene is > 0, the differential gene was considered to be up-regulated. On the contrary, if log2Foldchange was < 0, the differential gene was considered to be down-regulated.

Network analysis of the gut microbiota at genus level

Metastats software was used to confirm significant differences in the relative abundance of microbiota in three groups of samples. Results were considered significant if $P \leq 0.05$. Then used DEG seq for difference analysis, of which the threshold is $P < 0.05$ and $|\log2FC| > 1$. R psych package was used for partial Spearman correlations. $r > 0.8$ and $P < 0.05$ (strong correlation) were screened for mapping. Red edges indicate positive correlation and blue edges indicate negative correlation. The thicker the line, the stronger the correlation. The node size represents the node degree.

Statistical analysis

Differences were analyzed using a Student's *t*-test or one-way analysis of variance (ANOVA) with Graphpad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, United States). Comparison among groups was made using ANOVA analysis, and comparison between two groups were made using the independent-sample *t*-test. Results are shown as mean ± standard error; Values of $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ were considered as statistically significant.

RESULTS

FMT ameliorated DSS-induced colitis in mice

To explore the therapeutic effect of FMT on colitis, a mouse model of acute colitis induced by DSS was established. A flowchart of this study is shown in Figure 1. Five healthy mice were included as the control group. Other mice randomly drank 2.5% DSS for 7 d and were divided into the DSS and FMT groups ($n = 7$ each). The DSS group was gavaged with normal saline after 7 d of DSS, while the FMT group was gavaged fecal microbiota isolated from healthy mice for 7 d after DSS intervention (Figure 1A). Compared with the DSS group, FMT obviously alleviated body weight loss ($^{b}P < 0.01$; Figure 1B) and DAI ($^{c}P < 0.001$; Figure 1C). These findings suggested that FMT improved the clinical symptoms in DSS-induced colitis. Additionally, FMT significantly alleviated the colon shortening caused by DSS treatment ($^{c}P < 0.001$; Figure 1D). To evaluate further the anti-inflammatory role of FMT, HE staining was used to assess pathological features in DSS-induced colitis. In the DSS group, colonic mucosal epithelial cells were absent, glands had lost their integrity, extensive infiltration of inflammatory cells was apparent and typical inflammatory reactions were observed. However, in the FMT group, colonic structure was relatively intact, and the histological score was lower compared with that in the DSS group ($^{c}P < 0.001$; Figure 1E). These results indicated that FMT significantly attenuated the DSS-induced colonic tissue injury and inflammatory response in the mice.

FMT improved the gut microbiota of DSS-induced colitis

To ascertain the effects of FMT on the gut microbiota, 16S rRNA gene amplification and sequencing were conducted (Figure 2). In terms of bacterial composition at the phylum level, all samples shared similar taxonomic communities and exhibited a relatively high abundance of the phyla Bacteroidetes and Firmicutes (Figure 2A). FMT administration was associated with variations in the abundance of specific taxa at the genus level (Figure 2B). The linear discriminant analysis effect size analysis showed that Lactobacillales was enriched in the gut of untreated mice, while Clostridiales was enriched in DSS-induced colitis mice. After FMT, Lactobacillales became obviously enriched, compared with the DSS group (Figure 2C). At the genus level, *Clostridium_sensu_ stricto_1* ($^{c}P = 0.001$, one-way ANOVA), *Turicibacter* ($^{c}P < 0.001$, one-way ANOVA), *Alistipes* ($^{c}P = 0.001$, one-way ANOVA), *Acetatifactor* ($^{c}P = 0.001$, one-way ANOVA), *Ruminococcus_1* ($^{b}P < 0.01$, one-way ANOVA) and *Ruminiclostridium_6* ($^{a}P < 0.05$, one-way ANOVA), which were expanded in colitis, were reduced after FMT and returned to being comparable to those observed in healthy mice. On the contrary, *Lactobacillus* ($^{c}P < 0.001$, one-way ANOVA) and *Lachnospiraceae_UCG-001* ($^{a}P < 0.05$, one-way ANOVA) displayed a trend of high abundance in the FMT group (Figure 3), and there was no significant difference in *Lactobacillus* between control and transplanted mice. Collectively, these results showed that FMT significantly improved the imbalanced gut microbiota induced by DSS in mice.

FMT regulated T-cell in the colon

To investigate the molecular target of FMT, we used RNA sequencing in colonic tissue

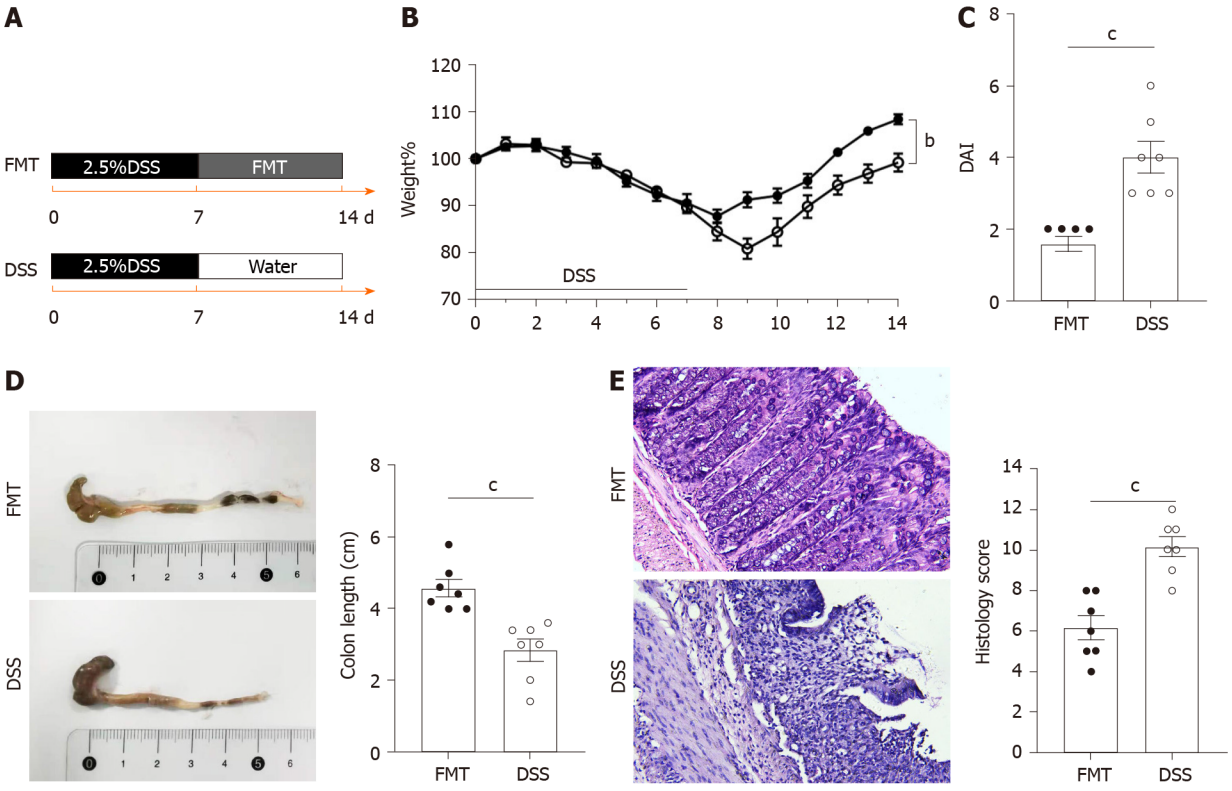


Figure 1 Therapeutic fecal microbiota transplantation ameliorates dextran sodium sulphate-induced inflammation. A: Schematic representation of fecal microbiota transplantation treatment in the dextran sodium sulphate colitis model (n = 7 per group); B: Daily changes of bodyweight of each group during the disease process; C: Disease activity index scores of each group were evaluated after sacrifice; D: Macroscopic view of colon was observed, colon length from each group was measured; E: Serial sections of colon tissues were stained with hematoxylin and eosin, original magnification 200 ×, Histological scores of each group were determined. Values of $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ were considered as statistically significant. DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation.

samples from control, DSS and FMT groups. We found that 1558 genes were differentially expressed in the DSS group compared to the control group, and 334 genes were differentially expressed in the FMT compared to the DSS group (Figure 4A). The GO enrichment histogram of DEGs showed the biological process, cellular component and molecular function. The bar chart shows the top 30 GO terms with the most significant enrichment (Figure 4B). The most enriched GO terms were immune system process and metabolic process. Additionally, there were 128 DEGs in the DSS group compared with the control and FMT groups. GO analysis showed that these genes mainly participated in T-cell activation induced by DSS and inhibited by FMT (Figure 5A). T-cell activation status was determined by surface marker staining, using flow cytometry. As expected, T-cell populations isolated from colons of FMT-treated mice showed reduced activity as compared to those isolated from colitis mice that received DSS (Figure 5B, C, $^{b}P < 0.01$). Phenotypically, mice receiving DSS displayed a higher proportion of $CD4^{+}$ and $CD8^{+}$ T cells. However, there was a significant decrease in $CD4^{+}$ and $CD8^{+}$ T cells in colons following FMT, supporting the observation that colonic T cells in FMT-treated mice manifested decreased activation. These results indicated that FMT alleviated DSS-induced colitis by inhibiting intestinal immunity related to T-cell modulation.

Correlation analysis between gut microbiota and transcriptome

The process by which the gut microbiota regulates intestinal immunity is complicated. The gut microbiota and T-cell immunity were both involved in the biological process of FMT treatment of colitis. Based on this, we tried to find the potential gut microbiota/immune axis-mediated signaling pathway. The correlation analysis between gut microbiota and transcriptome showed that *Clostridium_sensu_stricto_1* was the most enriched genus (Figure 6A). The DEGs related to gut microbiota are listed in Table 1. The DEGs, such as *IDO1*, *CCL8* and *REG3G*, were positively correlated with the relative abundance of *Clostridium_sensu_stricto_1* ($^{b}P < 0.01$). The DEGs such as *REG3G* were positively correlated with the relative abundance of *Turicibacter* ($^{b}P < 0.01$). Relative quantification of transcript level is shown in Figure 6B

Table 1 Correlation between gut microbiota and transcriptome

Number	Gene name	Target microbiota	*r* value	*P* value
1	*Ccl8*	*Clostridium_sensu_stricto_1*	0.87	< 0.001
2	*C6*	*Clostridium_sensu_stricto_1*	0.87	< 0.001
3	*Ido1*	*Clostridium_sensu_stricto_1*	0.87	< 0.001
4	*Abca12*	*Clostridium_sensu_stricto_1*	0.85	< 0.001
5	*Fcgr2b*	*Clostridium_sensu_stricto_1*	0.84	< 0.001
6	*Prap1*	*Clostridium_sensu_stricto_1*	0.84	< 0.001
7	*F5*	*Turicibacter*	0.84	< 0.001
8	*Ccr3*	*Clostridium_sensu_stricto_1*	0.84	< 0.001
9	*Fcer1g*	*Clostridium_sensu_stricto_1*	0.83	< 0.001
10	*Gbp5*	*Clostridium_sensu_stricto_1*	0.83	< 0.001
11	*Slc5a9*	*Clostridium_sensu_stricto_1*	0.82	= 0.001
12	*Reg3g*	*Turicibacter*	0.82	= 0.001
13	*Gbp4*	*Clostridium_sensu_stricto_1*	0.82	= 0.001
14	*Ifitm3*	*Clostridium_sensu_stricto_1*	0.82	= 0.001
15	*Car8*	*Turicibacter*	0.81	= 0.001
16	*Phf11d*	*Clostridium_sensu_stricto_1*	0.81	= 0.001
17	*Fcgr1*	*Clostridium_sensu_stricto_1*	0.8	= 0.002
18	*Gbp9*	*Clostridium_sensu_stricto_1*	0.8	= 0.002
19	*Iigp1*	*Clostridium_sensu_stricto_1*	0.8	= 0.002
20	*Gal3st2c*	*Clostridium_sensu_stricto_1*	-0.8	= 0.002
21	*Sall1*	*Erysipelatoclostridium*	-0.81	= 0.001
22	*B3gnt7*	*Turicibacter*	-0.81	= 0.001
23	*Mptx1*	*Clostridium_sensu_stricto_1*	-0.83	< 0.001
24	*Scin*	*Clostridium_sensu_stricto_1*	-0.83	< 0.001
25	*Itih2*	*Clostridium_sensu_stricto_1*	-0.84	< 0.001
26	*Cyp2f2*	*Clostridium_sensu_stricto_1*	-0.84	< 0.001
27	*B3gnt7*	*Clostridium_sensu_stricto_1*	-0.85	< 0.001
28	*Hhip*	*Clostridium_sensu_stricto_1*	-0.85	< 0.001
29	*Gal3st2b*	*Clostridium_sensu_stricto_1*	-0.86	< 0.001
30	*Ano1*	*Clostridium_sensu_stricto_1*	-0.87	< 0.001

(all $^{c}P < 0.001$, one-way ANOVA). These results provide a potential *Clostri dium_sensu_stricto_1*-mediated immune regulation for FMT to treat colitis.

Graphical display of therapeutic effect of FMT on colitis

FMT significantly modulated the dysbacteriosis induced by DSS and restored the disordered gut microbiota to normal level. Meanwhile, FMT protected intestinal epithelial barrier disruption induced by DSS *via* upregulating the relative abundance of *Lactobacillus* and downregulating the relative abundance of *Clostridium_sensu_stricto_1* and *Turicibacter*. Furthermore, after FMT treatment, the activation of T lymphocytes was significantly inhibited (Figure 7). Hypothetically, FMT may improve experimental colitis by increasing relative abundance of *Lactobacillus* and regulating T-cell function.

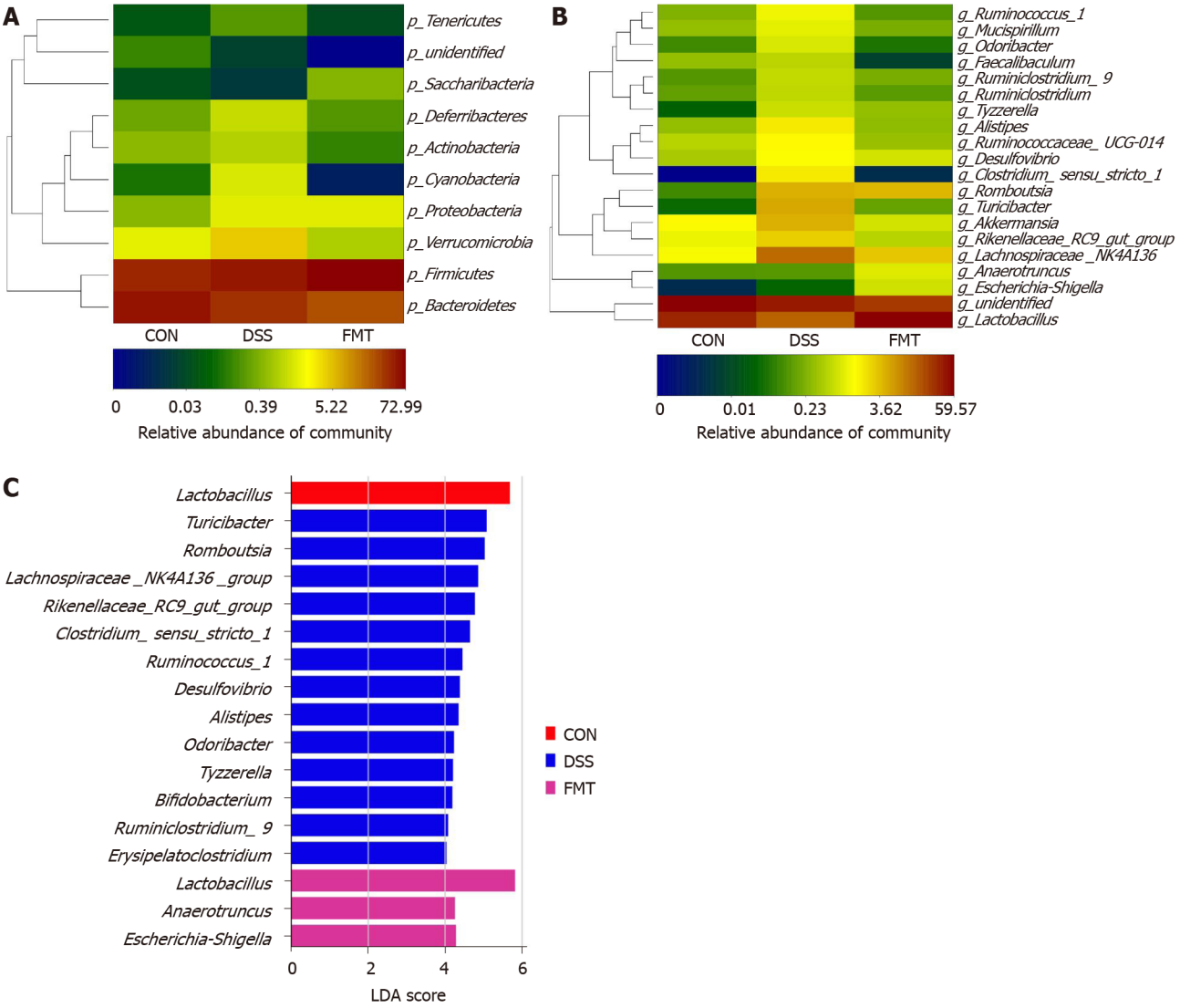


Figure 2 Fecal microbiota transplantation modulated the overall structure of gut microbiota. A and B: Heatmap of the relative abundance at the level of phylum and genus; C: The linear discriminant analysis effect size analysis at the level of genus in groups. CON: Control; DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation.

DISCUSSION

FMT has been shown to be effective in treating ulcerative colitis (UC), but its mechanism is still unclear. In this study, 16s rRNA and transcriptome sequencing were used to prove that FMT alleviated DSS-induced colitis in mice by improving the gut microbiota and regulating T-cell function.

Lactobacillus is considered a genus of beneficial bacteria in the gut and is abundant in healthy individuals. During the inflammation stage in the colon, the abundance of *Lactobacillus* decreased significantly[12]. Our data suggested that *Lactobacillus* was the main genus in healthy mice, but its abundance decreased significantly in DSS-induced colitis. However, this process was blocked by FMT. After transplanting the resident microbiota to colitis mice, the abundance of *Lactobacillus* increased significantly and it became the dominant genus in the FMT group. Similarly, an increase in *Lactobacillus* was observed after FMT treatment in UC patients[13]. Supplementing *Lactobacillus* significantly prevents colonic shortening and has a therapeutic effect on DSS-induced colitis[14]. The underlying mechanism of *Lactobacillus* in colitis is that it promotes beneficial metabolic pathways[15] that inhibit group 3 innate lymphoid cells[16], increasing induction of regulatory T lymphocytes[17,18], decreasing inflammatory pathways[19] and strengthening gut barrier function[20]. In UC mice, *Lactobacillus* induced regulatory T (Treg) cell differentiation and had an impact on intestinal expression of antimicrobial peptides[21]. In detail, the Treg cells expand with decreasing levels of adenosine monophosphate in colonic tissues and thus ameliorate

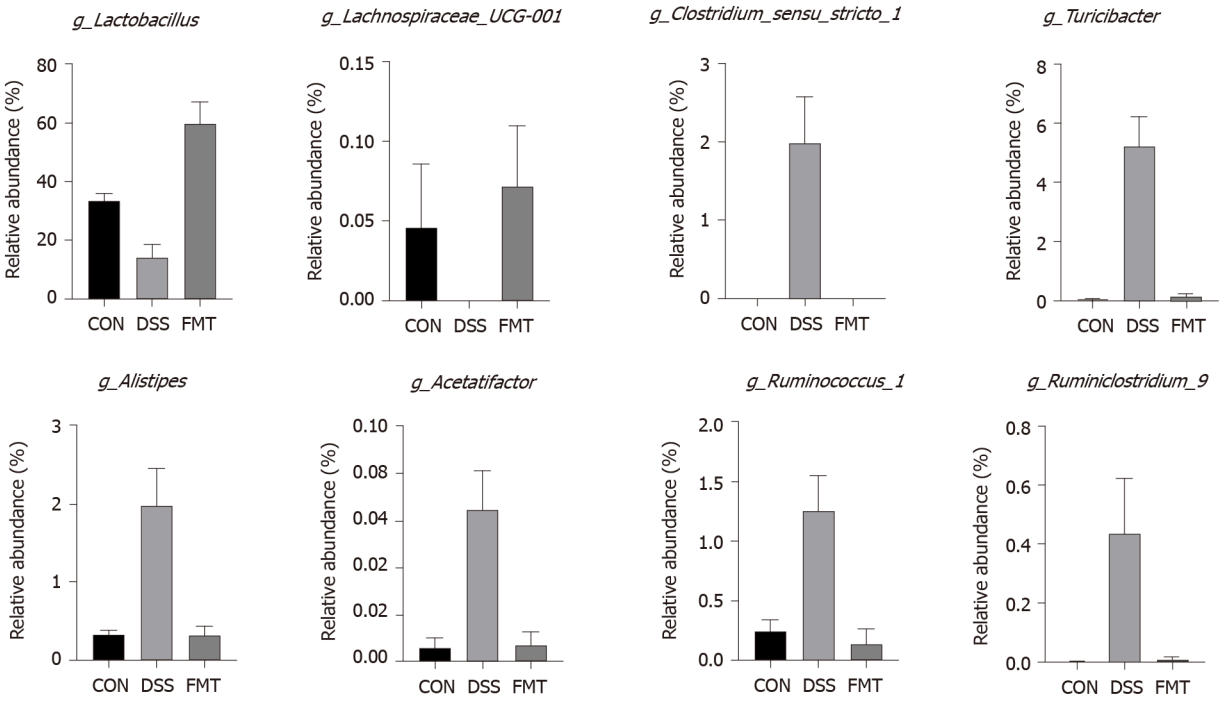


Figure 3 Fecal microbiota transplantation significantly regulated the relative abundance of eight genera compared with dextran sodium sulphate group. *Lactobacillus* [$^{c}P < 0.001$, one-way analysis of variance (ANOVA)]; *Lachnospiraceae_UCG-001* ($^{a}P < 0.05$, one-way ANOVA); *Clostridium_sensu_stricto_1* ($^{c}P = 0.001$, one-way ANOVA); *Turicibacter* ($^{c}P < 0.001$, one-way ANOVA); *Alistipes* ($^{c}P = 0.001$, one-way ANOVA); *Acetatifactor* ($^{c}P = 0.001$, one-way ANOVA); *Ruminococcus_1* ($^{b}P < 0.01$, one-way ANOVA); *Ruminiclostridium_6* ($^{a}P < 0.05$, one-way ANOVA). CON: Control; DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation.

UC in the mice[17]. *Lactobacillus* also promotes the release of anti-inflammatory interleukin (IL)-10 by T cells, which is pivotal for the resolution of intestinal inflammation[22,23] by reducing the proliferative capacity of proinflammatory T cells[24]. It should be noted that not all bacterial strains of *Lactobacillus* exert an immunoregulatory action on T cells[21]. Thus, the role of *Lactobacillus* in the pathogenesis of intestinal inflammation requires further study. Besides, *Clostridium_sensu_stricto_1*[25] and *Turicibacter*[26] are deemed to be opportunistic pathogens associated with colitis.

In our study, FMT significantly reduced the relative abundance of *Clostridium_sensu_stricto_1*, *Turicibacter* and *Ruminococcus*, which were increased after DSS treatment. In IBD patients, the abundance of these opportunistic pathogens also increased[27]. *Turicibacter* positively correlated with proinflammatory cytokines, such as IL-6, IL-1β, tumor necrosis factor-α and interferon-γ[28]. *Turicibacter* exhibited higher relative abundance in IBD patients and mouse models, further inducing more severe colitis[29,30]. As reported, *Turicibacter* was significantly less abundant in diarrhea-dominant irritable bowel syndrome patients[31]. Recent studies also indicate an beneficial role for *Turicibacter* in host serotonin metabolism[32]. These findings show the complex role of *Turicibacter* in chronic inflammatory settings. As recently reported, *Ruminococcus* may drive high neutrophil-to-lymphocyte ratios that are broadly characteristic of poor disease outcomes in IBDs[33]. *Ruminococcus* affects the efficacy of FMT, because the presence of higher levels of *Ruminococcus* in donors makes FMT more likely to fail[34]. To our knowledge, the relative abundance of *Clostridium_sensu_stricto_1*, *Turicibacter* and *Ruminococcus* was significantly decreased after FMT. Therefore, FMT may regulate the gut microbiota to exert an anti-inflammatory effect in colitis.

In UC, the balance of commensal microbes and the immune system is impaired. In IBD patients, $CD4^+$ T cells may exhibit significantly high pathogenicity, characterized by enhanced T-cell activation[35]. The reactivity to intestinal bacteria is a normal property of the human $CD4^+$ T-cell repertoire and does not necessarily indicate disrupted interactions between immune cells and the commensal microbiota[36]. Drugs commonly used in the treatment of IBD, such as anti-tumor necrosis factor-α agents, cyclosporin A and azathioprine, have been demonstrated to induce apoptosis of lamina propria T cells in IBD[37]. Therefore, targeting T cells shows potential therapeutic effects. In fact, FMT regulates intestinal immunity. Therapeutic FMT

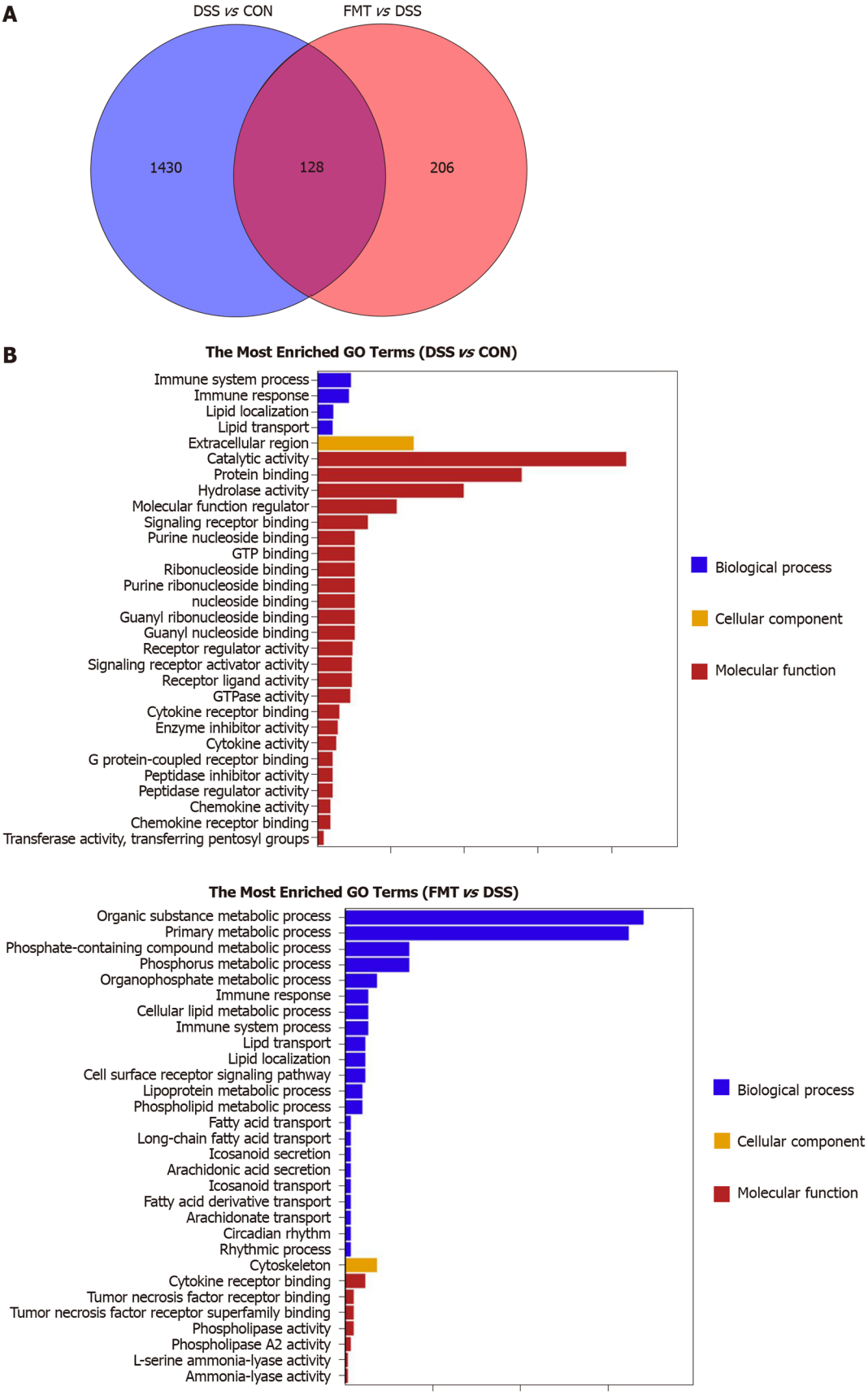


Figure 4 Effect of fecal microbiota transplantation on colonic transcriptome in colitis mice. A: Venn diagram with genes regulated between dextran sodium sulphate (DSS) and fecal microbiota transplantation (FMT) or shared between the two sections; B: The top 30 gene ontology terms with the most significant enrichment between DSS group and control (CON) group (left), FMT group and DSS group (right). After DSS treatment (left), the differentially expressed genes in mice colon were mainly associated with immune system process, while after FMT (right), the differentially expressed genes were mainly related to metabolic process and immune response. GO: Gene ontology.

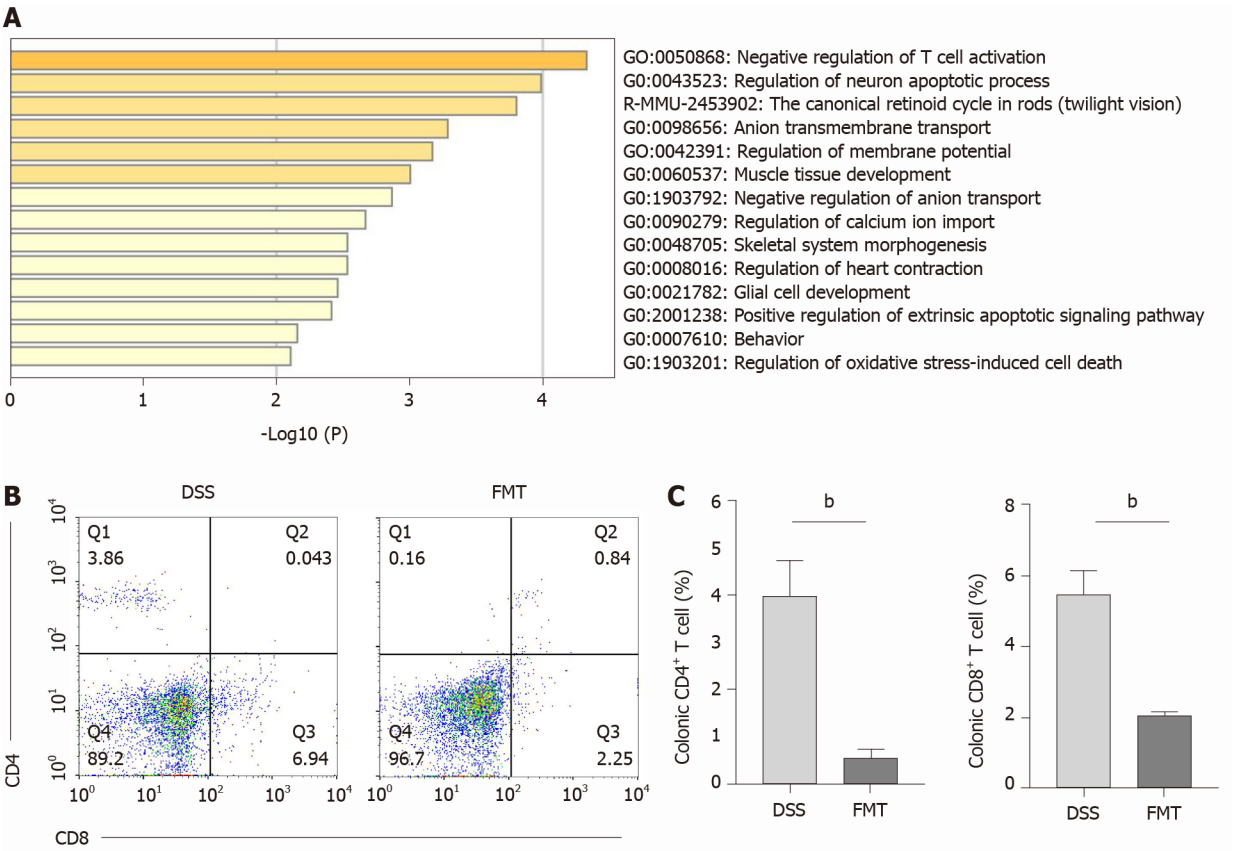


Figure 5 Therapeutic fecal microbiota transplantation modulates T cell phenotypes. A: Differentially expressed genes between control, dextran sodium sulphate (DSS) and fecal microbiota transplantation (FMT) group analyzed in Metascape; B: Colonic CD4$^+$ and CD8$^+$ T cells in DSS and FMT group; n = 4 mice per group; C: Bar charts of the percentage of T cells ($^bP < 0.01$). CON: Control.

administration during experimental colitis reduces colonic inflammation and initiates the restoration of intestinal homeostasis through regulating innate and adaptive immune responses in the intestinal mucosa[24]. In this work, a total of 128 DEGs were distinguished by comparing the control, DSS and FMT groups. Functional annotation analysis showed that these DEGs are mainly involved in the activation of T lymphocytes. After FMT, the activation of T lymphocytes was significantly inhibited and indoleamine 2,3-dioxygenase 1 (IDO1) was mainly involved in this process. In IBD patients and experimental colitis, overexpression of IDO1 had a positive correlation with the severity of the disease[38,39]. Likewise, there is a higher presence of IDO-producing DCs and Tregs in the lamina propria of IBD patients compared with healthy subjects, which may represent a role of regulating inflammatory mechanism[40]. Pharmacological inhibition of IDO1 alleviates DSS-induced colitis[39,41]. In a recent study, gut microbiota from IDO1-knockout mice could directly attenuate the severity of DSS-induced colitis[42]. These results suggested that IDO1 regulated the gut microbiota to maintain intestinal homeostasis in colitis. According to our results, IDO1 expression was increased by DSS but was significantly inhibited after FMT at the transcriptional level. The reduction of intestinal T-lymphocyte activation indicates reduction of intestinal pathogenic bacteria. This can explain how the gut microbiota improved after FMT, thereby reducing activation of T cells.

In this study, we investigated the relationship between the gut microbiota and colon transcriptome in colitis. There was a significant positive correlation between *Clostridium_sensu_stricto_1* and expression of antimicrobial C-type lectin regenerating islet-derived 3 gamma (REG3G), monocyte chemoattractant protein 2 (CCL8) and IDO1. REG3G belongs to the C-type lectin antimicrobial peptide family, which is closely regulated by T helper 17/IL-17A and prevents pathogenic bacteria from invading the colonic mucosa[43]. As previously described, the impact of *Lactobacillus* on antimicrobial peptides relied on the presence of the gut microbiota[21]. It is a defense mechanism widely produced by intestinal epithelial cells to regulate the dynamic balance between microbiota and host epithelium[44]. Colonic macrophages act as a major source of CCL8 chemokines that trigger further recruitment of their proinflammatory monocyte precursors[45]. After administering lipopolysaccharide,

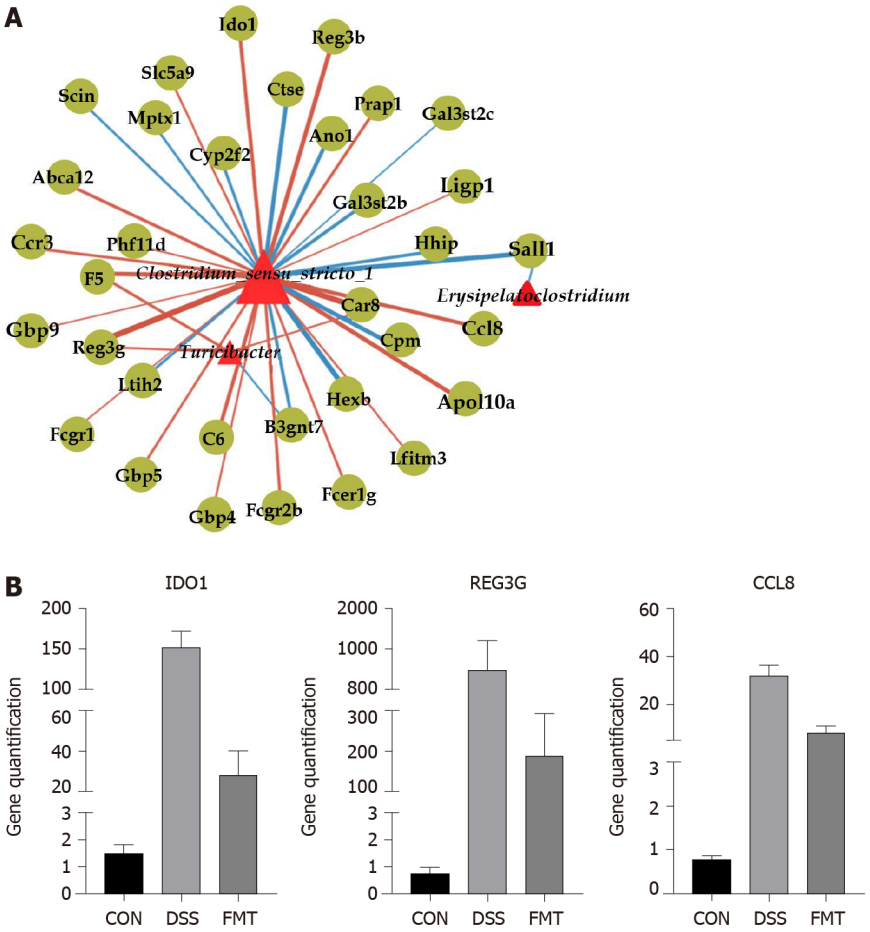


Figure 6 Network analysis of fecal microbiota transplantation in colitis mice. A: Network visualizing significant gene-microbe correlations ($r > 0.8$, $P < 0.05$). B: Relative quantification of transcript level of indoleamine 2,3-dioxygenase 1 (IDO1), antimicrobial C-type lectin regenerating islet-derived 3 gamma (REG3G) and monocyte chemoattractant protein 2 (CCL8) in groups (all the $P < 0.001$, one-way analysis of variance). CON: Control; DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation.

there was a marked increase in the expression of the monocyte chemoattractant CCL8[46]. As in animal colitis, CCL8 is significantly increased in active compared with quiescent IBD[45,47]. In our study, these highly expressed genes were positively correlated with *Clostridium_sensu_stricto_1* in DSS-induced colitis, but the expression was significantly lower in the FMT group. These results indicated that FMT alleviated mouse colitis *via* downregulation of the expression of these microbiota-related genes.

The current study had some limitations. Firstly, the current 16S sequencing mainly included bacterial and archaeal genomes, ignoring nonbacterial organisms, such as viruses, fungi and phages. Secondly, animal studies have some limitations in evaluating the mechanism of FMT. Administration of DSS to induce subclinical colitis, while convenient, does not yield a model that perfectly mimics the pathogenesis of IBD, since DSS causes a chemical colitis through epithelial injury. Thirdly, we analyzed the correlation between gut microbiota and gene expression, but the causal relationship was not further explained. In this study, the regulation of gut microbiota and colon transcriptome by FMT may be the key to the anti-inflammatory role in colitis. We will carry out clinical studies to verify the changes in gut microbiota and transcriptome in FMT treatment of UC.

CONCLUSION

In this study, we explored the mechanism of FMT in the treatment of experimental colitis by analysis of gut microbiota and colonic transcriptome. FMT contributed to alleviation of murine colitis with increased abundance of *Lactobacillus* and decreased *Clostridium_sensu_stricto_1* and *Turicibacter*. In transcriptomics, FMT inhibited activation of T lymphocytes in the colon by regulating gene expression related to the microbiota. Therefore, we propose that FMT regulates the gut microbiota and T

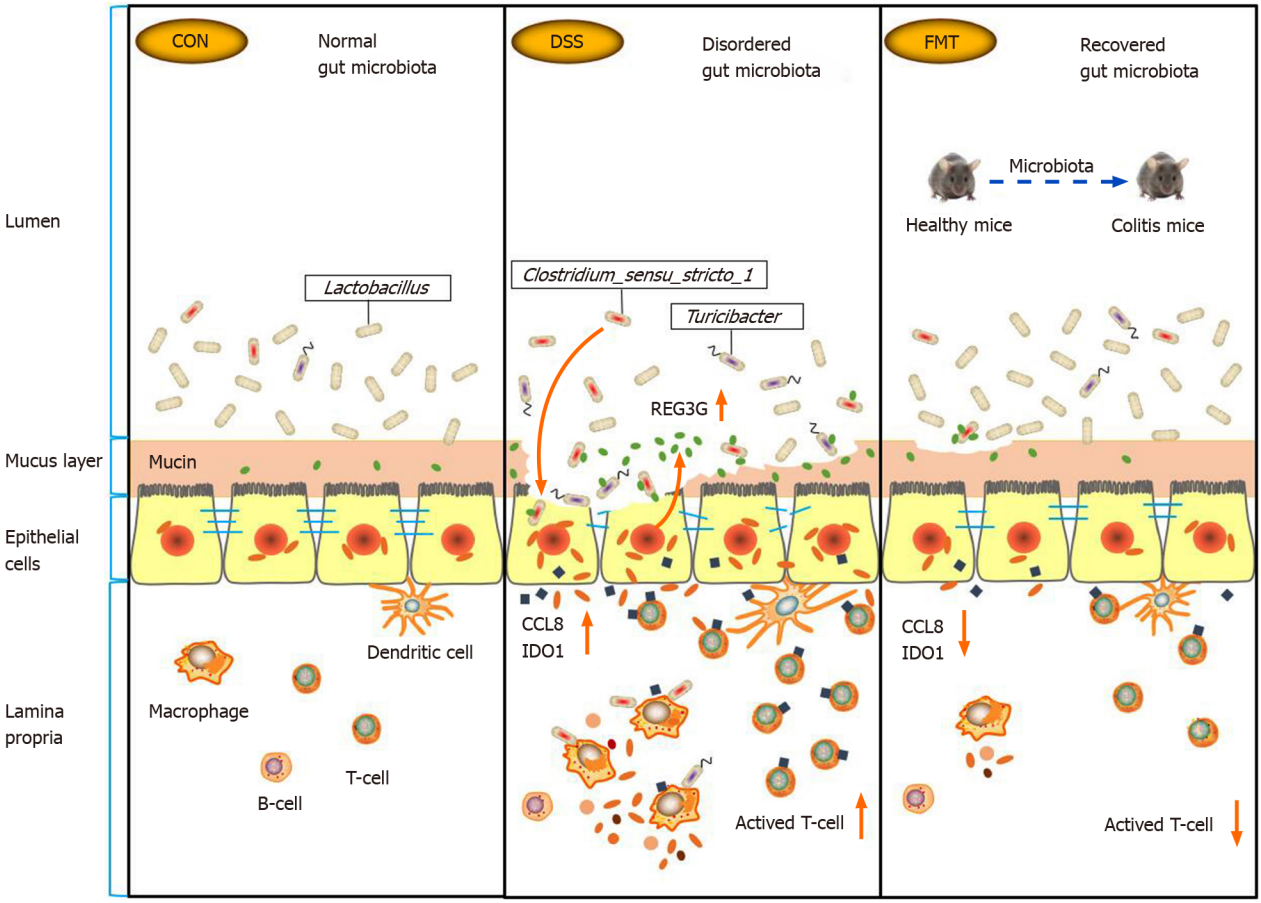


Figure 7 Summary scheme of the mechanisms underlying the therapeutic effect of fecal microbiota transplantation on experimental colitis. CCL8: Monocyte chemoattractant protein 2; CON: Control; DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation; IDO1: Indoleamine 2,3-dioxygenase 1; REG3G: Antimicrobial C-type lectin regenerating islet-derived 3 gamma.

lymphocytes function, and thereby alleviates colitis. The causal relationship between the gut microbiota and UC needs to be further studied.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is a growing global disease in which gut microbiota dysbiosis plays an important pathogenic role. However, the current drugs for UC treatment are far from optimal. Therefore, alternative safe and effective new treatments need to be developed.

Research motivation

Clinical practice has confirmed the therapeutic value of fecal microbiota transplantation (FMT) in UC, but its mechanism is unknown. Recently, few studies have indicated the regulatory effect of FMT on the expression of genes in intestinal mucosa, suggesting that the therapeutic effect of FMT on UC may be not only the reconstruction of gut microbiota but also the gene regulation after reconstruction.

Research objectives

The aim of the study was to explore the effect and mechanism of FMT on regulating the balance of gut microbiota and anti-inflammation in dextran sulfate sodium (DSS)-induced colitis.

Research methods

Experimental colitis was induced by DSS, then the severity of intestinal inflammation was evaluated by body weight, colon length, disease activity index and histological

scores. Gut microbiota alteration was analyzed through 16S rRNA sequencing. Transcriptome sequencing was used to screen differentially expressed genes in colon. The frequency of immune cells in lamina propria were phenotyped by flow cytometry.

Research results

DSS-induced weight loss, colon length shortening, disease activity index score and histological score were significantly alleviated after FMT treatment. 16S rRNA sequencing indicated that FMT up-regulated the relative abundance of *Lactobacillus* and down-regulated the relative abundance of *Clostridium_sensu_stricto_1* and *Turicibacter*. Transcriptomics-based differential gene analysis showed that FMT could regulate colonic T cell function. Further flow cytometry analysis showed that FMT downregulated the number of colonic $CD4^+$ and $CD8^+$ T cells to maintain intestinal homeostasis. Moreover, we found that the abundance of *Clostridium_sensu_stricto_1* was positively correlated with the expression of inflammation-related genes such as *REG3G*, *CCL8* and *IDO1*.

Research conclusions

FMT alleviated DSS-induced colitis in mice by improving the gut microbiota and regulating T-cell function.

Research perspectives

This study has initially revealed the mechanism of FMT in the treatment of colitis, which will be confirmed by human studies in the future.

REFERENCES

1 **Zhang T**, Lu G, Zhao Z, Liu Y, Shen Q, Li P, Chen Y, Yin H, Wang H, Marcella C, Cui B, Cheng L, Ji G, Zhang F. Washed microbiota transplantation vs. manual fecal microbiota transplantation: clinical findings, animal studies and *in vitro* screening. *Protein Cell* 2020; **11**: 251-266 [PMID: 31919742 DOI: 10.1007/s13238-019-00684-8]

2 **Tariq R**, Disbrow MB, Dibaise JK, Orenstein R, Saha S, Solanky D, Loftus EV, Pardi DS, Khanna S. Efficacy of Fecal Microbiota Transplantation for Recurrent C. Difficile Infection in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2020; **26**: 1415-1420 [PMID: 31821444 DOI: 10.1093/ibd/izz299]

3 **Costello SP**, Hughes PA, Waters O, Bryant RV, Vincent AD, Blatchford P, Katsikeros R, Makanyanga J, Campaniello MA, Mavrangelos C, Rosewarne CP, Bickley C, Peters C, Schoeman MN, Conlon MA, Roberts-Thomson IC, Andrews JM. Effect of Fecal Microbiota Transplantation on 8-Week Remission in Patients With Ulcerative Colitis: A Randomized Clinical Trial. *JAMA* 2019; **321**: 156-164 [PMID: 30644982 DOI: 10.1001/jama.2018.20046]

4 **Paramsothy S**, Kamm MA, Kaakoush NO, Walsh AJ, van den Bogaerde J, Samuel D, Leong RWL, Connor S, Ng W, Paramsothy R, Xuan W, Lin E, Mitchell HM, Borody TJ. Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet* 2017; **389**: 1218-1228 [PMID: 28214091 DOI: 10.1016/S0140-6736(17)30182-4]

5 **Yalchin M**, Segal JP, Mullish BH, Quraishi MN, Iqbal TH, Marchesi JR, Hart AL. Gaps in knowledge and future directions for the use of faecal microbiota transplant in the treatment of inflammatory bowel disease. *Therap Adv Gastroenterol* 2019; **12**: 1756284819891038 [PMID: 31803254 DOI: 10.1177/1756284819891038]

6 **Wang H**, Cui B, Li Q, Ding X, Li P, Zhang T, Yang X, Ji G, Zhang F. The Safety of Fecal Microbiota Transplantation for Crohn's Disease: Findings from A Long-Term Study. *Adv Ther* 2018; **35**: 1935-1944 [PMID: 30328062 DOI: 10.1007/s12325-018-0800-3]

7 **Li Q**, Ding X, Liu K, Marcella C, Liu X, Zhang T, Liu Y, Li P, Xiang L, Cui B, Wang J, Bai J, Zhang F. Fecal Microbiota Transplantation for Ulcerative Colitis: The Optimum Timing and Gut Microbiota as Predictors for Long-Term Clinical Outcomes. *Clin Transl Gastroenterol* 2020; **11**: e00224 [PMID: 32955197 DOI: 10.14309/ctg.0000000000000224]

8 **Li Q**, Zhang T, Ding X, Xiang L, Cui B, Buch H, Zhang F. Enhancing patient adherence to fecal microbiota transplantation maintains the long-term clinical effects in ulcerative colitis. *Eur J Gastroenterol Hepatol* 2020; **32**: 955-962 [PMID: 32282545 DOI: 10.1097/MEG.0000000000001725]

9 **Burrello C**, Giuffrè MR, Macandog AD, Diaz-Basabe A, Cribiù FM, Lopez G, Borgo F, Nezi L, Caprioli F, Vecchi M, Facciotti F. Fecal Microbiota Transplantation Controls Murine Chronic Intestinal Inflammation by Modulating Immune Cell Functions and Gut Microbiota Composition. *Cells* 2019; **8**: 517 [PMID: 31142049 DOI: 10.3390/cells8060517]

10 **Li C**, Ai G, Wang Y, Lu Q, Luo C, Tan L, Lin G, Liu Y, Li Y, Zeng H, Chen J, Lin Z, Xian Y, Huang X, Xie J, Su Z. Oxyberberine, a novel gut microbiota-mediated metabolite of berberine, possesses superior anti-colitis effect: Impact on intestinal epithelial barrier, gut microbiota profile and TLR4-MyD88-NF-κB pathway. *Pharmacol Res* 2020; **152**: 104603 [PMID: 31863867 DOI:

10.1016/j.phrs.2019.104603]
11 **Young MD**, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol* 2010; **11**: R14 [PMID: 20132535 DOI: 10.1186/gb-2010-11-2-r14]
12 **Miranda PM**, De Palma G, Serkis V, Lu J, Louis-Auguste MP, McCarville JL, Verdu EF, Collins SM, Bercik P. High salt diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production. *Microbiome* 2018; **6**: 57 [PMID: 29566748 DOI: 10.1186/s40168-018-0433-4]
13 **Mańkowska-Wierzbicka D**, Stelmach-Mardas M, Gabryel M, Tomczak H, Skrzypczak-Zielińska M, Zakerska-Banaszak O, Sowińska A, Mahadea D, Baturo A, Wolko Ł, Słomski R, Dobrowolska A. The Effectiveness of Multi-Session FMT Treatment in Active Ulcerative Colitis Patients: A Pilot Study. *Biomedicines* 2020; **8**: 268 [PMID: 32756350 DOI: 10.3390/biomedicines8080268]
14 **Hasannejad-Bibalan M**, Mojtahedi A, Eshaghi M, Rohani M, Pourshafie MR, Talebi M. The effect of selected Lactobacillus strains on dextran sulfate sodium-induced mouse colitis model. *Acta Microbiol Immunol Hung* 2020; **67**: 138-142 [PMID: 32554841 DOI: 10.1556/030.2020.00834]
15 **Tamura K**, Sasaki H, Shiga K, Miyakawa H, Shibata S. The Timing Effects of Soy Protein Intake on Mice Gut Microbiota. *Nutrients* 2019; **12** [PMID: 31892229 DOI: 10.3390/nu12010087]
16 **Wang T**, Zheng N, Luo Q, Jiang L, He B, Yuan X, Shen L. Probiotics *Lactobacillus reuteri* Abrogates Immune Checkpoint Blockade-Associated Colitis by Inhibiting Group 3 Innate Lymphoid Cells. *Front Immunol* 2019; **10**: 1235 [PMID: 31214189 DOI: 10.3389/fimmu.2019.01235]
17 **Tang C**, Kamiya T, Liu Y, Kadoki M, Kakuta S, Oshima K, Hattori M, Takeshita K, Kanai T, Saijo S, Ohno N, Iwakura Y. Inhibition of Dectin-1 Signaling Ameliorates Colitis by Inducing Lactobacillus-Mediated Regulatory T Cell Expansion in the Intestine. *Cell Host Microbe* 2015; **18**: 183-197 [PMID: 26269954 DOI: 10.1016/j.chom.2015.07.003]
18 **Kim DH**, Kim S, Ahn JB, Kim JH, Ma HW, Seo DH, Che X, Park KC, Jeon JY, Kim SY, Lee HC, Lee JY, Kim TI, Kim WH, Kim SW, Cheon JH. Lactobacillus plantarum CBT LP3 ameliorates colitis *via* modulating T cells in mice. *Int J Med Microbiol* 2020; **310**: 151391 [PMID: 32007342 DOI: 10.1016/j.ijmm.2020.151391]
19 **El-Baz AM**, Khodir AE, Adel El-Sokkary MM, Shata A. The protective effect of Lactobacillus *vs* 5-aminosalicylic acid in ulcerative colitis model by modulation of gut microbiota and Nrf2/Ho-1 pathway. *Life Sci* 2020; **256**: 117927 [PMID: 32526285 DOI: 10.1016/j.lfs.2020.117927]
20 **Wang Y**, Gu Y, Fang K, Mao K, Dou J, Fan H, Zhou C, Wang H. Lactobacillus acidophilus and Clostridium butyricum ameliorate colitis in murine by strengthening the gut barrier function and decreasing inflammatory factors. *Benef Microbes* 2018; **9**: 775-787 [PMID: 30014710 DOI: 10.3920/BM2017.0035]
21 **Hrdý J**, Alard J, Couturier-Maillard A, Boulard O, Boutillier D, Delacre M, Lapadatescu C, Cesaro A, Blanc P, Pot B, Ryffel B, Chamaillard M, Grangette C. Lactobacillus reuteri 5454 and Bifidobacterium animalis ssp. lactis 5764 improve colitis while differentially impacting dendritic cells maturation and antimicrobial responses. *Sci Rep* 2020; **10**: 5345 [PMID: 32210304 DOI: 10.1038/s41598-020-62161-1]
22 **Di Giacinto C**, Marinaro M, Sanchez M, Strober W, Boirivant M. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. *J Immunol* 2005; **174**: 3237-3246 [PMID: 15749854 DOI: 10.4049/jimmunol.174.6.3237]
23 **Jang YJ**, Kim WK, Han DH, Lee K, Ko G. *Lactobacillus fermentum* species ameliorate dextran sulfate sodium-induced colitis by regulating the immune response and altering gut microbiota. *Gut Microbes* 2019; **10**: 696-711 [PMID: 30939976 DOI: 10.1080/19490976.2019.1589281]
24 **Burrello C**, Garavaglia F, Cribiù FM, Ercoli G, Lopez G, Troisi J, Colucci A, Guglietta S, Carloni S, Guglielmetti S, Taverniti V, Nizzoli G, Bosari S, Caprioli F, Rescigno M, Facciotti F. Therapeutic faecal microbiota transplantation controls intestinal inflammation through IL10 secretion by immune cells. *Nat Commun* 2018; **9**: 5184 [PMID: 30518790 DOI: 10.1038/s41467-018-07359-8]
25 **Zou J**, Shen Y, Chen M, Zhang Z, Xiao S, Liu C, Wan Y, Yang L, Jiang S, Shang E, Qian D, Duan J. Lizhong decoction ameliorates ulcerative colitis in mice *via* modulating gut microbiota and its metabolites. *Appl Microbiol Biotechnol* 2020; **104**: 5999-6012 [PMID: 32418127 DOI: 10.1007/s00253-020-10665-1]
26 **Wan P**, Peng Y, Chen G, Xie M, Dai Z, Huang K, Dong W, Zeng X, Sun Y. Modulation of gut microbiota by Ilex kudingcha improves dextran sulfate sodium-induced colitis. *Food Res Int* 2019; **126**: 108595 [PMID: 31732076 DOI: 10.1016/j.foodres.2019.108595]
27 **Franzosa EA**, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, Vatanen T, Hall AB, Mallick H, McIver LJ, Sauk JS, Wilson RG, Stevens BW, Scott JM, Pierce K, Deik AA, Bullock K, Imhann F, Porter JA, Zhernakova A, Fu J, Weersma RK, Wijmenga C, Clish CB, Vlamakis H, Huttenhower C, Xavier RJ. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat Microbiol* 2019; **4**: 293-305 [PMID: 30531976 DOI: 10.1038/s41564-018-0306-4]
28 **Liang YN**, Yu JG, Zhang DB, Zhang Z, Ren LL, Li LH, Wang Z, Tang ZS. Indigo Naturalis Ameliorates Dextran Sulfate Sodium-Induced Colitis in Mice by Modulating the Intestinal Microbiota Community. *Molecules* 2019; **24** [PMID: 31726738 DOI: 10.3390/molecules24224086]
29 **Gu Z**, Zhu Y, Jiang S, Xia G, Li C, Zhang X, Zhang J, Shen X. Tilapia head glycolipids reduce inflammation by regulating the gut microbiota in dextran sulphate sodium-induced colitis mice. *Food Funct* 2020; **11**: 3245-3255 [PMID: 32219260 DOI: 10.1039/d0fo00116c]
30 **Liu A**, Lv H, Wang H, Yang H, Li Y, Qian J. Aging Increases the Severity of Colitis and the Related Changes to the Gut Barrier and Gut Microbiota in Humans and Mice. *J Gerontol A Biol Sci Med Sci* 2020; **75**: 1284-1292 [PMID: 32048723 DOI: 10.1093/gerona/glz263]

31 **Zhuang X**, Tian Z, Li L, Zeng Z, Chen M, Xiong L. Fecal Microbiota Alterations Associated With Diarrhea-Predominant Irritable Bowel Syndrome. *Front Microbiol* 2018; **9**: 1600 [PMID: 30090090 DOI: 10.3389/fmicb.2018.01600]

32 **Fung TC**, Vuong HE, Luna CDG, Pronovost GN, Aleksandrova AA, Riley NG, Vavilina A, McGinn J, Rendon T, Forrest LR, Hsiao EY. Intestinal serotonin and fluoxetine exposure modulate bacterial colonization in the gut. *Nat Microbiol* 2019; **4**: 2064-2073 [PMID: 31477894 DOI: 10.1038/s41564-019-0540-4]

33 **Schluter J**, Peled JU, Taylor BP, Markey KA, Smith M, Taur Y, Niehus R, Staffas A, Dai A, Fontana E, Amoretti LA, Wright RJ, Morjaria S, Fenelus M, Pessin MS, Chao NJ, Lew M, Bohannon L, Bush A, Sung AD, Hohl TM, Perales MA, van den Brink MRM, Xavier JB. The gut microbiota is associated with immune cell dynamics in humans. *Nature* 2020; **588**: 303-307 [PMID: 33239790 DOI: 10.1038/s41586-020-2971-8]

34 **Fuentes S**, Rossen NG, van der Spek MJ, Hartman JH, Huuskonen L, Korpela K, Salojärvi J, Aalvink S, de Vos WM, D'Haens GR, Zoetendal EG, Ponsioen CY. Microbial shifts and signatures of long-term remission in ulcerative colitis after faecal microbiota transplantation. *ISME J* 2017; **11**: 1877-1889 [PMID: 28398347 DOI: 10.1038/ismej.2017.44]

35 **Ma C**, Wu W, Lin R, Ge Y, Zhang C, Sun S, Cong Y, Li X, Liu Z. Critical Role of CD6highCD4+ T Cells in Driving Th1/Th17 Cell Immune Responses and Mucosal Inflammation in IBD. *J Crohns Colitis* 2019; **13**: 510-524 [PMID: 30395204 DOI: 10.1093/ecco-jcc/jjy179]

36 **Hegazy AN**, West NR, Stubbington MJT, Wendt E, Suijker KIM, Datsi A, This S, Danne C, Campion S, Duncan SH, Owens BMJ, Uhlig HH, McMichael A; Oxford IBD Cohort Investigators, Bergthaler A, Teichmann SA, Keshav S, Powrie F. Circulating and Tissue-Resident $CD4^+$ T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation. *Gastroenterology* 2017; **153**: 1320-1337. e16 [PMID: 28782508 DOI: 10.1053/j.gastro.2017.07.047]

37 **Giuffrida P**, Di Sabatino A. Targeting T cells in inflammatory bowel disease. *Pharmacol Res* 2020; **159**: 105040 [PMID: 32585338 DOI: 10.1016/j.phrs.2020.105040]

38 **Shi W**, Zou R, Yang M, Mai L, Ren J, Wen J, Liu Z, Lai R. Analysis of Genes Involved in Ulcerative Colitis Activity and Tumorigenesis Through Systematic Mining of Gene Co-expression Networks. *Front Physiol* 2019; **10**: 662 [PMID: 31214045 DOI: 10.3389/fphys.2019.00662]

39 **Zhang XJ**, Yuan ZW, Qu C, Yu XT, Huang T, Chen PV, Su ZR, Dou YX, Wu JZ, Zeng HF, Xie Y, Chen JN. Palmatine ameliorated murine colitis by suppressing tryptophan metabolism and regulating gut microbiota. *Pharmacol Res* 2018; **137**: 34-46 [PMID: 30243842 DOI: 10.1016/j.phrs.2018.09.010]

40 **Acovic A**, Gazdic M, Jovicic N, Harrell CR, Fellabaum C, Arsenijevic N, Volarevic V. Role of indoleamine 2,3-dioxygenase in pathology of the gastrointestinal tract. *Therap Adv Gastroenterol* 2018; **11**: 1756284818815334 [PMID: 30574192 DOI: 10.1177/1756284818815334]

41 **Shon WJ**, Lee YK, Shin JH, Choi EY, Shin DM. Severity of DSS-induced colitis is reduced in Ido1-deficient mice with down-regulation of TLR-MyD88-NF-kB transcriptional networks. *Sci Rep* 2015; **5**: 17305 [PMID: 26610689 DOI: 10.1038/srep17305]

42 **Shin JH**, Lee YK, Shon WJ, Kim B, Jeon CO, Cho JY, Morse HC 3rd, Choi EY, Shin DM. Gut microorganisms and their metabolites modulate the severity of acute colitis in a tryptophan metabolism-dependent manner. *Eur J Nutr* 2020; **59**: 3591-3601 [PMID: 32055962 DOI: 10.1007/s00394-020-02194-4]

43 **Kolls JK**, McCray PB Jr, Chan YR. Cytokine-mediated regulation of antimicrobial proteins. *Nat Rev Immunol* 2008; **8**: 829-835 [PMID: 18949018 DOI: 10.1038/nri2433]

44 **Hooper LV**, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* 2010; **10**: 159-169 [PMID: 20182457 DOI: 10.1038/nri2710]

45 **Jones GR**, Bain CC, Fenton TM, Kelly A, Brown SL, Ivens AC, Travis MA, Cook PC, MacDonald AS. Dynamics of Colon Monocyte and Macrophage Activation During Colitis. *Front Immunol* 2018; **9**: 2764 [PMID: 30542349 DOI: 10.3389/fimmu.2018.02764]

46 **Thomson CA**, McColl A, Graham GJ, Cavanagh J. Sustained exposure to systemic endotoxin triggers chemokine induction in the brain followed by a rapid influx of leukocytes. *J Neuroinflammation* 2020; **17**: 94 [PMID: 32213184 DOI: 10.1186/s12974-020-01759-8]

47 **Sato N**, Garcia-Castillo V, Yuzawa M, Islam MA, Albarracin L, Tomokiyo M, Ikeda-Ohtsubo W, Garcia-Cancino A, Takahashi H, Villena J, Kitazawa H. Immunobiotic *Lactobacillus jensenii* TL2937 Alleviates Dextran Sodium Sulfate-Induced Colitis by Differentially Modulating the Transcriptomic Response of Intestinal Epithelial Cells. *Front Immunol* 2020; **11**: 2174 [PMID: 33042131 DOI: 10.3389/fimmu.2020.02174]

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ORIGINAL ARTICLE

Basic Study

Insight into molecular mechanisms underlying hepatic dysfunction in severe COVID-19 patients using systems biology

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Abstract

BACKGROUND

The coronavirus disease 2019 (COVID-19), a pandemic contributing to more than 105 million cases and more than 2.3 million deaths worldwide, was described to be frequently accompanied by extrapulmonary manifestations, including liver dysfunction. Liver dysfunction and elevated liver enzymes were observed in about 53% of COVID-19 patients.

AIM

To gain insight into transcriptional abnormalities in liver tissue of severe COVID-19 patients that may result in liver dysfunction.

METHODS

Sharjah Research Academy, No: MED001; and Al-Jalila Foundation Seed Grant, No. AJF202019.

Institutional review board statement: The committee has granted an ethical approval for the above mentioned request and following documents.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: All the transcriptomic data is shared in Gene Expression Omnibus with data sets GSE150316 and GSE112356. All other processed data are shared in the manuscript as tables or figures.

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The transcriptome of liver autopsy samples from severe COVID-19 patients against those of non-COVID donors was analyzed. Differentially expressed genes were identified from normalized RNA-seq data and analyzed for the enrichment of functional clusters and pathways. The differentially expressed genes were then compared against the genetic signatures of liver diseases including cirrhosis, fibrosis, non-alcoholic fatty liver disease (NAFLD), and hepatitis A/B/C. Gene expression of some differentially expressed genes was assessed in the blood samples of severe COVID-19 patients with liver dysfunction using qRT-PCR.

RESULTS

Analysis of the differential transcriptome of the liver tissue of severe COVID-19 patients revealed a significant upregulation of transcripts implicated in tissue remodeling including G-coupled protein receptors family genes, *DNAJB1, IGF2, EGFR,* and *HDGF*. Concordantly, the differential transcriptome of severe COVID-19 liver tissues substantially overlapped with the disease signature of liver diseases characterized with pathological tissue remodeling (liver cirrhosis, Fibrosis, NAFLD, and hepatitis A/B/C). Moreover, we observed a significant suppression of transcripts implicated in metabolic pathways as well as mitochondrial function, including cytochrome P450 family members, *ACAD11, CIDEB, GNMT,* and *GPAM*. Consequently, drug and xenobiotics metabolism pathways are significantly suppressed suggesting a decrease in liver detoxification capacity. In correspondence with the RNA-seq data analysis, we observed a significant upregulation of *DNAJB1* and *HSP90AB1* as well as significant downregulation of *CYP39A1* in the blood plasma of severe COVID-19 patients with liver dysfunction.

CONCLUSION

Severe COVID-19 patients appear to experience significant transcriptional shift that may ensue tissue remodeling, mitochondrial dysfunction and lower hepatic detoxification resulting in the clinically observed liver dysfunction.

Key Words: COVID-19; Hepatic dysfunction; Tissue remodeling; Metabolic pathways; Drug metabolism; Hepatic detoxification

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Core Tip: Liver dysfunction was frequently observed in severe coronavirus disease 2019 (COVID-19) patients. However, the mechanism through which severe acute respiratory syndrome coronavirus 2 potentially elicits liver function abnormality is not fully understood. We report a thorough analysis of changes occurring at the gene expression level in liver tissue of severe COVID-19 patients. Our findings suggest that severe COVID-19 patients may have a lower hepatic detoxification capacity and may experience liver tissue remodeling resulting in liver dysfunction.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic resulted in 105 million coronavirus cases and more than 43 million coronavirus cases and more than 2.3 million deaths worldwide to date[1]. The presentation of COVID-19 varies in severity degree ranging from asymptomatic or mildly symptomatic to severe disease with pulmonary and extrapulmonary findings (*e.g.*, hepatic function impairment, kidney failure and heart failure)[2]. Multiple studies reported elevated levels of liver enzymes (*e.g.*

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aminotransferases and bilirubin) in up to 53% of COVID-19 patients[3], and others reported cases of acute liver injury and liver failure in response to syndrome coronavirus 2 (SARS-CoV-2) infection[2,4,5], Therefore, in consideration of the continuously rising number of COVID-19 cases worldwide, COVID-19 has contributed to a significant increase in the incidence of liver dysfunction globally.

The incidence of liver dysfunction in COVID-19 patients was found to correlate with the severity of the disease, with a higher prevalence in severe COVID-19 cases than non-severe COVID-19 cases and requiring the administration of liver protective drugs[6]. Moreover, therapeutic agents administered to COVID-19 patients were shown to independently correlate with increased elevation of aminotransferases levels indicating dysregulated hepatic drug metabolism and consequent hepatotoxicity[7]. Therefore, understanding the mechanism through which SARS-CoV-2 infection results in hepatic dysfunction can contribute to patient management and prognosis.

The observed hepatic dysfunction was speculated to be either a result of liver injury caused by direct effect of the virus, organ injury induced by drugs used to treat COVID-19 patients, cytokine storm consequence, or sequelae of multi organ damage caused by severe disease[8]. However, the mechanism through which SARS-CoV-2 potentially elicits liver function abnormality is yet to be clearly understood. Therefore, the main aim of this study is to gain some insight into the molecular mechanisms employed by SARS-CoV-2 infection to induce liver dysfunction. In this study, we analyzed the transcriptome of liver autopsy samples from severe COVID-19 patients to identify significantly affected cellular pathways and processes.

MATERIALS AND METHODS

RNA-seq Datasets retrieval

Datasets were retrieved from Gene Expression Omnibus for lung and liver autopsy samples from 12 patients severely infected with SARS-CoV-2 (GSE150316) (42 lung samples and 6 liver samples; Table 1), and lung and liver autopsy samples from 4 non-infected Caucasian male donors (GSE112356) (4 lung samples and 4 liver samples; Table 1). The COVID-19 autopsy samples were reported by the authors depositing the dataset[9] to be from donors with severe COVID-19 symptoms. The patients were hospitalized, required mechanical ventilation (9 out of 12 cases), and presented with elevated levels of D-Dimer (Cases 1-10 and 12), CRP (Cases 1-10 and 12), and AST (Cases 2-10 and 12).

Bioinformatics analysis and identification of differentially expressed genes

The general workflow is summarized in Figure 1. Initially, raw gene counts data were retrieved from the deposited datasets and the successfully mapped genes overlapping between the samples from both datasets (28131 genes) were filtered and normalized using the quantile normalization function in AltAnalyze software[10]. Differentially expressed genes (DEGs) were identified by comparing each SARS-CoV-2 infected tissue against a healthy control tissue according to cutoff values of > 2 or < -2 for the fold change and 0.25 for the adjusted *P* value (q-value), based on the *P* value cut-off described by Li *et al*[11] and Subramanian *et al*[12]. Commonly upregulated and downregulated DEGs between infected lung and liver tissues were intersected and identified using InteractiVenn[13].

In silico functional analysis

Functional clustering and pathways enrichment analysis of DEGs was performed using Metascape[14]. We further validated the significance of the differential expression of some DEGs by conducting two-tailed Student's *t* test statistical analysis using GraphPad Prism.

The transcriptomic signature that map to liver cirrhosis (C0023890, C0023893, C0156189, C0400943), liver fibrosis (C0239946), non-alcoholic fatty liver disease (NAFLD) (C0400966), hepatitis A (C0019159 and C0276434), hepatitis B (C0019163, C0524909, C4728019, and C0276609), and hepatitis C (C0019196, C0524910, C3872662, and C0400914) were retrieved from DisGeNET[15]. The signatures were then compared against the upregulated transcriptome of SARS-CoV-2 infected liver tissue samples to identify overlapping transcripts.

Quantitative real-time PCR (qRT-PCR) analysis of gene expression

Blood Serum was isolated from fresh blood samples collected from 4 healthy donors, 4

Table 1 List of analyzed non-coronavirus disease 2019 and severe coronavirus disease 2019 lung and liver autopsy samples retrieved from the datasets GSE112356 and GSE150316 deposited in Gene Expression Omnibus

Tissue source	Non-COVID-19 autopsy samples	COVID-19 autopsy samples	Total number of analyzed samples
Lung	H1Lu; H2Lu; H3Lu; H4Lu	Case1: Samples 1, 2, 3, 4; Case2: Samples 1, 2, 3; Case3: Samples 1, 2; Case4: Samples 1, 2; Case5: Samples 1, 2, 3, 4, 5; Case6: Samples 1, 2, 3, 4, 5; Case7: Samples 1, 2, 3, 4, 5; Case8: Samples 1, 2, 3, 4, 5; Case9: Samples 1, 2, 3, 4, 5; Case10: Samples 1, 2, 3; Case11: Samples 1, 2, 3	4 healthy samples and 42 COVID-19 samples
Liver	H1Li; H2Li; H3Li; H4Li	Case3: Sample 1; Case4: Sample 1; Case5: Sample 1; Case8: Sample 1; Case10: Sample 1; Case12: Sample1	4 healthy samples and 6 COVID-19 samples

COVID-19: Coronavirus disease 2019.

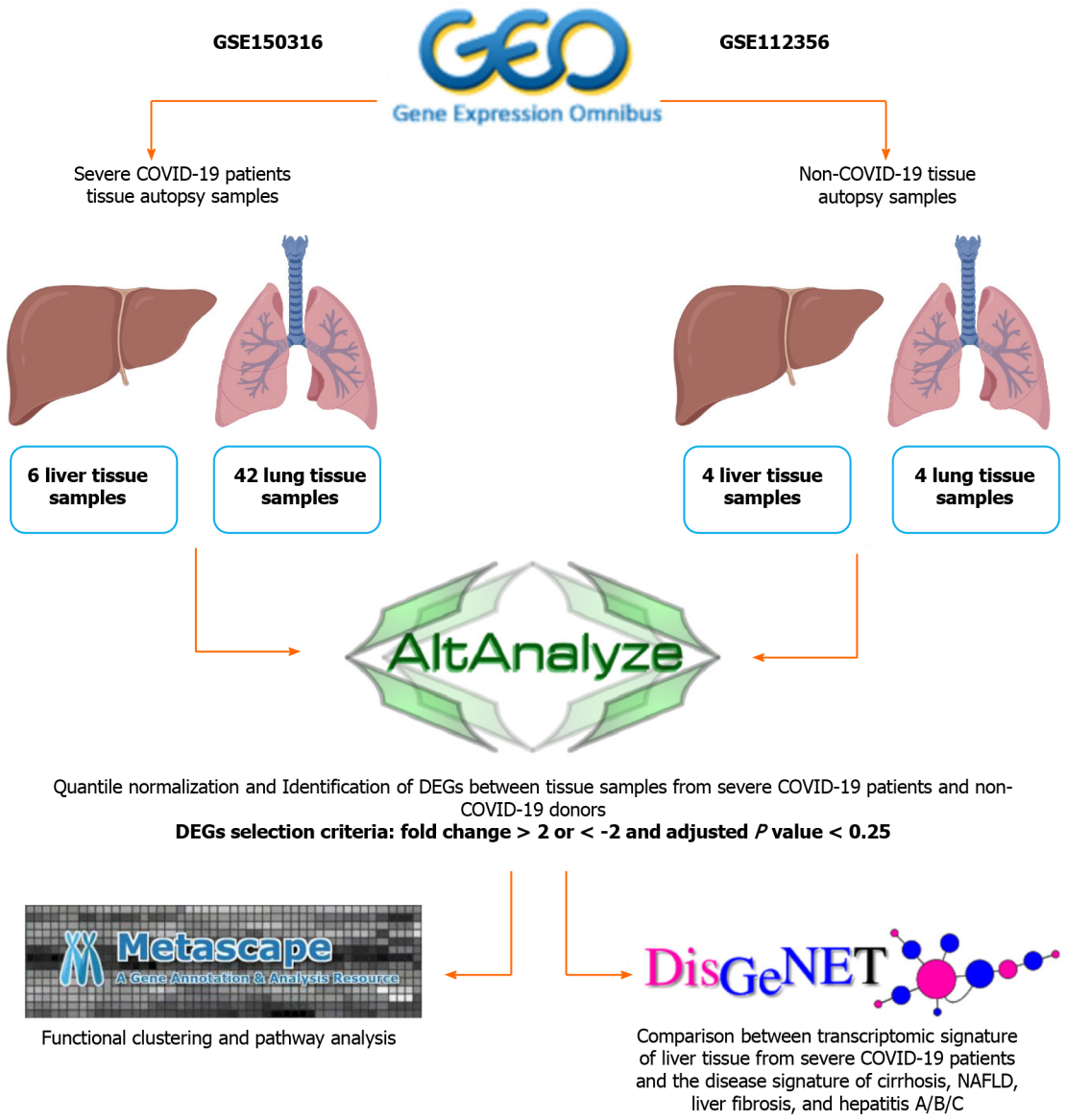


Figure 1 General Workflow of the in-silico analysis of the coronavirus disease 2019 and healthy tissue autopsy samples RNA-seq data.
Figure was created with BioRender.com. DEGs: Differentially expressed genes; DisGeNET: Disease Gene Network; NAFLD: Non-alcoholic fatty liver disease.

severe COVID-19 patients without liver dysfunction, and 9 severe COVID-19 patients with liver dysfunction (elevated alanine aminotransferase levels: > 41 U/L). The samples were collected following the approval of the ethical committee by Dubai Scientific Research Ethics Committee (DSREC-04/2020_09). Histopaque gradient

separation (Sigma) was used to extract blood plasma and the RNA content was extracted from 300 μl of plasma using QIAamp Viral RNA Mini Kit (Qiagen). cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit for RT-PCR (applied Biosystems) and qRT-PCR was performed using about 50 ng of cDNA in triplicates with the Maxima SYBR Green/ROX qPCR Master Mix (Thermoscientific) using QuantStudio3 real-time PCR instrument (applied biosystems). qRT-PCR was performed using primers for *18SrRNA, DNAJB1, HSP90A1* and *CYP39A1* as per the sequences in Table 2.

Statistical analysis

Unpaired, two-tailed *t* test statistical analysis was performed using GraphPad Prism (version 5.01) to analyze the statistical significance of the gene expression. The significance was taken to be $P < 0.05$. Heatmap representations were generated using R (version 3.6.0); boxplot representations were generated using GraphPad Prism (version 5.01).

RESULTS

General effect of SARS-CoV-2 infection on the transcriptome liver and lung tissues

Initially, we aimed at investigating the general transcriptomic changes in the liver tissues of severe COVID-19 patients in comparison to those of lung tissues. Our analysis of the differential transcriptome of the two tissue types revealed the dysregulation of a larger number of genes in liver tissue (8686 genes) in comparison to lung tissue (7349 genes) (Figure 2). 2085 genes were commonly differentially expressed in the two types of tissues (Figure 2A). Functional clustering and pathway analysis of the commonly upregulated transcripts revealed their contribution to transmembrane transport and vesicle formation, cell adhesions, tissue morphogenesis, development, and intracellular signaling pathways [*e.g.* G-coupled protein receptor (GPCR) and Hippo signaling] (Figure 2B). On the other hand, the commonly downregulated genes (2264 genes) were significantly implicated in cellular metabolic pathways and mitochondrial function (Figure 2C and D). The enrichment of these pathways suggests that SARS-CoV-2 elicits similar molecular effects in lung and liver tissue that may translate into similar phenotypic presentations (*e.g.* tissue remodeling and dysfunction). As the number of the lung tissue samples used in the analysis was relatively higher than that of the liver tissue samples, we repeated the analysis and comparison using subset with a smaller number of lung tissue samples and observed similar results.

Aberrant class A (rhodopsin-like) and class F (frizzled and smoothened) GPCRs signaling in the liver tissue of severe COVID-19 patients

We next aimed at analyzing the effect of SARS-CoV-2 infection on the dysregulation of liver function at advanced stages in more detail. Functional clustering and pathway analysis of the upregulated genes in the liver tissues of severe COVID-19 patients revealed the enrichment of transcripts implicated in DNA and RNA transcription mechanisms, GPCRs signaling, and transmembrane transport (Figure 3A).

Multiple GPCR classes are dysregulated in liver tissue collected from severe COVID-19 patient autopsies including class A-rhodopsin-like receptors (R-HSA-373076: class A/1 rhodopsin-like receptors) and class F-frizzled and smoothened receptors (R-HSA-3238698: WNT ligand biogenesis and trafficking; hsa04310: Wnt signaling pathway) (Figure 3B and C). Consequently to the dysregulation of these GPCRs, numerous downstream signaling pathways, including WNT signaling pathway, sonic hedgehog signaling and hippo signaling pathways, are dysregulated affecting thereby cell proliferation, cell survival, host response to infection, immune response, chemotactic pathways, and tissue and local microenvironment remodeling [16-19]. Moreover, Wnt signaling pathway through frizzled binding has been shown to be hijacked by viral infections (*e.g.*, influenza virus) to increase viral production[20]. GPCRs were found as well to be exploited by viruses (*e.g.*, Filoviruses) to facilitate viral cellular entry, they can be explored as potential facilitators of SARS-CoV-2 entry into hepatocytes in the absence of ACE2 and TMPRSS2[21]. Therefore, the significant dysregulation of GPCRs and Wnt signaling, coupled with the significant upregulation of transmembrane and intracellular transport, in liver tissue of severe COVID-19 patients may indicate that SARS-CoV-2 may hijack these mechanisms to facilitate and enhance viral infectivity and production.

Table 2 List of primer designs used in the qRT-PCR validation of some differentially expressed genes (DNAJB1, HSP90AB1, and CYP39A1) and housekeeping gene (18SrRNA)

Gene symbol	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
18SrRNA	TGACTCAACACGGGAAACC	TCGCTCCACCAACTAAGAAC	114
DNAJB1	GTTTTAAAGGACAAGCCCCACA	TCCAGAGTGGGGACGTTCA	119
HSP90AB1	CTCTGTCAGAGTATGTTTCTCGC	GTTTCCGCACTCGCTCCACAAA	114
CYP39A1	GTTCCAGTGTCCTGCAAGGT	TGGGTAATGGGTCCAGAAGAC	101

bp: base pair; *18SrRNA*: 18S ribosomal RNA; *DNAJB1*: DnaJ heat shock protein family (Hsp40) member B1; *HSP90AB1*: Heat shock protein 90 alpha family class B member 1; *CYP39A1*: Cytochrome P450 family 39 subfamily A member 1.

One of the top 10 upregulated genes in the liver tissue of severe COVID-19 patients was the DnaJ heat shock protein family (Hsp40) member B1, *DNAJB1* (Figure 3D), which was found to contribute to liver inflammation, activation of uncontrolled fibrosis, and development of fibrolamellar carcinoma as a part of the DNAJB1-PRKACA fusion[22]. Another top upregulated gene was insulin like growth factor 2 (*IGF2*), a pro-steatosis and tissue remodeling factor[23]. Hepatoma-derived growth factor, *HDGF*, a pro-fibrogenic protein implicated in liver fibrosis through TGF-beta pathway, was as well amongst the top 10 upregulated genes[24]. Another significantly upregulated gene was the epidermal growth factor receptor (*EGFR*), a transmembrane receptor implicated in the regulation of various downstream intracellular signaling pathways including tissue remodeling pathways[25,26]. The upregulation of these genes suggests that SARS-CoV-2 infection may elicit tissue remodeling and fibrosis-like changes in liver tissue at advanced stages.

SARS-CoV-2 infection activates tissue remodeling mechanisms observed in liver steatosis, cirrhosis, fibrosis, and hepatitis infections

Based on the enrichment of transcripts implicated in pathways central to the activation and regulation of tissue remodeling, we next compared the differential transcriptome of the liver tissues from COVID-19 patients with the disease signature of liver cirrhosis, liver fibrosis, and NAFLD, which are commonly associated with pathological tissue remodeling.

The comparison revealed that 1027 genes (318 upregulated and 709 downregulated) from the upregulated transcriptome of COVID-19 patients' liver samples overlap with liver cirrhosis disease signature; 630 genes (206 upregulated and 424 downregulated) overlap with the signature of liver fibrosis; and 583 genes (190 upregulated and 393 downregulated) overlap with the signature of NAFLD (top 20 genes overlapping with every disease signature are presented in Figure 4A).

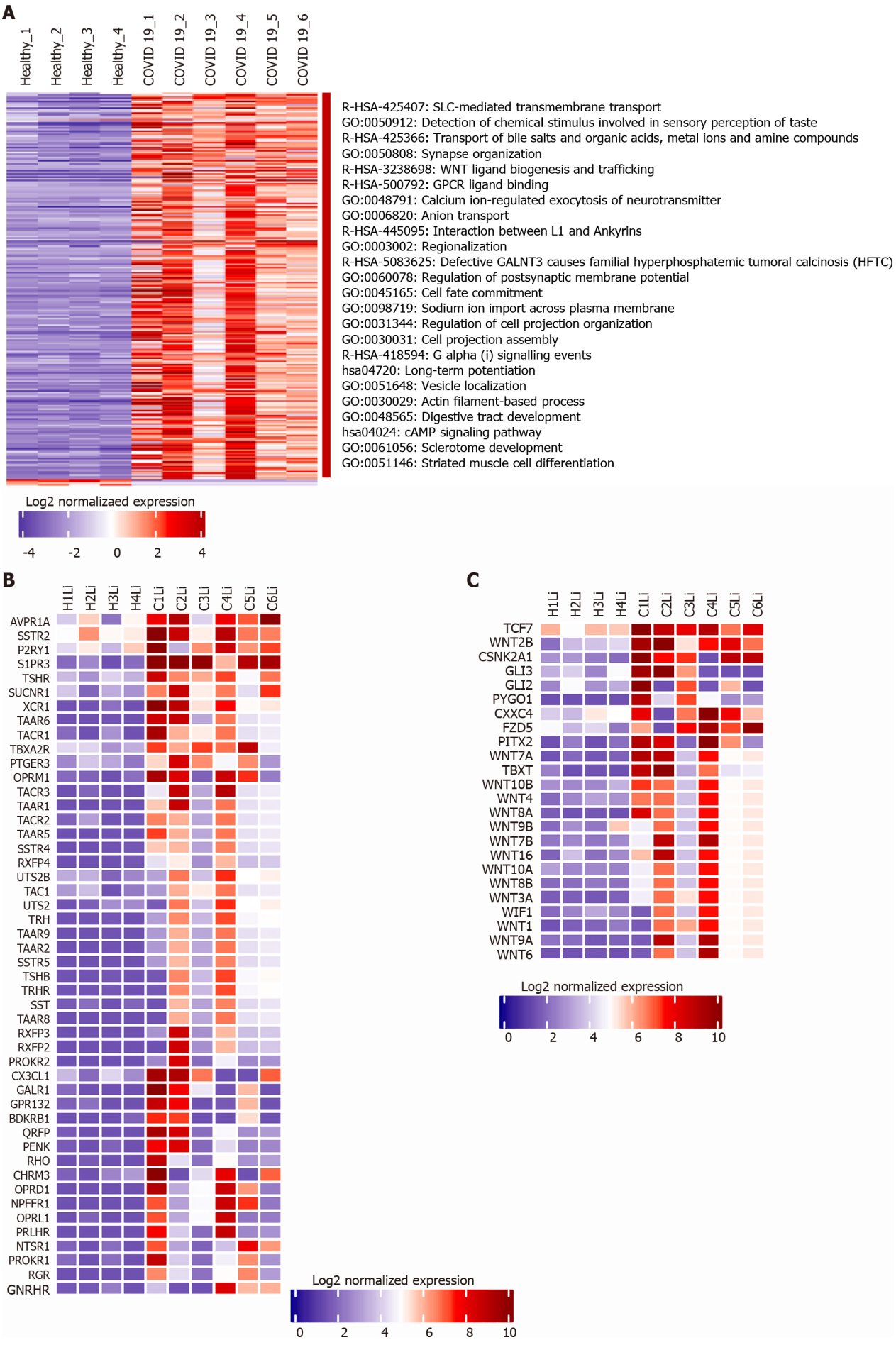
The transcriptome shared with the liver cirrhosis, liver fibrosis and NAFLD signature was significantly enriched in transcripts implicated in response to wounding and wound healing, cellular response to external stimulus (*e.g.*, peptides, inorganic substance, growth factors, and hormone stimulus), response to pathogens from bacterial and viral origins (*e.g.*, human papillomavirus infection, response to lipopolysaccharides), metabolic pathways, hormone and insulin mediated signaling pathways, cytokine-mediated signaling, MAPK signaling cascade, regulation of cell adhesions, regulation of cell differentiation, tissue/organ development and morphogenesis, blood vessels morphogenesis and development, regulation of programmed cell death, cell chemotaxis (*e.g.*, leukocytes, endothelial cells, and epithelial cells), and immune response activation (Figure 4B).

One of the identified overlapping genes with the signature of liver cirrhosis and liver fibrosis was the vascular endothelial growth factor receptor 1 (*FLT1*), shown to be significantly upregulated during the pathogenesis of liver cirrhosis in which it centrally contributes to the tissue remodeling and increased vascularization of liver tissue[27]. Another overlapping gene with the signatures of the three diseases was leptin (*LEP*), a central mediator of hepatic fibrosis and tissue remodeling in response to chronic liver injury[28]. PNPLA3, overexpressed in the liver tissue of COVID-19 patients, has been previously shown to be implicated in the pathogenesis of non-alcoholic fatty liver disease, hepatic steatosis, liver cirrhosis and fibrosis[29].

Moreover, we observed the upregulation of a number of metalloproteinases (*e.g.*, *MMP3, MMP16, MMP17, TIMP1, TIMP2*, and *TIMP4*), collagens (*COL6A3, COL18A1-AS1, COL20A1, COL24A1, COLEC12, COL13A1, COL22A1, COLGALT2*), and *VCAM1*



Figure 2 General overview of the commonly dysregulated transcriptome in lung and liver tissues in severe coronavirus disease 2019 patients. A and B: Venn diagram representation of the overlapping (A) upregulated and (C) downregulated genes in response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infection in lung and liver tissues; C and D: Top 20 pathways and functional clusters enriched in the commonly (B) upregulated and (D) downregulated genes in SARS-CoV-2 infected lung and liver tissues.


A
Healthy_1
Healthy_2
Healthy_3
Healthy_4
COVID 19_1
COVID 19_2
COVID 19_3
COVID 19_4
COVID 19_5
COVID 19_6
R-HSA-425407: SLC-mediated transmembrane transport
GO:0050912: Detection of chemical stimulus involved in sensory perception of taste
R-HSA-425366: Transport of bile salts and organic acids, metal ions and amine compounds
GO:0050808: Synapse organization
R-HSA-3238698: WNT ligand biogenesis and trafficking
R-HSA-500792: GPCR ligand binding
GO:0048791: Calcium ion-regulated exocytosis of neurotransmitter
GO:0006820: Anion transport
R-HSA-445095: Interaction between L1 and Ankyrins
GO:0003002: Regionalization
R-HSA-5083625: Defective GALNT3 causes familial hyperphosphatemic tumoral calcinosis (HFTC)
GO:0060078: Regulation of postsynaptic membrane potential
GO:0045165: Cell fate commitment
GO:0098719: Sodium ion import across plasma membrane
GO:0031344: Regulation of cell projection organization
GO:0030031: Cell projection assembly
R-HSA-418594: G alpha (i) signalling events
hsa04720: Long-term potentiation
GO:0051648: Vesicle localization
GO:0030029: Actin filament-based process
GO:0048565: Digestive tract development
hsa04024: cAMP signaling pathway
GO:0061056: Sclerotome development
GO:0051146: Striated muscle cell differentiation
Log2 normalizaed expression
-4
-2
0
2
4
B
H1Li
H2Li
H3Li
H4Li
C1Li
C2Li
C3Li
C4Li
C5Li
C6Li
AVPR1A
SSTR2
P2RY1
S1PR3
TSHR
SUCNR1
XCR1
TAAR6
TACR1
TBXA2R
PTGER3
OPRM1
TACR3
TAAR1
TACR2
TAAR5
SSTR4
RXFP4
UTS2B
TAC1
UTS2
TRH
TAAR9
TAAR2
SSTR5
TSHB
TRHR
SST
TAAR8
RXFP3
RXFP2
PROKR2
CX3CL1
GALR1
GPR132
BDKRB1
QRFP
PENK
RHO
CHRM3
OPRD1
NPFFR1
OPRL1
PRLHR
NTSR1
PROKR1
RGR
GNRHR
Log2 normalized expression
0
2
4
6
8
10
C
H1Li
H2Li
H3Li
H4Li
C1Li
C2Li
C3Li
C4Li
C5Li
C6Li
TCF7
WNT2B
CSNK2A1
GLI3
GLI2
PYGO1
CXXC4
FZD5
PITX2
WNT7A
TBXT
WNT10B
WNT4
WNT8A
WNT9B
WNT7B
WNT16
WNT10A
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WIF1
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Log2 normalized expression
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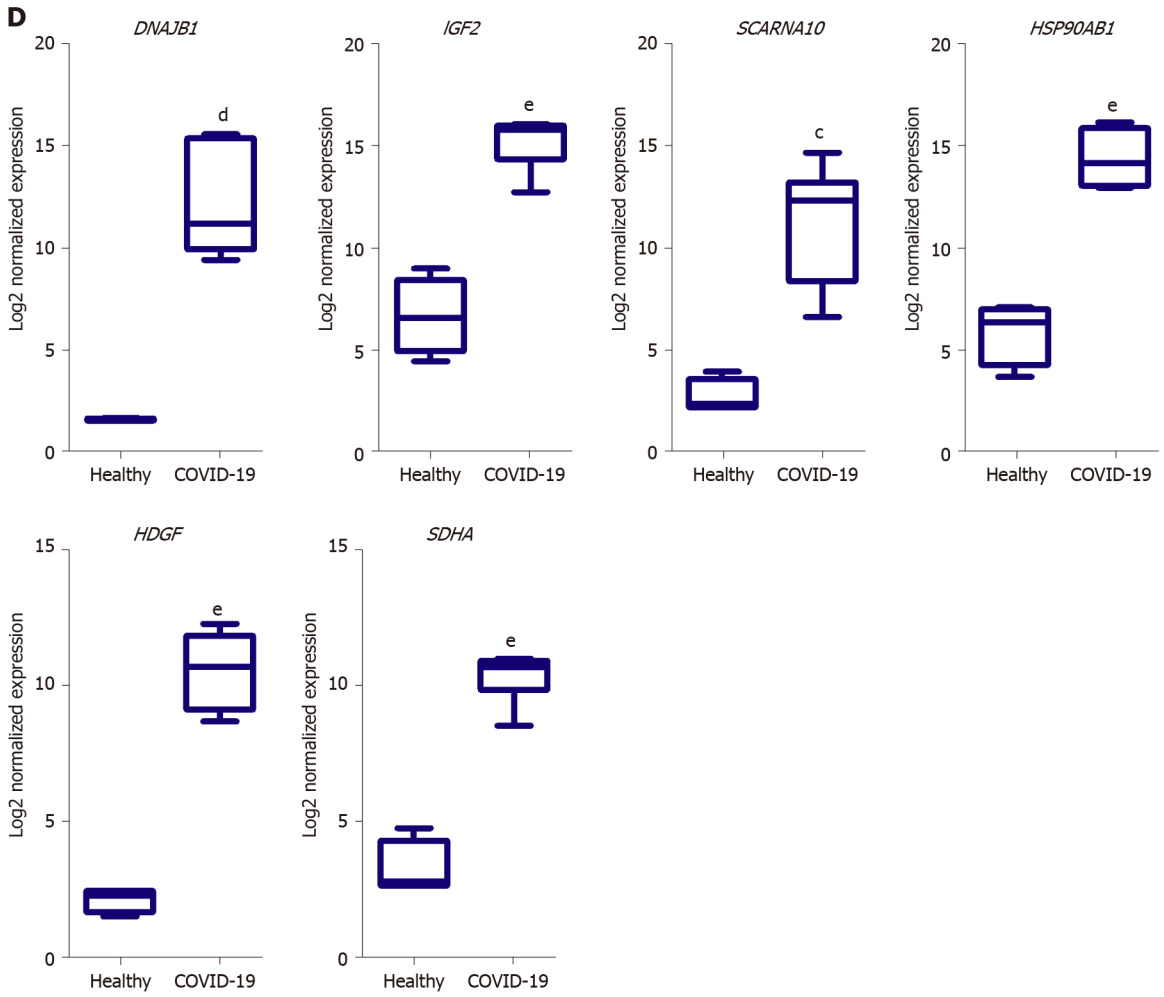


Figure 3 functional clustering analysis of the upregulated transcriptome of severe coronavirus disease 2019 patients liver autopsy tissues. A: Heatmap representation of the differentially upregulated genes and subsequently enriched pathways and functional clusters in severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infected liver samples in comparison to baseline expression in healthy tissue samples; B and C: Heatmap representation of the log2 of normalized expression of transcripts implicated signaling through (B) Class A-rhodopsin-like receptors and (C) Class F-frizzled and smoothened receptors; D: Box plot representation of some of the top upregulated transcripts in SARS-CoV-2 infected liver tissue samples. Data presented as mean ± SE; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0005$, $^{e}P < 0.0001$ coronavirus disease 2019 (COVID-19) *vs* Healthy. *DNAJB1*: DnaJ heat shock protein family (hsp40) member b1; *IGF2*: Insulin growth factor 2; *SCARNA10*: Small cajal body-specific rna 10; *HSP90AB1*: Heat shock protein 90 alpha family class B member 1; *HDGF*: Hepatoma-derived growth factor; *SDHA*: Succinate dehydrogenase complex flavoprotein subunit A.

which were proposed to contribute to pathological tissue remodeling in liver diseases (*e.g.* non-alcoholic fatty liver disease and liver fibrosis)[30]. Furthermore, we observed a significant upregulation in the expression of *TREM2* which was shown to be a protective mechanism activated in response to liver damage (*e.g.*, immune-mediated hepatocellular damage and hepatotoxic injuries) (acts as a natural brake on inflammation during hepatocellular injury)[31]; which supports the potential occurrence of liver inflammation and injury in response to SARS-CoV-2 infection. Altogether, these results suggest the potential activation of tissue remodeling pathways (*e.g.*, fibrosis, increased vascularization, and steatosis) in the liver tissue of severe COVID-19 patients at advanced stages in response to liver injury and inflammation that may result in cirrhosis/fibrosis-like phenotype.

We then compared the differential transcriptome of liver samples from severe COVID-19 patients with the disease signature of hepatitis A, hepatitis B and hepatitis C infection. We observed the overlap of 84 genes from the upregulated transcriptome of COVID-19 liver samples overlap with hepatitis A infection signature; 278 genes overlap with the signature of hepatitis B infection; and 336 genes overlap with the signature of hepatitis C infection (top overlapping genes are presented in Figure 5A). The overlapping signatures between the COVID-19 samples and the three types of hepatitis infections were enriched in transcripts contributing to response to infection, activation of immune response mechanisms, cytokines-mediated signaling, tissue remodeling and homeostasis (Figure 5B).

A

Liver cirrhosis			Liver fibrosis			Non-alcoholic fatty liver disease		
Gene	GDA score	F.C.	Gene	GDA score	F.C.	Gene	GDA score	F.C.
FLT1	0.51	6.02	GLIS3	0.42	4.88	PNPLA3	0.5	4.21
TGFB1	0.5	4.28	TGFB1	0.4	4.28	LEP	0.4	5.33
LEP	0.5	5.33	PTEN	0.38	3.27	TGFB1	0.34	4.28
NOS2	0.5	12.02	FGF7	0.32	14.13	PRKCA	0.33	6.03
TGFB1	0.4	4.28	THBS1	0.31	5.40	PTEN	0.32	3.27
KRT8	0.34	20.56	SLC22A8	0.3	4.94	PRKACA	0.3	2.79
FGF7	0.32	14.13	FLT1	0.3	6.02	RDX	0.3	4.70
PTEN	0.31	3.27	CHRM3	0.3	8.93	SERPINB2	0.3	5.61
THBS1	0.31	5.40	TNFRSF1B	0.3	9.12	STC2	0.3	6.23
EIF4E3	0.3	2.07	KRT8	0.3	20.56	B3GAT1	0.3	8.35
ARHGAP30	0.3	2.38	INPP5E	0.11	4.99	TNFRSF1B	0.3	9.12
ITGAL	0.3	2.41	TNFSF15	0.11	45.20	NCAN	0.18	7.85
SGCB	0.3	2.62	WDPCP	0.1	3.14	PLPP7	0.1	4.35
ZDHHC13	0.3	2.67	PNPLA3	0.1	4.21	PRKAA2	0.1	5.12
TCF20	0.3	2.87	LEP	0.1	5.33	PIK3CG	0.1	5.93
SOX4	0.3	3.01	PIK3CG	0.1	5.93	GLP1R	0.1	9.14
CD163	0.3	3.04	SMAD2	0.1	7.65	TP63	0.1	10.99
SP140	0.3	3.06	POU2AF1	0.1	10.37	SIRT3	0.08	2.84
KANK1	0.3	3.15	TCF4	0.1	15.99	UCP2	0.07	3.47
SPSB4	0.3	3.15	MAPK1	0.07	3.40	MTHFR	0.06	4.36

B

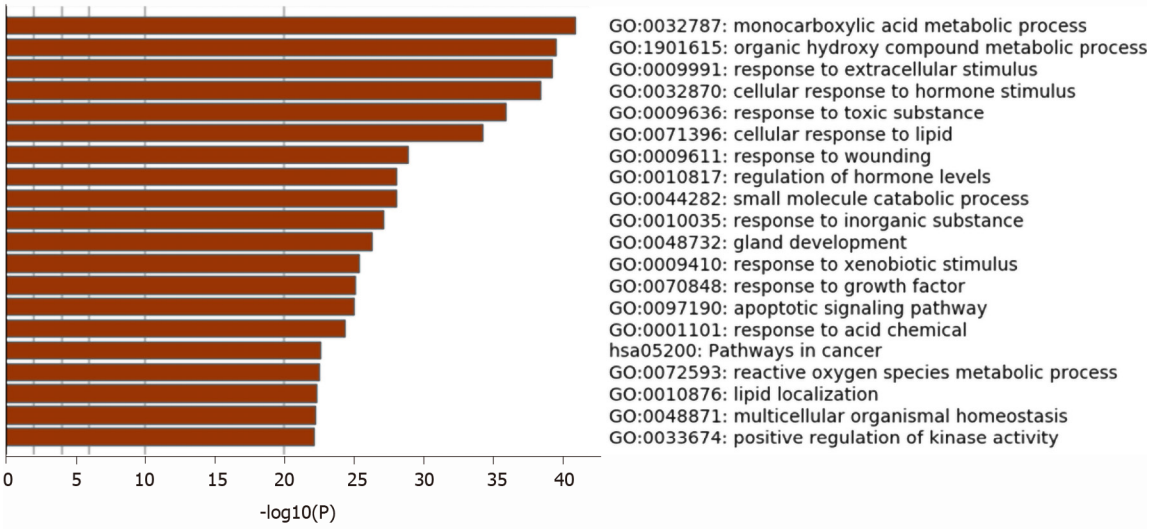


Figure 4 Comparative analysis of the overlapping transcriptomes mapping to severe coronavirus disease 2019 liver tissue, liver cirrhosis, liver fibrosis, and non-alcoholic fatty liver disease. A: Top 20 Genes overlapping between the upregulated transcriptome of severe coronavirus disease 2019 (COVID-19) patients' liver tissue samples and the signatures of liver cirrhosis, liver fibrosis, and non-alcoholic fatty liver disease retrieved from DisGeNET; gene disease association score was retrieved from DisGeNET and fold change is calculated using AltAnalyze to represent the difference in expression in COVID-19 liver tissue samples *vs* non-COVID-19 liver samples; B: functional clustering and pathway analysis of the overlapping transcriptome (upregulated and downregulated) of the severe COVID-19 patients' liver tissues with the disease signature of liver cirrhosis, liver fibrosis, and non-alcoholic fatty liver disease analyzed using Metascape. GDA score: Gene disease association score; F.C.: Fold change.

Dysregulation of mitochondrial function, metabolic and biosynthetic pathways in the liver tissue of patients with severe COVID-19

Analysis of the downregulated genes in the liver tissues of severe COVID-19 patients revealed a significant suppression of various metabolic and biosynthetic pathways including the metabolism of amines, aromatic compounds, amino acids, steroids, glycoproteins, lipids, and flavones (Figure 6A). The suppression of these metabolic pathways is concordant with the dysregulation of mitochondrial function reflected by the suppression of oxidation-reduction process, electron transport and respiratory chain, and the assembly of mitochondrial structures. These findings are consistent

A

Hepatitis A			Hepatitis B			Hepatitis C		
Gene	GDA score	F.C.	Gene	GDA score	F.C.	Gene	GDA score	F.C.
TGFB1	0.07	4.28	HP	0.35	6.63	HP	0.31	6.63
HAVCR2	0.04	6.73	HLA-B	0.17	9.68	OPRM1	0.3	20.46
SOCS3	0.04	65.79	KIF1B	0.16	2.65	PAEP	0.1	2.22
PAEP	0.03	2.22	PNPLA3	0.14	4.21	TLR7	0.1	3.13
TLR9	0.03	2.85	TRIM31	0.11	9.02	MAPK1	0.1	3.40
MAVS	0.03	5.54	PAEP	0.1	2.22	PNPLA3	0.1	4.21
NTSR1	0.03	9.41	TLR7	0.1	3.13	TGFB1	0.1	4.28
TLR7	0.02	3.13	TGFB1	0.1	4.28	OXTR	0.1	4.61
RIT2	0.02	5.67	HAVCR2	0.1	6.73	MAVS	0.1	5.54
TNFAIP8L2	0.02	6.14	TERT	0.1	10.87	RIT2	0.1	5.67
SLCO6A1	0.02	6.97	IGF2	0.1	370.76	PIK3CG	0.1	5.93
NOS2	0.02	12.02	TLR9	0.09	2.85	SERPINB6	0.1	6.04
TNFRSF9	0.02	12.24	VDR	0.09	51.17	SPATA16	0.1	6.51
EMB	0.02	16.72	TGFB1	0.08	4.28	BRD4	0.1	8.12
TRIM21	0.01	2.09	SLCO6A1	0.08	6.97	EGFR	0.1	8.29
IRS1	0.01	2.25	AR	0.08	8.16	EIF2AK2	0.1	8.76
ITGAL	0.01	2.41	PTEN	0.07	3.27	HLA-B	0.1	9.68
KIF1B	0.01	2.65	SLC26A5	0.07	3.33	AGO2	0.1	9.88
TBX21	0.01	2.84	MDM2	0.07	11.14	SDK2	0.1	11.14
MAPK1	0.01	3.40	SOCS3	0.06	65.79	PTPN11	0.1	24.40

B

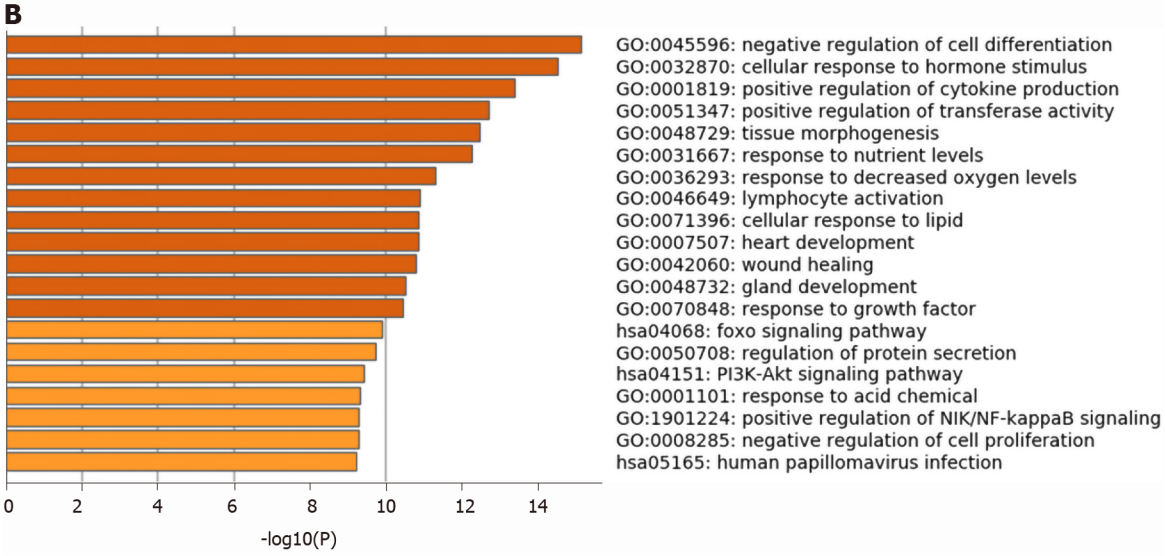


Figure 5 Comparative analysis of the overlapping transcriptomes mapping to severe coronavirus disease 2019 liver tissue, hepatitis A, hepatitis B, and hepatitis C. A: Table representing the genes overlapping between the upregulated signature of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infected liver tissue samples and the signatures of hepatitis A, B, and C retrieved from DisGeNET; gene disease association score was retrieved from DisGeNET and fold change is calculated using AltAnalyze to represent the difference in expression in COVID-19 liver tissue samples *vs* non-COVID-19 liver samples; B: Top 20 pathways and functional clusters enriched in the commonly upregulated genes between SARS-CoV-2 infected liver tissues and hepatitis A, B, and C disease signatures analyzed using Metascape. GDA score: Gene disease association score; F.C.: Fold change.

with previously published data on the accumulation of amino acids and steroids in the sera of COVID-19 patients in consequence to the suppression of lipid and amino acid metabolism[32]. These data show that metabolic pathways are potentially hijacked in severe COVID-19 patients' liver tissue through the dysregulating of mitochondrial function. Analysis of key transcriptional regulators of the dysregulated gene implicated in mitochondrial function using TRRUST database suggested *SP1*, *GATA6*, *SP3*, *HNF4A*, and *SMAD3* as candidates. *SP1* has been previously implicated in pathogenesis of diseases characterized by mitochondrial dysfunction such as schizophrenia[33].

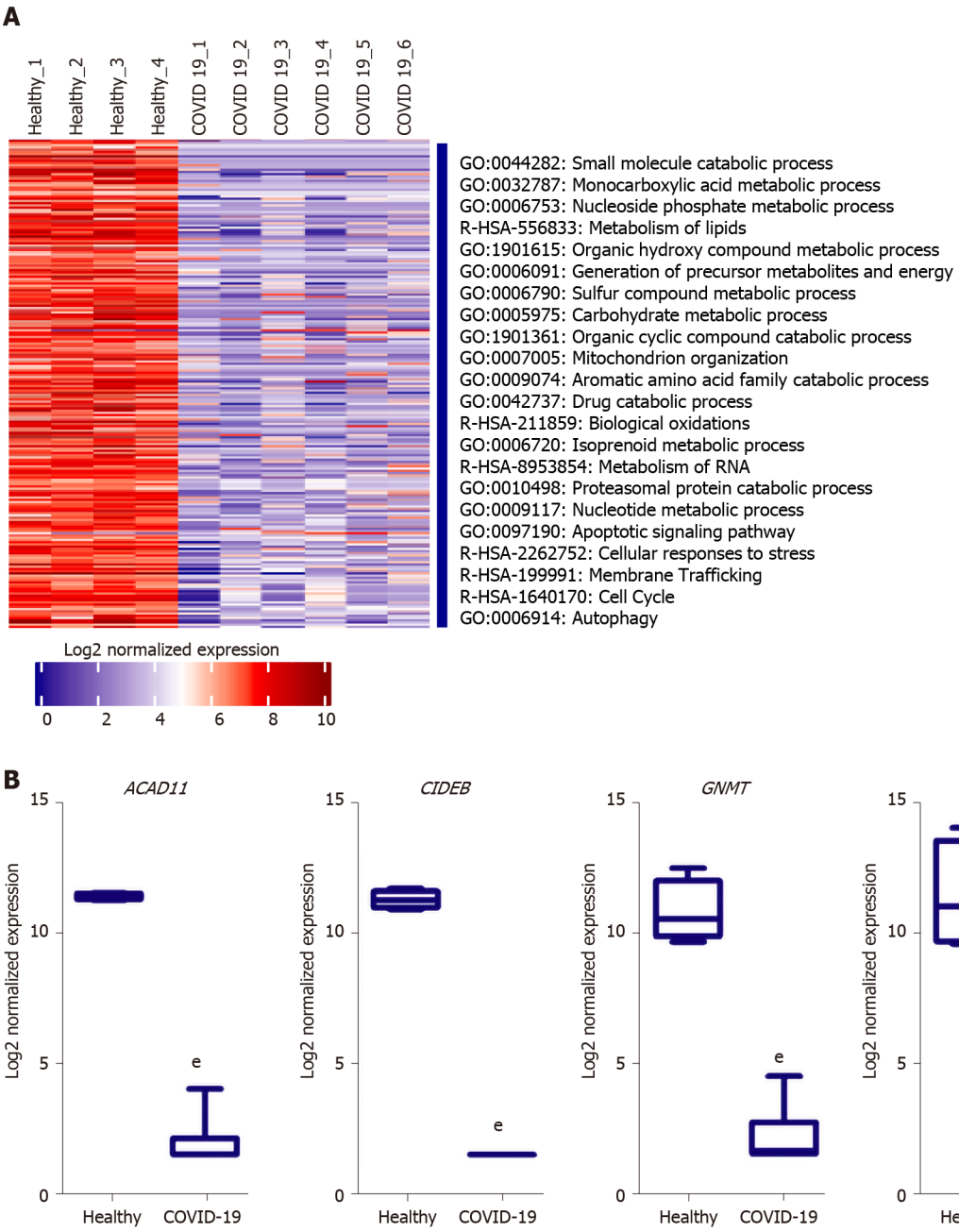


Figure 6 functional clustering analysis of the downregulated transcriptome of severe coronavirus disease 2019 patients liver autopsy tissues. A: Heatmap representation of the differentially downregulated genes and subsequently enriched pathways and functional clusters in severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infected liver samples in comparison to baseline expression in healthy tissue samples; B: box plot representation of some of the top downregulated transcripts in SARS-CoV-2 infected liver tissue samples. Data presented as mean ± SE; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0005$, $^{e}P < 0.0001$ COVID-19 *vs* Healthy. *ACAD11*: acyl-CoA dehydrogenases 11; *CIDEB*: cell death-inducing DNA fragmentation factor alpha-like effector B; *GNMT*: glycine N-methyltransferase; *GPAM*: glycerol-3-phosphate acyltransferase and metabolism.

One of the top downregulated genes was acyl-CoA dehydrogenases 11 (*ACAD11*) (Figure 6B), a mitochondrial flavoenzyme involved in mitochondrial β-oxidation and metabolism of long chain fatty acyl-CoAs[34]. Another top downregulated gene was the cell death-inducing DNA fragmentation factor alpha-like effector B (*CIDEB*), a liver-specific regulator of lipid droplet dynamics and lipids metabolism[35]. Glycine N-methyltransferase (*GNMT*), another top downregulated gene in SARS-CoV-2 infected liver samples, was found to contribute to the development of liver steatosis and fibrosis when suppressed due to the metabolic dysregulation and accumulation of S-adenosylmethionine[36]. Glycerol-3-phosphate acyltransferase and metabolism (*GPAM*), another of the top 10 downregulated genes, is implicated triglyceride biosynthesis[37]. Moreover, 17β-hydroxysteroid dehydrogenase type 4 (*HSD17B4*), a regulator of fatty acid β-oxidation and steroid metabolism[38], was downregulated. Altogether, the downregulation of these genes suggests metabolic and biosynthetic

pathways are suppressed in the liver tissue of severe COVID-19 patients.

Moreover, our analysis showed a significant enrichment of downregulated transcripts implicated in iron, copper, sodium, potassium, zinc, and calcium binding, transport, homeostasis and signaling. Imbalance of some of these ions (*e.g.* calcium and potassium ions) has been shown to induce mitochondrial swelling through shifting osmotic pressure[39].

COVID-19 inhibits drug metabolism potentially resulting in potential hepatotoxicity

The downregulated transcriptome of COVID-19 patients' liver tissue samples was enriched in members of the cytochromes P450 family enzymes (CYPs), including *CYP39A1, CYP11A1, CYP4X1, CYP2U1*, and *CYP1A2*, which play central roles in various metabolic pathways including the metabolism of drugs and xenobiotics (Figure 7A). Moreover, we observed the downregulation of the CYPs, *CYP2C8* (fold change -4.18) and *CYP3A4/5* (fold change -2.44), which have been shown to be centrally implicated in the metabolism of chloroquine and hydroxychloroquine; therapeutic agents administered to COVID-19 patients[40,41]. Functional clustering analysis revealed the enrichment of transcripts implicated in xenobiotic and drug metabolism processes (GO:0006805 and GO:0017144) in the downregulated transcriptome of SARS-CoV-2 infected liver samples (adjusted *P* values 0.01215 and 0.06111, respectively for xenobiotic and drug metabolism processes). KEGG pathway enrichment analysis confirmed the suppression of the metabolism of various types of drugs through CYPs as well as other enzymes (Figure 7B).

Cross-validation of some differentially expressed genes in severe COVID-19 patients' blood plasma

We next aimed at investigating the potential of utilizing some of the identified DEGs as putative biomarkers for liver dysfunction in COVID-19 patients. Therefore, we assessed the gene expression of some of the differentially expressed genes in the liver tissue of severe COVID-19 patients using blood plasma samples of severe COVID-19 patients with liver dysfunction. In correlation with the RNA-seq data analysis, qRT-PCR analysis confirmed the significant elevation in the gene expression of *DNAJB1* and *HSP90AB1* and the significant downregulation of *CYP39A1* in response to liver dysfunction in severe COVID-19 patients (Figure 8).

DISCUSSION

Manifestations of SARS-CoV-2 infection were observed systemically and included indications of liver dysfunction. However, the molecular changes underlying the phenotypic systemic observations linking to liver dysfunction at advanced stages of COVID-19 are yet to be thoroughly explored. Therefore, in this study, we explored an RNA-seq dataset of severe COVID-19 patients' liver tissues to get some insight on the dysregulated molecular mechanisms resulting in the observed phenotypic manifestations (summary of findings in Figure 9).

Our analysis of the RNA-seq data revealed that severe COVID-19 patients experience transcriptional shifts in their liver tissue that supersede those observed in lung tissue at advanced stages. These transcriptional shifts include the significant dysregulation of signaling through GPCRs [class A-rhodopsin-like receptors (R-HSA-373076: class A/1 Rhodopsin-like receptors] and class F-frizzled and smoothened receptors). Subsequently, various intracellular signaling pathways (*e.g.*, WNT, hippo and SHH signaling pathways) are dysregulated resulting in the transcriptional dysregulation of viral reproduction, cell proliferation, cells survival, cellular chemotaxis as well as local environment, inflammation, and tissue remodeling[16,19,20].

A number of the top upregulated transcripts, including *DNAJB1*, *IGF2*, and *HDGF*, were implicated in the activation of tissue remodeling pathways[22-24]. The upregulation of these genes suggests that severe COVID-19 patients may experience tissue remodeling of the liver tissue. *IGF2* overexpression was proposed to induce fatty liver and hepatic steatosis by increasing lipid accumulation in liver tissue[23]. In our analysis of the SARS-CoV-2 infected liver samples, we observed an increase in the pro-steatosis factor, *IGF2*, and a concordant decrease of the steatosis and tissue remodeling preventive factors, *IGF1* and *GHR* (growth hormone receptor)[42]. In addition to *IGF2*, numerous genes proposed to contribute to the pathogenesis of liver diseases characterized by tissue remodeling (*e.g.* liver steatosis, liver fibrosis, and liver cirrhosis) were upregulated in the liver tissue of severe COVID-19 patients (*e.g. FLT1, LEP, PNPLA3, VCAM1, TREM2*, metalloproteinases-expressing genes, and collagen-expressing

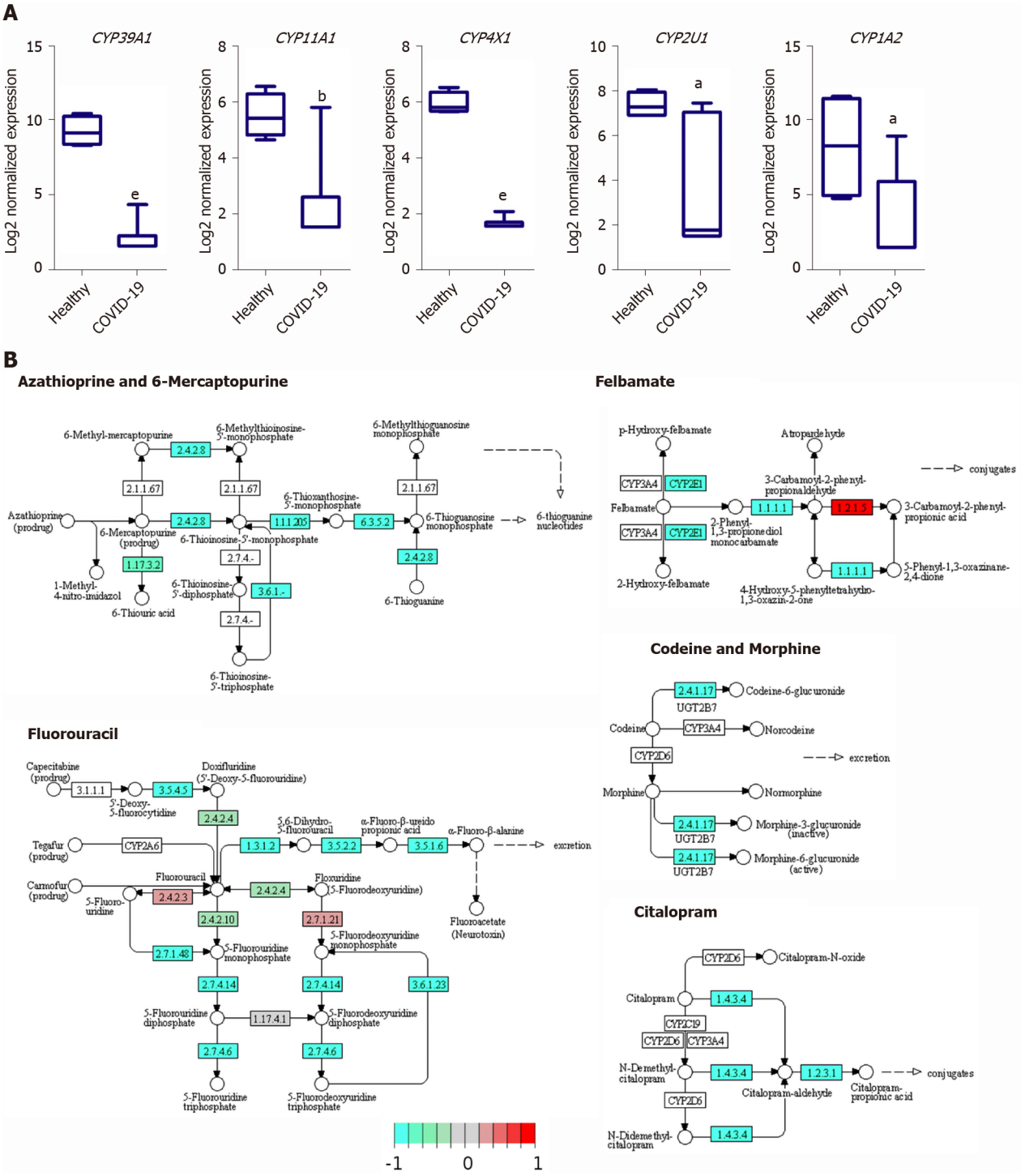


Figure 7 Enrichment of cytochromes P450 family members involved in drug and xenobiotics metabolism in the downregulated transcriptome of severe coronavirus disease 2019 patients liver tissues. A: Box plot representation of some of the significantly downregulated cytochrome P450 family members; B: Kegg pathway representations of the suppressive effect of severe acute respiratory syndrome coronavirus 2 infection in liver on xenobiotic and drug metabolism through cytochrome P450 and other enzymes. Data presented as mean ± SE; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0005$, $^{e}P < 0.0001$ coronavirus disease 2019 (COVID-19) *vs* Healthy. *CYP39A1*: Cytochrome P450 family 39 subfamily A member 1; CYP11A1: Cytochrome P450 family 11 subfamily A member 1; CYP4X1: Cytochrome P450 family 4 subfamily X member 1; CYP2U1: Cytochrome P450 family 2 subfamily U member 1; CYP1A2: Cytochrome P450 family 1 subfamily A member 2.

genes)[27-31].

Furthermore, consistently with the upregulation of these genes implicated in liver tissue remodeling, we observed the significant upregulation of *EGFR* which was proposed to contribute to the development of pulmonary fibrosis[25]. The enrichment of these transcripts in the transcriptome of SARS-CoV-2 infected liver samples suggests the potential activation of tissue remodeling and fat accumulation in liver tissue. Consistently with these findings, immunohistochemical analysis of liver tissue

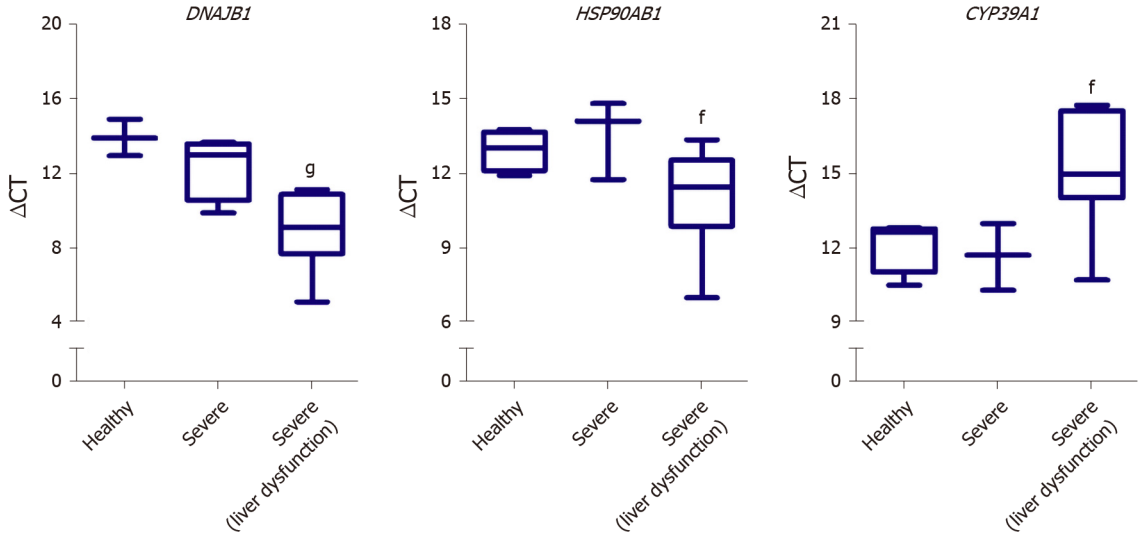


Figure 8 Validation of differentially expressed genes identified from the RNA-seq analysis of the severe coronavirus disease 2019 patients liver samples using blood plasma samples of severe coronavirus disease 2019 patients. Box plot representation of the gene expression of *DNAJB1*, *HSP90AB1*, and *CYP39A1* in the blood plasma of severe coronavirus disease 2019 (COVID-19) patients without liver dysfunction (Severe) or with liver dysfunction (Severe (liver dysfunction)) in comparison to non-COVID-19 controls (Healthy); The expression was detected in 4 healthy samples (DNAJB1 expression was only detected in 3 samples), 4 severe COVID-19 samples, and 9 severe COVID-19 samples with liver dysfunction (*CYP39A1* expression was only detected in 8 samples); Data presented as mean ± SE; $^{f}P < 0.05$, $^{g}P < 0.001$ Severe with liver dysfunction *vs* Healthy. *DNAJB1*: DnaJ heat shock protein family (Hsp40) member B1; *HSP90AB1*: Heat shock protein 90 alpha family class B member 1; *CYP39A1*: Cytochrome P450 family 39 subfamily A member 1.

from COVID-19 patients revealed signs of hepatic steatosis and hepatic siderosis [43,44]. These morphologic changes might be attributed to the activation of the tissue remodeling mechanisms in response to inflammation; to the dysregulation of intracellular signaling pathways (*e.g.*, EFGR, WNT, and SHH signaling pathways) in response to direct viral infection; or to the dysregulation of metabolic and biosynthetic pathways resulting the accumulation of lipids and other metabolic substrates in liver tissue and subsequently the development of liver steatosis.

Altogether, the dysregulation of these transcripts and pathways in the liver tissue of severe COVID-19 patients may indicate that the patients may experience pathological tissue remodeling similar to that observed in liver steatosis/cirrhosis/fibrosis that potentially contributes to the phenotypically observed liver dysfunction. Moreover, despite the observed recovery of liver function in some patients in response to treatment, the activation of tissue remodeling mechanisms may have long-term consequences in patients experiencing advanced stages of COVID-19 and would require long term follow-up.

On the other hand, the downregulated transcriptome of the SARS-CoV-2 infected liver tissue samples was enriched in transcripts implicated in metabolic and biosynthetic pathways regulation, including *ACAD11, CIDEB, GNMT, GPAM,* and *HSD17B4*. Previous investigations in the metabolomic profile of the SARS-CoV-2 infected patients' sera similarly revealed the suppressive effect of SARS-CoV-2 infection on lipid and amino acid metabolism[32]. Moreover, accumulating evidence suggested the localization of SARS-CoV-2 viral RNA in the mitochondrial matrix and the direct viral interaction with mitochondrial proteins and metabolic regulators (*e.g.* MRPS2, MRPS5, MRPS25, MRPS27, NDUFAF1, NDUFB9, NDUFAF2, ATP1B1, ATP6V1A, ACADM, AASS, PMPCB, PITRM1, COQ8B, and PMPCA)[45-47]. By localizing to the mitochondria, SARS-CoV-2 was speculated to usurp mitochondrial machineries to generate hide-out like double membrane vesicles, regulate intra-organelle crosstalk (*e.g.* ER-mitochondrial cross-talk), modulate immune response, and modulate cellular metabolic and biosynthetic processes. These speculations are consistent with the dysregulation of mitochondrial, metabolic and biosynthetic pathways revealed in our analysis. Altogether, these findings suggest that SARS-CoV-2 infection hijacks cellular metabolism resulting in the suppression of metabolic and biosynthetic pathways potentially through dysregulating mitochondrial function. Intriguingly, one of the mechanisms of action through which hydroxychloroquine might counteract SARS-CoV-2 infection is through the regulation of dysregulated metabolic pathways and protecting against the development of liver steatosis and subsequent tissue remodeling [48].

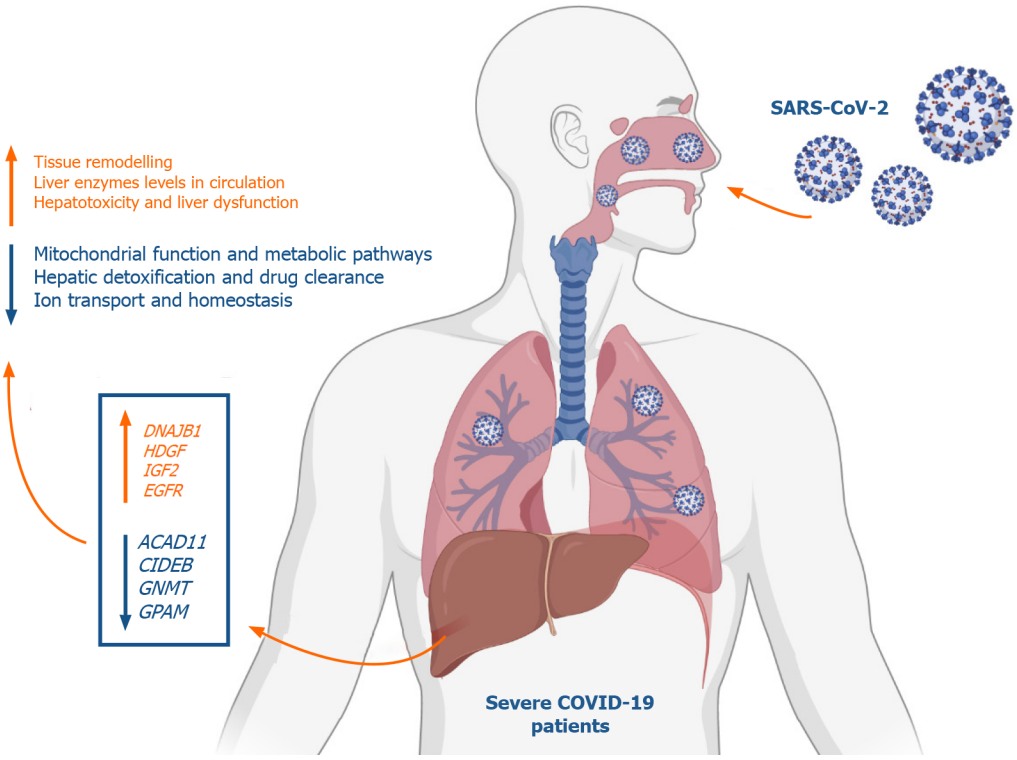


Figure 9 Summary of the study findings. At advanced stages, severe acute respiratory syndrome coronavirus 2 infection appears to elicit transcriptional shifts in liver tissue resulting in increased G-coupled protein receptors signaling, tissue remodeling, transmembrane transport, vesicle formation and cell projections morphogenesis. On the other hand, metabolic and biosynthetic pathways including metabolism of drugs and xenobiotics as well as mitochondrial processes and ions homeostasis regulatory mechanisms appear to be suppressed in coronavirus disease 2019 (COVID-19) patients. In response to these transcriptional shifts, we speculate that severe COVID-19 patients would be subjected to hepatic injury and tissue remodeling which may results in the clinically observed liver dysfunction, low liver detoxification capacity and increased liver enzymes. Figure was created with BioRender.com. *DNAJB1*: DnaJ heat shock protein family (Hsp40) member B1; *HDGF*: Hepatoma-derived growth factor; *IGF2*: Insulin growth factor 2; *EGFR*: Epidermal growth factor receptor; *ACAD11*: Acyl-CoA dehydrogenases 11; *CIDEB*: Cell death-inducing DNA fragmentation factor alpha-like effector B; *GNMT*: Glycine N-methyltransferase; *GPAM*: Glycerol-3-phosphate acyltransferase and metabolism.

Further investigations on the potential secondary mechanisms resulting in mitochondrial dysfunction divulged the imbalance in ions (*e.g.* calcium, potassium, sodium) due to dysregulated ionic transport and homeostasis as a potential underlying mechanism. Ionic imbalance can shift the osmotic pressure resulting in mitochondrial swelling and consequent dysfunction[39]. Moreover, ionic imbalance can have critical consequences on intracellular signaling and cellular morphology which can be another mechanism through which SARS-CoV-2 induces the observed transcriptomic shifts[49,50].

Clinical assessment of SARS-CoV-2 infected liver tissues revealed mitochondrial swelling which can be an underlying cause for the mitochondrial dysfunction observed in the infected cells. One of the potential mechanisms resulting in the mitochondrial swelling is the dysregulation of ions binding, transport and balance within the cell and its compartments. Our analysis showed a significant enrichment of downregulated transcripts implicated in iron, copper, sodium, potassium, zinc, and calcium binding, transport, homeostasis and signaling. Imbalance of some of these ions (*e.g.*, calcium and potassium ions) have been shown to induce mitochondrial swelling through shifting osmotic pressure[39]. Ionic imbalances were similarly found to be induced by SARS-CoV and Influenza virus resulting in cellular morphology changes as well as stimulation of the inflammasome and subsequent inflammatory mechanisms such as IL-1β production and signaling[49,50]. Potassium levels imbalance and absorption dysregulation has been reported in Covid-19 patients in the form of hypokalemia and the patients were found to respond well to potassium supplements during recovery[51]. Taken together, we speculate that SARS-CoV-2 hijacks cellular metabolic and biosynthetic pathways by disrupting ion imbalance and mitochondrial function.

One of the main targets that appear to be suppressed in response to SARS-CoV-2 infection is the CYPs family which plays a central role in xenobiotic and drug metabolism in liver tissues. The suppression of the members of this family of metabolic enzymes may potentially result in hepatoxicity and acute liver injury upon

the administration of treatments. Currently used therapeutic agents for the treatment of COVID-19 patients including chloroquine and hydroxychloroquine are metabolized by members of the CYPs family enzymes (*e.g.*, CYP2C8 and CYP3A4/5)[40,41], which we observed to be downregulated in SARS-CoV-2 infected liver samples. Therefore, the downregulation of these enzymes might dysregulate the metabolism of these therapeutic agents resulting in hepatotoxicity, autoimmune hepatitis, and liver injury as observed histologically[44]. In concordance with these findings, metabolomic profiling of covid-19 patients' sera suggested a decline in liver detoxification function [32]. Moreover, the downregulation of CYPs has been implicated in the dysregulation of metabolic pathways in cirrhotic patients[52]. Therefore, the downregulation of the CYPs may contribute to the liver injury through the dysregulation of drug metabolism as well as the activation of tissue remodeling mechanisms that ensure the pathogenesis of other liver diseases (*e.g.*, cirrhosis).

Whilst the main limitation of this study is the lack of validation on patients' liver tissue samples, we cross-validated and confirmed the dysregulation of some DEGs (*DNAJB1*, *HSP90AB1*, and *CYP39A1*) using blood plasma samples of severe COVID-19 patients with liver dysfunction. The systems biology analysis carried out in this study highlighted some molecular mechanisms which may potentially elucidate the clinically observed haptic dysfunction in severe COVID-19 patients. Moreover, the findings of this study shed the light on the potentially activation of fibrosis/cirrhosis/steatosis patterns in response to the activation of wound healing and tissue remodeling mechanisms in severe COVID-19 patients which may require long-term follow up. These findings may prove to be critical in cases presenting with long COVID, as the chronic exposure to the infection and disease symptoms may induce irreversible tissue remodeling of the liver. Furthermore, based on the observed significant reduction in the metabolic pathways and hepatic detoxification in our study, serious consideration is required for the administered hepatotoxic treatments to severe COVID-19 patients (*e.g.*, chloroquine, hydroxychloroquine, and remdesivir). However, it remains to be ascertained whether the observed transcriptional shifts observed in the liver tissue of severe COVID-19 patients are the result of viral infection, systemic inflammation, or drug induced liver injury and toxicity.

CONCLUSION

In conclusion, we aimed at getting some insight into the molecular mechanisms underlying liver dysfunction in severe COVID-19 patients using RNA-seq data of severe COVID-19 liver autopsy samples. Through systems biology analysis, we observed a significant upregulation in transcripts implicated in GPCRs signaling, tissue remodeling, and intracellular/transmembrane transport (*e.g.*, *DNAJB1, IGF2, EGFR,* and *HDGF)*. On the other hand, we observed a significant downregulation in transcripts implicated in metabolic pathways and mitochondrial function (*e.g.*, *ACAD11, CIDEB, GNMT,* and *GPAM)*. Moreover, we observed a significant suppression of CYPs, which play central roles in the metabolism of drugs and xenobiotics metabolism suggesting a compromised hepatic detoxification capacity. Moreover, the dysregulation of some genes (*e.g.*, *DNAJB1, HSP90AB1,* and *CYP39A1)* in the tissue autopsies correlated with their dysregulation in the blood plasma of severe COVID-19 patients with hepatic dysfunction suggesting their potential as putative biomarkers of liver dysfunction.

ARTICLE HIGHLIGHTS

Research background

The coronavirus disease 2019 (COVID-19), a pandemic contributing to more than 105 million cases and more than 2.3 million deaths worldwide is associated with extrapulmonary manifestations, including liver dysfunction. Liver dysfunction and elevated liver enzymes were observed in about 53% of COVID-19 patients. However, the molecular mechanistic aspect of this is not clear but can be of importance in understanding how the virus infects the hepatocytes and the infection leads to liver dysfunction. Understanding this can help in the diagnosis and treatment of liver dysfunction caused by COVID-19.

Research motivation

Liver is an important organ in human health as it is involved in the detoxification of many compounds accumulated through pollution or using the wrong diet. Liver dysfunction can lead to many systemic diseases in the body. One of the organs affected by SARS-CoV-2 infection is the liver and therefore understanding the mechanism of infection and the cause of liver dysfunction can help COVID-19 patients whose liver is affected but also the results can shed light on the molecular mechanism of liver dysfunction that may results from other liver diseases including cirrhosis, fibrosis, non-alcoholic fatty liver disease (NAFLD), and hepatitis A/B/C.

Research objectives

The main objectives of this research are: (1) Obtain genetic data from COVID-19 patients who had hepatic dysfunction; (2) Compare the genetic signature of COVID-19 patients with hepatic failure with that of other liver diseases including cirrhosis, fibrosis, NAFLD, and hepatitis A/B/C; and (3) Carry out *in vitro* validation of the biomarkers identified using blood samples from severe COVID-19 patients with liver dysfunction.

Research methods

The transcriptome of liver autopsy samples from severe COVID-19 patients against those of non-COVID donors was analyzed. Differentially expressed genes were identified from normalized RNA-seq data and analyzed for the enrichment of functional clusters and pathways. The differentially expressed genes were then compared against the genetic signatures of liver diseases including cirrhosis, fibrosis, NAFLD, and hepatitis A/B/C. Gene expression of some differentially expressed genes was assessed in the blood samples of severe COVID-19 patients with liver dysfunction using qRT-PCR.

Research results

Analysis of the differential transcriptome of the liver tissue of severe COVID-19 patients revealed a significant upregulation of transcripts implicated in tissue remodeling including *GPCRs*, *DNAJB1, IGF2, EGFR,* and *HDGF*. Concordantly, the differential transcriptome of severe COVID-19 liver tissues substantially overlapped with the disease signature of liver diseases characterized with pathological tissue remodeling (liver cirrhosis, Fibrosis, NAFLD, and hepatitis A/B/C). Moreover, we observed a significant suppression of transcripts implicated in metabolic pathways as well as mitochondrial function, including cytochrome P450 family members, *ACAD11, CIDEB, GNMT,* and *GPAM*. Consequently, drug and xenobiotics metabolism pathways are significantly suppressed suggesting a decrease in liver detoxification capacity. In correspondence with the RNA-seq data analysis, we observed a significant upregulation of *DNAJB1* and *HSP90AB1* as well as significant downregulation of *CYP39A1* in the blood plasma of severe COVID-19 patients with liver dysfunction.

Research conclusions

Some insights into the molecular mechanisms underlying liver dysfunction in severe COVID-19 patients was obtained using RNA-seq data of severe COVID-19 liver autopsy samples. Through systems biology analysis, we observed a significant upregulation in transcripts implicated in GPCRs signaling, tissue remodeling, and intracellular/transmembrane transport (*e.g.*, *DNAJB1, IGF2, EGFR,* and *HDGF*). On the other hand, we observed a significant downregulation in transcripts implicated in metabolic pathways and mitochondrial function (*e.g.*, *ACAD11, CIDEB, GNMT,* and *GPAM*). Moreover, we observed a significant suppression of cytochrome P450 family members, which play central roles in the metabolism of drugs and xenobiotics metabolism suggesting a compromised hepatic detoxification capacity. Moreover, the dysregulation of some genes (*e.g.*, *DNAJB1, HSP90AB1,* and *CYP39A1*) in the tissue autopsies correlated with their dysregulation in the blood plasma of severe COVID-19 patients with hepatic dysfunction suggesting their potential as putative biomarkers of liver dysfunction.

Research perspectives

This study identified key cellular pathways involved in liver dysfunction in general but also liver dysfunction linked to COVID-19 patients. The analysis identified key molecular biomarkers which can be used in future to general a biomarker panel that can assess the degree of hepatic dysfunction in patients from a blood test since the biomarkers identified in this study was shown to follow the same trend when

compared with blood samples from COVID-19 patients with hepatic dysfunction. Mechanistically, the study showed that severe COVID-19 patients appear to experience significant transcriptional shift that may ensue tissue remodeling, mitochondrial dysfunction and apparent hepatic toxification resulting in the clinically observed liver dysfunction. The finding from this study can be used in future to assess the degree of hepatic dysfunction but also to differentiate patients with COVID-19 related hepatic dysfunction from hepatic dysfunction caused by other liver diseases. This can be of importance in the management of COVID-19 patients including those with long COVID who have experienced hepatic dysfunction.

REFERENCES

1 **Utunen H**, Ndiaye N, Piroux C, George R, Attias M, Gamhewage G. Global Reach of an Online COVID-19 Course in Multiple Languages on OpenWHO in the First Quarter of 2020: Analysis of Platform Use Data. *J Med Internet Res* 2020; **22**: e19076 [PMID: 32293580 DOI: 10.2196/19076]

2 **Lin L**, Jiang X, Zhang Z, Huang S, Fang Z, Gu Z, Gao L, Shi H, Mai L, Liu Y, Lin X, Lai R, Yan Z, Li X, Shan H. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. *Gut* 2020; **69**: 997-1001 [PMID: 32241899 DOI: 10.1136/gutjnl-2020-321013]

3 **Shi H**, Han X, Jiang N, Cao Y, Alwalid O, Gu J, Fan Y, Zheng C. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. *Lancet Infect Dis* 2020; **20**: 425-434 [PMID: 32105637 DOI: 10.1016/S1473-3099(20)30086-4]

4 **Weber S**, Mayerle J, Irlbeck M, Gerbes AL. Severe liver failure during SARS-CoV-2 infection. *Gut* 2020; **69**: 1365-1367 [PMID: 32327526 DOI: 10.1136/gutjnl-2020-321350]

5 **Chen T**, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, Ding C, Zhang X, Huang J, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ* 2020; **368**: m1091 [PMID: 32217556 DOI: 10.1136/bmj.m1091]

6 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]

7 **Yip TC**, Lui GC, Wong VW, Chow VC, Ho TH, Li TC, Tse YK, Hui DS, Chan HL, Wong GL. Liver injury is independently associated with adverse clinical outcomes in patients with COVID-19. *Gut* 2021; **70**: 733-742 [PMID: 32641471 DOI: 10.1136/gutjnl-2020-321726]

8 **Jothimani D**, Venugopal R, Abedin MF, Kaliamoorthy I, Rela M. COVID-19 and the liver. *J Hepatol* 2020; **73**: 1231-1240 [PMID: 32553666 DOI: 10.1016/j.jhep.2020.06.006]

9 **Desai N**, Neyaz A, Szabolcs A, Shih AR, Chen JH, Thapar V, Nieman LT, Solovyov A, Mehta A, Lieb DJ, Kulkarni AS, Jaicks C, Xu KH, Raabe MJ, Pinto CJ, Juric D, Chebib I, Colvin RB, Kim AY, Monroe R, Warren SE, Danaher P, Reeves JW, Gong J, Rueckert EH, Greenbaum BD, Hacohen N, Lagana SM, Rivera MN, Sholl LM, Stone JR, Ting DT, Deshpande V. Temporal and spatial heterogeneity of host response to SARS-CoV-2 pulmonary infection. *Nat Commun* 2020; **11**: 6319 [PMID: 33298930 DOI: 10.1038/s41467-020-20139-7]

10 **Emig D**, Salomonis N, Baumbach J, Lengauer T, Conklin BR, Albrecht M. AltAnalyze and DomainGraph: analyzing and visualizing exon expression data. *Nucleic Acids Res* 2010; **38**: W755-W762 [PMID: 20513647 DOI: 10.1093/nar/gkq405]

11 **Li J**, Witten DM, Johnstone IM, Tibshirani R. Normalization, testing, and false discovery rate estimation for RNA-sequencing data. *Biostatistics* 2012; **13**: 523-538 [PMID: 22003245 DOI: 10.1093/biostatistics/kxr031]

12 **Subramanian A**, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005; **102**: 15545-15550 [PMID: 16199517 DOI: 10.1073/pnas.0506580102]

13 **Heberle H**, Meirelles GV, da Silva FR, Telles GP, Minghim R. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics* 2015; **16**: 169 [PMID: 25994840 DOI: 10.1186/s12859-015-0611-3]

14 **Zhou Y**, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda SK. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun* 2019; **10**: 1523 [PMID: 30944313 DOI: 10.1038/s41467-019-09234-6]

15 **Piñero J**, Ramírez-Anguita JM, Saüch-Pitarch J, Ronzano F, Centeno E, Sanz F, Furlong LI. The DisGeNET knowledge platform for disease genomics: 2019 update. *Nucleic Acids Res* 2020; **48**: D845-D855 [PMID: 31680165 DOI: 10.1093/nar/gkz1021]

16 **Sodhi A**, Montaner S, Gutkind JS. Viral hijacking of G-protein-coupled-receptor signalling networks. *Nat Rev Mol Cell Biol* 2004; **5**: 998-1012 [PMID: 15573137 DOI: 10.1038/nrm1529]

17 **Wang Z**, Lu W, Zhang Y, Zou F, Jin Z, Zhao T. The Hippo Pathway and Viral Infections. *Front Microbiol* 2019; **10**: 3033 [PMID: 32038526 DOI: 10.3389/fmicb.2019.03033]

18 **Ljungberg JK**, Kling JC, Tran TT, Blumenthal A. Functions of the WNT Signaling Network in Shaping Host Responses to Infection. *Front Immunol* 2019; **10**: 2521 [PMID: 31781093 DOI: 10.3389/fimmu.2019.02521]

19 **Smelkinson MG**. The Hedgehog Signaling Pathway Emerges as a Pathogenic Target. *J Dev Biol* 2017; **5**: 14 [PMID: 29214147 DOI: 10.3390/jdb5040014]

20 **More S**, Yang X, Zhu Z, Bamunuarachchi G, Guo Y, Huang C, Bailey K, Metcalf JP, Liu L. Regulation of influenza virus replication by Wnt/β-catenin signaling. *PLoS One* 2018; **13**: e0191010 [PMID: 29324866 DOI: 10.1371/journal.pone.0191010]

21 **Cheng H**, Lear-Rooney CM, Johansen L, Varhegyi E, Chen ZW, Olinger GG, Rong L. Inhibition of Ebola and Marburg Virus Entry by G Protein-Coupled Receptor Antagonists. *J Virol* 2015; **89**: 9932-9938 [PMID: 26202243 DOI: 10.1128/JVI.01337-15]

22 **Graham RP**, Jin L, Knutson DL, Kloft-Nelson SM, Greipp PT, Waldburger N, Roessler S, Longerich T, Roberts LR, Oliveira AM, Halling KC, Schirmacher P, Torbenson MS. DNAJB1-PRKACA is specific for fibrolamellar carcinoma. *Mod Pathol* 2015; **28**: 822-829 [PMID: 25698061 DOI: 10.1038/modpathol.2015.4]

23 **Kessler SM**, Laggai S, Van Wonterg E, Gemperlein K, Müller R, Haybaeck J, Vandenbroucke RE, Ogris M, Libert C, Kiemer AK. Transient Hepatic Overexpression of Insulin-Like Growth Factor 2 Induces Free Cholesterol and Lipid Droplet Formation. *Front Physiol* 2016; **7**: 147 [PMID: 27199763 DOI: 10.3389/fphys.2016.00147]

24 **Kao YH**, Chen CL, Jawan B, Chung YH, Sun CK, Kuo SM, Hu TH, Lin YC, Chan HH, Cheng KH, Wu DC, Goto S, Cheng YF, Chao D, Tai MH. Upregulation of hepatoma-derived growth factor is involved in murine hepatic fibrogenesis. *J Hepatol* 2010; **52**: 96-105 [PMID: 19913322 DOI: 10.1016/j.jhep.2009.10.002]

25 **Venkataraman T**, Frieman MB. The role of epidermal growth factor receptor (EGFR) signaling in SARS coronavirus-induced pulmonary fibrosis. *Antiviral Res* 2017; **143**: 142-150 [PMID: 28390872 DOI: 10.1016/j.antiviral.2017.03.022]

26 **Enomoto Y**, Orihara K, Takamasu T, Matsuda A, Gon Y, Saito H, Ra C, Okayama Y. Tissue remodeling induced by hypersecreted epidermal growth factor and amphiregulin in the airway after an acute asthma attack. *J Allergy Clin Immunol* 2009; **124**: 913-20. e1-7 [PMID: 19895983 DOI: 10.1016/j.jaci.2009.08.044]

27 **Corpechot C**, Barbu V, Wendum D, Kinnman N, Rey C, Poupon R, Housset C, Rosmorduc O. Hypoxia-induced VEGF and collagen I expressions are associated with angiogenesis and fibrogenesis in experimental cirrhosis. *Hepatology* 2002; **35**: 1010-1021 [PMID: 11981751 DOI: 10.1053/jhep.2002.32524]

28 **Leclercq IA**, Farrell GC, Schriemer R, Robertson GR. Leptin is essential for the hepatic fibrogenic response to chronic liver injury. *J Hepatol* 2002; **37**: 206-213 [PMID: 12127425 DOI: 10.1016/s0168-8278(02)00102-2]

29 **Dong XC**. PNPLA3-A Potential Therapeutic Target for Personalized Treatment of Chronic Liver Disease. *Front Med (Lausanne)* 2019; **6**: 304 [PMID: 31921875 DOI: 10.3389/fmed.2019.00304]

30 **Cazanave S**, Podtelezhnikov A, Jensen K, Seneshaw M, Kumar DP, Min HK, Santhekadur PK, Banini B, Mauro AG, M Oseini A, Vincent R, Tanis KQ, Webber AL, Wang L, Bedossa P, Mirshahi F, Sanyal AJ. The Transcriptomic Signature Of Disease Development And Progression Of Nonalcoholic Fatty Liver Disease. *Sci Rep* 2017; **7**: 17193 [PMID: 29222421 DOI: 10.1038/s41598-017-17370-6]

31 **Perugorria MJ**, Esparza-Baquer A, Oakley F, Labiano I, Korosec A, Jais A, Mann J, Tiniakos D, Santos-Laso A, Arbelaiz A, Gawish R, Sampedro A, Fontanellas A, Hijona E, Jimenez-Agüero R, Esterbauer H, Stoiber D, Bujanda L, Banales JM, Knapp S, Sharif O, Mann DA. Non-parenchymal TREM-2 protects the liver from immune-mediated hepatocellular damage. *Gut* 2019; **68**: 533-546 [PMID: 29374630 DOI: 10.1136/gutjnl-2017-314107]

32 **Shen B**, Yi X, Sun Y, Bi X, Du J, Zhang C, Quan S, Zhang F, Sun R, Qian L, Ge W, Liu W, Liang S, Chen H, Zhang Y, Li J, Xu J, He Z, Chen B, Wang J, Yan H, Zheng Y, Wang D, Zhu J, Kong Z, Kang Z, Liang X, Ding X, Ruan G, Xiang N, Cai X, Gao H, Li L, Li S, Xiao Q, Lu T, Zhu Y, Liu H, Guo T. Proteomic and Metabolomic Characterization of COVID-19 Patient Sera. *Cell* 2020; **182**: 59-72. e15 [PMID: 32492406 DOI: 10.1016/j.cell.2020.05.032]

33 **Ben-Shachar D**, Karry R. Sp1 expression is disrupted in schizophrenia; a possible mechanism for the abnormal expression of mitochondrial complex I genes, NDUFV1 and NDUFV2. *PLoS One* 2007; **2**: e817 [PMID: 17786189 DOI: 10.1371/journal.pone.0000817]

34 **He M**, Pei Z, Mohsen AW, Watkins P, Murdoch G, Van Veldhoven PP, Ensenauer R, Vockley J. Identification and characterization of new long chain acyl-CoA dehydrogenases. *Mol Genet Metab* 2011; **102**: 418-429 [PMID: 21237683 DOI: 10.1016/j.ymgme.2010.12.005]

35 **Slayton M**, Gupta A, Balakrishnan B, Puri V. CIDE Proteins in Human Health and Disease. *Cells* 2019; **8**: 238 [PMID: 30871156 DOI: 10.3390/cells8030238]

36 **Varela-Rey M**, Martínez-López N, Fernández-Ramos D, Embade N, Calvisi DF, Woodhoo A, Rodríguez J, Fraga MF, Julve J, Rodríguez-Millán E, Frades I, Torres L, Luka Z, Wagner C, Esteller M, Lu SC, Martínez-Chantar ML, Mato JM. Fatty liver and fibrosis in glycine N-methyltransferase knockout mice is prevented by nicotinamide. *Hepatology* 2010; **52**: 105-114 [PMID: 20578266 DOI: 10.1002/hep.23639]

37 **Chen YQ**, Kuo MS, Li S, Bui HH, Peake DA, Sanders PE, Thibodeaux SJ, Chu S, Qian YW, Zhao Y, Bredt DS, Moller DE, Konrad RJ, Beigneux AP, Young SG, Cao G. AGPAT6 is a novel microsomal glycerol-3-phosphate acyltransferase. *J Biol Chem* 2008; **283**: 10048-10057 [PMID: 18238778 DOI: 10.1074/jbc.M708151200]

38 **Pierce SB**, Walsh T, Chisholm KM, Lee MK, Thornton AM, Fiumara A, Opitz JM, Levy-Lahad E,

Klevit RE, King MC. Mutations in the DBP-deficiency protein HSD17B4 cause ovarian dysgenesis, hearing loss, and ataxia of Perrault Syndrome. *Am J Hum Genet* 2010; **87**: 282-288 [PMID: 20673864 DOI: 10.1016/j.ajhg.2010.07.007]

39 **Javadov S**, Chapa-Dubocq X, Makarov V. Different approaches to modeling analysis of mitochondrial swelling. *Mitochondrion* 2018; **38**: 58-70 [PMID: 28802667 DOI: 10.1016/j.mito.2017.08.004]

40 **Kim KA**, Park JY, Lee JS, Lim S. Cytochrome P450 2C8 and CYP3A4/5 are involved in chloroquine metabolism in human liver microsomes. *Arch Pharm Res* 2003; **26**: 631-637 [PMID: 12967198 DOI: 10.1007/BF02976712]

41 **Wahie S**, Daly AK, Cordell HJ, Goodfield MJ, Jones SK, Lovell CR, Carmichael AJ, Carr MM, Drummond A, Natarajan S, Smith CH, Reynolds NJ, Meggitt SJ. Clinical and pharmacogenetic influences on response to hydroxychloroquine in discoid lupus erythematosus: a retrospective cohort study. *J Invest Dermatol* 2011; **131**: 1981-1986 [PMID: 21734714 DOI: 10.1038/jid.2011.167]

42 **Adamek A**, Kasprzak A. Insulin-Like Growth Factor (IGF) System in Liver Diseases. *Int J Mol Sci* 2018; **19**: 1308 [PMID: 29702590 DOI: 10.3390/ijms19051308]

43 **Deinhardt-Emmer S**, Wittschieber D, Sanft J, Kleemann S, Elschner S, Haupt KF, Vau V, Häring C, Rödel J, Henke A, Ehrhardt C, Bauer M, Philipp M, Gaßler N, Nietzsche S, Löffler B, Mall G. Early postmortem mapping of SARS-CoV-2 RNA in patients with COVID-19 and the correlation with tissue damage. *Elife* 2021; **10**: e60361 [PMID: 33781385 DOI: 10.7554/eLife.60361]

44 **Abenavoli L**, Gentile I, Maraolo AE, Negro F. SARS-CoV-2 and liver damage: a possible pathogenetic link. *Hepatobiliary Surg Nutr* 2020; **9**: 322-324 [PMID: 32509818 DOI: 10.21037/hbsn-20-437]

45 **Singh KK**, Chaubey G, Chen JY, Suravajhala P. Decoding SARS-CoV-2 hijacking of host mitochondria in COVID-19 pathogenesis. *Am J Physiol Cell Physiol* 2020; **319**: C258-C267 [PMID: 32510973 DOI: 10.1152/ajpcell.00224.2020]

46 **Wu KE**, Fazal FM, Parker KR, Zou J, Chang HY. RNA-GPS Predicts SARS-CoV-2 RNA Residency to Host Mitochondria and Nucleolus. *Cell Syst* 2020; **11**: 102-108. e3 [PMID: 32673562 DOI: 10.1016/j.cels.2020.06.008]

47 **Gordon DE**, Jang GM, Bouhaddou M, Xu J, Obernier K, White KM, O'Meara MJ, Rezelj VV, Guo JZ, Swaney DL, Tummino TA, Hüttenhain R, Kaake RM, Richards AL, Tutuncuoglu B, Foussard H, Batra J, Haas K, Modak M, Kim M, Haas P, Polacco BJ, Braberg H, Fabius JM, Eckhardt M, Soucheray M, Bennett MJ, Cakir M, McGregor MJ, Li Q, Meyer B, Roesch F, Vallet T, Mac Kain A, Miorin L, Moreno E, Naing ZZC, Zhou Y, Peng S, Shi Y, Zhang Z, Shen W, Kirby IT, Melnyk JE, Chorba JS, Lou K, Dai SA, Barrio-Hernandez I, Memon D, Hernandez-Armenta C, Lyu J, Mathy CJP, Perica T, Pilla KB, Ganesan SJ, Saltzberg DJ, Rakesh R, Liu X, Rosenthal SB, Calviello L, Venkataramanan S, Liboy-Lugo J, Lin Y, Huang XP, Liu Y, Wankowicz SA, Bohn M, Safari M, Ugur FS, Koh C, Savar NS, Tran QD, Shengjuler D, Fletcher SJ, O'Neal MC, Cai Y, Chang JCJ, Broadhurst DJ, Klippsten S, Sharp PP, Wenzell NA, Kuzuoglu-Ozturk D, Wang HY, Trenker R, Young JM, Cavero DA, Hiatt J, Roth TL, Rathore U, Subramanian A, Noack J, Hubert M, Stroud RM, Frankel AD, Rosenberg OS, Verba KA, Agard DA, Ott M, Emerman M, Jura N, von Zastrow M, Verdin E, Ashworth A, Schwartz O, d'Enfert C, Mukherjee S, Jacobson M, Malik HS, Fujimori DG, Ideker T, Craik CS, Floor SN, Fraser JS, Gross JD, Sali A, Roth BL, Ruggero D, Taunton J, Kortemme T, Beltrao P, Vignuzzi M, García-Sastre A, Shokat KM, Shoichet BK, Krogan NJ. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 2020; **583**: 459-468 [PMID: 32353859 DOI: 10.1038/s41586-020-2286-9]

48 **Qiao X**, Zhou ZC, Niu R, Su YT, Sun Y, Liu HL, Teng JL, Ye JN, Shi H, Yang CD, Cheng XB. Hydroxychloroquine Improves Obesity-Associated Insulin Resistance and Hepatic Steatosis by Regulating Lipid Metabolism. *Front Pharmacol* 2019; **10**: 855 [PMID: 31427967 DOI: 10.3389/fphar.2019.00855]

49 **Nieto-Torres JL**, DeDiego ML, Verdiá-Báguena C, Jimenez-Guardeño JM, Regla-Nava JA, Fernandez-Delgado R, Castaño-Rodriguez C, Alcaraz A, Torres J, Aguilella VM, Enjuanes L. Severe acute respiratory syndrome coronavirus envelope protein ion channel activity promotes virus fitness and pathogenesis. *PLoS Pathog* 2014; **10**: e1004077 [PMID: 24788150 DOI: 10.1371/journal.ppat.1004077]

50 **Ichinohe T**, Pang IK, Iwasaki A. Influenza virus activates inflammasomes *via* its intracellular M2 ion channel. *Nat Immunol* 2010; **11**: 404-410 [PMID: 20383149 DOI: 10.1038/ni.1861]

51 **Chen D**, Li X, Song Q, Hu C, Su F, Dai J, Ye Y, Huang J, Zhang X. Assessment of Hypokalemia and Clinical Characteristics in Patients With Coronavirus Disease 2019 in Wenzhou, China. *JAMA Netw Open* 2020; **3**: e2011122 [PMID: 32525548 DOI: 10.1001/jamanetworkopen.2020.11122]

52 **Dietrich CG**, Götze O, Geier A. Molecular changes in hepatic metabolism and transport in cirrhosis and their functional importance. *World J Gastroenterol* 2016; **22**: 72-88 [PMID: 26755861 DOI: 10.3748/wjg.v22.i1.72]

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ORIGINAL ARTICLE

Clinical and Translational Research

Survival-associated alternative splicing events interact with the immune microenvironment in stomach adenocarcinoma

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Author contributions: Ye ZS designed the project, analyzed data, prepared figures, and wrote the manuscript; Chen LC conceived the study, supervised the results, critically revised/wrote the manuscript, and was responsible for its financial support and the corresponding works; all the other authors conceived the study, and critically revised the manuscript; all authors approved the final manuscript, Ye ZS, Zheng M, Zeng Y, Wei SH and Liu QY contributed equally to the work.

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Abstract

BACKGROUND

Alternative splicing (AS) increases the diversity of mRNA during transcription; it might play a role in alteration of the immune microenvironment, which could influence the development of immunotherapeutic strategies against cancer.

AIM

To obtain the transcriptomic and clinical features and AS events in stomach adenocarcinoma (STAD) from the database. The overall survival data associated with AS events were used to construct a signature prognostic model for STAD.

METHODS

Differentially expressed immune-related genes were identified between subtypes on the basis of the prognostic model. In STAD, 2042 overall-survival-related AS events were significantly enriched in various pathways and influenced several cellular functions. Furthermore, the network of splicing factors and overall-survival-associated AS events indicated potential regulatory mechanisms underlying the AS events in STAD.

RESULTS

An eleven-AS-signature prognostic model (CD44|14986|ES, PPHLN1|21214|AT,

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RASSF4 | 11351 | ES, KIAA1147 | 82046 | AP, PPP2R5D | 76200 | ES, LOH12CR1 | 20507 | ES, CDKN3 | 27569 | AP, UBA52 | 48486 | AD, CADPS | 65499 | AT, SRSF7 | 53276 | RI, and WEE1 | 14328 | AP) was constructed and significantly related to STAD overall survival, immune cells, and cancer-related pathways. The differentially expressed immune-related genes between the high- and low-risk score groups were significantly enriched in cancer-related pathways.

CONCLUSION

This study provided an AS-related prognostic model, potential mechanisms for AS, and alterations in the immune microenvironment (immune cells, genes, and pathways) for future research in STAD.

Key Words: Stomach adenocarcinoma; Alternative splicing; Tumor microenvironment; Immune-related genes and pathways

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Core Tip: In this study, we performed a systematic analysis of prognostic splicing events in stomach adenocarcinoma (STAD), constructed an elevated Alternative splicing (AS)-signature prognostic model, and explored the association between AS and cancer immunity. The final overall survival-related AS events, differentially expressed immune-related genes, and the enriched pathways may play an important role in tumorigenesis in STAD; they deserve further study in clinical applications as diagnostic biomarkers and therapeutic targets.

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INTRODUCTION

Stomach cancer is a multifactorial cancer with a relatively high mortality (the third leading cause of death) worldwide. Each year, approximately one million new cases are reported, and the disease kills approximately 700000 men annually[1]. Around 90%-95% of all stomach cancers are stomach adenocarcinoma (STAD), which develops from cells that form in the mucosa[2]. Despite progress in surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, improvement in survival remains a major challenge in STAD therapy[3]. Several patients with early-stage gastric cancer are misdiagnosed with chronic gastritis or gastric ulcers owing to insidious clinical characteristics related to early-stage symptoms. Most gastric cancer patients lose the chance to undergo radical surgery, which decreases the 5-year survival rate in STAD[4]. Moreover, gastric cancer tends to lead to local infiltration and distant metastasis, spreading from the stomach to other organs of the body, especially the liver, peripheral vascular system, lungs, bones, supraclavicular lymph nodes, lining of the abdomen, and lymph nodes[5]. In patients with advanced stages of STAD, development of novel effective biomarkers and exploration of the most reliable mechanism for predicting the development and progression would be beneficial for improving the accuracy and specificity of disease prevention, risk prediction, and favorable STAD prognosis[6].

Alternative splicing (AS) increases the diversity of mRNA during transcription, which indicates that a single gene could code multiple proteins. A total of 20000 protein-coding genes might generate a mass of variants, even more than we expected in the traditional way of thinking[7]. Abundant AS production has broad implications for complex biological processes. AS occurs as a normal and common phenomenon in humans. Traditional classification of AS modes includes exon skip (ES), alternate promoters (AP), alternate terminators (AT), alternate acceptors (AA), alternate donors

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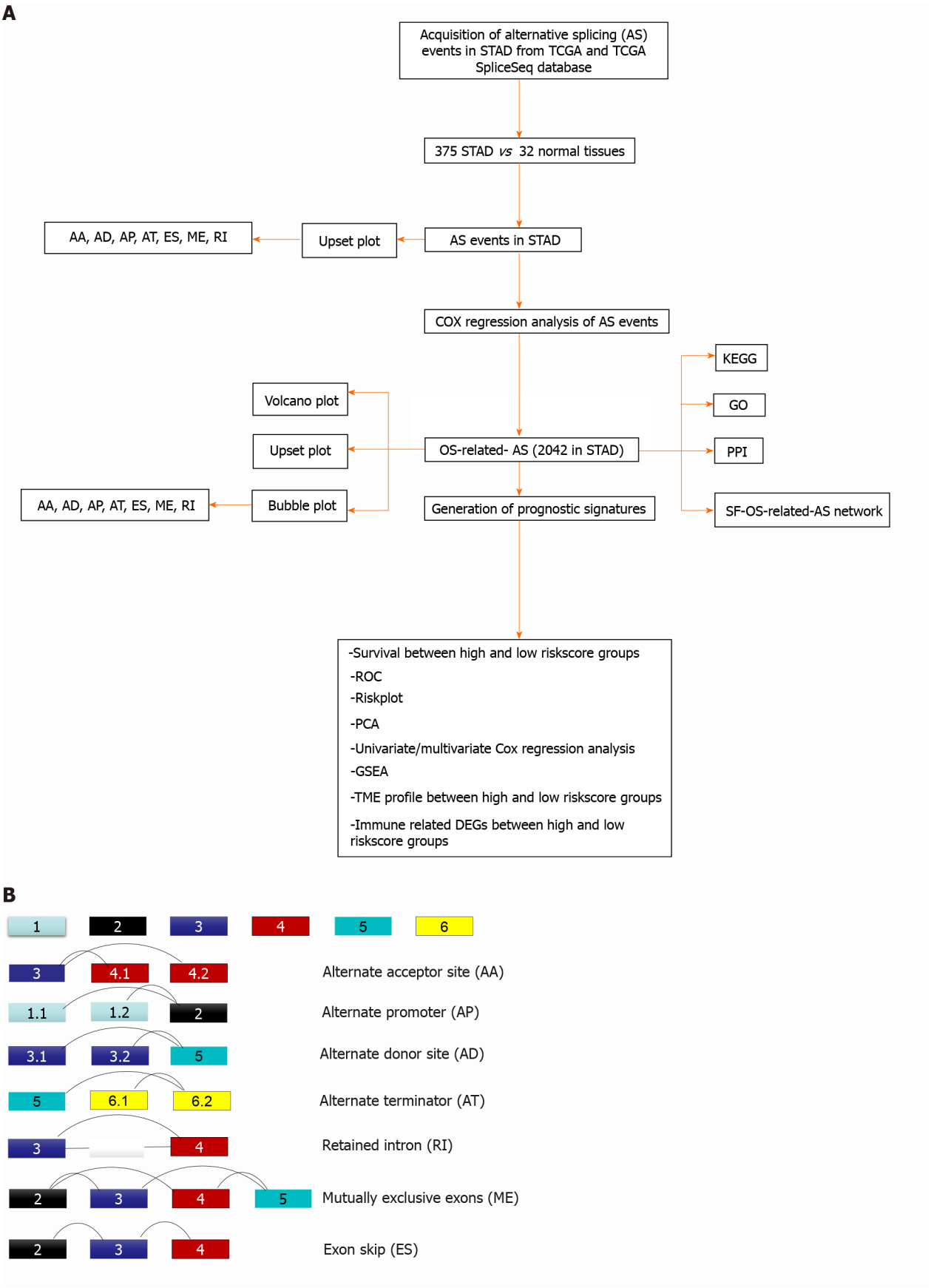
(AD), retained introns (RI), and exclusive exons (ME)[8]. The relative frequencies of the AS modes vary among humans; moreover, new AS events have been consistently identified using high-throughput techniques[9]. Abnormal AS events are frequently implicated in genetic disorders that result in human diseases, particularly, tumorigenesis and cancer progression. A comparison of cancerous and normal cells through combined RNA-Seq and proteomics analyses indicated significantly different expressions or types of splice events for key proteins in cancer-associated pathways [10]. For instance, cancerous cells exhibit higher levels of RI and lower levels of ES compared with normal cells[11]. Changes in trans-acting splicing factors (SFs) are general splicing mechanisms, including posttranslational modification, chromatin structure and histone modifications[12], DNA methylation[13], and gene mutation. The remarkable developments in immunotherapy strategies against STAD have yielded good results and almost changed the landscape of STAD treatment. The immune checkpoint molecules programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1), that focus on the role of T cells and associated co-inhibitory signals, hamper the immune escape of tumor cells[14]. Through clinical trials, researchers have evaluated the safety and efficacy of novel immunotherapies that target new immune checkpoint molecules, such as mucin domain containing-3, lymphocyte-activation gene 3, indoleamine 2,3-dioxygenase, and T cell immunoglobulin[15]. In a previous study, immunotherapeutically relevant gene signatures were constructed on the basis of tumor microenvironment characterization in gastric cancer; they have great potential for improving STAD patient outcome[16]. Researchers have observed that new antigens generated through AS mechanisms are common in most cancer patients; these new antigens may serve as potential therapeutic targets for patients with low tumor mutation burden[17]. Studies have demonstrated that the tumor microenvironment profile undergoes specific changes that are induced by SFs[18]. AS-influenced immune processes have captured great attention, including the B cell receptor signaling pathway, T cell receptor signaling pathway, antigen processing and presentation pathways, interleukins and interleukin receptors, chemokines and chemokine receptors, natural killer cell cytotoxicity, transforming growth factor (TGF) family members and TGF family member receptors, and interferons and interferon receptors[19]. Survival-associated AS events interact with the immune microenvironment and could influence the development of immunotherapeutic strategies against STAD.

Here, AS signatures were systematically analyzed on the basis of The Cancer Genome Atlas (TCGA) database for elucidating the survival-associated AS event profile, cancer-related pathways of AS genes, and potential mechanisms of SF-AS interactions in STAD. The overall survival (OS)-related AS signature prognostic model-based high and low risk score groups were significantly related to STAD OS. Furthermore, the significantly different distribution of immune cells between the high- and low-risk score groups according to the AS signatures induced further exploration of the differentially expressed immune-related genes (DEIRGs) between the two groups. The enriched pathways of these DEIRGs suggested that survival-associated AS events might influence the immune microenvironment. These observations need further experimental verification and clinical research for the development of clinical applications utilizing the identified AS-associated biomarkers and immune-related genes (IRGs). A flow chart of the study is presented in Figure 1A.

MATERIALS AND METHODS

Data collection in STAD

The RNA transcriptome (level 3) profiles and corresponding clinical variables (including age, sex, pathologic stage, pathologic T, pathologic M, and pathologic N) of the STAD cohorts were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/). AS events in STAD were obtained from TCGA SpliceSeq (https://bioinformatics.mdanderson.org/public-software/tcgaspliceseq/). The Percent Spliced In values, which represent the ratio between reads excluding or including exons, were used to calculate splice events, including AA, AD, AP, AT, ES, ME, and RI. Each splicing event has a unique code consisting of the gene symbol, ID number, and splicing type to ensure accuracy. In addition, an UpSet plot (https://www.rdocumentation.org/packages/UpSetR/versions/1.4.0/topics/upset) was generated using the R package to summarize overlapping sets among the seven types (AA, AD, AT, AP, ES, ME, and RI) of AS events in STAD. IRGs were matched with the ImmPort database (https://www.immport.org/shared/home). The proportion of 22 human


A
Acquisition of alternative splicing (AS) events in STAD from TCGA and TCGA SpliceSeq database
375 STAD *vs* 32 normal tissues
AS events in STAD
Upset plot
AA, AD, AP, AT, ES, ME, RI
COX regression analysis of AS events
OS-related- AS (2042 in STAD)
Volcano plot
Upset plot
Bubble plot
AA, AD, AP, AT, ES, ME, RI
KEGG
GO
PPI
SF-OS-related-AS network
Generation of prognostic signatures
-Survival between high and low riskscore groups
-ROC
-Riskplot
-PCA
-Univariate/multivariate Cox regression analysis
-GSEA
-TME profile between high and low riskscore groups
-Immune related DEGs between high and low riskscore groups
B
Alternate acceptor site (AA)
Alternate promoter (AP)
Alternate donor site (AD)
Alternate terminator (AT)
Retained intron (RI)
Mutually exclusive exons (ME)
Exon skip (ES)

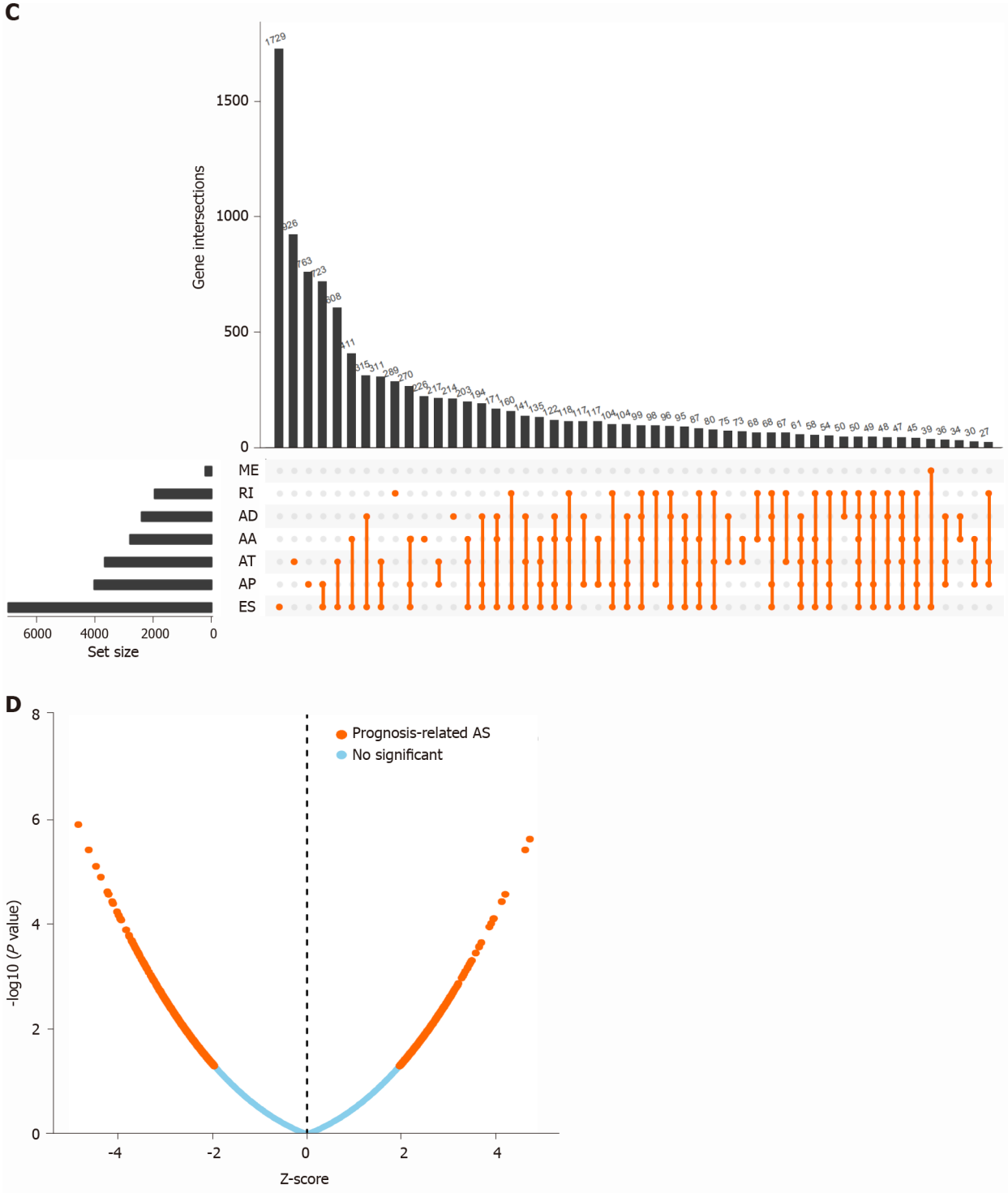


Figure 1 Overview of alternative splicing events profiling in stomach adenocarcinoma. A: The flow chart for identification of overall survival (OS)-related alternative splicing (AS) signatures in stomach adenocarcinoma (STAD); B: Schematic diagram of seven splicing modes; C: UpSet plot of different types of AS events in STAD; D: Volcano plot of OS-related AS events in STAD. Upregulated OS-related AS events are indicated by red points and downregulated OS-related AS events by green points. TCGA: The Cancer Genome Atlas; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; PPI: Protein-protein interaction; ES: Exon skip; AP: Alternate promoters; AT: Alternate terminators; AA: Alternate acceptors; AD: Alternate donors; RI: Retained introns; ME: Exclusive exons.

immune cells in STAD was evaluated with the CIBERSORT (http://cibersort.stanford.edu/) algorithm-based LM22 gene signature using 1000 permutations.

Cox regression analysis of IRG AS events in STAD

The Cox proportional hazard regression model was constructed using the survival R package (https://www.rdocumentation.org/packages/survival/versions/3.2-3) to select OS-related AS events (P value < 0.05) in STAD. Additionally, we generated an UpSet plot (https://www.rdocumentation.org/packages/UpSetR/versions

/1.4.0/topics/upset) and bubble plot (https://www.rdocumentation.org/ggplot2/versions/3.3.2) using the R package to summarize OS-related AS event modes (AA, AD, AT, AP, ES, ME, and RI) in STAD.

Functional and pathway enrichment analyses of OS-related AS events in STAD

The identified OS-related AS event genes in STAD were input into the DAVID functional annotation bioinformatics microarray database (https://david.ncifcrf.gov/) to analyze the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [$P < 0.05$ and false discovery rate (FDR) < 0.05]. Gene Ontology (GO) terms analysis according to biological process (BP) was performed using Cytoscape ClueGO (two-sided hypergeometric test, adjusted P value < 0.05, corrected using the Benjamini-Hochberg method). All OS-related AS events were used to construct a protein-protein interaction (PPI) network in the STRING database (https://string-db.org/), which had a combined score > 0.9 and which was confirmed through experiments.

Construction of the SF-OS-related AS network in STAD

The SF protein is involved in removing introns from strings of mRNA, following which the exons combine again; the AS process occurs in the spliceosomes. The SF gene list was matched and selected from the Splice Aid database (http://www.introni.it/splicing.html). Furthermore, the correlation between the SFs and OS-related-AS was analyzed using the Corrplot R package (https://cran.r-project.org/web/packages/corrplot/vignettes/corrplot-intro.html). The network of OS-related AS events and SFs was constructed using Cytoscape on the basis of the cutoff value (correlation coefficient > 0.6 and P value < 0.05).

Lasso regression construction and verification in STAD

The OS-related AS events were selected for constructing a prognostic model in STAD with lasso regression using the glmnet R package (https://cran.r-project.org/web/packages/glmnet/index.html). Lasso regression achieves dimension reduction of high-dimensional data by restricting the sum of the absolute value of coefficients to be smaller than a predetermined value. As a result, variables with a relatively small contribution would be conferred a coefficient of zero. The best model was determined by maximizing model performance and minimizing the number of features (*i.e.*, genes). Only genes with nonzero coefficients in the lasso regression model were chosen to further calculate the risk score. We computed the risk score for each patient using the following formula: Risk scores =∑j=1nCoefj × Xj, with Coefj indicating the coefficient and Xj representing the relative expression levels of each AS-related gene standardized by the z-score. The median risk score was chosen as a cutoff value to dichotomize TCGA-STAD cohorts. The STAD samples were divided into two subtypes (high-risk-score group and low-risk-score group) according to the median risk score obtained through the OS-related AS events prognostic model. We plotted the receiver operating characteristic (ROC) curves using the R package (https://www.rdocumentation.org/packages/pROC/versions/1.16.2/topics/roc) to show the specificity and sensitivity of the risk score for predicting prognosis in STAD. The Kaplan-Meier curve was used to evaluate the relevance of the OS between the risk score groups. Additionally, univariate and multivariate Cox regression models were used to analyze the association between OS and clinical characteristics of STAD, including age, sex, pathologic stage, pathologic T, pathologic M, pathologic N, and risk score. Additionally, gene set enrichment analysis (GSEA) (version 4.1.0) was used to identify different gene sets in the high-risk-score group and low-risk-score group using 1000 permutations (P value < 0.05 and FDR Q value < 0.05, as calculated using the Benjamini-Hochberg multiple testing).

Identification of different immune cells and DEIRGs between high- and low-risk-score groups in STAD

The distribution of immune cells (P value < 0.05) in STAD tissue samples was compared between the high- and low-risk-score groups on the basis of the OS-related AS prognostic model using the Kolmogorov-Smirnov test with the R package (https://stat.ethz.ch/R-manual/R-devel/Library/stats/html/ks.test.html). Furthermore, the correlation of different immune cells was performed using the Corrplot R package (https://cran.r-project.org/web/packages/corrplot/vignettes/corrplot-intro.html). We used the Limma package (https://www.bioconductor.org/packages/release/bioc/html) to compare DEIRGs (P value < 0.05, FDR ≤ 0.05, and $\log^{(\text{fold change})}$ filter ≥ 0.58) between the high- and low-risk-score groups. The DEIRGs in STAD were input into the DAVID functional annotation bioinformatics microarray database (

https://david.ncifcrf.gov/) for analyzing significant KEGG pathways ($P < 0.05$ and FDR < 0.05).

RESULTS

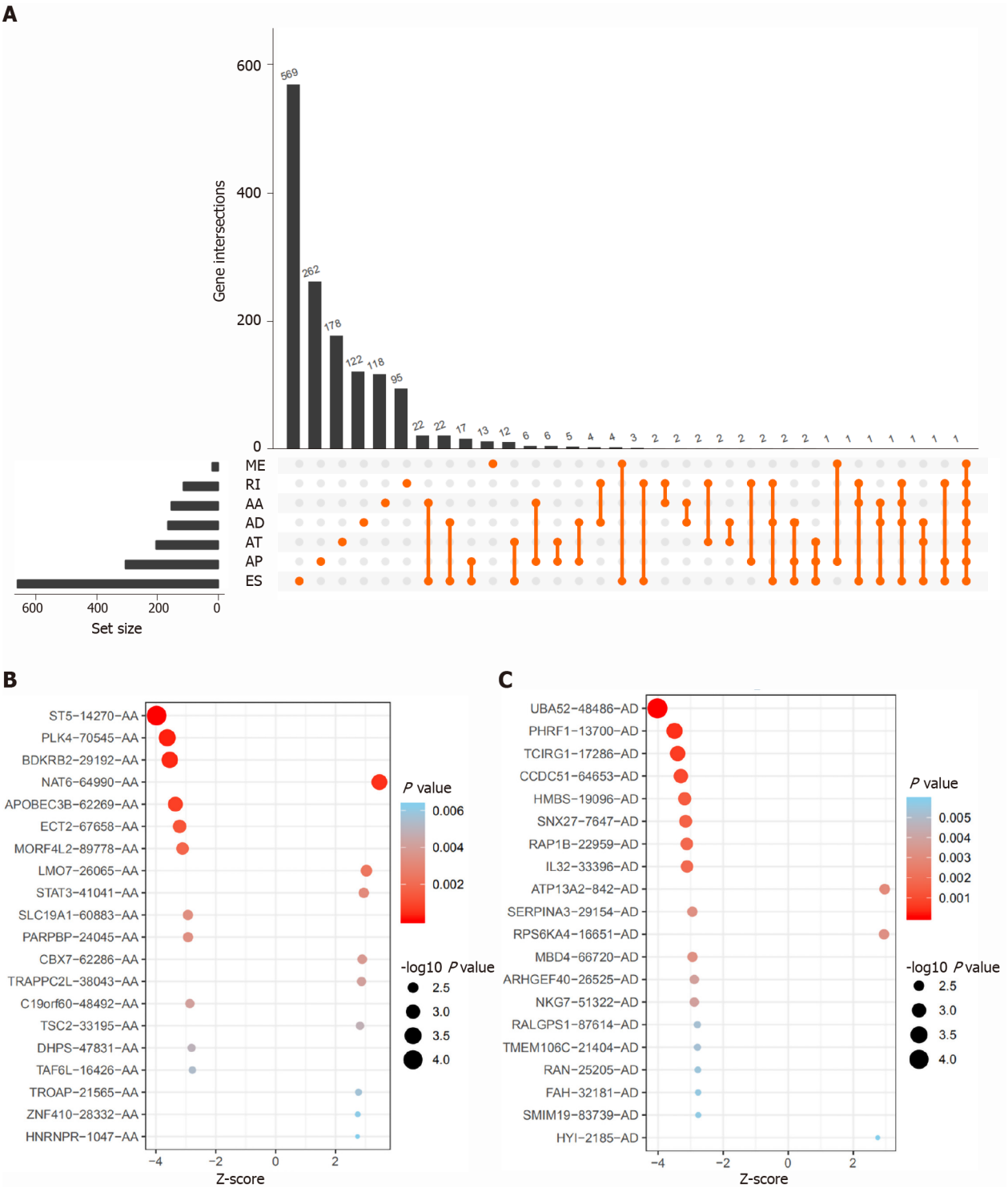
Identification of OS-related AS events in STAD

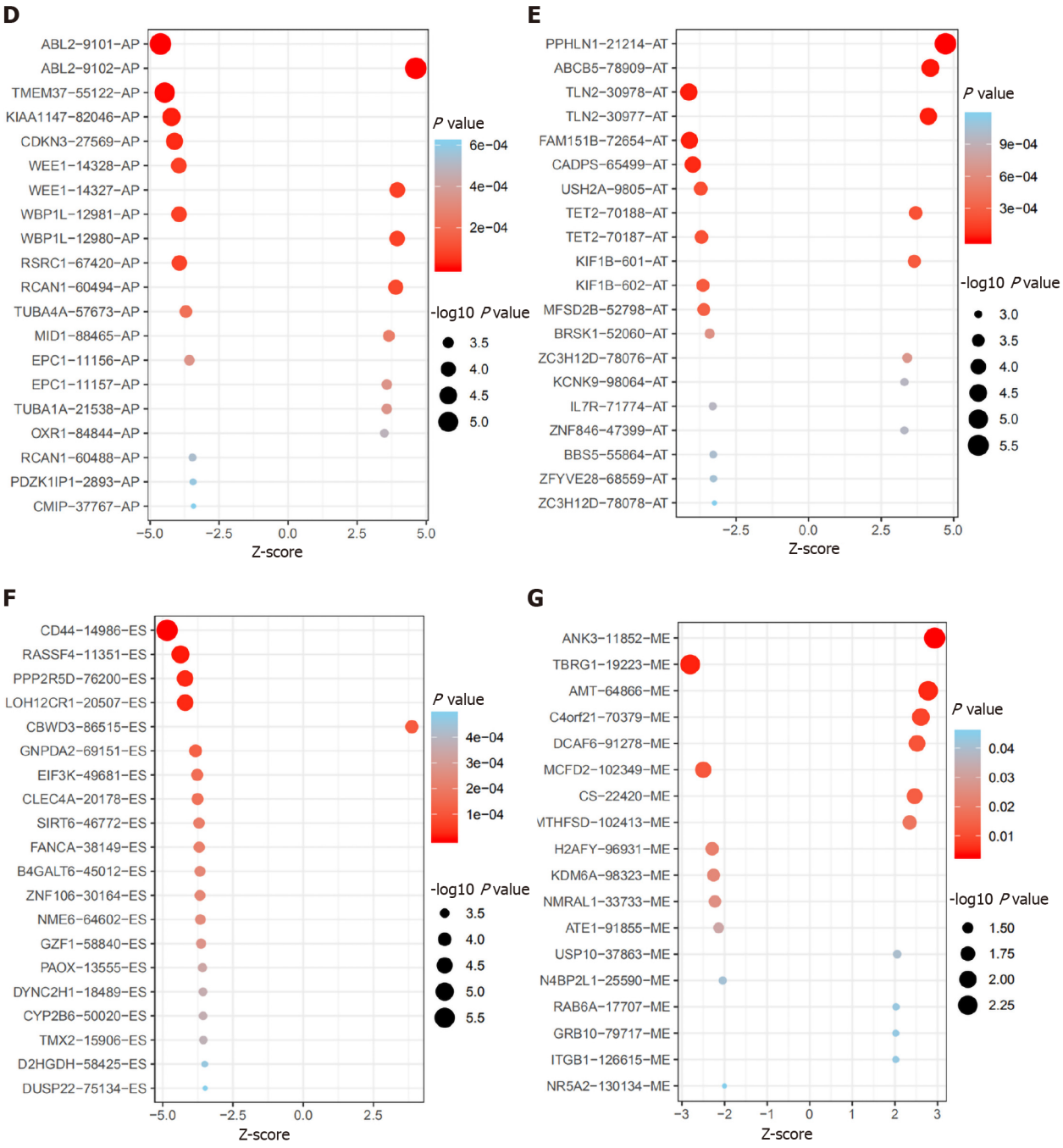
The AS event modes included seven traditional types, namely ES, AA, AT, AP, AD, ME, and RI (Figure 1B). The diagrammatic drawing of different AS event modes described different splicing locations in the pre-mRNAs. For example, the ES form is the most common splicing mode in mammalian pre-mRNAs, where an exon may be spliced out of the primary transcript or retained. In the AA mode, an alternative 3' splice junction is used to change the downstream exon 5' boundary. In the AD mode, an alternative 5' splice junction is used to change the upstream exon 3' boundary. In the ME mode, one of the two exons would be retained in the mRNAs after splicing but not both. RI is the rarest mode in mammalian pre-mRNAs, where a sequence might be spliced out as an intron or simply retained.

In total, 375 STAD patients and 32 normal tissues were included in the analysis of the AS events. The AS profile of STAD is shown in the UpSet plot (Figure 1C). A total of 48141 AS events were detected, and the original data were obtained from the TCGA SpliceSeq database (https://bioinformatics.mdanderson.org/TCGASpliceSeq/). Among these AS events, 2042 OS-related AS events were identified using the Cox survival regression analysis (Figure 1D), including 157 AA, 174 AD, 297 AT, 461 AP, 805 ES, 18 ME, and 130 RI (Figure 2A). There were 569 genes that only occurred in ES; 262 genes, in AP; 178 genes, in AT; 122 genes, in AD; 118 genes, in AA; 95 genes, in RI; and 13 genes, in ME. Some genes exhibited multiple OS-related AS events; for example, four types of AS events were detected for IL32, including IL32|33378|RI, IL32|33396|AD, IL32|33397|ES, IL32|33399|ES, IL32|33402|ES, IL32|33403|ES, IL32|33406|ES, IL32|33407|ES, IL32|33412|ES, IL32|33413|ES, IL32|33426|RI, IL32|33435|AA, and IL32|33439|AD. Three types of AS events were detected for the following: ANAPC11, which included ANAPC11|44201|AP, ANAPC11|44207|ES, ANAPC11|44211|ES, ANAPC11|44214|AD, and ANAPC11|44217|ES; CARD8, which included CARD8|50711|ES, CARD8|50713|ES, CARD8|50715|ES, CARD8|50719|AD, and CARD8|50721|RI; IDS, which included IDS|90286|AP, IDS|90287|AP, IDS|90290|AT, IDS|90291|AT, and IDS|90294|ES; KIAA1217, which included KIAA1147|82046|AP, KIAA1217|10993|AP, KIAA1217|11000|RI, KIAA1217|11002|ES, KIAA1217|11003|ES, and KIAA1217|11004|ES; MIB2, which included MIB2|183|AP, MIB2|184|AP, MIB2|192|ES, MIB2|196|ES, and MIB2|198|AD; NUDT22, which included NUDT22|16584|AT, NUDT22|16587|ES, and NUDT22|16591|AD; PHRF1, which included PHRF1|13697|AA, PHRF1|13699|ES, and PHRF1|13700|AD; SRSF7, which included SRSF7|53276|RI, SRSF7|53277|RI, SRSF7|53278|AA, SRSF7|53279|RI, SRSF7|53280|RI, and SRSF7|53284|ES; and TMEM205, which included TMEM205|47656|RI, TMEM205|47657|RI, TMEM205|47665|AD, TMEM205|47673|RI, TMEM205|47675|ES, and TMEM205|47678|AD. Some genes had multiple AS locations with the same AS events, for example, CD44 (CD44|14985|ES, CD44|14986|ES, CD44|14989|ES, CD44| 15133|ES, and CD44|15142|ES), COL1A1 (COL1A1|1058233|ES, COL1A1|1557945|ES, COL1A1|1569777|ES, COL1A1|316114|ES, COL1A1|316125| ES, COL1A1|316133|ES, COL1A1| 409264|ES, COL1A1|941205|ES, and COL1A1|941210|ES), SRSF2 (SRSF2|43661|RI, SRSF2|43662| RI, and SRSF2|43663|RI), and ZNF436 (ZNF436|1050|AP, ZNF436|1051|AP, ZNF439|47755|AP, and ZNF439|47756|AP). The distribution of OS-related AS events in STAD is shown in the bubble plot, including AAs, ADs, ATs, APs, ESs, ME, and RIs (Figure 2B-H). The difference in the distribution of OS-related AS events in STAD might indicate specific splicing locations in the genome and could enable the development of novel treatment strategies for STAD.

OS-related-AS genes were significantly enriched in certain cancer-related pathways and BPs in STAD

The KEGG enrichment analysis was used to analyze pathways involved in the identified OS-related-AS events. A total of 27 statistically significant KEGG pathways were enriched in STAD (Figure 3A); most of the pathways were closely associated with tumorigenesis and cancer development. Some of the OS-related-AS genes played important roles in the signaling pathways and served as hub molecules.


A
Gene intersections
600
400
200
0
569
262
178
122
118
95
22
22
17
13
12
6
6
5
4
4
3
2
2
2
2
2
2
2
2
1
1
1
1
1
1
ME
RI
AA
AD
AT
AP
ES
600
400
200
0
Set size
B
ST5−14270−AA
PLK4−70545−AA
BDKRB2−29192−AA
NAT6−64990−AA
APOBEC3B−62269−AA
ECT2−67658−AA
MORF4L2−89778−AA
LMO7−26065−AA
STAT3−41041−AA
SLC19A1−60883−AA
PARPBP−24045−AA
CBX7−62286−AA
TRAPPC2L−38043−AA
C19orf60−48492−AA
TSC2−33195−AA
DHPS−47831−AA
TAF6L−16426−AA
TROAP−21565−AA
ZNF410−28332−AA
HNRNPR−1047−AA
−4
−2
0
2
Z-score
P value
0.006
0.004
0.002
-log10 P value
2.5
3.0
3.5
4.0
C
UBA52−48486−AD
PHRF1−13700−AD
TCIRG1−17286−AD
CCDC51−64653−AD
HMBS−19096−AD
SNX27−7647−AD
RAP1B−22959−AD
IL32−33396−AD
ATP13A2−842−AD
SERPINA3−29154−AD
RPS6KA4−16651−AD
MBD4−66720−AD
ARHGEF40−26525−AD
NKG7−51322−AD
RALGPS1−87614−AD
TMEM106C−21404−AD
RAN−25205−AD
FAH−32181−AD
SMIM19−83739−AD
HYI−2185−AD
−4
−2
0
2
Z-score
P value
0.005
0.004
0.003
0.002
0.001
-log10 P value
2.5
3.0
3.5
4.0


D
ABL2−9101−AP
ABL2−9102−AP
TMEM37−55122−AP
KIAA1147−82046−AP
CDKN3−27569−AP
WEE1−14328−AP
WEE1−14327−AP
WBP1L−12981−AP
WBP1L−12980−AP
RSRC1−67420−AP
RCAN1−60494−AP
TUBA4A−57673−AP
MID1−88465−AP
EPC1−11156−AP
EPC1−11157−AP
TUBA1A−21538−AP
OXR1−84844−AP
RCAN1−60488−AP
PDZK1IP1−2893−AP
CMIP−37767−AP
−5.0 −2.5 0.0 2.5 5.0
Z-score
P value
6e−04
4e−04
2e−04
-log10 P value
3.5
4.0
4.5
5.0
E
PPHLN1−21214−AT
ABCB5−78909−AT
TLN2−30978−AT
TLN2−30977−AT
FAM151B−72654−AT
CADPS−65499−AT
USH2A−9805−AT
TET2−70188−AT
TET2−70187−AT
KIF1B−601−AT
KIF1B−602−AT
MFSD2B−52798−AT
BRSK1−52060−AT
ZC3H12D−78076−AT
KCNK9−98064−AT
IL7R−71774−AT
ZNF846−47399−AT
BBS5−55864−AT
ZFYVE28−68559−AT
ZC3H12D−78078−AT
−2.5 0.0 2.5 5.0
Z-score
P value
9e−04
6e−04
3e−04
-log10 P value
3.0
3.5
4.0
4.5
5.0
5.5
F
CD44−14986−ES
RASSF4−11351−ES
PPP2R5D−76200−ES
LOH12CR1−20507−ES
CBWD3−86515−ES
GNPDA2−69151−ES
EIF3K−49681−ES
CLEC4A−20178−ES
SIRT6−46772−ES
FANCA−38149−ES
B4GALT6−45012−ES
ZNF106−30164−ES
NME6−64602−ES
GZF1−58840−ES
PAOX−13555−ES
DYNC2H1−18489−ES
CYP2B6−50020−ES
TMX2−15906−ES
D2HGDH−58425−ES
DUSP22−75134−ES
−5.0 −2.5 0.0 2.5
Z-score
P value
4e−04
3e−04
2e−04
1e−04
-log10 P value
3.5
4.0
4.5
5.0
5.5
G
ANK3−11852−ME
TBRG1−19223−ME
AMT−64866−ME
C4orf21−70379−ME
DCAF6−91278−ME
MCFD2−102349−ME
CS−22420−ME
MTHFSD−102413−ME
H2AFY−96931−ME
KDM6A−98323−ME
NMRAL1−33733−ME
ATE1−91855−ME
USP10−37863−ME
N4BP2L1−25590−ME
RAB6A−17707−ME
GRB10−79717−ME
ITGB1−126615−ME
NR5A2−130134−ME
−3 −2 −1 0 1 2 3
Z-score
P value
0.04
0.03
0.02
0.01
-log10 P value
1.50
1.75
2.00
2.25

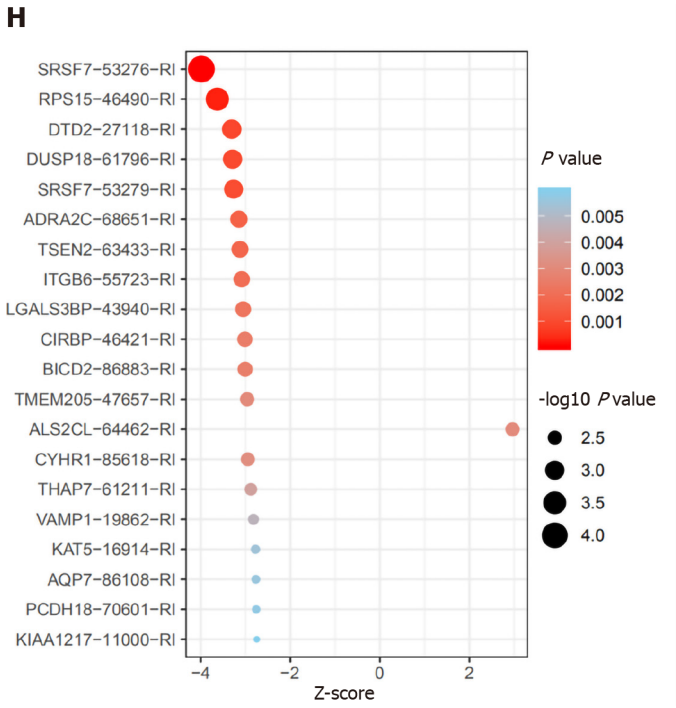


Figure 2 Overall survival-related alternative splicing events and the distribution in stomach adenocarcinoma. A: UpSet plot of different types of overall survival (OS)-related alternative splicing (AS) events in stomach adenocarcinoma (STAD); B-H: The distribution of OS-related AS events in STAD are illustrated by the bubble plot, including exon skip, alternate promoters, alternate terminators, alternate acceptors, alternate donors, retained introns, and exclusive exons.

We used the GO enrichment analysis according to BP to analyze the identified OS-related-AS genes. A total of 72 statistically significant BPs were obtained in STAD (Figure 3B), which primarily included cellular processes involved in positive and negative regulation of gene expression (positive and negative regulation of transcription, positive regulation of nucleic-acid-templated transcription), regulation of RNA metabolic processes (negative regulation of transcription by RNA polymerase II, RNA processing, mRNA processing, RNA splicing, positive and negative regulation of RNA metabolic process, and positive and negative regulation of RNA biosynthetic process), regulation of metabolic processes (protein kinase activity, regulation of protein modification process, establishment of protein localization to organelles, cellular protein modification process, intracellular protein transport, enzyme-linked receptor protein signaling pathway, transmembrane receptor protein tyrosine kinase signaling pathway, protein transport, regulation of protein transport, positive regulation of protein localization, kinase activity, negative regulation of phosphorylation, and positive regulation of protein transport), and regulation of intracellular signal transduction (transcription coregulator activity, Wnt signaling pathway, regulation of cell migration, regulation of apoptotic process, and regulation of cell cycle phase transition). These BP enrichments of the identified OS-related-AS genes have broad implications in STAD cells, influencing the process of DNA, RNA, protein, and related signal transductions.

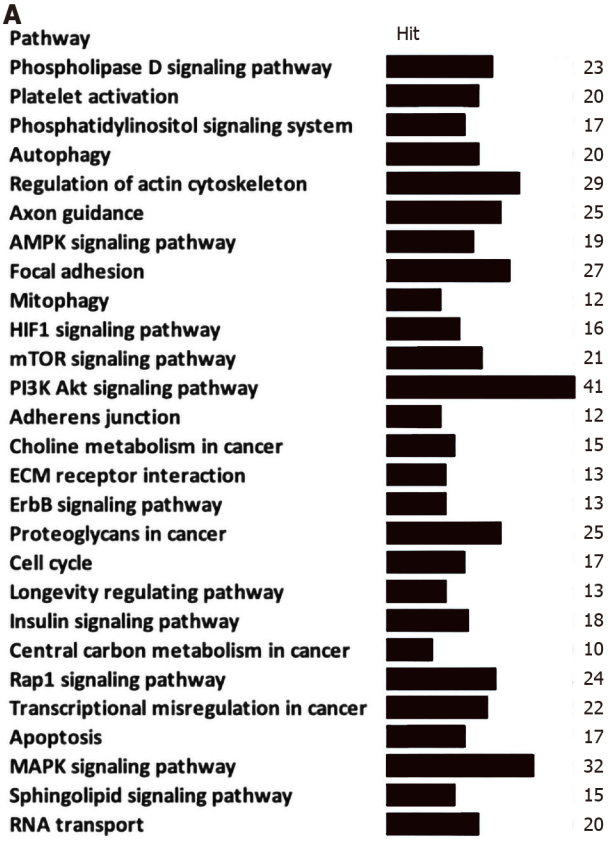
The identified OS-related-AS genes were used for constructing a PPI network for STAD (Figure 3C). Stringent filter conditions were set for the PPI network, which included a combined score more than 0.9; the interactions were experimentally determined in previous studies. A total of 170 protein pairs (132 proteins) were obtained.

Key modules in the PPI network of STAD include CASP8, CREBBP, NDUFS4, CDC27, PTTG1, FZR1, ANAPC11, UBA52, RPS23, RPS6, RPL13, RPL36, GNB2L1, RPL26L1, FAU, RPS15, RPS21, RPS24, RPS5, RPLP0, and RPL18A. Several ribosome-associated proteins were present in the PPI network.

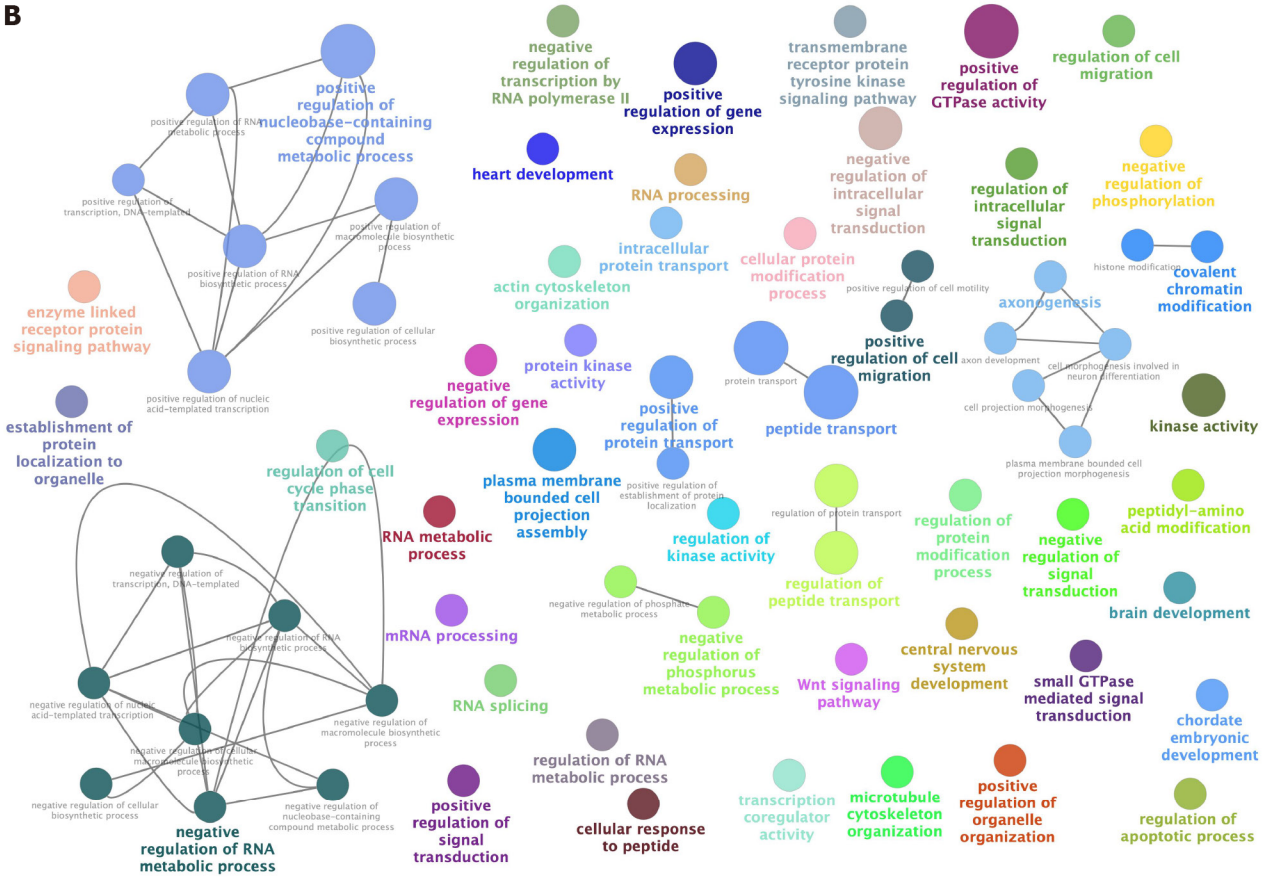
Construction of potential SF-AS regulatory network in STAD

For exploring the potential upstream regulatory mechanisms of the SFs of OS-related-AS events in STAD, the RNA expression of the SFs was extracted from the TCGA database based on the Splice Aid database in STAD. The regulatory mechanisms between SF and AS with a correlation coefficient greater than 0.6 and a *P* value less

A


Pathway
Hit
Phospholipase D signaling pathway 23
Platelet activation 20
Phosphatidylinositol signaling system 17
Autophagy 20
Regulation of actin cytoskeleton 29
Axon guidance 25
AMPK signaling pathway 19
Focal adhesion 27
Mitophagy 12
HIF1 signaling pathway 16
mTOR signaling pathway 21
PI3K Akt signaling pathway 41
Adherens junction 12
Choline metabolism in cancer 15
ECM receptor interaction 13
ErbB signaling pathway 13
Proteoglycans in cancer 25
Cell cycle 17
Longevity regulating pathway 13
Insulin signaling pathway 18
Central carbon metabolism in cancer 10
Rap1 signaling pathway 24
Transcriptional misregulation in cancer 22
Apoptosis 17
MAPK signaling pathway 32
Sphingolipid signaling pathway 15
RNA transport 20

B


positive regulation of nucleobase-containing compound metabolic process
negative regulation of transcription by RNA polymerase II
positive regulation of gene expression
transmembrane receptor protein tyrosine kinase signaling pathway
positive regulation of GTPase activity
regulation of cell migration
heart development
RNA processing
negative regulation of intracellular signal transduction
regulation of intracellular signal transduction
negative regulation of phosphorylation
intracellular protein transport
cellular protein modification process
actin cytoskeleton organization
histone modification
covalent chromatin modification
enzyme linked receptor protein signaling pathway
positive regulation of cell motility
axonogenesis
positive regulation of cell migration
protein transport
peptide transport
protein kinase activity
negative regulation of gene expression
positive regulation of protein transport
establishment of protein localization to organelle
kinase activity
regulation of cell cycle phase transition
plasma membrane bounded cell projection assembly
RNA metabolic process
regulation of protein transport
regulation of peptide transport
regulation of protein modification process
negative regulation of signal transduction
peptidyl-amino acid modification
brain development
mRNA processing
negative regulation of phosphorus metabolic process
central nervous system development
small GTPase mediated signal transduction
Wnt signaling pathway
RNA splicing
chordate embryonic development
regulation of RNA metabolic process
transcription coregulator activity
microtubule cytoskeleton organization
positive regulation of organelle organization
cellular response to peptide
positive regulation of signal transduction
regulation of apoptotic process
negative regulation of RNA metabolic process

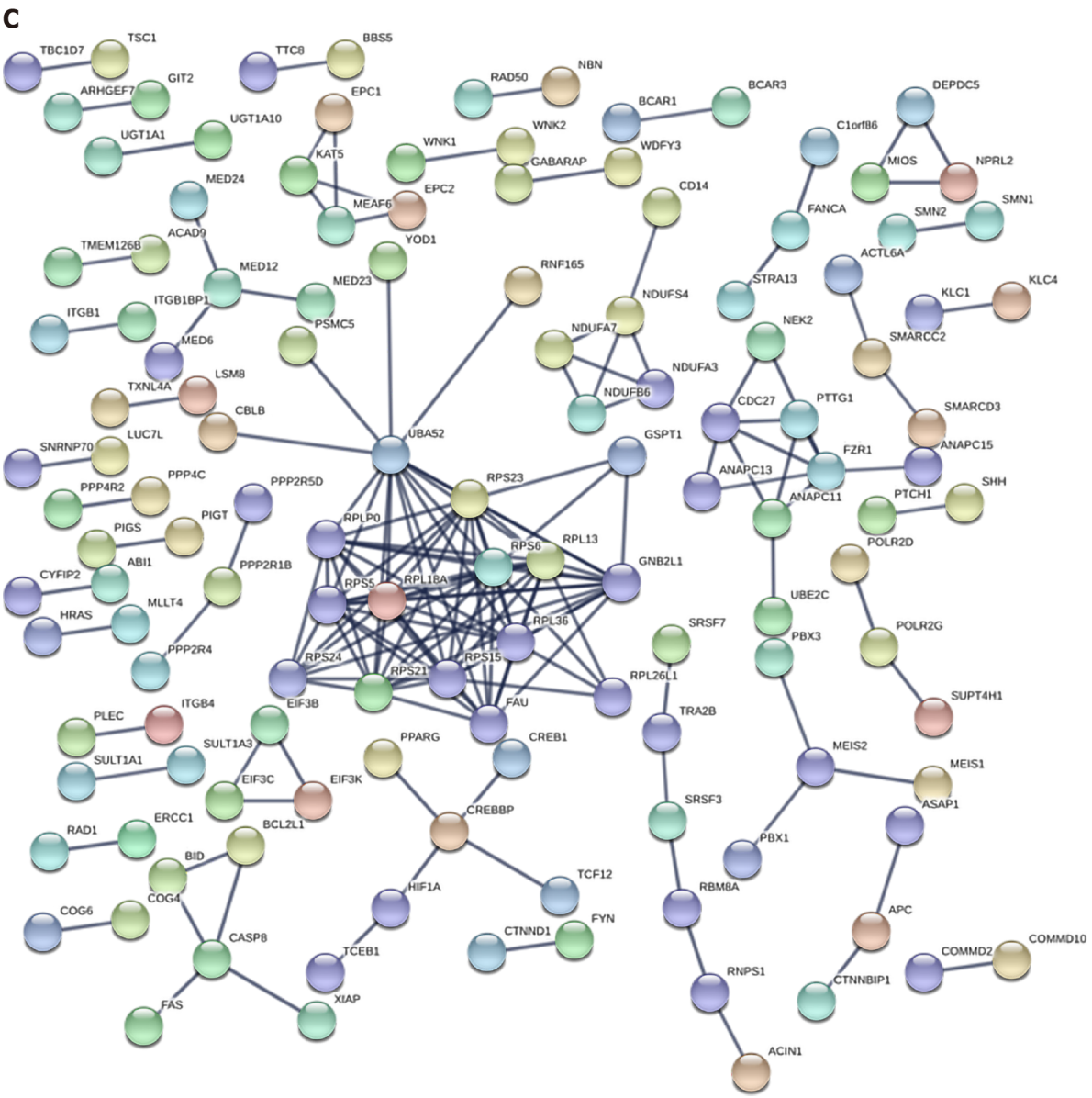


Figure 3 Functional enrichment and pathway analysis of overall survival-related alternative splicing events in stomach adenocarcinoma. A: Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of overall survival (OS)-related alternative splicing (AS) events in stomach adenocarcinoma (STAD) ($P < 0.05$); B: GO enrichment analysis (biological process) of OS-related AS events in STAD. Only gene sets with natural organic matter $P < 0.05$ and false discovery rate (FDR) Q < 0.05 (adjusted P value using the Benjamini-Hochberg procedure to control the FDR) were considered significant. The lower P value and higher significant enrichment are shown with the greater node size. The same color indicated the same function group. Among the groups, we chose a representative for the most significant term and lag highlighted; C: Protein-protein interaction network of OS-related AS events in STAD.

than 0.05 were utilized to construct the SF-AS regulatory network (Figure 4). A total of 26 SFs were included in the network, including RBM25, SREK1, HSPA1A, SNRNP200, LUC7L3, SNRPN, PPIG, RBMS1, QKI, SNRPF, BAG2, RBFOX2, BUD31, RBM5, PRPF38B, RBM15, DHX9, CCAR1, SRSF2, DDX17, CELF2, SRSF11, TIA1, ARGLU1, RBM47, and CLK1. A total of 81 AS events were included in the network, of which 33 AS events were negatively correlated with SFs and 48 AS events were positively correlated with SFs. Certain potential SF-AS pairs showed a correlation coefficient greater than 0.7, for example, QKI with CLSTN1-575-ES, PLEKHM2-767-ES, KIF1B-601-AT, ACOT9-88694-ES, ARHGEF11-8338-ES, LRRFIP2-63960-ES, KIF1B-602-AT, SEPT11-69616-AT, MAP3K7-77020-ES, and CD47-66013-ES; BAG2 and DAB2IP-87442-AP; BUD31 and MFSD7-68391-AP; CELF2 and ARHGEF11-8338-ES; and RBFOX2 and CCSER2-12409-ES. These SF-AS pairs would be important when exploring the upstream SFs and downstream regulatory mechanisms of the OS-related AS events in STAD.

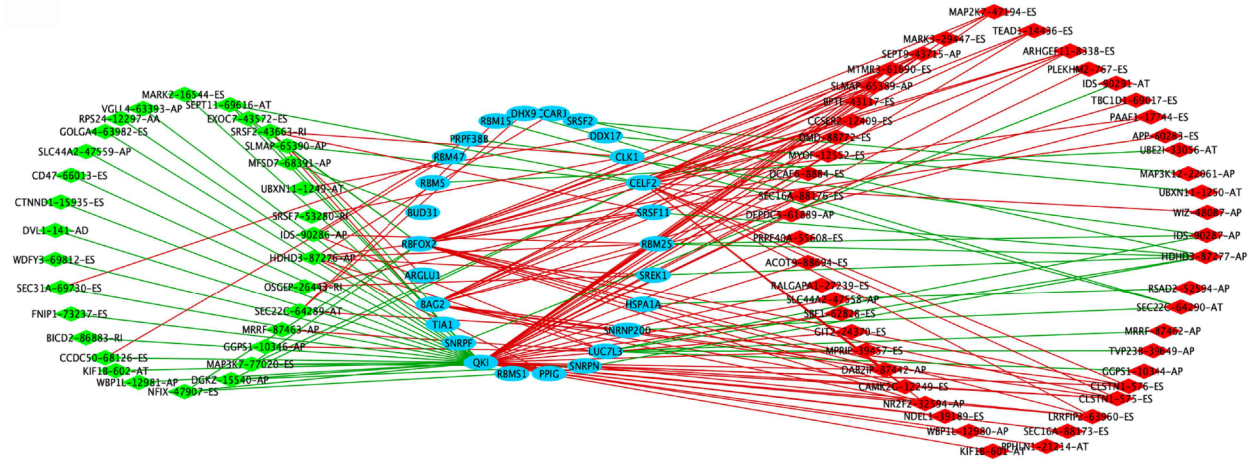


Figure 4 Splicing factor-alternative splicing interaction network in stomach adenocarcinoma. The favorable prognosis of overall survival (OS)-related alternative splicing (AS) events are indicated in green; the unfavorable prognosis of OS-related AS events, red; the upstream splicing factors (SFs), blue; the negative correlation between SFs and OS-related AS events, green lines; the positive correlation between SFs and OS-related AS events, red lines.

Construction of the OS-related AS prognostic model for STAD patients

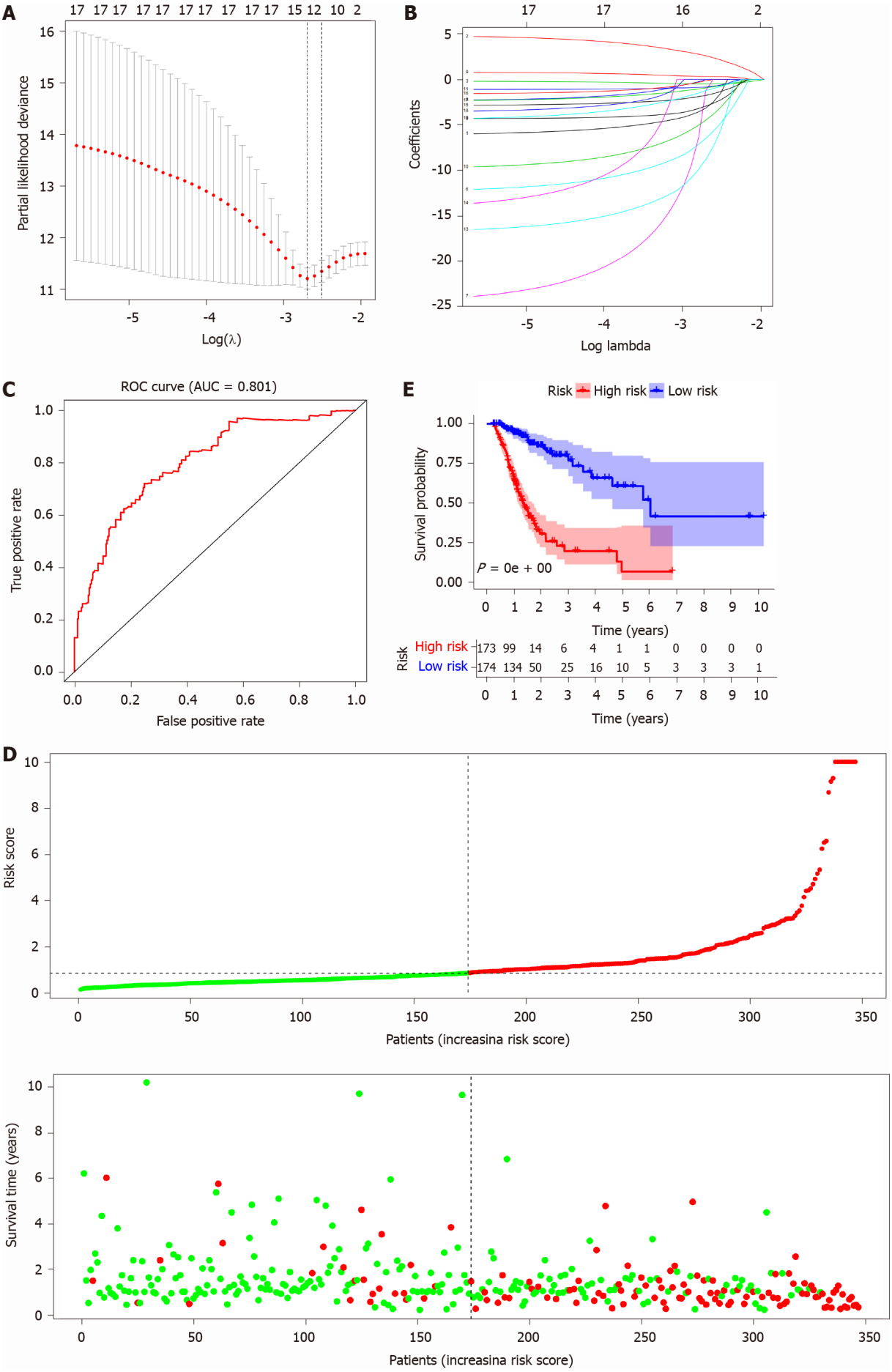
The OS-related AS events were selected to construct a prognostic model for STAD patients using lasso regression. Furthermore, when log (lambda) was set within -2 and -3 (Figure 5A and B), a signature model of 11 OS-related AS events (CD44 | 14986 | ES, PPHLN1 | 21214 | AT, RASSF4 | 11351 | ES, KIAA1147 | 82046 | AP, PPP2R5D | 76200 | ES, LOH12CR1 | 20507 | ES, CDKN3 | 27569 | AP, UBA52 | 48486 | AD, CADPS | 65499 | AT, SRSF7 | 53276 | RI, and WEE1 | 14328 | AP) was obtained for STAD. On the basis of the OS-related AS-event signature model, the STAD samples were clustered into high- and low-risk-score groups based on the median risk score (median risk score = 0.87). In addition, the ROC revealed that the constructed prognostic model exhibited high sensitivity and specificity; the area under the curve was equal to 0.81 (Figure 5C). OS analysis revealed that high mortality occurred in the high-risk-score group (Figure 5D), and the survival probability was significantly different between the high- and low-risk-score groups (Figure 5E). Univariate analysis indicated that age, pathologic stage, pathologic T, pathologic N, and risk score were significantly associated with OS (Figure 5F). Multivariate analysis demonstrated that age, pathologic stage, pathologic N, and risk score might be independent risk factors for STAD (Figure 5G).

Gene-set enrichments between high- and low-risk-score groups based on the OS-related AS prognostic model in STAD

GSEA revealed 27 statistically significant gene sets between the high- and low-risk score groups in STAD. These included ECM receptor interaction (Figure 6A), cytokine and cytokine receptor interaction (Figure 6B), MAPK signaling pathway (Figure 6C), focal adhesion, neuroactive ligand receptor interaction, calcium signaling pathway, hematopoietic cell lineage, cell adhesion molecules, gap junction, leukocyte transendothelial migration, TGF beta signaling pathway, hedgehog signaling pathway, and pathways in cancer, which were enriched in the high-risk-score group, and spliceosome (Figure 6D), cell cycle (Figure 6E), proteasome (Figure 6F), homologous recombination, nucleotide excision repair, one carbon pool by folate, DNA replication, glyoxylate and dicarboxylate metabolism, peroxisome, aminoacyl tRNA biosynthesis, alanine aspartate and glutamate metabolism, pyrimidine metabolism, RNA degradation, and the citrate cycle, which were enriched in the low-risk-score group.

Difference in distribution of immune cells, expressed IRGs, and immune-related pathways between the high- and low-risk-score groups

The proportion of immune cells in STAD was significantly different between the high- and low-risk groups, including naïve B cells, CD8 T cells, memory activated CD4 T cells, activated natural killer (NK) cells, monocytes, M0 macrophages, and resting mast cells, activated mast cells, and neutrophils (Figure 7A). Additionally, certain immune cells that were distributed differently between the risk-score subtypes were correlated


A
17 17 17 17 17 17 17 17 17 17 15 12 10 2
Partial likelihood deviance
Log(λ)
B
17 17 16 2
Coefficients
Log lambda
C
ROC curve (AUC = 0.801)
True positive rate
False positive rate
E
Risk High risk Low risk
Survival probability
P = 0e + 00
Time (years)
Risk
High risk 173 99 14 6 4 1 1 0 0 0 0
Low risk 174 134 50 25 16 10 5 3 3 3 1
Time (years)
D
Risk score
Patients (increasina risk score)
Survival time (years)
Patients (increasina risk score)

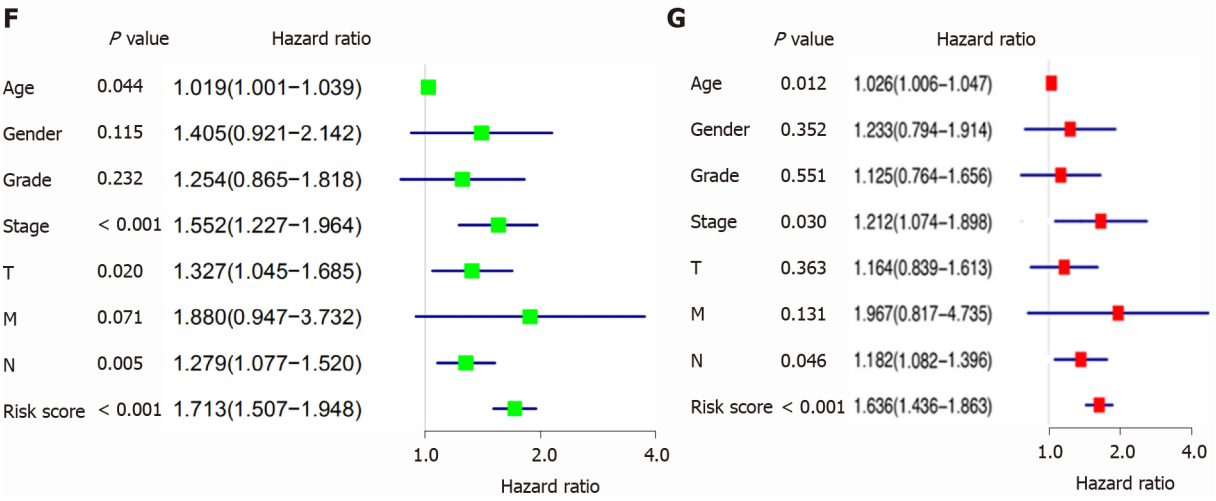
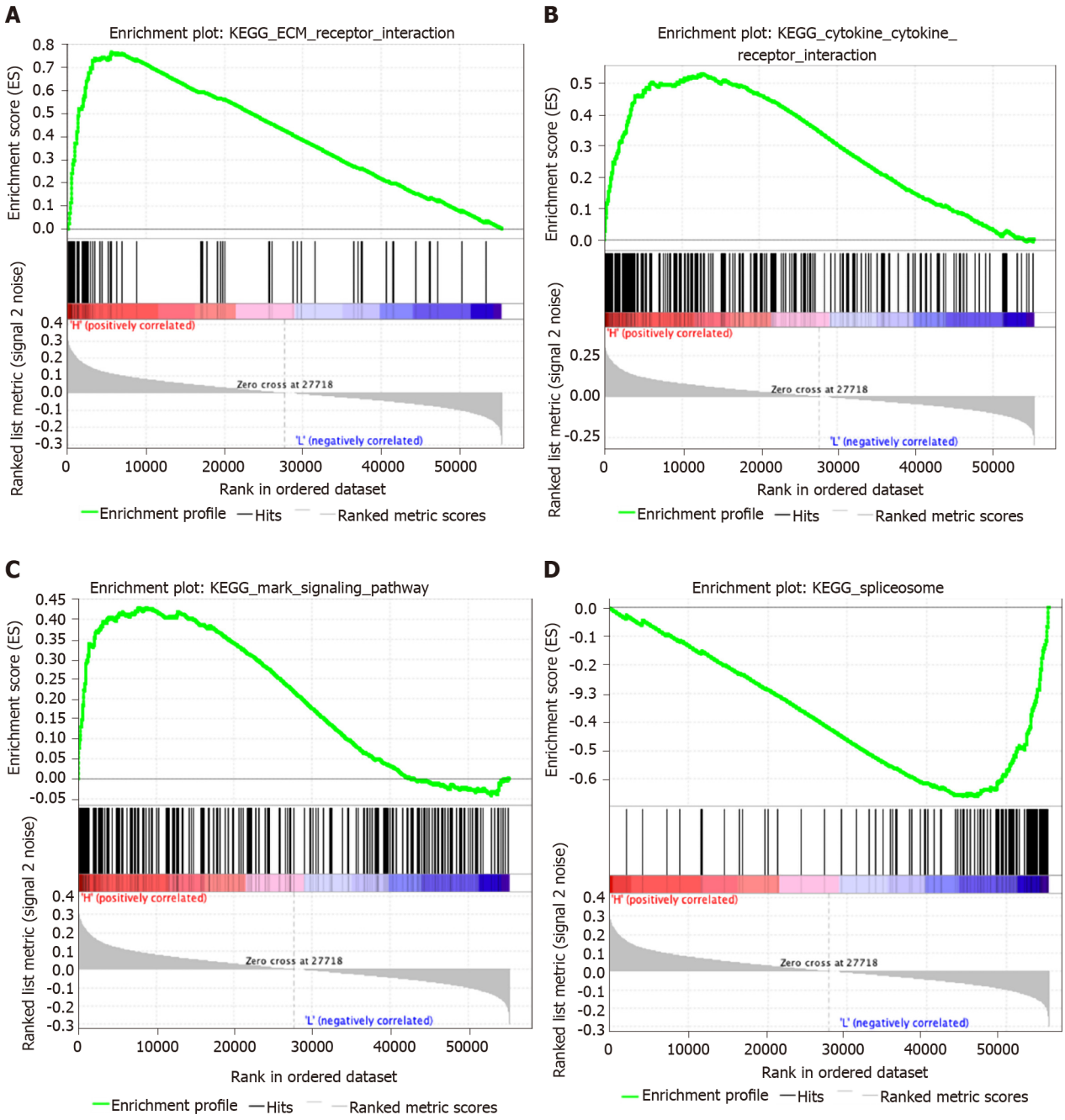


Figure 5 Lasso regression identified the prognostic model in stomach adenocarcinoma. A and B: Lasso regression complexity was controlled by lambda using the glmnet R package; C: The receiver operating characteristic of risk score in stomach adenocarcinoma (STAD); D: Risk plot between the high- and low-risk-score groups; E: Overall survival analysis between the high- and low-risk-score groups; F: The univariate Cox regression analysis of risk factors in STAD; G: The multivariate Cox regression analysis of risk factors in STAD. ROC: Receiver operating characteristic; AUC: Area under the curve.

with each other, for example, neutrophils and activated mast cells, CD8 T cells and activated mast cells, CD8 T cells and activated CD4 memory T cells, CD8 T cells and M0 macrophages, and resting mast cells and activated mast cells (Figure 7B). Furthermore, 112 DEIRGs were identified between the high-risk-score group and low-risk score group, including 2 down-regulated IRGs and 110 up-regulated IRGs (Figure 7C). The identified DEIRGs were enriched in 13 significant KEGG pathways, such as pathways in cancer (Figure 7D), cytokine-cytokine receptor interaction (Figure 7E), neuroactive ligand-receptor interaction, Ras signaling pathway, TGF-beta signaling pathway, ErbB signaling pathway, Jak-STAT signaling pathway, PI3K-Akt signaling pathway, melanoma, hematopoietic cell lineage, Rap1 signaling pathway, transcriptional misregulation in cancer, and MAPK signaling pathway. Certain DEIRGs were located at the key points of signaling pathways, for example, AR, EGF, FGF, FGFR, FLT3, and GPCR in pathways in cancer and CCL19, CCR9, GHR, NGFR, and BMPR1B in the cytokine-cytokine receptor interaction pathway.

DISCUSSION

Early clinical signs and symptoms of STAD might include continuous upper abdominal pain, heartburn, loss of appetite, emesis, and nausea; however, these nonspecific clinical manifestations commonly result in misdiagnosis of STAD[20]. Most STAD patients progress to advanced stages with symptoms including yellowing of the skin and the whites of the eyes, vomiting, difficulty in swallowing, malnutrition, weight loss, intolerable pain, and blood in the stool[21]. The development of undiscovered biomarkers could improve the accuracy and specificity of disease prevention, risk prediction, and early diagnosis, promoting a more favorable prognosis in STAD[22]. AS events have been shown to play an essential role as both oncogenes and tumor suppressors in various human cancers, including STAD[23]. An increasing number of studies have shown that AS events could regulate tumor cell proliferation, cell death, the immune microenvironment, energy metabolism, epigenetic regulation, invasion, metastasis, angiogenesis, and drug resistance[24]. For example, RhoA, a member of the Rho family of small GTPases, has been identified as a driver of STAD through recent large-scale sequencing studies. The aberrant splicing variants of RHOA exclusively occurred in gastric carcinoma cell lines; however, wild-type RhoA was nearly undetectable using quantitative real-time polymerase chain reaction (qRT-PCR) analysis, which indicated that aberrant AS induced the loss of the activity and expression of RhoA in STAD cells[25]. A previous study demonstrated that splicing variants (M-RIP, CDCA1, HYAL2, and MSMB) showed differential expression between 38 cancer cell lines from various organs and 9 corresponding normal tissues. Furthermore, RT-PCR verified that 2 splicing variants of CDCA1 were upregulated in surgically resected gastric cancer tissues, while the variants of MSMB


A
Enrichment plot: KEGG_ECM_receptor_interaction
Enrichment score (ES)
Ranked list metric (signal 2 noise)
'H' (positively correlated)
'L' (negatively correlated)
Zero cross at 27718
Rank in ordered dataset
Enrichment profile
Hits
Ranked metric scores
B
Enrichment plot: KEGG_cytokine_cytokine_receptor_interaction
C
Enrichment plot: KEGG_mark_signaling_pathway
D
Enrichment plot: KEGG_spliceosome

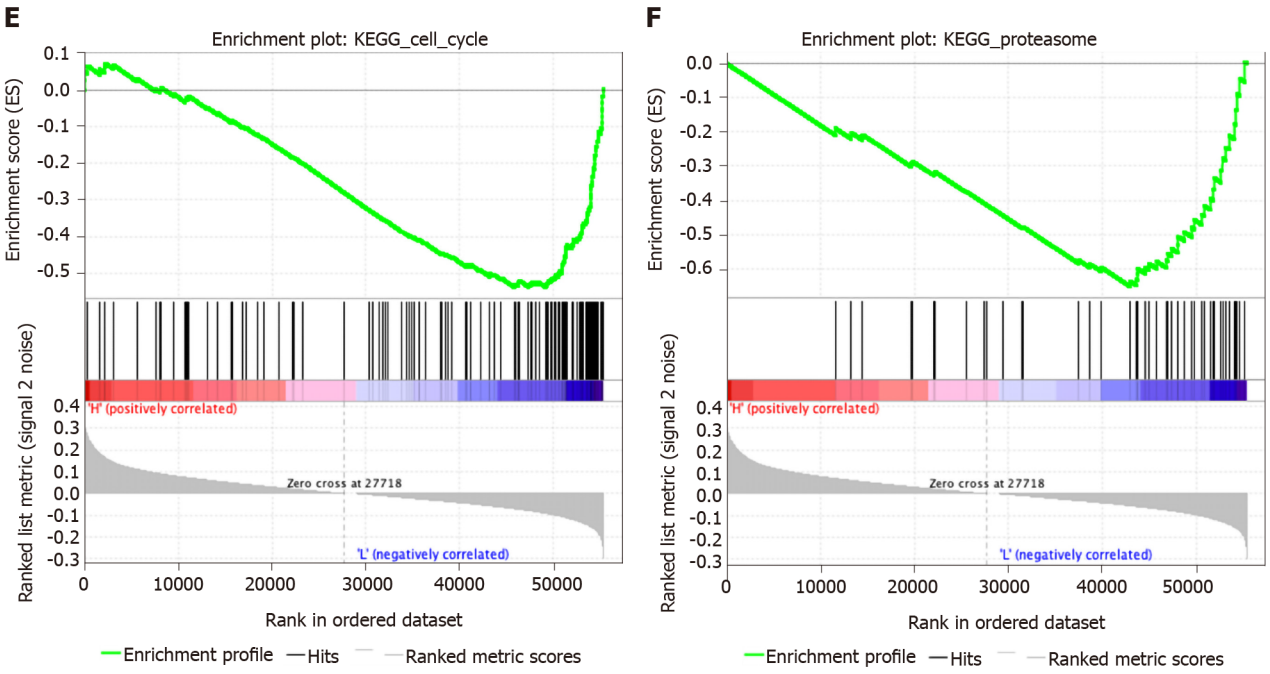
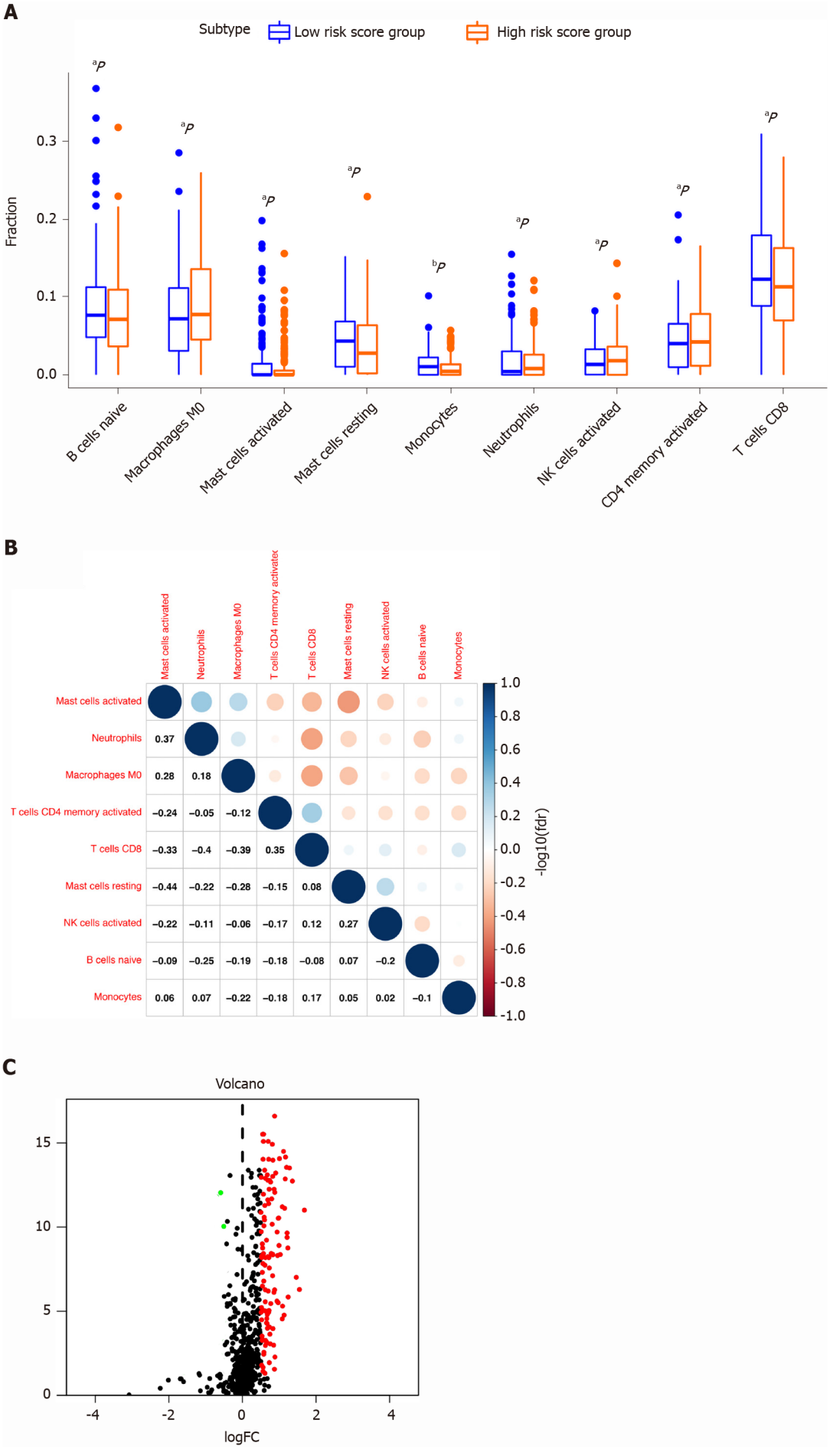


Figure 6 Gene set enrichment analysis identified gene sets that were different between the high-risk-score group and low-risk-score group in stomach adenocarcinoma. A: The ECM receptor interaction pathway was significantly different between the high-risk-score group and low-risk-score group; B: The cytokine and cytokine receptor interaction pathway was significantly different between the high-risk-score group and low-risk-score group; C: The MAPK signaling pathway was significantly different between the high-risk-score group and low-risk-score group; D: The spliceosome pathway was significantly different between the high-risk-score group and low-risk-score group; E: The cell cycle pathway was significantly different between the high-risk-score group and low-risk-score group; F: The proteasome pathway was significantly different between the high-risk-score group and low-risk-score group.

were predominantly determined in the corresponding normal tissues[26]. Immunotherapy with checkpoint inhibitors, such as targeting PD-1 or PD-L1, is a novel treatment approach in clinical applications for various cancers, including STAD. Available reports regarding IRG expression and immune cell alteration in gastric cancer would be helpful in promoting potential clinical implications of immunotherapy for STAD patients[27]. Certain novel immune checkpoints on the T cell surface were observed to have the ability to inhibit T cell responses. For example, the T cell surface molecule TIGIT plays a crucial role in the development and progression of STAD. The percentage of TIGIT$^+$CD8 T cells was increased in STAD patients compared with that in healthy individuals, and the TIGIT$^+$CD8 T cells showed functional exhaustion with proliferation, cytokine production, impaired activation, and metabolism. Targeting of TIGIT enhances CD8 T cell activation and significantly improves the chances of survival of tumor-bearing mice[28]. Certain subtypes of immune cells have also been shown to be pivotal in cancer progression and prognosis in STAD. For example, prognosis and treatment outcomes in STAD patients can be predicted by analyzing cytokines and the proportion of immune cells. The proportions of CD45RO$^+$ HLA-DR$^+$CD8 T lymphocytes, FoxP3$^+$Tregs, and PD-1$^+$CD3$^+$T lymphocytes were identified as independent prognostic factors in STAD[29]. AS increases the diversity of the mRNA during transcription. Recently, AS was reported to play a role in the alteration of the immune microenvironment, thus influencing the development of immunotherapeutic strategies against cancer; therefore, the novel splicing variants discovered may be potentially useful targets for cancer therapy and could be utilized in immunotherapy[30].

Some of the OS-related AS events identified in this study have been reported to exhibit unique functions in tumorigenesis and prognosis in STAD. For example, CD44 is a cell-surface glycoprotein that is involved in a wide variety of cellular processes, such as cell-cell interactions, lymphocyte activation, cell adhesion and migration, recirculation and homing, tumor metastasis, hematopoiesis, and interaction with matrix metalloproteinases. The complex alternative splicing variants of CD44 have been determined to be related to tumor metastasis[31]. Certain variants of CD44 are associated with gastric cancer. Among the CD44 splicing variants, CD44v8-10 has been proven to be the most promising biomarker for theranostic agents in STAD. The differentially expressed CD44 splicing variant (CD44v8-10) has been verified in stomach cancer cell lines and 74 STAD patient tissues by exon-specific qRT-PCR[32]. TRA2B, as


A
Subtype
Low risk score group
High risk score group
Fraction
0.0
0.1
0.2
0.3
aP
bP
B cells naive
Macrophages M0
Mast cells activated
Mast cells resting
Monocytes
Neutrophils
NK cells activated
CD4 memory activated
T cells CD8
B
Mast cells activated
Neutrophils
Macrophages M0
T cells CD4 memory activated
T cells CD8
Mast cells resting
NK cells activated
B cells naive
Monocytes
0.37
0.28 0.18
-0.24 -0.05 -0.12
-0.33 -0.4 -0.39 0.35
-0.44 -0.22 -0.28 -0.15 0.08
-0.22 -0.11 -0.06 -0.17 0.12 0.27
-0.09 -0.25 -0.19 -0.18 -0.08 0.07 -0.2
0.06 0.07 -0.22 -0.18 0.17 0.05 0.02 -0.1
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-log10(fdr)
C
Volcano
15
10
5
0
-4
-2
0
2
4
logFC

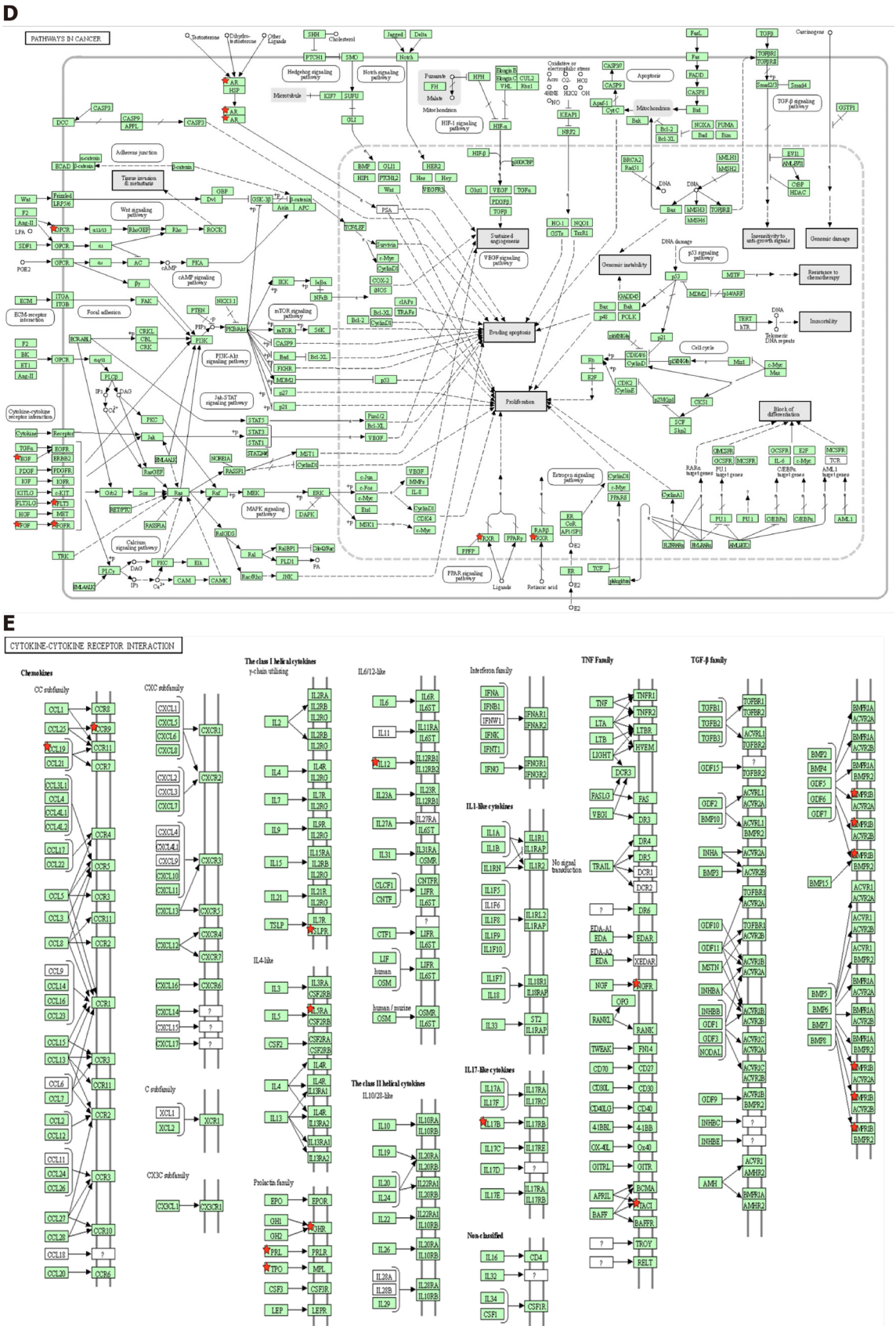


Figure 7 Difference in distribution of immune cells, expressed immune-related genes, and immune-related pathways between high- and low-risk score groups. A: Boxplot shows the differences in the ratio of nine immune cells between the high- and low-risk-score groups in stomach adenocarcinoma (STAD); B: The correlation between those nine immune cells in STAD; C: Volcano plot of differentially expressed immune-related genes (DEIRGs) in STAD between the high- and low-risk-score groups. Upregulated DEIRGs are indicated in red points and downregulated DEIRGs in green points; D: The pathways in cancer was significantly enriched among DEIRGs in STAD ($P < 0.05$); E: The cytokine and cytokine receptor interaction pathway was significantly enriched among DEIRGs in STAD ($P < 0.05$). $^{a}P < 0.05$, and $^{b}P < 0.01$.

a sequence-specific serine/arginine SF, plays a role in splicing patterns, mRNA processing, and gene expression. Alternative splicing generates multiple transcript variants of TRA2B (tra2beta1-5). The functions of different transcript variants vary in gastric cancer cell lines. In a previous study, a gastric cancer cell line was treated with arsenite (100 μM) for generation of oxidative stress. In this process, the isoforms tra2beta4 and tra2beta1 continued to be upregulated at the level of mRNA expression; simultaneously, higher amounts of full-length tra2beta were translocated from the nucleus to the cytoplasm during the initial 4-6 h (acute phase)[33]. TACC, a centrosome and microtubule-associated protein, is essential for mitotic spindle function. At least six different transcript variants of TACC1 have been found, and splicing variants of TACC1 with abnormal expression in gastric cancer cells might contribute to genetic instability. TACC1-D showed relatively strong expression in 50% of gastric cancer tissue samples but not in other normal tissues. Additionally, TACC1-F was observed to show predominant expression in gastric tumors[34]. Additionally, the identified OS-related AS events in our study were enriched in a wide variety of cancer-related pathways. Most of these pathways are crucial in the development and progression of STAD, and certain pathways have been reported to be affected by AS of important genes in pathways. For example, the autophagy pathway is critical for maintaining homeostasis; disruption of this pathway can mediate both tumorigenesis and tumor cell survival. The alternative mRNA splicing of the autophagy-related protein ATG5 specifically disrupted the binding pocket of ATG5-ATG16L1 interactions and blocked the conjugation of ATG12-ATG5. Therefore, the assembly and stability of the ATG12-ATG5-ATG16L1 complex in the autophagy pathway was damaged[35]. Mammalian cells sense oxygen and target multiple associated genes in response to hypoxia. The hypoxia signaling pathway is a hallmark of various solid tumors, due to the rapid proliferation of cancer cells and the alternative angiogenesis within tumors. Hypoxia-induced factor (HIF)-1 was identified as a key component in the hypoxia signaling pathway. One study suggested that HIF-1 isoforms modulated the expression of targeted genes with different activities under hypoxia. Both HIF-1alpha (736) and HIF-1alpha (FL) could combine with HIF-1beta to activate the VEGF promoter. However, the shorter HIF-1alpha isoform, which lacks the C-terminal transactivation domain, was 3-fold less active than HIF-1alpha (FL) under hypoxia [36]. In terms of the mechanism, a potential SF-AS regulatory network was constructed in our study, which explored the upstream SFs and downstream regulatory mechanisms of the OS-related AS events in STAD. Certain SFs were consistent with previous studies. For example, SRSF2 is a member of the serine/ arginine-rich family of pre-mRNA SFs and is critical for mRNA splicing. The reported data suggested that abnormal SRSF2 could set off a cascade of gene regulatory events that collectively drive cancer, known as the "splicing-cascade" phenotype, indicating that abnormal SFs could lead to widespread modifications in multiple proteins involved in RNA processing and splicing[37]. In our study, the identification of meaningful OS-related AS events in STAD and their upstream SFs or enriched cancer-related pathways involved in the development and progression of STAD would provide valuable prospects for clinical diagnosis and new therapeutic strategies.

Furthermore, a single gene for predicting the prognosis of STAD patients would be limited; therefore, the eleven-AS-signature prognostic model was constructed and significantly related to the OS in STAD. Most OS-associated AS genes in prognostic models have been proven to be related to cancers. For example, the upregulated CD44 variant isoforms are often observed in cancers and are significantly related to the more aggressive tumor phenotypes. CD44v9 is a major protein splicing variant of CD44, which is differentially expressed in human STAD cells. These findings indicate that targeting the cancer-related CD44v9 isoform has significant implications in clinical applications in STAD patients[38]. CDKN3, a dual-specificity protein tyrosine phosphatase, can dephosphorylate CDK1/CDK2 and other proteins. The expression and alternative splicing of CDKN3 are often abnormal in human cancers, which correlates with survival in several types of cancer. Certain CDKN3 transcript variants induce CDKN3 overexpression or CDKN3 activity, offering an advantage in tumorigenesis[39]. Alternative splicing of WEE1 has also been identified as a novel oncogene and prognostic biomarker to promote proliferation ability through regulating the cell cycle[40]. In a previous semiquantitative immunoblot analysis, HNRNPA1 and SRSF7 levels were significantly higher in gastric cancer than in gastric normal mucosa, and SRSF7 levels were higher in intestinal-type compared with diffuse-type gastric adenocarcinoma[41]. Our study demonstrates new findings that have not been reported in previous studies, including data on PPHLN1|21214|AT, RASSF4|11351|ES, KIAA1147|82046|AP, PPP2R5D|76200|ES, LOH12CR1|20507|ES, UBA52|48486|AD, and CADPS|65499|AT. The functions and expression of

the OS-associated AS genes in the prognostic model need to be verified in further experiments. In terms of mechanisms, the GSEA analysis demonstrated the differential enrichment of several gene sets between the high- and low-risk score groups. These gene sets indicated potential regulatory mechanisms of the prognostic model in STAD. Further study and verification of the identified OS-associated AS genes in a prognostic model is necessary.

Immunotherapy has achieved favorable effects in the management of advanced human cancers, and immune checkpoint inhibitors targeting CTLA-4 or PD-1 have revolutionized cancer immunotherapy[42]. Increasing evidence suggests that AS plays a crucial role in promoting tumorigenesis, progression, invasion, and metastasis in various cancer cells[43]. The process of AS on pre-mRNAs would increase the diversity of RNAs and proteins, and neo-antigens derived from different AS variants might alter the antigen-presenting mechanism. However, a limited number of cancer patients have been shown to respond to immunotherapy using cancer-specific RNA splicing[44]. This prompted us to explore whether STAD, which presents OS-related AS events, may exhibit a favorable response to immunotherapy. In this study, the proportion of immune cells in STAD was significantly different between the high- and low-risk groups, including naïve B cells, CD8 T cells, memory-activated CD4 T cells, activated NK cells, monocytes, M0 macrophages, resting mast cells, activated mast cells, and neutrophils. Furthermore, the IRGs that were expressed differently between the high-risk score group and low-risk score group were enriched in 13 significant cancer-related KEGG pathways. We conducted an in-depth investigation of IRGs driven by AS variants. For example, AR-V7 is an AS variant of the androgen receptor, and it has been shown to be associated with resistance against hormone therapies in prostate cancer[45]. Additionally, AS controlled the expression of human interleukin-5 receptor alpha, which generated two different AS variants encoding a membrane-anchored and a soluble form of the IL-5 receptors, respectively[46]. A systematic analysis of IRGs between AS-event subtypes would be beneficial for clarifying the role of splice variants in cancer immunotherapy.

CONCLUSION

In summary, this study included a systematic analysis of prognostic splicing events in STAD, constructed an elevated AS-signature prognostic model, and explored the association between AS and cancer immunity. The final OS-related AS events, DEIRGs, and the enriched pathways may play an important role in tumorigenesis in STAD; they deserve further study in clinical applications as diagnostic biomarkers and therapeutic targets.

ARTICLE HIGHLIGHTS

Research background

Alternative splicing (AS) increases the diversity of mRNA during transcription; it might play a role in alteration of the immune microenvironment, which could influence the development of immunotherapeutic strategies against cancer.

Research motivation

To explore survival-associated AS events which interact with immune microenvironment in stomach adenocarcinoma (STAD).

Research objectives

The overall survival data associated with AS events were used to construct a signature prognostic model for STAD.

Research methods

In STAD, 2042 overall-survival-related AS events were significantly enriched in various pathways and influenced several cellular functions.

Research results

An eleven-AS-signature prognostic model (CD44|14986|ES, PPHLN1|21214|AT, RASSF4|11351|ES, KIAA1147|82046|AP, PPP2R5D|76200|ES, LOH12CR1|205

07 | ES, CDKN3 | 27569 | AP, UBA52 | 48486 | AD, CADPS | 65499 | AT, SRSF7 | 53276 | RI, and WEE1 | 14328 | AP) was constructed and significantly related to STAD overall survival, immune cells, and cancer-related pathways.

Research conclusions

This study provided an AS-related prognostic model, potential mechanisms for AS, and alterations in the immune microenvironment (immune cells, genes, and pathways) for future research in STAD.

Research perspectives

This study provided a paradigm in STAD.

REFERENCES

1 **Brenner H**, Rothenbacher D, Arndt V. Epidemiology of stomach cancer. *Methods Mol Biol* 2009; **472**: 467-477 [PMID: 19107449 DOI: 10.1007/978-1-60327-492-0_23]

2 **Abdi E**, Latifi-Navid S, Zahri S, Yazdanbod A, Pourfarzi F. Risk factors predisposing to cardia gastric adenocarcinoma: Insights and new perspectives. *Cancer Med* 2019; **8**: 6114-6126 [PMID: 31448582 DOI: 10.1002/cam4.2497]

3 **Nagaraja AK**, Kikuchi O, Bass AJ. Genomics and Targeted Therapies in Gastroesophageal Adenocarcinoma. *Cancer Discov* 2019; **9**: 1656-1672 [PMID: 31727671 DOI: 10.1158/2159-8290.CD-19-0487]

4 **Triantafillidis JK**, Cheracakis P. Diagnostic evaluation of patients with early gastric cancer--a literature review. *Hepatogastroenterology* 2004; **51**: 618-624 [PMID: 15086218]

5 **Polom K**, Marrelli D, D'Ignazio A, Roviello F. Hereditary diffuse gastric cancer: how to look for and how to manage it. *Updates Surg* 2018; **70**: 161-166 [PMID: 29869323 DOI: 10.1007/s13304-018-0545-1]

6 **Matsuoka T**, Yashiro M. Biomarkers of gastric cancer: Current topics and future perspective. *World J Gastroenterol* 2018; **24**: 2818-2832 [PMID: 30018477 DOI: 10.3748/wjg.v24.i26.2818]

7 **Tress ML**, Abascal F, Valencia A. Alternative Splicing May Not Be the Key to Proteome Complexity. *Trends Biochem Sci* 2017; **42**: 98-110 [PMID: 27712956 DOI: 10.1016/j.tibs.2016.08.008]

8 **Demircioğlu D**, Cukuroglu E, Kindermans M, Nandi T, Calabrese C, Fonseca NA, Kahles A, Lehmann KV, Stegle O, Brazma A, Brooks AN, Rätsch G, Tan P, Göke J. A Pan-cancer Transcriptome Analysis Reveals Pervasive Regulation through Alternative Promoters. *Cell* 2019; **178**: 1465-1477. e17 [PMID: 31491388 DOI: 10.1016/j.cell.2019.08.018]

9 **Omenn GS**, Guan Y, Menon R. A new class of protein cancer biomarker candidates: differentially expressed splice variants of ERBB2 (HER2/neu) and ERBB1 (EGFR) in breast cancer cell lines. *J Proteomics* 2014; **107**: 103-112 [PMID: 24802673 DOI: 10.1016/j.jprot.2014.04.012]

10 **He C**, Zhou F, Zuo Z, Cheng H, Zhou R. A global view of cancer-specific transcript variants by subtractive transcriptome-wide analysis. *PLoS One* 2009; **4**: e4732 [PMID: 19266097 DOI: 10.1371/journal.pone.0004732]

11 **Kim E**, Goren A, Ast G. Insights into the connection between cancer and alternative splicing. *Trends Genet* 2008; **24**: 7-10 [PMID: 18054115 DOI: 10.1016/j.tig.2007.10.001]

12 **Luco RF**, Allo M, Schor IE, Kornblihtt AR, Misteli T. Epigenetics in alternative pre-mRNA splicing. *Cell* 2011; **144**: 16-26 [PMID: 21215366 DOI: 10.1016/j.cell.2010.11.056]

13 **Li S**, Zhang J, Huang S, He X. Genome-wide analysis reveals that exon methylation facilitates its selective usage in the human transcriptome. *Brief Bioinform* 2018; **19**: 754-764 [PMID: 28334074 DOI: 10.1093/bib/bbx019]

14 **Kim ST**, Cristescu R, Bass AJ, Kim KM, Odegaard JI, Kim K, Liu XQ, Sher X, Jung H, Lee M, Lee S, Park SH, Park JO, Park YS, Lim HY, Lee H, Choi M, Talasaz A, Kang PS, Cheng J, Loboda A, Lee J, Kang WK. Comprehensive molecular characterization of clinical responses to PD-1 inhibition in metastatic gastric cancer. *Nat Med* 2018; **24**: 1449-1458 [PMID: 30013197 DOI: 10.1038/s41591-018-0101-z]

15 **Kwak Y**, Seo AN, Lee HE, Lee HS. Tumor immune response and immunotherapy in gastric cancer. *J Pathol Transl Med* 2020; **54**: 20-33 [PMID: 31674166 DOI: 10.4132/jptm.2019.10.08]

16 **Zeng D**, Li M, Zhou R, Zhang J, Sun H, Shi M, Bin J, Liao Y, Rao J, Liao W. Tumor Microenvironment Characterization in Gastric Cancer Identifies Prognostic and Immunotherapeutically Relevant Gene Signatures. *Cancer Immunol Res* 2019; **7**: 737-750 [PMID: 30842092 DOI: 10.1158/2326-6066.CIR-18-0436]

17 **Payer LM**, Steranka JP, Ardeljan D, Walker J, Fitzgerald KC, Calabresi PA, Cooper TA, Burns KH. Alu insertion variants alter mRNA splicing. *Nucleic Acids Res* 2019; **47**: 421-431 [PMID: 30418605 DOI: 10.1093/nar/gky1086]

18 **Brosseau JP**, Lucier JF, Nwilati H, Thibault P, Garneau D, Gendron D, Durand M, Couture S, Lapointe E, Prinos P, Klinck R, Perreault JP, Chabot B, Abou-Elela S. Tumor microenvironment-associated modifications of alternative splicing. *RNA* 2014; **20**: 189-201 [PMID: 24335142 DOI:

10.1261/rna.042168.113]
19 **Bhattacharya S**, Andorf S, Gomes L, Dunn P, Schaefer H, Pontius J, Berger P, Desborough V, Smith T, Campbell J, Thomson E, Monteiro R, Guimaraes P, Walters B, Wiser J, Butte AJ. ImmPort: disseminating data to the public for the future of immunology. *Immunol Res* 2014; **58**: 234-239 [PMID: 24791905 DOI: 10.1007/s12026-014-8516-1]
20 **Ito M**, Tanaka S, Chayama K. Characteristics and Early Diagnosis of Gastric Cancer Discovered after *Helicobacter pylori* Eradication. *Gut Liver* 2020 [PMID: 32321202 DOI: 10.5009/gnl19418]
21 **Lopez A**, Harada K, Mizrak Kaya D, Ajani JA. Current therapeutic landscape for advanced gastroesophageal cancers. *Ann Transl Med* 2018; **6**: 78 [PMID: 29666801 DOI: 10.21037/atm.2017.10.29]
22 **Hamashima C**. Current issues and future perspectives of gastric cancer screening. *World J Gastroenterol* 2014; **20**: 13767-13774 [PMID: 25320514 DOI: 10.3748/wjg.v20.i38.13767]
23 **Li Y**, Yuan Y. Alternative RNA splicing and gastric cancer. *Mutat Res* 2017; **773**: 263-273 [PMID: 28927534 DOI: 10.1016/j.mrrev.2016.07.011]
24 **Urbanski LM**, Leclair N, Anczuków O. Alternative-splicing defects in cancer: Splicing regulators and their downstream targets, guiding the way to novel cancer therapeutics. *Wiley Interdiscip Rev RNA* 2018; **9**: e1476 [PMID: 29693319 DOI: 10.1002/wrna.1476]
25 **Miyamoto S**, Nagamura Y, Nakabo A, Okabe A, Yanagihara K, Fukami K, Sakai R, Yamaguchi H. Aberrant alternative splicing of RHOA is associated with loss of its expression and activity in diffuse-type gastric carcinoma cells. *Biochem Biophys Res Commun* 2018; **495**: 1942-1947 [PMID: 29247652 DOI: 10.1016/j.bbrc.2017.12.067]
26 **Ohnuma S**, Miura K, Horii A, Fujibuchi W, Kaneko N, Gotoh O, Nagasaki H, Mizoi T, Tsukamoto N, Kobayashi T, Kinouchi M, Okabe M, Sasaki H, Shiiba K, Miyagawa K, Sasaki I. Cancer-associated splicing variants of the CDCA1 and MSMB genes expressed in cancer cell lines and surgically resected gastric cancer tissues. *Surgery* 2009; **145**: 57-68 [PMID: 19081476 DOI: 10.1016/j.surg.2008.08.010]
27 **Vrána D**, Matzenauer M, Neoral Č, Aujeský R, Vrba R, Melichar B, Rušarová N, Bartoušková M, Jankowski J. From Tumor Immunology to Immunotherapy in Gastric and Esophageal Cancer. *Int J Mol Sci* 2018; **20** [PMID: 30577521 DOI: 10.3390/ijms20010013]
28 **He W**, Zhang H, Han F, Chen X, Lin R, Wang W, Qiu H, Zhuang Z, Liao Q, Zhang W, Cai Q, Cui Y, Jiang W, Wang H, Ke Z. CD155T/TIGIT Signaling Regulates $CD8^+$ T-cell Metabolism and Promotes Tumor Progression in Human Gastric Cancer. *Cancer Res* 2017; **77**: 6375-6388 [PMID: 28883004 DOI: 10.1158/0008-5472.CAN-17-0381]
29 **Park HS**, Kwon WS, Park S, Jo E, Lim SJ, Lee CK, Lee JB, Jung M, Kim HS, Beom SH, Park JY, Kim TS, Chung HC, Rha SY. Comprehensive immune profiling and immune-monitoring using body fluid of patients with metastatic gastric cancer. *J Immunother Cancer* 2019; **7**: 268 [PMID: 31639056 DOI: 10.1186/s40425-019-0708-8]
30 **Martinez NM**, Lynch KW. Control of alternative splicing in immune responses: many regulators, many predictions, much still to learn. *Immunol Rev* 2013; **253**: 216-236 [PMID: 23550649 DOI: 10.1111/imr.12047]
31 **Prochazka L**, Tesarik R, Turanek J. Regulation of alternative splicing of CD44 in cancer. *Cell Signal* 2014; **26**: 2234-2239 [PMID: 25025570 DOI: 10.1016/j.cellsig.2014.07.011]
32 **Choi ES**, Kim H, Kim HP, Choi Y, Goh SH. CD44v8-10 as a potential theranostic biomarker for targeting disseminated cancer cells in advanced gastric cancer. *Sci Rep* 2017; **7**: 4930 [PMID: 28694503 DOI: 10.1038/s41598-017-05247-7]
33 **Takeo K**, Kawai T, Nishida K, Masuda K, Teshima-Kondo S, Tanahashi T, Rokutan K. Oxidative stress-induced alternative splicing of transformer 2beta (SFRS10) and CD44 pre-mRNAs in gastric epithelial cells. *Am J Physiol Cell Physiol* 2009; **297**: C330-C338 [PMID: 19439532 DOI: 10.1152/ajpcell.00009.2009]
34 **Line A**, Slucka Z, Stengrevics A, Li G, Rees RC. Altered splicing pattern of TACC1 mRNA in gastric cancer. *Cancer Genet Cytogenet* 2002; **139**: 78-83 [PMID: 12547166 DOI: 10.1016/s0165-4608(02)00607-6]
35 **Wible DJ**, Chao HP, Tang DG, Bratton SB. *ATG5* cancer mutations and alternative mRNA splicing reveal a conjugation switch that regulates ATG12-ATG5-ATG16L1 complex assembly and autophagy. *Cell Discov* 2019; **5**: 42 [PMID: 31636955 DOI: 10.1038/s41421-019-0110-1]
36 **Gothié E**, Richard DE, Berra E, Pagès G, Pouysségur J. Identification of alternative spliced variants of human hypoxia-inducible factor-1alpha. *J Biol Chem* 2000; **275**: 6922-6927 [PMID: 10702253 DOI: 10.1074/jbc.275.10.6922]
37 **Liang Y**, Tebaldi T, Rejeski K, Joshi P, Stefani G, Taylor A, Song Y, Vasic R, Maziarz J, Balasubramanian K, Ardasheva A, Ding A, Quattrone A, Halene S. SRSF2 mutations drive oncogenesis by activating a global program of aberrant alternative splicing in hematopoietic cells. *Leukemia* 2018; **32**: 2659-2671 [PMID: 29858584 DOI: 10.1038/s41375-018-0152-7]
38 **Moreira IB**, Pinto F, Gomes C, Campos D, Reis CA. Impact of Truncated *O*-glycans in Gastric-Cancer-Associated CD44v9 Detection. *Cells* 2020; **9** [PMID: 31973075 DOI: 10.3390/cells9020264]
39 **Cress WD**, Yu P, Wu J. Expression and alternative splicing of the cyclin-dependent kinase inhibitor-3 gene in human cancer. *Int J Biochem Cell Biol* 2017; **91**: 98-101 [PMID: 28504190 DOI: 10.1016/j.biocel.2017.05.013]
40 **Kouyama Y**, Masuda T, Fujii A, Ogawa Y, Sato K, Tobo T, Wakiyama H, Yoshikawa Y, Noda M, Tsuruda Y, Kuroda Y, Eguchi H, Ishida F, Kudo SE, Mimori K. Oncogenic splicing abnormalities

induced by DEAD-Box Helicase 56 amplification in colorectal cancer. *Cancer Sci* 2019; **110**: 3132-3144 [PMID: 31390121 DOI: 10.1111/cas.14163]

41 **Park WC**, Kim HR, Kang DB, Ryu JS, Choi KH, Lee GO, Yun KJ, Kim KY, Park R, Yoon KH, Cho JH, Lee YJ, Chae SC, Park MC, Park DS. Comparative expression patterns and diagnostic efficacies of SR splicing factors and HNRNPA1 in gastric and colorectal cancer. *BMC Cancer* 2016; **16**: 358 [PMID: 27282379 DOI: 10.1186/s12885-016-2387-x]

42 **Haanen JB**, Robert C. Immune Checkpoint Inhibitors. *Prog Tumor Res* 2015; **42**: 55-66 [PMID: 26382943 DOI: 10.1159/000437178]

43 **Climente-González H**, Porta-Pardo E, Godzik A, Eyras E. The Functional Impact of Alternative Splicing in Cancer. *Cell Rep* 2017; **20**: 2215-2226 [PMID: 28854369 DOI: 10.1016/j.celrep.2017.08.012]

44 **Frankiw L**, Baltimore D, Li G. Alternative mRNA splicing in cancer immunotherapy. *Nat Rev Immunol* 2019; **19**: 675-687 [PMID: 31363190 DOI: 10.1038/s41577-019-0195-7]

45 **Ferraldeschi R**, Welti J, Powers MV, Yuan W, Smyth T, Seed G, Riisnaes R, Hedayat S, Wang H, Crespo M, Nava Rodrigues D, Figueiredo I, Miranda S, Carreira S, Lyons JF, Sharp S, Plymate SR, Attard G, Wallis N, Workman P, de Bono JS. Second-Generation HSP90 Inhibitor Onalespib Blocks mRNA Splicing of Androgen Receptor Variant 7 in Prostate Cancer Cells. *Cancer Res* 2016; **76**: 2731-2742 [PMID: 27197266 DOI: 10.1158/0008-5472.CAN-15-2186]

46 **Pérez C**, Vandesompele J, Vandenbroucke I, Holtappels G, Speleman F, Gevaert P, Van Cauwenberge P, Bachert C. Quantitative real time polymerase chain reaction for measurement of human interleukin-5 receptor alpha spliced isoforms mRNA. *BMC Biotechnol* 2003; **3**: 17 [PMID: 14519208 DOI: 10.1186/1472-6750-3-17]

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ORIGINAL ARTICLE

Retrospective Study

Clinicopathological characteristics and prognosis of 232 patients with poorly differentiated gastric neuroendocrine neoplasms

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Abstract

BACKGROUND

Poorly differentiated gastric neuroendocrine neoplasms (PDGNENs) include gastric neuroendocrine carcinoma (NEC) and mixed adenoneuroendocrine carcinoma, which are highly malignant and rare tumors, and their incidence has increased over the past few decades. However, the clinicopathological features and outcomes of patients with PDGNENs have not been completely elucidated.

AIM

To investigate the clinicopathological characteristics and prognostic factors of patients with PDGNENs.

METHODS

The data from seven centers in China from March 2007 to November 2019 were analyzed retrospectively.

RESULTS

Among the 232 patients with PDGNENs, 191 (82.3%) were male, with an average age of 62.83 ± 9.11 years. One hundred and thirteen (49.34%) of 229 patients had a stage III disease and 86 (37.55%) had stage IV disease. Three (1.58%) of 190 patients had no clinical symptoms, while 187 (98.42%) patients presented clinical symptoms. The tumors were mainly (89.17%) solitary and located in the upper third of the stomach (cardia and fundus of stomach: 115/215, 53.49%). Most lesions were ulcers (157/232, 67.67%), with an average diameter of 4.66 ± 2.77 cm. In terms of tumor invasion, the majority of tumors invaded the serosa (116/198, 58.58%). The median survival time of the 232 patients was 13.50 mo (7, 31 mo), and the overall 1-year, 3-year, and 5-year survival rates were 49%, 19%, and 5%, respectively. According to univariate analysis, tumor number, tumor diameter, gastric invasion status, American Joint Committee on Cancer (AJCC) stage, and distant metastasis status were prognostic factors for patients with PDGNENs. Multivariate analysis showed that tumor number, tumor diameter, AJCC stage, and distant metastasis status were independent prognostic factors for patients with PDGNENs.

CONCLUSION

The overall prognosis of patients with PDGNENs is poor. The outcomes of patients with a tumor diameter > 5 cm, multiple tumors, and stage IV tumors are worse than those of other patients.

Key Words: Poorly differentiated gastric neuroendocrine neoplasms; Clinicopathological characteristics; Prognosis; Distant metastasis; Tumor diameter

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Core Tip: Most patients with poorly differentiated gastric neuroendocrine neoplasms (PDGNENs) present with lymph node metastasis or distant metastasis at the time of diagnosis, and the 5-year overall survival rate of patients with PDGNENs is only 5%. The malignancy of PDGNENs is very high, and the onset is relatively unclear. Routine gastroscopy may help detect PDGNENs as early as possible. Patients with a tumor diameter > 5 cm, multiple tumors, and with American Joint Committee on Cancer stage III or IV have a poor prognosis.

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INTRODUCTION

Gastric neuroendocrine neoplasms (G-NENs) are a group of heterogeneous and rare malignant tumors originating from peptidergic neurons and neuroendocrine cells. Neuroendocrine neoplasms can occur throughout the body, such as in the gastrointestinal tract, pancreas, liver and gallbladder, thymus, and lung, but the gastrointestinal tract is the most commonly affected site[1]. According to the Surveillance, Epidemiology, and End Results report, the incidence of G-NENs has increased 15-fold in recent decades and reached 4.85/1000000 in 2014, mainly due to the wide application of gastroscopy and the improvement of pathological diagnosis techniques[2,3]. The incidence of poorly differentiated gastric neuroendocrine neoplasms (PDGNENs) is approximately 1/1000000 people, accounting for 16.4% of G-NENs, while the incidence of other tumors in the stomach shows a decreasing trend compared with G-NENs[4].

PDGNENs are divided into functional and nonfunctional tumors according to whether the tumor secretes active hormones and causes characteristic clinical manifestations, and the most common clinical tumors are nonfunctional tumors. The clinical symptoms of nonfunctional PDGNENs lack specificity, and early diagnosis is difficult. Generally, the clinical symptoms are caused by the tumor size or metastasis, mainly including abdominal pain and abdominal distension. Few functional PDGNENs secrete bioactive amines that cause carcinoid syndrome, including skin flushing, diarrhea, wheezing, *etc*. PDGNENs have a worse prognosis than neuroendocrine tumors (NETs), and tumor diameter, stage, location, and treatment are significantly correlated with the prognosis[4]. The 5-year survival rate of patients with well-differentiated G-NENs presenting with distant metastases may reach 35%, while the 5-year survival rate of patients with PDGNENs accompanied by distant metastasis is only 4%[5].

Relatively few large-cohort studies have assessed PDGNENs and limited data is available on their clinicopathological features and outcomes. Therefore, we collected the data of 232 patients with PDGNENs from multiple centers in China and analyzed the clinicopathological characteristics and prognostic factors of these patients, aiming to provide a reference for clinical work on PDGNENs.

MATERIALS AND METHODS

Patient selection

From March 2007 to November 2019, a total of 232 patients with PDGNENs from seven centers in China were enrolled (China-Japan Friendship Hospital, n = 71; Sun Yat-Sen University Cancer Center, n = 54; The Fourth Affiliated Hospital of Hebei Medical University, n = 49; The First Affiliated Hospital Sun Yat-Sen University, n = 39; Fudan University Shanghai Cancer Center, n = 10; The Fifth Medical Center of the PLA General Hospital, n = 8; and Yunnan Tumor Hospital, n = 1). The inclusion criteria were as follows: (1) All patients were confirmed to have neuroendocrine carcinoma (NEC) or mixed adenoneuroendocrine carcinoma (MANEC) based on pathology; (2) The clinical data of the patients were relatively complete; (3) The patients underwent regular follow-up; and (4) The patients had no other tumors. This study was conducted in accordance with the provisions of the Declaration of Helsinki and approved by the Clinical Research Ethics Committee of China-Japan Friendship Hospital (No. 2019-24-K18-1).

Tumor grade and stage

The pathological grading standard used in this study adopted the 2019 World Health Organization classification and grading criteria for gastrointestinal pancreatic neuroendocrine tumors[6] and updated the pathological classification and grading of gastroenteropancreatic neuroendocrine tumors. Well-differentiated gastric neuroendocrine tumors were classified into three types. NEC was no longer graded and was divided into only two subtypes: Large cell NEC (LCNEC) and small cell NEC (SCNEC). In addition, MANEC has also been replaced by mixed neuroendocrine and nonneuroendocrine tumors (MiNENs), which contain a wider range of contents. Most of the NEN components in MiNEN are NEC and may also be NETs; in addition to adenocarcinoma, other components, such as squamous cell carcinoma, may also occur in non-NEN components, but each component must account for more than 30% and be classified when reporting[7]. The 8th edition gastric cancer tumor-node-metastasis staging system was used for staging[8].

Follow-up

The patients were followed regularly by an outpatient review, inpatient medical record review, and telephone interview. The starting point was the time when the patient's histopathology yielded a diagnosis of PDGNENs. The end point of the follow-up was the time of death.

Statistical methods

Measurement data are presented as the mean ± SD, and the follow-up time is reported as the median (interquartile range). Count data are presented as numbers of cases (percentages). The Kaplan-Meier method was used for the survival analysis, and comparisons were performed using the log-rank test. Multivariable survival analyses were also performed to exclude dependent variables using Cox proportional hazards regression models. When the two-tailed *P* value was less than 0.05, the difference was considered statistically significant. Data were analyzed using SPSS 25.0 statistical analysis software (IBM, Chicago, IL, United States).

RESULTS

Clinicopathological characteristics

Among the 232 patients with PDGNENs, 191 (82.3%) were male, with an average age of 62.83 years (range: 30-85 years), and the elderly accounted for 65.5% of the patients (Table 1). The average diameter of the tumor was 4.66 ± 2.77 cm, and the lesions mainly invaded the serosa (116/198, 58.58%). The lymph nodes were positive in 175 (79.78%) of 225 patients, and 86 (37.39%) of 230 patients exhibited distant metastases. In addition, 113 (49.34%) patients presented with a stage II disease and 86 (37.55%) presented with stage IV disease. In terms of endoscopic performance, most of the tumors were mainly located in the cardia (65/215, 30.23%). The majority of tumors were solitary (215/219, 89.17%). In addition, the lesions were mainly ulcers (157/232, 67.67%). Typical gastroscopic findings are shown in Figure 1.

The pathological classification of 232 patients was G3. Of these patients, 41 had LCNEC (17.67%) (Figure 2), 26 had SCNEC (12.21%) (Figure 3), and 38 had MANEC (16.38%) (Figure 4); the subtype was not reported for 127 (54.74%) patients. The average Ki-67 index was 65.34% (Table 1).

The clinical symptoms of 190 patients were recorded. One hundred and eighty-seven (98.42%) patients experienced symptoms, while three (1.58%) patients had no clinical symptoms. The main symptoms were abdominal pain (105/190, 55.26%), abdominal distension (67/190, 35.26%), weight loss (41/190, 21.58%), poor appetite (38/190, 20.00%), and gastrointestinal bleeding (31/190, 16.32%) (Figure 5).

Treatment

Among the 232 patients with PDGNENs, 86 (37.07%) were treated by surgery, 40 (17.24%) were treated with chemotherapy, 92 (38.79%) were treated by surgery plus chemotherapy, and 14 (6.03%) were treated with other treatments (somatostatin analogs, targeted therapy, immunotherapy, traditional Chinese medicine treatment, *etc.*). One hundred and forty-three patients had no distant metastasis or resectable tumors (tumor stage I: 5 cases; stage II: 25 cases; and stage III: 113 cases), of whom 75 were treated by surgery, 6 were treated by chemotherapy, 55 were treated by surgery combined with chemotherapy, and 7 were treated with other treatments. This study analyzed patients without distant metastases and found that the median survival time of the surgery alone group was 18 mo, while the median survival time of the chemotherapy alone group was 11 mo and that of the surgery combined with chemotherapy group was 23 mo ($P < 0.001$) (Figure 6).

Follow-up and outcomes

With a median follow-up time of 13.50 mo (range, 7-31 mo), the overall 1-year, 3-year, and 5-year survival rates were 47%, 15%, and 5%, respectively. The median survival time was 14 mo. The univariate analysis showed that tumor number, tumor diameter, gastric invasion status, American Joint Committee on Cancer (AJCC) stage, and distant metastasis status were correlated with the prognosis (log-rank test $P < 0.05$; Table 1), while age, sex, lymph node metastasis, pathological type, Ki-67 index, and tumor site were not related to the prognosis (log-rank test $P > 0.05$; Table 1 and Figure 6). In the multivariate analysis, the tumor number {multiple *vs* solitary, hazard ratio (HR) [95% confidence interval (CI)]: 3.89 (1.66-9.11), $P < 0.001$}, tumor diameter [5 cm *vs* 0-5 cm,

Table 1 Clinicopathological characteristics of 232 patients with gastric neuroendocrine carcinoma and univariate analysis

Classification	*n*	Median survival time/mo	*P* value
Age (yr)			0.26
< 60	80	12.56 (9.42-14.57)	
≥ 60	152	14.85 (11.80-18.11)	
Gender			0.32
Male	191	14.50 (11.78-16.21)	
Female	41	11.50 (7.94-12.05)	
Tumor number			0.01
Solitary	5	14.02 (11.93-16.06)	
Multiple	214	5.98 (3.40-6.60)	
NR	13	NR	NR
Tumor diameter (cm)			0.01
0-5	105	18.10 (9.35-20.64)	
> 5	99	11.50 (9.41-16.58)	
NR	28	NR	NR
Invasion			0.02
M^1/S/MP^2	82	16.27 (12.51-17.49)	
Serosa	116	11.54 (7.54-14.54)	
NR	34	NR	NR
Tumor site			0.09
Cardia	65	16.12 (11.53-18.46)	
Fundus	15	19.88 (3.99-28.00)	
Body	33	15.98 (9.46-20.53)	
Antrum	27	11.80 (8.73-17.26)	
C + F	35	12.50 (7.96-16.03)	
Other	40	15.23 (8.59-23.40)	
NR	17	NR	NR
Pathological type			0.68
Large cell NEC	41	12.96 (12.11-13.84)	
Small cell NEC	26	16.33 (11.30-20.69)	
MANEC	38	12.33 (8.80-15.19)	
NR	127	14.31 (10.40-17.59)	
Ki-67 index			0.61
20%-50%	60	12.69 (7.50-16.49)	
50%-70%	114	14.10 (11.74-16.25)	
> 70%	38	13.23 (0.00-26.80)	
NR	20	NR	NR
Endoscopic performance			0.90
Polypoid	7	15.00 (11.85-16.14)	
Mucosal elevation	28	17.19 (6.99-25.00)	
Ulcer	157	13.69 (10.66-15.33)	
Other	40	11.00 (9.09-10.90)	

Tumor N staging			0.16
N0	50	20.00 (5.30-30.70)	
N1-N3	174	13.11 (9.95-16.00)	
NR	8	NR	NR
Distant metastasis			< 0.001
No	157	17.43 (12.94-21.05)	
Yes	73	11.64 (9.04-12.95)	
NR	2	NR	NR
AJCC stage			< 0.001
I	5	88.26 (36.06-139.94)	
II	25	31.34 (13.56-48.43)	
III	113	16.12 (13.43-18.56)	
IV	86	11.52 (9.05-12.94)	
NR	3	NR	NR
Treatment			0.01
Surgery	86	15.47 (10.87-19.12)	
Chemotherapy	40	12.10 (10.24-13.75)	
Surgery + chemotherapy	92	15.70 (9.99-20.00)	
Other	14	11.00 (0.00-21.68)	

NR: Not reported.
M[1]: Mucosa; S: Submucosa.
MP[2]: Muscularis propria.
C + F: Cardia + Fundus; NEC: Neuroendocrine carcinoma; MANEC: Mixed adenoneuroendocrine carcinoma; AJCC: American Joint Committee on Cancer.

HR (95%CI): 1.56 (1.01-2.41), $P = 0.04$], tumor stage [IV *vs* I, HR (95%CI): 5.98 (1.78-20.60), $P < 0.001$; III *vs* I, HR (95%CI): 3.582 (1.07-11.88), $P = 0.03$], and distant metastasis status [yes *vs* no, HR (95%CI): 2.16 (1.41-3.31), $P < 0.001$] were independent risk factors affecting the prognosis (Table 2). The overall 1-year, 3-year, and 5-year survival rates of the 232 patients with PDGNENs are shown in Table 3.

DISCUSSION

PDGNENs are rare tumors that are highly malignant, accounting for 6.9% of gastroenteropancreatic neuroendocrine tumors and 0.3%-1.8% of all malignant gastric tumors[9,10]. According to the Korean literature, PDGNENs account for 2.84% of all NENs and 40% of G-NENs[11]. In this article, the male-to-female ratio reached 4.66:1, and significantly more male patients were identified than female patients. Kim *et al*[12] reported that among 63 patients with G-NECs, 48 were male and 15 were female. Other studies[13-15] have indicated that G-NECs have a sex bias favoring males, but the reason has not been clarified.

PDGNENs are mostly nonfunctional and often detected incidentally. Among the 232 patients with PDGNENs analyzed in our study, all patients had nonfunctional lesions. The main symptoms were similar to those reported in several other studies[15-17]. Early PDGNENs are asymptomatic or have no specific symptoms, such as anemia, abdominal pain, and dyspepsia, and they are unintentionally identified through routine upper gastrointestinal endoscopy. Indeed, regular upper gastrointestinal endoscopy is important and can help to detect PDGNENs, especially in patients with early-stage disease. Advanced PDGNENs are easily detected, mainly because patients show tumor-related features, such as obstruction, bleeding, weight loss, and pain due to infiltration or distant metastasis[16].

Most patients with PDGNENs exhibit advanced tumors at the time of diagnosis, and patients with advanced tumors have worse outcomes than those with early-stage tumors. In our study, the majority of patients had advanced stage tumors at the time of

Table 2 Multivariate Cox regression analysis of poorly differentiated gastric neuroendocrine neoplasms

Parameter	*n*	HR (95%CI)	*P* value
Tumor number			
Solitary	5	1	
Multiple	214	3.89 (1.66-9.11)	< 0.001
Tumor diameter			
0-5	105	1	
> 5	99	1.56 (1.01-2.41)	0.04
AJCC stage			
I	5	1	
II	25	2.71 (0.56-8.44)	0.26
III	113	3.582 (1.07-11.88)	0.03
IV	86	5.98 (1.78-20.06)	< 0.001
Distant metastasis			
No	157	1	
Yes	73	2.16 (1.41-3.31)	< 0.001

AJCC: American Joint Committee on Cancer.

diagnosis; 199 (85.78%) patients exhibited lymph node metastasis or distant metastasis, and these values are similar to those of several other studies[18]. Additionally, the survival analysis showed significant differences between patients with stages I-IV. The median survival time of patients with stage I disease was 88 mo, while that of stage IV patients was only 11 mo, and the 3-year overall survival rates were 100% and 11%, respectively. Patients with PDGNENs often benefit if the disease is detected at an early stage. Ishida *et al*[18] reported that the 5-year survival rates of 51 patients with stage I, II, III, or IV G-NECs were 66.7%, 49.3%, 64.3%, and 7.7%, respectively. Tierney *et al*[19] found that the median survival times of patients with stages I-II, III, and IV tumors were 40 mo, 31 mo, and 6 mo, respectively[19]. Furthermore, our study suggested that distant metastasis is an independent prognostic factor, which is consistent with relevant reports[18-21].

Tumor diameter may be relevant to the outcomes in our study. According to the study conducted by Liang *et al*[20], a gastric neuroendocrine tumor diameter greater than 4.2 cm was a poor prognostic factor for patients. Fang *et al*[22] analyzed 156 patients with G-NECs. Univariate analysis revealed a significant difference between patients with a tumor diameter less than 4.5 cm and those with a tumor diameter greater than 4.5 cm, and the 5-year survival rates were 57.9% and 29.3%, respectively. Our data suggest that a tumor diameter greater than 5 cm was a risk factor affecting the prognosis. In clinical practice, the tumor diameter of PDGNENs may be useful to predict the outcome, and patients with a tumor diameter greater than 5 cm should receive more attention.

Tumor site may also be related to the prognosis. In our study, PDGNENs were mainly located in the upper third of the stomach (total: 53.95%), while only 27 (12.56%) PDGNENs were found in the antrum. The values are similar to those of other reports[23-25]. In addition, our data showed a longer median survival time of patients with lesions in the cardia (15.97 mo) and fundus of the stomach (20.00 mo) than that of patients with lesions in the gastric antrum (12.50 mo). Hu *et al*[4] reported a median survival time of patients with G-NECs in the cardia and fundus of 20 mo, which was longer than that of patients with tumors in the antrum (13 mo). Bukhari *et al*[14] observed a better prognosis for patients with tumors in the cardia region than that of patients with tumors in the gastric antrum (median survival: 48.0 mo *vs* 19.0 mo)[14]. To a certain extent, we postulate that the prognosis of PDGNENs in the upper part of the stomach is better than that of tumors in the lower part.

The European Neuroendocrine Tumor Society proposed guidelines for the treatment of NEC in 2016. For patients with no distant metastasis or resectable tumors, surgical treatment can be selected, and adjuvant chemotherapy (AC) or radiotherapy

Table 3 Survival analysis of related factors of 232 patients with poorly-differentiated gastric neoplasms

Classification	Median survival time (mo)	1-yr survival rate (%)	3-yr survival rate (%)	5-yr survival rate (%)
Age (yr)				
< 60	12	37	11	5
≥ 60	15	52	18	5
Gender				
Male	14	52	16	6
Female	10	29	13	3
Tumor number				
Solitary	14	49	17	6
Multiple	5	33	0	0
Tumor diameter (cm)				
0-5	19	58	23	9
> 5	12	40	13	5
Invasion				
M^1/S/MP^2	17	60	23	6
Serosal	12	42	13	5
Tumor site				
Cardia	16	58	9	0
Fundus	20	67	17	17
Body	15	57	30	11
Antrum	12	33	0	0
C + F	13	41	6	0
Other	15	57	26	13
Pathological type				
Large cell NEC	16	54	18	9
Small cell NEC	13	34	19	9
MANEC	12	37	11	0
NR	14	52	16	5
Ki-67 index				
20%-50%	12	47	26	6
50%-70%	14	57	12	1
> 70%	13	29	7	0
Endoscopic				
Performance	14	67	17	0
Polypoid	16	54	25	8
Mucosal elevation				
Ulcer	13	48	15	6
Other	10	36	14	3
LN metastasis				
No	18	57	18	6
Yes	13	47	15	5
Distant metastasis				

No	17	62	22	7
Yes	11	28	7	2
AJCC stage				
I	60	100	100	14
II	31	73	41	8
III	16	58	17	5
IV	11	33	11	4
Treatment				
Surgery	15	55	17	9
Chemotherapy	12	29	4	0
S + C	16	57	23	6
Other	10	33	11	0

S + C: Surgery plus Chemotherapy; C + F: Cardia + Fundus.
M^1: Mucosa; S: Submucosa.
MP^2: Muscularis propria.
NEC: Neuroendocrine carcinoma; MANEC: Mixed adenoneuroendocrine carcinoma; NR: Not reported; AJCC: American Joint Committee on Cancer.

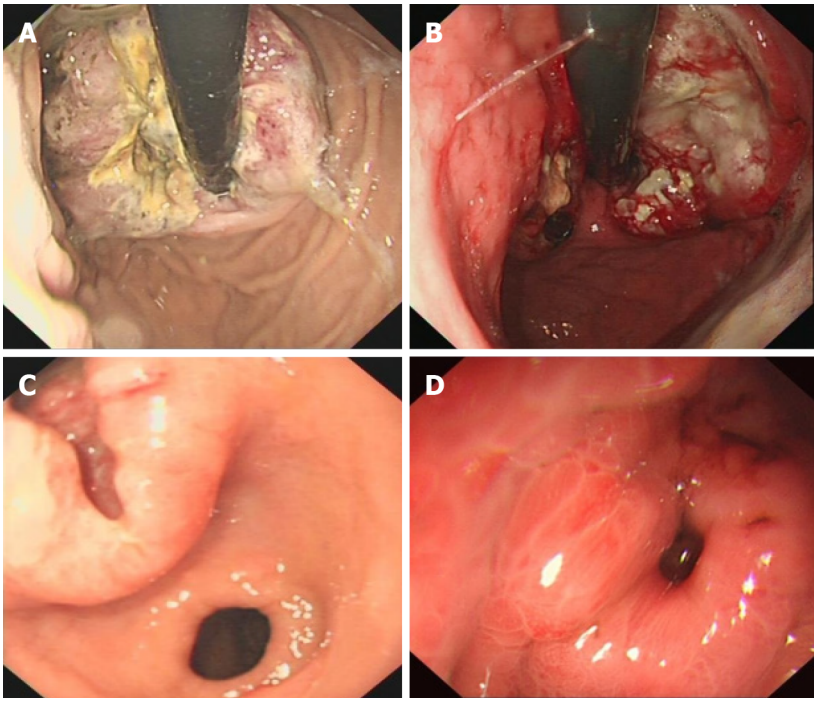


Figure 1 Endoscopic detection of poorly differentiated gastric neuroendocrine neoplasms. A: Circumferential raised lesions on the cardia with uneven surfaces; B: Irregular bumps on the side of the minor curvature of the cardia, accompanied by erosions, ulcers, and unclear boundaries that bled easily when contacted; C: A raised ulcer with a diameter of 4 cm was observed in the small curvature of the antrum; D: A deep ulcer with a diameter of approximately 0.5 cm on the posterior wall of the gastric fundus was observed, and the base was not clear.

can be selected after surgery. Surgery is the only curative treatment for resectable PDGNENs, but the prognosis of patients who undergo surgery alone remains very poor[26]. This study analyzed patients without distant metastases and found that the median survival time of patients treated by surgery combined with chemotherapy (23 mo) was longer than that of patients who were treated by surgery alone (18 mo) or chemotherapy alone (11 mo). Their 3-year overall survival rates were 35%, 22%, and 4%, respectively. Patients with early-stage tumors should choose the proper treatment method, which may improve the quality of life and prolong the survival time. According to a retrospective study including 69 patients with G-NECs in China, the overall 3-year survival rate of patients receiving surgery combined with chemotherapy

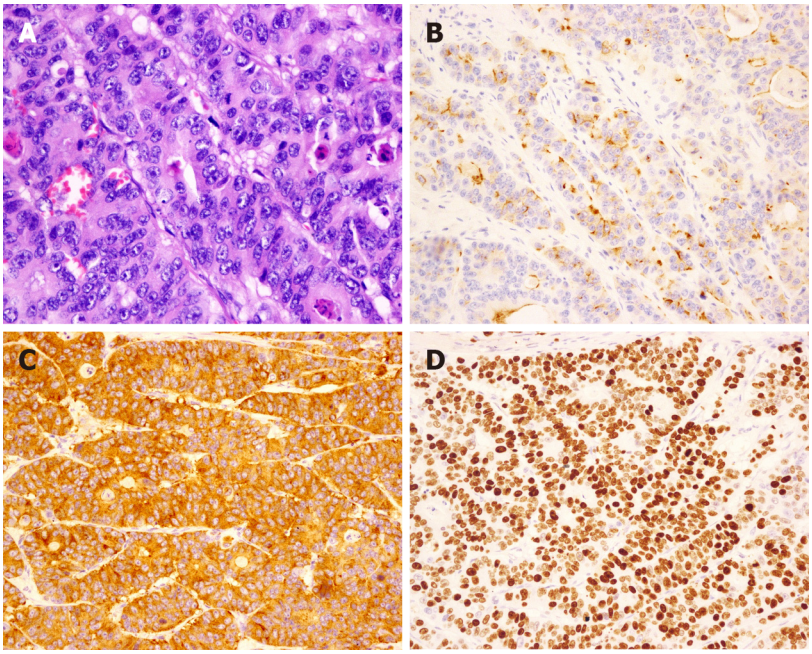


Figure 2 Morphology and immunohistochemical staining of large cell neuroendocrine carcinoma in the cardia. A: Morphology (hematoxylin and eosin staining, × 400); B: Chromogranin A-positive staining [immunohistochemical (IHC), × 200]; C: Synaptophysin-positive staining (IHC, × 200); D: Ki-67 index: 90% (IHC, × 200).

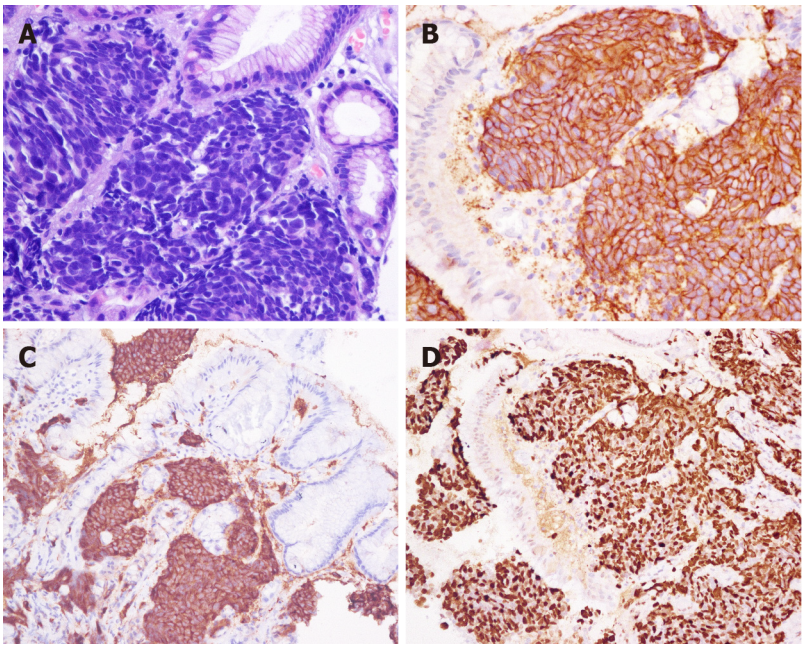


Figure 3 Morphology and immunohistochemical staining of gastric small cell neuroendocrine carcinoma. A: Morphology (hematoxylin and eosin staining, × 400); B: CD56-positive staining [immunohistochemical (IHC), × 400]; C: Synaptophysin-positive staining (IHC, × 200); D: Ki-67 index: 90% (IHC, × 200).

was 68.8%, while that of patients who received surgery alone was only 3.8%[27]. Bukhari *et al*[14] assessed 43 patients with G-NECs. Five patients did not undergo postoperative chemotherapy, and the median survival time of these patients was 15 mo. The median survival time of the remaining 34 patients who received postoperative chemotherapy was 44 mo[14]. Mao *et al*[28] analyzed 806 patients diagnosed with nonmetastatic poorly differentiated colorectal NECs, and 394 (48.9%) of these patients received AC. Kaplan-Meier curves showed that the median overall survival (OS) was significantly longer for patients treated with AC *vs* observation (57.4 mo *vs* 38.2 mo; $P = 0.007$). The Cox proportional hazards regression analysis showed that AC was associated with a significant OS benefit [HR = 0.73, $P < 0.001$][28]. Surgery combined with chemotherapy has advantages and may improve the prognosis of patients

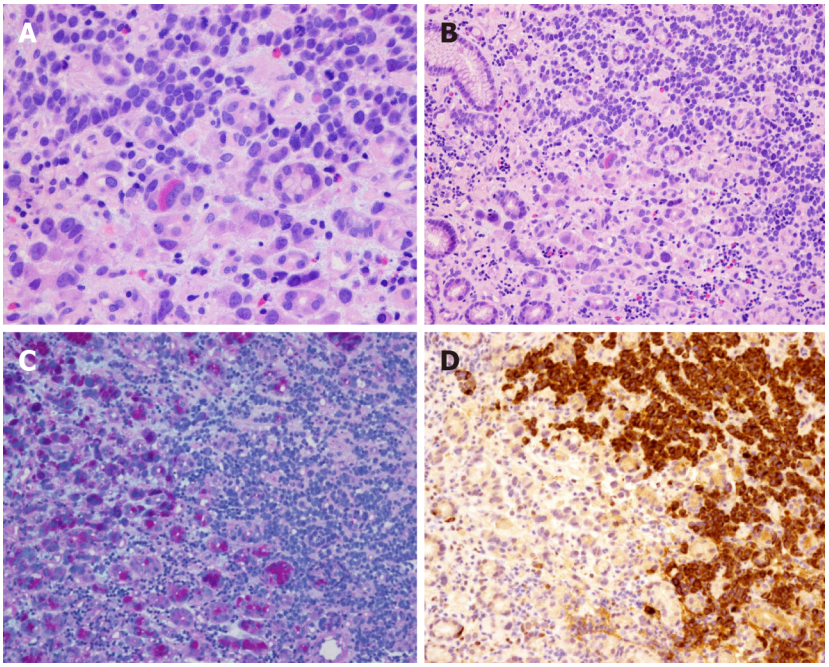


Figure 4 Morphology and immunohistochemical staining of mixed adenoneuroendocrine carcinoma. A and B: Morphology of gastric small cell neuroendocrine carcinoma mixed with adenocarcinoma (hematoxylin and eosin staining, × 400); C: Gastric small cell neuroendocrine carcinoma mixed with adenocarcinoma (hematoxylin and eosin staining, × 200). Alcian blue/periodic acid–Schiff (AB-PAS) staining (× 200): The left side of the picture shows adenocarcinoma (AB-PAS positive); D: Chromogranin A-positive staining (× 200). The right side of the picture shows small cell neuroendocrine carcinoma.

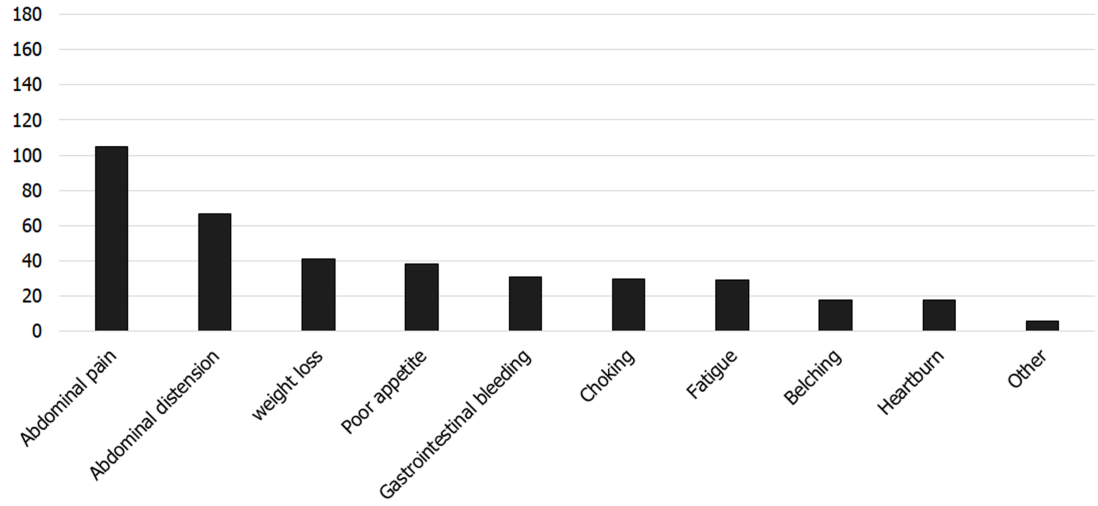


Figure 5 Distribution of nonspecific clinical symptoms in 190 patients with poorly differentiated gastric neuroendocrine neoplasms.

compared with treatment with either approach alone.

As a retrospective study, this study provides novel insights into the diagnosis and treatment of PDGNENs and the risk factors related to the prognosis. However, some limitations should be noted. First, some patients had incomplete basic information. Second, some patients had poor compliance, and a certain number of patients were lost to follow-up (30%). In the future, prospective, multicenter, large-scale trials are still needed to identify independent risk factors that affect the prognosis of patients with PDGNENs.

CONCLUSION

In summary, the majority of patients with PDGNENs had already developed lymph node or distant metastasis at the time of diagnosis, and the prognosis was poor, with a

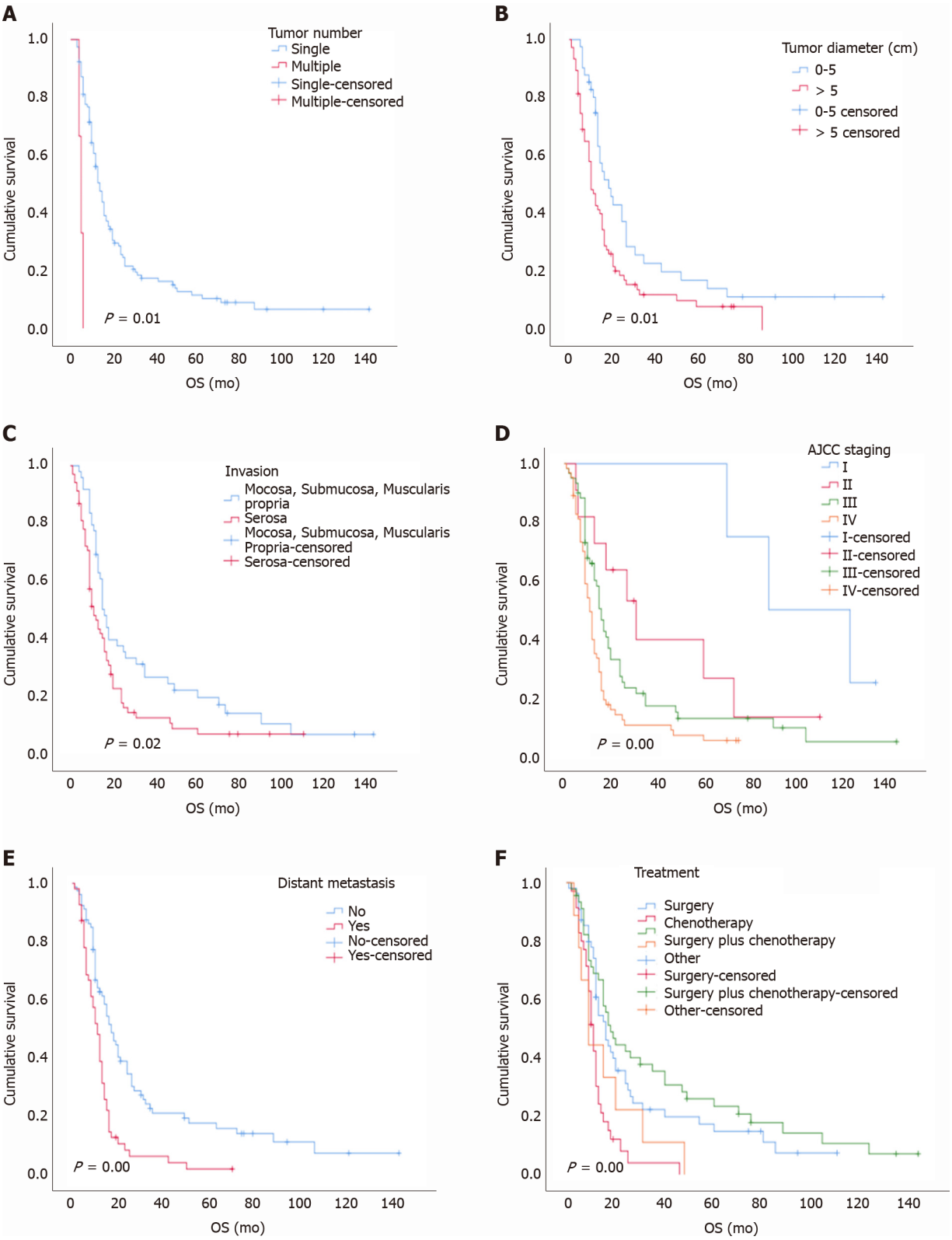


Figure 6 Kaplan-Meier survival analysis (*P* < 0.05). A: Tumor number (*P* = 0.01); B: Tumor diameter (*P* = 0.01); C: Invasion (*P* = 0.02); D: American Joint Committee on Cancer stage (*P* < 0.001); E: Distant metastasis (*P* < 0.001); F: Treatment for patients without distant metastases (*P* < 0.001). AJCC: American Joint Committee on Cancer; OS: Overall survival.

5-year survival rate of 5% and a median survival time of 13.50 mo. Electronic gastroscopy and pathological diagnostic technology have been widely popularized. When people experience the aforementioned symptoms, gastroscopy should be performed in a timely manner. If a lesion is detected, pathology should be performed to determine a clear diagnosis. Clinicians should pay more attention to patients with lesions greater than 5 cm because their prognosis is the worst, with a 5-year survival

rate of 5%. Patients are recommended to undergo AC after surgery. In addition, tumor number, tumor diameter, AJCC stage, and distant metastasis are independent factors affecting the prognosis of patients with PDGNENs.

ARTICLE HIGHLIGHTS

Research background

Poorly differentiated gastric neuroendocrine neoplasm (PDGNEN) is a rare tumor, but its incidence is gradually increasing. The clinical understanding of PDGNENs is limited, and a completely unified conclusion is difficult to determine. This study included 232 patients with PDGNENs from multiple centers in China and statistically analyzed the clinicopathological characteristics and prognostic factors of the patients, aiming to further standardize and systemize the diagnosis and treatment of PDGNENs.

Research motivation

This study aimed to explore the clinicopathological characteristics and prognostic factors for patients with PDGNENs, improve clinicians' awareness of this disease, reduce the misdiagnosis and missed diagnosis of PDGNENs, and achieve early detection, early diagnosis, and early treatment.

Research objectives

This study analyzed the clinicopathological characteristics and prognostic factors of patients with PDGNENs to identify independent risk factors that potentially affect prognosis. By interfering with independent prognostic risk factors for PDGNENs, the quality of life of patients can be further improved and the survival period be prolonged.

Research methods

This study was a retrospective study and included 232 patients with PDGNENs treated at seven centers in China from March 2007 to November 2019. The data were analyzed using the Kaplan-Meier method to evaluate the survival of patients. Single-factor analysis was performed using the log-rank test, and the Cox proportional hazard regression model was used to explore the risk factors that affect patient prognosis.

Research results

Among the 232 patients with PDGNENs, 113 (49.34%) patients had stage III tumors and 86 (37.55%) had stage IV tumors. The tumors were mainly (89.17%) solitary and located in the upper third of the stomach (cardia and fundus of stomach: 115/215, 53.49%). Most lesions were ulcers (157/232, 67.67%), with an average diameter of 4.66 cm. In terms of tumor invasion, the majority of tumors invaded the serosa (116/198, 58.58%). The median survival time of the 232 patients was 13.50 mo (range, 7-31 mo), and the overall 1-year, 3-year, and 5-year survival rates were 49%, 19%, and 5%, respectively.

Research conclusions

According to univariate analysis, tumor number, tumor diameter, gastric invasion status, American Joint Committee on Cancer (AJCC) stage, and distant metastasis status were prognostic factors for patients with PDGNENs. Multivariate analysis showed that tumor number, tumor diameter, AJCC stage, and distant metastasis status were independent prognostic factors for patients with PDGNENs.

Research perspectives

This study provides novel insights into the diagnosis and treatment of PDGNENs and the risk factors related to the prognosis. In the future, prospective, multicenter, large-scale research is still needed.

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REFERENCES

1 **Andreasi V**, Partelli S, Muffatti F, Manzoni MF, Capurso G, Falconi M. Update on gastroenteropancreatic neuroendocrine tumors. *Dig Liver Dis* 2021; **53**: 171-182 [PMID: 32912771 DOI: 10.1016/j.dld.2020.08.031]

2 **Dasari A**, Shen C, Halperin D, Zhao B, Zhou S, Xu Y, Shih T, Yao JC. Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States. *JAMA Oncol* 2017; **3**: 1335-1342 [PMID: 28448665 DOI: 10.1001/jamaoncol.2017.0589]

3 **Yang Z**, Wang W, Lu J, Pan G, Pan Z, Chen Q, Liu W, Zhao Y. Gastric Neuroendocrine Tumors (G-Nets): Incidence, Prognosis and Recent Trend Toward Improved Survival. *Cell Physiol Biochem* 2018; **45**: 389-396 [PMID: 29402806 DOI: 10.1159/000486915]

4 **Hu P**, Bai J, Liu M, Xue J, Chen T, Li R, Kuai X, Zhao H, Li X, Tian Y, Sun W, Xiong Y, Tang Q. Trends of incidence and prognosis of gastric neuroendocrine neoplasms: a study based on SEER and our multicenter research. *Gastric Cancer* 2020; **23**: 591-599 [PMID: 32026156 DOI: 10.1007/s10120-020-01046-8]

5 **Oronsky B**, Ma PC, Morgensztern D, Carter CA. Nothing But NET: A Review of Neuroendocrine Tumors and Carcinomas. *Neoplasia* 2017; **19**: 991-1002 [PMID: 29091800 DOI: 10.1016/j.neo.2017.09.002]

6 **Nagtegaal ID**, Odze RD, Klimstra D, Paradis V, Rugge M, Schirmacher P, Washington KM, Carneiro F, Cree IA; WHO Classification of Tumours Editorial Board. The 2019 WHO classification of tumours of the digestive system. *Histopathology* 2020; **76**: 182-188 [PMID: 31433515 DOI: 10.1111/his.13975]

7 **Assarzadegan N**, Montgomery E. What is New in 2019 World Health Organization (WHO) Classification of Tumors of the Digestive System: Review of Selected Updates on Neuroendocrine Neoplasms, Appendiceal Tumors, and Molecular Testing. *Arch Pathol Lab Med* 2020 [PMID: 32233993 DOI: 10.5858/arpa.2019-0665-RA]

8 **Doescher J**, Veit JA, Hoffmann TK. [The 8th edition of the AJCC Cancer Staging Manual : Updates in otorhinolaryngology, head and neck surgery]. *HNO* 2017; **65**: 956-961 [PMID: 28717958 DOI: 10.1007/s00106-017-0391-3]

9 **Tian FX**, Cai YQ, Zhuang LP, Chen MF, Xiu ZB, Zhang Y, Liu H, Liu ZH, Liu GP, Zeng C, Lin FL, Liu J, Huang ST, Zhang LZ, Lin HY. Clinicopathological features and prognosis of patients with gastric neuroendocrine tumors: A population-based study. *Cancer Med* 2018; **7**: 5359-5369 [PMID: 30311450 DOI: 10.1002/cam4.1683]

10 **Delle Fave G**, O'Toole D, Sundin A, Taal B, Ferolla P, Ramage JK, Ferone D, Ito T, Weber W, Zheng-Pei Z, De Herder WW, Pascher A, Ruszniewski P; Vienna Consensus Conference participants. ENETS Consensus Guidelines Update for Gastroduodenal Neuroendocrine Neoplasms. *Neuroendocrinology* 2016; **103**: 119-124 [PMID: 26784901 DOI: 10.1159/000443168]

11 **Gastrointestinal Pathology Study Group of Korean Society of Pathologists**. Cho MY, Kim JM, Sohn JH, Kim MJ, Kim KM, Kim WH, Kim H, Kook MC, Park DY, Lee JH, Chang H, Jung ES, Kim HK, Jin SY, Choi JH, Gu MJ, Kim S, Kang MS, Cho CH, Park MI, Kang YK, Kim YW, Yoon SO, Bae HI, Joo M, Moon WS, Kang DY, Chang SJ. Current Trends of the Incidence and Pathological Diagnosis of Gastroenteropancreatic Neuroendocrine Tumors (GEP-NETs) in Korea 2000-2009: Multicenter Study. *Cancer Res Treat* 2012; **44**: 157-165 [PMID: 23091441 DOI: 10.4143/crt.2012.44.3.157]

12 **Kim BS**, Park YS, Yook JH, Kim BS. Comparison of relapse-free survival in gastric neuroendocrine carcinoma (WHO grade 3) and gastric carcinoma. *Therap Adv Gastroenterol* 2017; **10**: 407-415 [PMID: 28507599 DOI: 10.1177/1756283X17697870]

13 **Liu DJ**, Fu XL, Liu W, Zheng LY, Zhang JF, Huo YM, Li J, Hua R, Liu Q, Sun YW. Clinicopathological, treatment, and prognosis study of 43 gastric neuroendocrine carcinomas. *World J Gastroenterol* 2017; **23**: 516-524 [PMID: 28210088 DOI: 10.3748/wjg.v23.i3.516]

14 **Bukhari MH**, Coppola D, Nasir A. Clinicopathologic analysis of primary gastroenteropancreatic poorly differentiated neuroendocrine carcinoma; A ten year retrospective study of 68 cases at Moffit Cancer Center. *Pak J Med Sci* 2020; **36**: 265-270 [PMID: 32063972 DOI: 10.12669/pjms.36.2.1336]

15 **Sekerci A**, Turk HM, Demir T, Seker M, Akcakaya A, Arici DS. Clinicopathological Features of Gastroenteropancreatic Neuroendocrine Neoplasms. *J Coll Physicians Surg Pak* 2020; **30**: 863-867 [PMID: 32893801 DOI: 10.29271/jcpsp.2020.08.863]

16 **Xie J**, Zhao Y, Zhou Y, He Q, Hao H, Qiu X, Zhao G, Xu Y, Xue F, Chen J, Su G, Li P, Zheng CH, Huang CM. Predictive Value of Combined Preoperative Carcinoembryonic Antigen Level and Ki-67 Index in Patients With Gastric Neuroendocrine Carcinoma After Radical Surgery. *Front Oncol* 2021; **11**: 533039 [PMID: 33738246 DOI: 10.3389/fonc.2021.533039]

17 **Ma F**, Wang B, Xue L, Kang W, Li Y, Li W, Liu H, Ma S, Tian Y. Neoadjuvant chemotherapy improves the survival of patients with neuroendocrine carcinoma and mixed adenoneuroendocrine carcinoma of the stomach. *J Cancer Res Clin Oncol* 2020; **146**: 2135-2142 [PMID: 32306127 DOI: 10.1007/s00432-020-03214-w]

18 **Ishida M**, Sekine S, Fukagawa T, Ohashi M, Morita S, Taniguchi H, Katai H, Tsuda H, Kushima R.

Neuroendocrine carcinoma of the stomach: morphologic and immunohistochemical characteristics and prognosis. *Am J Surg Pathol* 2013; **37**: 949-959 [PMID: 23759931 DOI: 10.1097/PAS.0b013e31828ff59d]

19 **Tierney JF**, Poirier J, Chivukula S, Pappas SG, Hertl M, Schadde E, Keutgen X. Primary Tumor Site Affects Survival in Patients with Gastroenteropancreatic and Neuroendocrine Liver Metastases. *Int J Endocrinol* 2019; **2019**: 9871319 [PMID: 30992703 DOI: 10.1155/2019/9871319]

20 **Liang WQ**, Zhang W, Qiao S, Wang BH, Wang C, Zhuang ZW, Xi HQ, Cai AZ, Wei B, Chen L. [Clinicopathologic features and prognostic analysis of 240 patients with gastric neuroendocrine neoplasms]. *Zhonghua Wei Chang Wai Ke Za Zhi* 2020; **23**: 38-43 [PMID: 31958929 DOI: 10.3760/cma.j.issn.1671-0274.2020.01.007]

21 **Zhang P**, Wang W, Lu M, Zeng C, Chen J, Li E, Tan H, Yu X, Tang Q, Zhao J, Shen L, Li J. Clinicopathological features and outcome for neuroendocrine neoplasms of gastroesophageal junction: A population-based study. *Cancer Med* 2018; **7**: 4361-4370 [PMID: 30062861 DOI: 10.1002/cam4.1702]

22 **Fang C**, Wang W, Zhang Y, Feng X, Zeng Y, Li Y, Chen J, Chen Y, Zhou Z. [Clinicopathological features and prognosis of patients with gastric neuroendocrine carcinoma from multiple centers in southern China]. *Zhonghua Wei Chang Wai Ke Za Zhi* 2016; **19**: 1230-1234 [PMID: 27928791]

23 **Li Y**, Wang YF, Tan BB, Er LM, Zhao Q, Fan LQ, Zhang ZD, Liu Y. [Pathological characteristics and survival analysis of 355 patients with gastroenteropancreatic neuroendocrine neoplasms]. *Zhonghua Zhong Liu Za Zhi* 2020; **42**: 426-431 [PMID: 32482035 DOI: 10.3760/cma.j.cn112152-112152-20191011-00663]

24 **Morita M**, Taguchi K, Kagawa M, Nakanoko T, Uehara H, Sugiyama M, Ota M, Ikebe M, Sugimachi K, Esaki T, Toh Y. Treatment strategies for neuroendocrine carcinoma of the upper digestive tract. *Int J Clin Oncol* 2020; **25**: 842-850 [PMID: 32036480 DOI: 10.1007/s10147-020-01631-y]

25 **Lu J**, Zhao YJ, Zhou Y, He Q, Tian Y, Hao H, Qiu X, Jiang L, Zhao G, Huang CM. On behalf of the Study Group for Gastric Neuroendocrine Tumours. Modified staging system for gastric neuroendocrine carcinoma based on American Joint Committee on Cancer and European Neuroendocrine Tumor Society systems. *Br J Surg* 2020; **107**: 248-257 [PMID: 31971627 DOI: 10.1002/bjs.11408]

26 **Garcia-Carbonero R**, Sorbye H, Baudin E, Raymond E, Wiedenmann B, Niederle B, Sedlackova E, Toumpanakis C, Anlauf M, Cwikla JB, Caplin M, O'Toole D, Perren A; Vienna Consensus Conference participants. ENETS Consensus Guidelines for High-Grade Gastroenteropancreatic Neuroendocrine Tumors and Neuroendocrine Carcinomas. *Neuroendocrinology* 2016; **103**: 186-194 [PMID: 26731334 DOI: 10.1159/000443172]

27 **Shen C**, Chen H, Yin Y, Han L, Chen J, Tang S, Yin X, Zhou Z, Zhang B, Chen Z. Surgical treatment and prognosis of gastric neuroendocrine neoplasms: a single-center experience. *BMC Gastroenterol* 2016; **16**: 111 [PMID: 27613657 DOI: 10.1186/s12876-016-0505-5]

28 **Mao R**, Li K, Cai JQ, Luo S, Turner M, Blazer D 3rd, Zhao H. Adjuvant Chemotherapy Versus Observation Following Resection for Patients With Nonmetastatic Poorly Differentiated Colorectal Neuroendocrine Carcinomas. *Ann Surg* 2019 [PMID: 31478977 DOI: 10.1097/SLA.0000000000003562]

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ORIGINAL ARTICLE

Observational Study

Prediction of hepatic inflammation in chronic hepatitis B patients with a random forest-backward feature elimination algorithm

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Author contributions: Zhou JY designed the study, analyzed the data, and contributed to writing the manuscript; Song LW and Lu XP performed the ELISA experiments and contributed to discussions; Yuan R performed the RF-BFE analysis; Wang GQ provided patient data and overall direction; all authors gave final approval of the version to be published and agree to be accountable for all aspects of the work.

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Informed consent statement: All patients gave informed consent.

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Abstract

BACKGROUND

Persistent liver inflammatory damage is the main risk factor for developing liver fibrosis, cirrhosis, and even hepatocellular carcinoma in chronic hepatitis B (CHB) patients. Thus, accurate prediction of the degree of liver inflammation is a high priority and a growing medical need.

AIM

To build an effective and robust non-invasive model for predicting hepatitis B-related hepatic inflammation.

METHODS

A total of 650 treatment-naïve CHB (402 HBeAg-positive and 248 HBeAg-negative) patients who underwent liver biopsy were enrolled in this study. Histological inflammation grading was assessed by the Ishak scoring system. Serum quantitative hepatitis B core antibody (qAnti-HBc) levels and 21 immune-related inflammatory factors were measured quantitatively using a chemiluminescent microparticle immunoassay. A backward feature elimination (BFE) algorithm utilizing random forest (RF) was used to select optional features and construct a combined model. The diagnostic abilities of the model or variables were evaluated based on the estimated area under the receiver operating characteristics curve (AUROC) and compared using the DeLong test.

benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of item.

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RESULTS

Four features were selected to predict moderate-to-severe inflammation in CHB patients using the RF-BFE method. These predictive features included qAnti-HBc, ALT, AST, and CXCL11. Spearman's correlation analysis indicated that serum qAnti-HBc, ALT, AST, and CXCL11 levels were positively correlated with the histology activity index (HAI) score. These selected features were incorporated into the model to establish a novel model named I-3A index. The AUROC [0.822; 95% confidence interval (CI): 0.790-0.851] of the I-3A index was significantly increased compared with qAnti-HBc alone (0.760, 95%CI: 0.724-0.792, $P < 0.0001$) in all CHB patients. The use of an I-3A index cutoff value of 0.41 produced a sensitivity of 69.17%, specificity of 81.44%, and accuracy of 73.8%. Additionally, the I-3A index showed significantly improved diagnostic performance for predicting moderate-to-severe inflammation in HBeAg-positive and HBeAg-negative CHB patients (0.829, 95%CI: 0.789-0.865 and 0.810, 95%CI: 0.755-0.857, respectively).

CONCLUSION

The selected features of the I-3A index constructed using the RF-BFE algorithm can effectively predict moderate-to-severe liver inflammation in CHB patients.

Key Words: Hepatic inflammation; Machine learning; Quantitative hepatitis B core antibody; CXCL11; Diagnostic efficiency

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Core Tip: We aimed to propose an effective backward feature elimination algorithm utilizing random forest to select optimal features and construct a novel non-invasive model for predicting hepatitis B-related hepatic inflammation based on a large, multicenter cohort. The results indicated that the I-3A index constructed based on the selected features significantly improved the diagnostic efficiency of quantitative hepatitis B core antibody alone for predicting moderate-to-severe inflammation. Additionally, the I-3A index showed high diagnostic accuracy for moderate-to-severe inflammation in both HBeAg-positive and HBeAg-negative chronic hepatitis B patients.

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INTRODUCTION

It is estimated that greater than 257 million people are chronically infected with hepatitis B virus (HBV), and over 880000 deaths are caused by HBV infection annually worldwide due to complications of the disease, such as liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)[1]. In general, hepatocellular lesions are not caused by HBV directly but are the result of the induction of the body's autoimmune response to inflammatory damage in hepatocytes. The burden of continuous inflammatory injury of hepatocytes not only hinders the body to clear HBV but is also the main risk factor for the development of liver fibrosis, cirrhosis, and even HCC[2]. Thus, it is essential to accurately evaluate the degree of hepatic inflammation and effectively reverse disease progression in chronic hepatitis B (CHB) patients.

Hepatitis B core antibody (anti-HBc) is a specific, nonprotective antibody against hepatitis B core antigen (HBcAg) that can be detected in almost all patients who have been exposed to HBV[3]. Recent studies have identified serum quantitative hepatitis B core antibody (qAnti-HBc) levels as an important immunological indicator that reflects immune activation status[4-7]. Furthermore, we previously reported that serum qAnti-HBc levels increase significantly with increasing histology activity index (HAI) score

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and have potential clinical value in assessing the degree of hepatitis B-related hepatic inflammation in CHB patients[8]. However, the optimal diagnostic efficacy may not be obtained by using qAnti-HBc alone, and its combination with other biomarkers potentially offers great clinical application value.

In this study, we aimed to assess the correlation between inflammatory immune-related factors and the degree of liver inflammation in CHB patients. Furthermore, we aimed to establish a novel model of serum qAnti-HBc combined with other immune inflammatory factors using a machine learning strategy to improve the diagnostic efficiency of qAnti-HBc in predicting the degree of hepatitis B-related hepatic inflammation.

MATERIALS AND METHODS

Patients

Patients with chronic HBV infection were enrolled in this study from 24 hospitals between October 2013 and December 2015. Patients recruited in the cohort study met the following criteria: (1) Age 18-75 years; (2) Hepatitis B surface antigen (HBsAg) seropositive status beyond 6 mo; (3) Being treatment naïve; (4) Serum negative for anti-hepatitis Avirus (HAV) IgM, anti-HCV, anti-hepatitis E virus (HEV) IgM/IgG, anti-Epstein–Barr virus (EBV) IgM, and anti-cytomegalovirus (CMV) IgM; and (5) Withdrawal from potential transaminase-lowering agents such as bicyclol for at least 2 wk prior to blood biochemistry analysis.

The exclusion criteria for this study included overlapping etiologies for hepatitis, including HCV, HIV, alcoholism, autoimmune disease, genetic disease, drug-induced causes, and nonalcoholic fatty liver. Patients with decompensated cirrhosis or HCC were also excluded. All patents provided written informed consent for the scientific use of their clinical data and samples. The complete protocol for the clinical trial has been registered at clinicaltrials.gov (NCT01962155) and chictr.org (ChiCTR-DDT-13003724).

Data acquisition

All patients underwent ultrasonographic-guided liver biopsy according to a standardized protocol. Pathological interpretations were conducted at the Department of Pathology of Youan Hospital affiliated with Capital Medical University. Each section was blindly and independently assessed by two pathologists. When discrepancies occurred, the samples were reviewed by experienced pathologists who were also responsible for reassessing in 10% of the samples selected randomly. The modified HAI was used to grade disease inflammatory activity. HAI ≥ 5 represented moderate-to-severe inflammation, and fibrosis score ≥ 3 was considered significant fibrosis[9,10].

At the time of liver biopsy, biochemical, blood cell, and coagulation tests were further performed using routine automated analyzers. Serum HBsAg and HBeAg levels were quantified by commercially available enzyme immunoassays (Roche Diagnostics, Penzberg, Germany). Serum HBV DNA levels (range 2.0×10^1-1.7×10^8 IU/mL) were measured *via* a COBAS AmpliPrep/COBAS TaqMan method. Serum qAnti-HBc levels were measured using double-sandwich enzyme-linked immunosorbent assays (Wantai, Xiamen, China).

Six cytokines (CXCL9, CXCL10, CXCL11, IL-2R, IL-33, and IL-34) were measured with a Luminex screening system (LXSAHM-6, R&D, Minneapolis, MN, United States). Additionally, 15 cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17A, CCL2, CCL3, IL-12 p70, IFN-γ, TNF-α, TGF-α, and granulocyte-monocyte colony stimulating factor) were measured with human cytokines panel I (Cat. No. HCYTOMAG-60K, EMD Millipore, Billerica, MA, United States). The results were analyzed with a Luminex 200 system (EMD Millipore, Billerica, MA, United States) according to the manufacturer's instructions. The coefficient of variation between the duplicate wells was controlled within 10%, and R^2 of the standard curve was at least 0.999.

Selection of significant features

In this study, a random forest (RF)-backward feature elimination (BFE) algorithm was used to automatically select the optimal feature subset, and the main procedure of the RF-BFE algorithm is illustrated in Figure 1. First, the entire dataset was shuffled and the features were ranked by RF importance measures. Then, least absolute shrinkage and selection operator (LASSO) regression was iteratively used to perform both

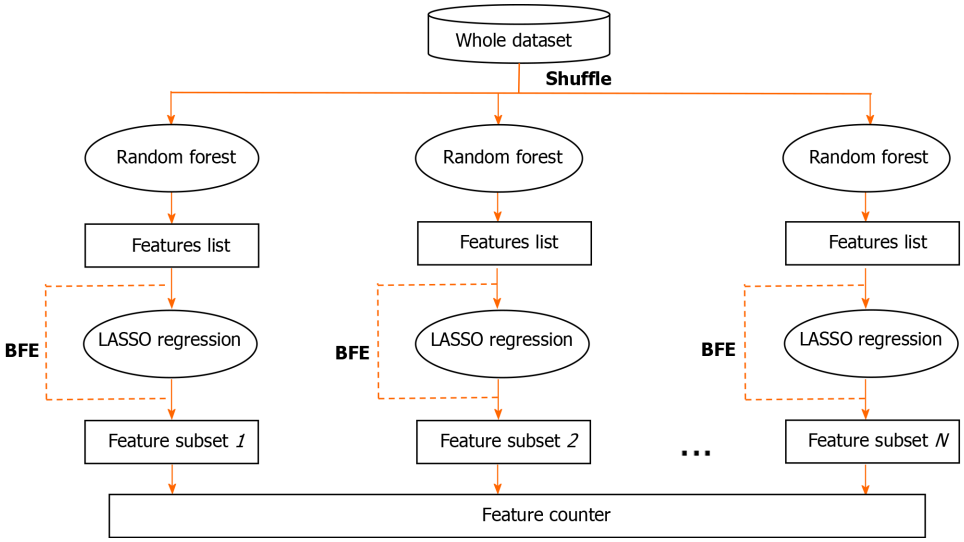


Figure 1 The main procedure of the random forest-backward feature elimination algorithm. BFE: Backward feature elimination; LASSO: Least absolute shrinkage and selection operator.

regularization and calculation of accuracy in the BFE. LASSO constructs a linear model, which penalizes the regression coefficients with an L1 penalty, shrinking many of them to zero. It performs both variable selection and regularization in order to enhance the prediction accuracy and interpretability of the resulting statistical model. The iterations ended when further removals did not result in an improvement, and produced a series of feature subsets. Finally, all of the feature subsets were gathered in a feature counter, and the final feature subset was determined by hard voting with equal weight.

To provide more robust results, the RF importance measure of each feature was calculated in every iteration. The detailed feature selection scheme in an iteration is illustrated in Figure 2. The least important feature was eliminated, and then the updated features were used to retrain the LASSO regression. The BFE process ended when the accuracy no longer improved. The accuracy was calculated as follows:

$$Sensitivity = TP/(TP + FN)$$
$$Specificity = TN/(TN + FP)$$
$$Accuracy = (Sensitivity + Specificity)/2 \quad (1)$$

where TP, TN, FP, and FN represent true positive, true negative, false positive, and false negative, respectively.

In our study, 100 iterations were implemented. Therefore, we used a hard vote strategy to determine the final features after the 100 subsets of the features were obtained. All of the selected features were gathered in a feature counter. The final feature subset *Fs* with *k* features was determined as follows:

$$Fs = Fsub:\{f_1, f_2, ..., f_k\} \mid v_f > v_0$$
$$v_f, v_0 \in (0, N) \quad (2)$$

where F_{sub} is the set of features addressed in all subsets, v_f is the vote of the feature, and v_0 is the threshold. Given that we used 100 iterations, v_f and v_0 ranged from 0 to 100.

Statistical analysis

Statistical analyses were conducted with SPSS (version16.0) and Python (version 3.5). Quantitative variables are expressed as the mean ± SD. Student's *t* test was used to analyze single specific differences of biological interest, and the chi-square test was used to analyze relationships between categorical variables. Spearman's rank tests were used to analyze associations between features and the HAI score. The LASSO logistic regression was performed using the "glmnet" package. The AUROC of models for diagnosing moderate-to-severe inflammation was analyzed using MedCalc version 15.6 (Ostend, Belgium). The diagnostic abilities of the different models were compared using the DeLong test. Additionally, the sensitivity, specificity, positive predictive value, (PPV) and negative predictive value (NPV) for the cut-off values were calculated. The level of statistical significance was defined as $P < 0.05$ (two-tailed).

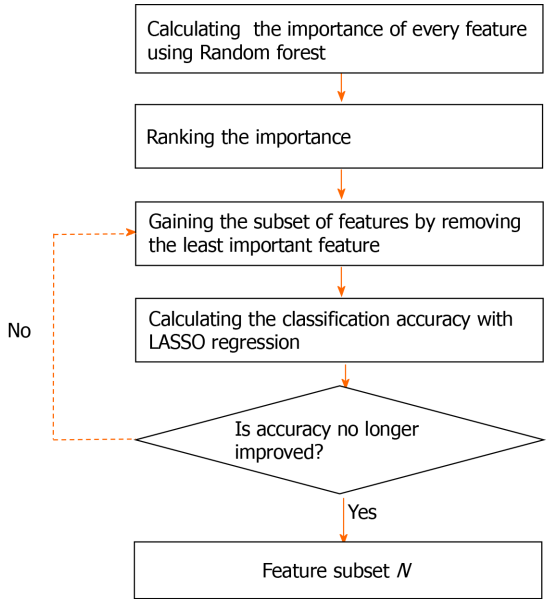


Figure 2 Feature selection scheme in an iteration. LASSO: Least absolute shrinkage and selection operator.

RESULTS

Clinical characteristics of the study population

In this study, a total of 650 patients were included in the statistical analysis, including 402 HBeAg-positive CHB patients and 248 HBeAg-negative CHB patients. Among the HBeAg-positive patients, 248 (61.69%) had at least moderate inflammation, and 249 (61.49%) had significant fibrosis. Among the HBeAg-negative patients, 138 (55.64%) had at least moderate inflammation, and 101 (40.73%) had significant fibrosis. The specific demographic characteristics are shown in Table 1. Serum levels of 4 (IL-2, IL-4, IL-12 p70, and CXCL9) of 21 cytokines were below the limits of detection in CHB patients. The remaining 17 cytokines were detected.

Features selection and model building

In this study, we set the threshold *v0* to 100, and four features (qAnti-HBc, ALT, AST, and CXCL11) were selected using the RF-BFE method (Figure 3 and Table 2). To validate the correlation between the selected features and HAI score, Spearman's rank tests were further performed. In HBeAg-positive patients, serum qAnti-HBc, ALT, AST, and CXCL11 levels were positively correlated with the HAI score (r = 0.55, 0.45, 0.51, and 0.34, respectively, P < 0.001). In HBeAg-negative patients, serum qAnti-HBc, ALT, AST, and CXCL11 Levels were also positively correlated with the HAI score (r = 0.49, 0.44, 0.49, and 0.33, respectively, all P < 0.001) (Table 2). Therefore, these selected features were incorporated into model construction to predict moderate-to-severe inflammation. Furthermore, the selected features were normalized using LASSO regression according to the following formula: ALT′ = (ALT-100.1)/130.25; AST′ = (AST-64.28)/78.33; Log(qAnti-HBc)′ = [log(qAnti-HBc) - 4.408]/0.7835; CXCL11′ = (CXCL11-55.02)/68.80. The established model, namely, the I-3A score, was calculated as follows:

Logit P = 1/[1+ exp(0.4986 × ALT′ + 1.190 × AST′ + 0.9154 × log(qAnti-HBc)′ + 0.5333 × CXCL11′ + 0.8099)]. (3)

Predictive performance

The ROC curves for predicting moderate-to-severe inflammation are shown in Figure 4 and Table 3. In all patients, the AUROC of the I-3A index to differentiate patients with moderate-to-severe inflammation was 0.822 [95% confidence interval (CI): 0.790-0.851], showing a significant increase compared with the value of 0.760 (95%CI: 0.724-0.792) for qAnti-HBc alone (P < 0.0001). The use of an I-3A index cutoff value of 0.41 (< 0.41) for predicting moderate-to-severe liver inflammation produced a sensitivity of 69.17%, specificity of 81.44%, and accuracy of 73.8%. The use of a qAnti-HBc cutoff value of 4.20 Log_{10}IU/mL produced a sensitivity of 83.94%, specificity of 55.68%, and accuracy of 72.13% (Table 3). Similar results were obtained in diagnostic efficiency analyses of both in HBeAg-positive and HBeAg-negative CHB patients.

Table 1 Clinical characteristics of patients with chronic hepatitis B virus infection

Parameter	HBeAg-positive patients (*n* = 402)	HBeAg-negative patients (*n* = 248)	*P* value
Gender, male	79.4.4% (319/402)	76.2% (189/248)	0.38
Age (yr)	36.35 ± 10.36	41.67 ± 9.65	< 0.001
BMI (kg/m^2)	23.12 ± 3.31	23.62 ± 2.84	0.01
HBsAg ($\log_{10}$ IU/mL)	3.83 ± 0.81	3.16 ± 0.73	< 0.001
HBV DNA ($\log_{10}$IU/mL)	6.93 ± 1.66	4.82 ± 1.64	< 0.001
ALT (U/L)	106.74 ± 139.40	80.58 ± 105.61	< 0.001
AST (U/L)	65.48 ± 78.67	56.69 ± 70.06	0.029
TC (mmol/L)	4.61 ± 3.05	4.44 ± 0.98	0.691
TBil (μmol/L)	16.54 ± 15.48	18.49 ± 27.32	0.009
DBil (μmol/L)	5.61 ± 7.54	5.77 ± 6.59	0.527
Albumin (g/L)	43.72 ± 5.14	44.91 ± 6.07	0.011
PT (s)	12.81 ± 1.49	12.55 ± 1.49	0.035
Cr (μmol/L)	70.21 ± 25.41	69.19 ± 15.93	0.818
BUN (mmol/L)	5.28 ± 5.57	4.90 ± 1.33	0.681
Platelet counts (× 10^9/L)	178.6 ± 56.18	158.5 ± 55.28	< 0.001
Histology (*n*, %)			
HAI 0-4	38.31% (154/402)	44.35% (110/248)	0.14
HAI ≥ 5	61.69% (248/402)	55.64% (138/248)	
Fibrosis score 0-2	38.06% (153/402)	59.27% (147/248)	< 0.001
Fibrosis score ≥ 3	61.49% (249/402)	40.73% (101/248)	

BMI: Body mass index; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TC: Total cholesterol; TBil: Total bilirubin; DBil: Direct bilirubin; PT: Prothrombin time; Cr: Creatinine; BUN: Blood urea nitrogen; HAI: Histology activity index.

Thus, these results indicate that the I-3A index has better diagnostic efficiency for predicting moderate-to-severe inflammation.

DISCUSSION

The BFE algorithm is widely used to reduce the dimensionality of feature space and is commonly employed using the support vector machine (SVM). RF is an importance measure that reveals the impact of each variable and has several advantages compared with SVM, as RF is an unbiased estimator, easy to parallel, and effectively and efficiently employed[11]. In this study, we proposed an effective BFE algorithm utilizing RF to select optional features and constructed a novel non-invasive model for predicting the degree of hepatitis B-related hepatic inflammation based on serum qAnti-HBc and other immune inflammatory factors. The results indicated that the I-3A index constructed based on the selected features significantly improved the diagnostic efficiency of qAnti-HBc alone for predicting moderate-to-severe inflammation. Additionally, the I-3A index showed high diagnostic accuracy for moderate-to-severe inflammation in both HBeAg-positive and HBeAg-negative CHB patients (AUROC = 0.829 and 0.810, respectively).

Histological analysis based on liver biopsy is the gold standard for evaluating the severity of hepatic inflammation. However, the practical use of this diagnostic method is limited by the traumatic nature of the procedures, which has inherent risks such as bleeding and sampling error[12,13]. ALT is the most widely used parameter of liver inflammation in clinical practice[14]. However, the diagnostic efficiency of ALT is limited due to its poor performance in CHB patients[15,16]. Therefore, non-invasive biomarkers that can accurately assess hepatic inflammation are still urgently needed. Our previous study of large, multicenter cohorts revealed that the serum qAnti-HBc

Table 2 Selected features for predicting moderate-to-severe inflammation in patients with chronic hepatitis B virus infection (more than 40 votes)

Feature name	HBeAg-positive patients			HBeAg-negative patients		
	Votes	*Spearman'r* (95%CI)[1]	*P* value[1]	Votes	Spearman *r* (95%CI)[1]	*P* value[1]
ALT	100	0.45 (0.36-0.53)	< 0.001	100	0.44 (0.33-0.53)	< 0.001
AST	100	0.51 (0.43-0.58)	< 0.001	100	0.49 (0.38-0.58)	< 0.001
qAnti-HBc	100	0.55 (0.47-0.62)	< 0.001	100	0.49 (0.39-0.58)	< 0.001
CXCL10	46	0.34 (0.25-0.43)	< 0.001	51	0.35 (0.23-0.46)	< 0.001
CXCL11	100	0.34 (0.25-0.43)	< 0.001	100	0.33 (0.21-0.44)	< 0.001
IL-2R	71	0.30 (0.20-0.39)	< 0.001	44	0.27 (0.14-0.38)	< 0.001
CCL2	42	-0.01 (-0.09-0.07)	0.79	52	0.03 (-0.10-0.16)	0.65
TNF-α	56	0.01 (-0.17-0.09)	0.78	62	0.01 (-0.17-0.09)	0.78
IL-1β	27	-0.10 (-0.23-0.03)	0.12	54	0.15 (0.03-0.28)	0.02

[1]Correlation between features and scores of histological activity in patients with chronic hepatitis B virus infection. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; qAnti-HBc: Quantitative hepatitis B core antibody; IL: Interleukin; TNF: Tumor necrosis factor; CI: Confidence interval.

Table 3 Receiver operating characteristic curve analysis of inflammation scores in patients with chronic hepatitis B virus infection

	Cutoff value	Se (%)	Spe (%)	PPV (%)	NPV (%)	Accuracy (%)	AUROC (95%CI)	*P* value
All patients								
I-3A	< 0.41	69.17	81.44	84.49	64.37	73.8	0.82 (0.79-0.85)	< 0.0001
qAnti-HBc	> 4.20 $\log_{10}$ IU/mL	83.94	55.68	73.47	70.33	72.13	0.76 (0.72-0.79)	
HBeAg-positive patients								
I-3A	< 0.41	70.16	80.52	85.29	62.63	74.13	0.83 (0.79-0.87)	< 0.0001
qAnti-HBc	> 4.20 $\log_{10}$ IU/ml	83.06	60.39	77.15	68.89	74.38	0.77 (0.73-0.81)	
HBeAg-negative patients								
I-3A	< 0.44	73.19	78.18	80.8	69.92	75.4	0.81 (0.76-0.86)	0.002
qAnti-HBc	> 4.40 $\log_{10}$ IU/mL	76.81	61.13	71.62	68	70.16	0.75 (0.70-0.81)	

Se: Sensitivity; Spe: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; AUROC: Area under the receiver operating characteristics curve; CI: Confidence interval; HBeAg: Hepatitis B e antigen; qAnti-HBc: Quantitative hepatitis B core antibody.

level was remarkably correlated with the degree of liver inflammation in CHB patients[8]. Further analysis indicated that qAnti-HBc was an independent risk factor for moderate-to-severe inflammation in CHB patients and exhibited high diagnostic accuracy for hepatic inflammation in patients with normal ALT levels[8]. A recent study by Chen *et al*[17] reported that serum qAnti-HBc levels could reflect the degree of hepatic inflammation in children infected with HBV who have normal ALT levels. Together, these findings suggest that serum qAnti-HBc is a promising biomarker of hepatic inflammation. In this study, the AUROC value of serum qAnti-HBc levels for predicting CHB patients with moderate-to-severe inflammation was 0.760, similar to the value of 0.768 reported by Li *et al*[18]. To improve the diagnostic efficiency of qAnti-HBc in the CHB population, a combined model was constructed. Li *et al*[18] reported a novel model (AC index) combing qAnti-HBc and ALT that exhibited improved diagnostic efficiency for predicting patients with moderate-to-severe inflammation with an AUROC of 0.810. In this study, the RF-BFE algorithm was used to evaluate immune inflammatory factors that may be related to the degree of CHB liver inflammation, and the importance of each factor was evaluated. The results showed that in addition to qAnti-HBc, CXCL11, ALT, and AST exhibited relatively high

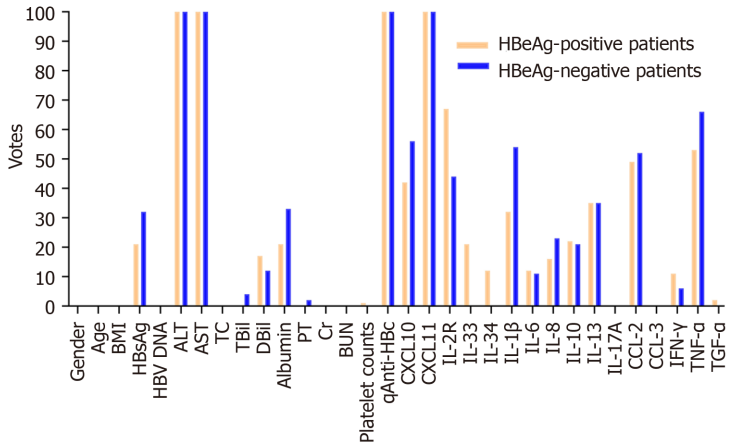


Figure 3 istogram of the votes in the feature counter. BMI: Body mass index; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TC: Total cholesterol; TBil: Total bilirubin; DBil: Direct bilirubin; PT: Prothrombin time; Cr: Creatinine; BUN: Blood urea nitrogen; qAnti-HBc: Quantitative hepatitis B core antibody; IL: Interleukin, IFN: Interferon; TNF: Tumor necrosis factor; TGF: Transforming growth factor.

significance in measuring liver inflammation. The AUROC value of the combined I-3A model based on LASSO regression was as high as 0.822, showing a significant increase compared with the diagnostic value of qAnti-HBc alone (LeLong $P < 0.0001$). Furthermore, the AUROC value of the I-3A index was significantly higher than that of the AC index reported by Li *et al*[18] (0.822 *vs* 0.810, DeLong $P = 0.041$).

Of note, serum CXCL11 levels increased significantly as the HAI score increased in CHB patients. CXCL11, which is also known as human interferon-inducible T cell alpha chemokine (I-TAC), is a member of the chemokine family and is involved in various inflammatory and angiogenic processes[19]. In liver disease, hepatocytes and sinusoidal cells release CXCL11, selectively recruit activated T cells to sites of inflammation, and are involved in the progression of hepatic damage[20]. In our study, CXCL11 was selected using the RF-BFE method and represents a potential biomarker for predicting moderate-to-severe inflammation in CHB patients. Serum CXCL10 levels were also positively correlated with the severity of histological inflammation, consistent with the findings of previous studies[21,22]. However, in our study, CXCL10 was not selected and incorporated into the model. CXCR3 (CD183) is a surface marker of B lymphocyte activation, and its ligands include CXCL10 and CXCL11. Increased CXCR3 expression is observed in chronic viral hepatitis and correlates with increases in CXCL10 and CXCL11 expression[21,23]. In our study, serum CXCL10 and CXCL11 levels were positively correlated not only with the degree of liver inflammation but also with the level of serum qAnti-HBc in CHB patients (data not shown). This finding suggests that a large proportion of activated B lymphocytes may be present in CHB patients with hepatic inflammatory injury and that the specific level of qAnti-HBc secreted by B lymphocytes in circulating blood may indirectly reflect the degree of immune inflammatory injury of the liver. However, the specific mechanisms of B lymphocytes, Anti-HBc, and liver immune injury must be further explored.

There are several limitations to our study. First, this study examined only serological inflammatory factors, but imaging techniques or transient elastography may improve performance. Radiomics is a promising tool that extracts high-throughput quantitative features from medical images and can provide valuable information for the diagnosis of liver damage. Second, it was a cross-sectional study and longitudinal data were not obtained to verify our results. Finally, our previous studies reported that qAnti-HBc alone has high diagnostic accuracy for predicting liver inflammation in CHB patients with normal ALT levels, and the I-3A index does not remarkably improve the diagnostic value of qAnti-HBc. Therefore, future prospective studies are needed to achieve increased diagnostic accuracy.

CONCLUSION

In conclusion, the selected features of the I-3A index constructed using the RF-BFE method can effectively predict liver inflammation in CHB patients.

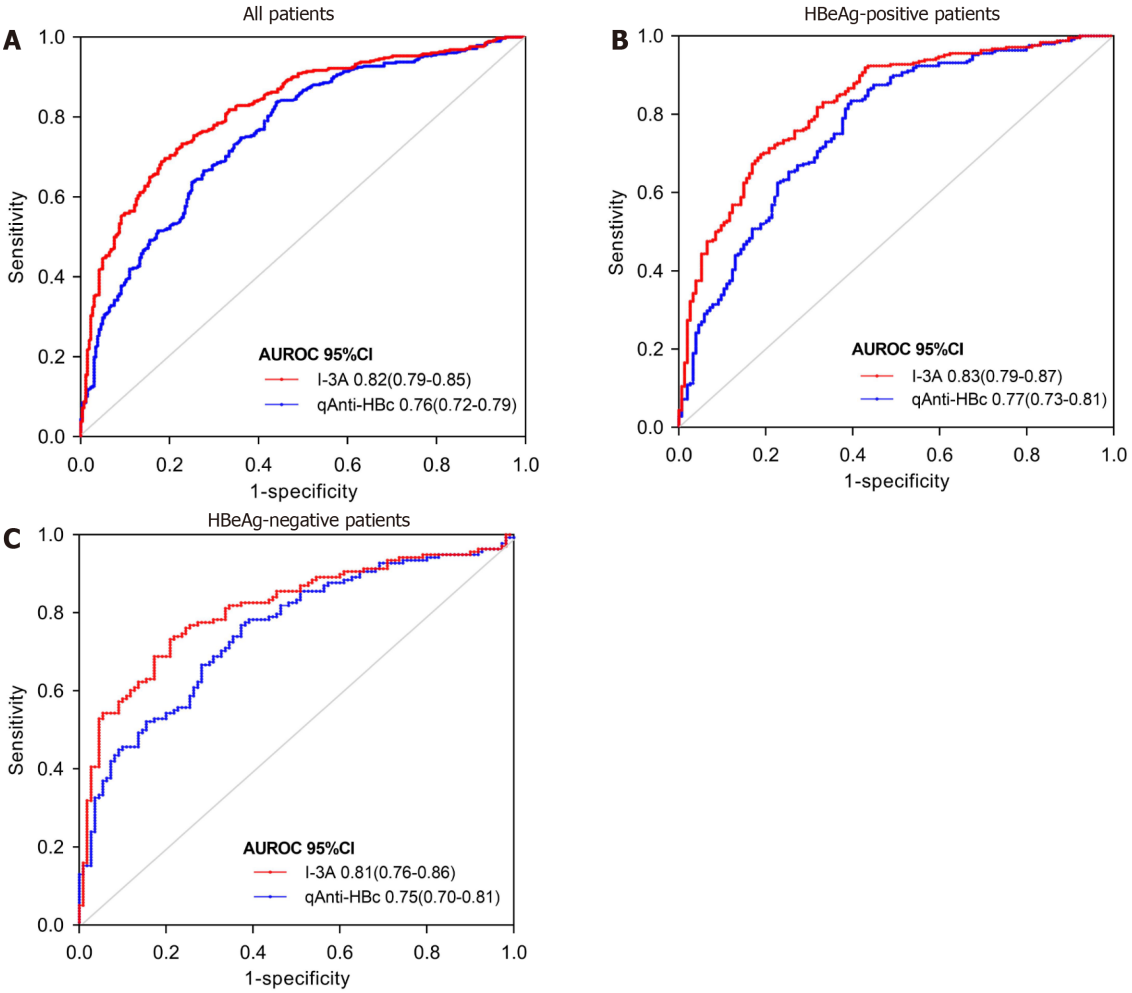


Figure 4 Receiver operating characteristic curve analysis for predicting moderate-to-severe inflammation in all patients with chronic hepatitis B virus infection, HBeAg-positive patients with chronic hepatitis B virus infection, and HBeAg-negative patients with chronic hepatitis B virus infection. A: All patients with chronic hepatitis B virus infection; B: HBeAg-positive patients with chronic hepatitis B virus infection; C: HBeAg-negative patients with chronic hepatitis B virus infection. AUROC: Area under the receiver operating characteristics curve; CI: Confidence interval; qAnti-HBc: Quantitative hepatitis B core antibody.

ARTICLE HIGHLIGHTS

Research background

The burden of continuous inflammatory injury of hepatocytes is the main risk factor for the development of liver fibrosis, cirrhosis, and even hepatocellular carcinoma. Thus, it is essential to accurately evaluate the degree of hepatic inflammation and effectively reverse disease progression in chronic hepatitis B (CHB) patients.

Research motivation

Recent studies have identified that serum quantitative hepatitis B core antibody (qAnti-HBc) levels have potential clinical value in assessing the degree of hepatitis B-related hepatic inflammation in CHB patients. However, the optimal diagnostic efficacy may not be obtained by using qAnti-HBc alone, and its combination with other biomarkers potentially offers great clinical application value.

Research objectives

The objective of this study was to build an effective and robust noninvasive model for predicting hepatitis B-related hepatic inflammation.

Research methods

Serum qAnti-HBc levels and 21 immune-related inflammatory factors were measured quantitatively in 650 treatment-naïve CHB patients who underwent liver biopsy. A backward feature elimination (BFE) algorithm utilizing Random Forest (RF) was used

to select optional features and construct a combined model. The diagnostic abilities of the model or variables were evaluated based on estimated area under the receiver operating characteristics curve (AUROC) and compared using the DeLong test.

Research results

Four features (qAnti-HBc, ALT, AST, and CXCL11) were selected and incorporated into the model to establish a novel I-3A index. The AUROC of the I-3A index to predict moderate-to-severe liver inflammation was significantly increased compared with qAnti-HBc alone in all CHB patients. The I-3A index showed significantly improved diagnostic performance for predicting moderate-to-severe inflammation in HBeAg-positive and HBeAg-negative CHB patients.

Research conclusions

The selected features of the I-3A index constructed using the RF-BFE algorithm can effectively predict moderate-to-severe liver inflammation in CHB patients.

Research perspectives

The novel I-3A index is a promising non-invasive tool to predict liver inflammation. A longitudinal study is needed to verify our results and more emerging strategies such as radiomics are needed to further achieve increased diagnostic efficiency.

REFERENCES

1 **Polaris Observatory Collaborators**. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol* 2018; **3**: 383-403 [PMID: 29599078 DOI: 10.1016/S2468-1253(18)30056-6]

2 **Berasain C**, Castillo J, Perugorria MJ, Latasa MU, Prieto J, Avila MA. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci* 2009; **1155**: 206-221 [PMID: 19250206 DOI: 10.1111/j.1749-6632.2009.03704.x]

3 **Wu T**, Kwok RM, Tran TT. Isolated anti-HBc: The Relevance of Hepatitis B Core Antibody-A Review of New Issues. *Am J Gastroenterol* 2017; **112**: 1780-1788 [PMID: 29087395 DOI: 10.1038/ajg.2017.397]

4 **Fan R**, Sun J, Yuan Q, Xie Q, Bai X, Ning Q, Cheng J, Yu Y, Niu J, Shi G, Wang H, Tan D, Wan M, Chen S, Xu M, Chen X, Tang H, Sheng J, Lu F, Jia J, Zhuang H, Xia N, Hou J; Chronic Hepatitis B Study Consortium. Baseline quantitative hepatitis B core antibody titre alone strongly predicts HBeAg seroconversion across chronic hepatitis B patients treated with peginterferon or nucleos(t)ide analogues. *Gut* 2016; **65**: 313-320 [PMID: 25586058 DOI: 10.1136/gutjnl-2014-308546]

5 **Song LW**, Liu PG, Liu CJ, Zhang TY, Cheng XD, Wu HL, Yang HC, Hao XK, Yuan Q, Zhang J, Kao JH, Chen DS, Chen PJ, Xia NS. Quantitative hepatitis B core antibody levels in the natural history of hepatitis B virus infection. *Clin Microbiol Infect* 2015; **21**: 197-203 [PMID: 25658546 DOI: 10.1016/j.cmi.2014.10.002]

6 **Yang HC**, Tsou HH, Pei SN, Chang CS, Chen JH, Yao M, Lin SJ, Lin J, Yuan Q, Xia N, Liu TW, Chen PJ, Cheng AL, Hsu C; Taiwan Cooperative Oncology Group. Quantification of HBV core antibodies may help predict HBV reactivation in patients with lymphoma and resolved HBV infection. *J Hepatol* 2018; **69**: 286-292 [PMID: 29551710 DOI: 10.1016/j.jhep.2018.02.033]

7 **Chi H**, Li Z, Hansen BE, Yu T, Zhang X, Sun J, Hou J, Janssen HLA, Peng J. Serum Level of Antibodies Against Hepatitis B Core Protein Is Associated With Clinical Relapse After Discontinuation of Nucleos(t)ide Analogue Therapy. *Clin Gastroenterol Hepatol* 2019; **17**: 182-191.e1 [PMID: 29902645 DOI: 10.1016/j.cgh.2018.05.047]

8 **Zhou J**, Song L, Zhao H, Yan L, Ma A, Xie S, Zhang X, Zhang D, Xie Q, Zhang G, Shang J, Cheng J, Zhao W, Zou Z, Zhang M, Xia N, Wang G. Serum hepatitis B core antibody as a biomarker of hepatic inflammation in chronic hepatitis B patients with normal alanine aminotransferase. *Sci Rep* 2017; **7**: 2747 [PMID: 28584279 DOI: 10.1038/s41598-017-03102-3]

9 **Wai CT**, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 518-526 [PMID: 12883497 DOI: 10.1053/jhep.2003.50346]

10 **Hui AY**, Chan HL, Wong VW, Liew CT, Chim AM, Chan FK, Sung JJ. Identification of chronic hepatitis B patients without significant liver fibrosis by a simple noninvasive predictive model. *Am J Gastroenterol* 2005; **100**: 616-623 [PMID: 15743360 DOI: 10.1111/j.1572-0241.2005.41289.x]

11 **Breiman L**. Random Forests. *Mach Learn* 2001; **45**: 5-32 [DOI: 10.1023/A:1010933404324]

12 **Cadranel JF**, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology* 2000; **32**: 477-481 [PMID: 10960438 DOI: 10.1053/jhep.2000.16602]

13 **Regev A**, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, Feng ZZ, Reddy KR, Schiff ER. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am J Gastroenterol* 2002; **97**: 2614-2618 [PMID: 12385448 DOI:

10.1111/j.1572-0241.2002.06038.x]

14 **Kim WR**, Flamm SL, Di Bisceglie AM, Bodenheimer HC; Public Policy Committee of the American Association for the Study of Liver Disease. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology* 2008; **47**: 1363-1370 [PMID: 18366115 DOI: 10.1002/hep.22109]

15 **Chao DT**, Lim JK, Ayoub WS, Nguyen LH, Nguyen MH. Systematic review with meta-analysis: the proportion of chronic hepatitis B patients with normal alanine transaminase ≤ 40 IU/L and significant hepatic fibrosis. *Aliment Pharmacol Ther* 2014; **39**: 349-358 [PMID: 24387289 DOI: 10.1111/apt.12590]

16 **Sarin SK**, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS, Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int* 2016; **10**: 1-98 [PMID: 26563120 DOI: 10.1007/s12072-015-9675-4]

17 **Chen HS**, Wu JF, Su TH, Chen HL, Hsu HY, Xia NS, Chen PJ, Chang MH. Baseline Level of Hepatitis B Core Antibody Predicts Spontaneous Hepatitis B e Antigen (HBeAg) Seroconversion in HBeAg-Positive Children With a Normal Alanine Aminotransferase Level. *Hepatology* 2019; **70**: 1903-1912 [PMID: 31121067 DOI: 10.1002/hep.30788]

18 **Li J**, Zhang TY, Song LW, Qi X, Yu XP, Li FH, Zhou P, Qin YL, Yang L, Zhao JH, Mao RC, Zhang YM, Wang JY, Yang FF, Zhu HX, Yang SS, Huang YX, Yuan Q, Zhang J, Zhang JM, Xia NS. Role of quantitative hepatitis B core antibody levels in predicting significant liver inflammation in chronic hepatitis B patients with normal or near-normal alanine aminotransferase levels. *Hepatol Res* 2018; **48**: E133-E145 [PMID: 28707778 DOI: 10.1111/hepr.12937]

19 **Lacotte S**, Brun S, Muller S, Dumortier H. CXCR3, inflammation, and autoimmune diseases. *Ann N Y Acad Sci* 2009; **1173**: 310-317 [PMID: 19758167 DOI: 10.1111/j.1749-6632.2009.04813.x]

20 **Helbig KJ**, Ruszkiewicz A, Semendric L, Harley HA, McColl SR, Beard MR. Expression of the CXCR3 ligand I-TAC by hepatocytes in chronic hepatitis C and its correlation with hepatic inflammation. *Hepatology* 2004; **39**: 1220-1229 [PMID: 15122750 DOI: 10.1002/hep.20167]

21 **Zeremski M**, Petrovic LM, Chiriboga L, Brown QB, Yee HT, Kinkhabwala M, Jacobson IM, Dimova R, Markatou M, Talal AH. Intrahepatic levels of CXCR3-associated chemokines correlate with liver inflammation and fibrosis in chronic hepatitis C. *Hepatology* 2008; **48**: 1440-1450 [PMID: 18798334 DOI: 10.1002/hep.22500]

22 **Chalin A**, Lefevre B, Devisme C, Barget N, Amiot L, Samson M. Circulating levels of CXCL11 and CXCL12 are biomarkers of cirrhosis in patients with chronic hepatitis C infection. *Cytokine* 2019; **117**: 72-78 [PMID: 30826602 DOI: 10.1016/j.cyto.2019.02.006]

23 **Butera D**, Marukian S, Iwamaye AE, Hembrador E, Chambers TJ, Di Bisceglie AM, Charles ED, Talal AH, Jacobson IM, Rice CM, Dustin LB. Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C. *Blood* 2005; **106**: 1175-1182 [PMID: 15860662 DOI: 10.1182/blood-2005-01-0126]

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FRONTIER

Fecal microbiota transplantation for irritable bowel syndrome: An intervention for the 21st century

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Abstract

Irritable bowel syndrome (IBS) affects about 12% of the global population. Although IBS does not develop into a serious disease or increase mortality, it results in a considerable reduction in the quality of life. The etiology of IBS is not known, but the intestinal microbiota appears to play a pivotal role in its pathophysiology. There is no effective treatment for IBS, and so the applied treatments clinically focus on symptom relief. Fecal microbiota transplantation (FMT), an old Chinese treatment, has been applied to IBS patients in seven randomized controlled trials (RCTs). Positive effects on IBS symptoms in various degrees were obtained in four of these RCTs, while there was no effect in the remaining three. Across the seven RCTs there were marked differences in the selection processes for the donor and treated patients, the transplant dose, the route of administration, and the methods used to measure how the patients responded to FMT. The present frontier discusses these differences and proposes: (1) criteria for selecting an effective donor (superdonor); (2) selection criteria for patients that are suitable for FMT; (3) the optimal FMT dose; and (4) the route of transplant administration. FMT appears to be safe, with only mild, self-limiting side effects of abdominal pain, cramping, tenderness, diarrhea, and constipation. Although it is early to speculate about the mechanisms underlying the effects of FMT, the available data suggest that changes in the intestinal bacteria accompanied by changes in fermentation patterns and fermentation products (specifically short-chain fatty acids) play an important role in improving the IBS symptoms seen after FMT. FMT appears to be a promising treatment for IBS, but further studies are needed before it can be applied in everyday clinical practice.

Key Words: Butyric acid; Enteroendocrine cells; Etiology; Microbiota; Short-chain fatty acids; Superdonor; Therapy

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Core Tip: Irritable bowel syndrome (IBS) is a common gastrointestinal disorder. There is no effective treatment for IBS, but fecal microbiota transplantation (FMT) seems promising. Some randomized controlled trials (RCTs) have shown that FMT improved the symptoms and quality of life of IBS patients, but others have found no such effects. The discrepancies between the outcomes of these RCTs are due to the selection processes for the donor and treated patients, the dose of transplant used, and the route of administration. Further studies are needed to optimize the FMT conditions in IBS patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder with a prevalence of 12.1% worldwide[1,2]. Although IBS does not increase mortality, it considerably reduces the quality of life[3]. The etiology of IBS is not known, but several factors appear to be involved in its pathophysiology, including genetics, diet, the intestinal microbiota, enteroendocrine cells, low-grade inflammation, and stress[2,4]. An effective treatment for IBS is lacking, and so the interventions applied to IBS patients clinically are aimed at symptom relief[5].

The human intestine is colonized by more than 10 microorganisms, including archaea, fungi, viruses, and bacteria[6,7], spanning 2172 bacteria species belonging to 12 different bacteria phyla[8]. Bacteria belonging to *Firmicutes* and *Bacteroidetes* phyla dominate the bacterial population in the intestine of healthy subjects, and they combine with a few members from the *Proteobacteria* and *Actinobacteria* phyla[8,9]. The intestinal bacterial composition varies between individuals due to both genetic and environmental factors[6,10]. These environmental factors include diet, the frequency of antibiotic treatment, the intake of nonantibiotic drugs, geographical location, surgery, smoking, and depression[6,10,11]. A low intestinal bacterial diversity (dysbiosis) is found in several diseases[10,12].

The intestinal bacterial composition in IBS patients deviates from that of healthy subjects[10,13-15]. Compared with healthy subjects, IBS patients have a lower abundance of butyrate-producing bacteria (*Erysipelotrichaceae*, *Ruminococcaceae*, *Bifidobacterium*, *Faecalibacterium*, and *Erysipelotrichaceae* spp.) and methanogenic bacteria, and a higher abundance of bacteria belonging to *Proteobacteria*, *Veillonella*, and Firmicutes such as *Lactobacillus* and *Ruminococcus* spp.[7,16]. In addition, the diversity of intestinal bacteria is lower (dysbiosis) in IBS patients than in healthy sub-jects[10,13-15,17].

Transplanting the intestinal microbiome (*via* feces) from a healthy subject with normal bowel function to patients — so-called fecal microbiota transplantation (FMT) — was applied for the first time by the Chinese physician Ge Hong in the fourth century for treating severe diarrhea and malaria[18]. In the present era, FMT represents an effective treatment for *Clostridium difficile* infection (CDI) and is also a promising invention for other diseases[19-21].

The use of FMT as a treatment for IBS has been investigated in seven randomized controlled trials (RCTs)[11,22-27], which have produced different outcomes. The present frontier aimed at clarifying these differences, highlighting the gaps in our knowledge that need to be filled, and proposing a guideline for successful FMT in IBS patients.

FACTORS THAT AFFECT THE OUTCOME OF FMT

FMT reduced the symptoms and improved the quality of life of the treated IBS patients in four of the seven RCTs[11,22,26,27], but had no effect in the remaining three RCTs[23-25]. These differences in the outcomes of RCTs of FMT in IBS could be explained by differences in the protocols, donor selection, included IBS patients, dose of fecal transplant used, and administration route (Table 1)[11,22-27]. Several suggestions have been made about how to improve the efficacy of FMT in IBS[28,29].

Donor selection

The outcomes of FMT in inflammatory bowel disease (IBD) have varied considerably between studies[20], which has been attributed to differences in the donor used[10,30]. A donor that produces a large response to FMT in IBD patients is called a superdonor[10]. Since it was not possible to predict who is a superdonor, the feces from several donors were pooled to increase the likelihood of patients receiving superdonor feces[31]. However, this approach was not successful, which was probably due to the dilution of the eventual superdonor feces resulting in an insufficient dose from the superdonor to the recipients[32].

Similar to IBD, discrepancies in the results of the RCTs of FMT in IBS could be mainly attributed to differences in the criteria used for donor selection (Table 1)[11,22-27]. An RCT performed by El-Salhy *et al*[11] that obtained good responses to FMT (Figure 1) established clinical criteria and the bacterial profile for selecting the superdonor. In contrast, the less-successful RCTs did not establish either clinical criteria or a bacterial profile for the donor[22-27] (Table 1). The stability of the donor intestinal bacterial composition over time is also an important factor to consider when selecting a superdonor[27].

The clinical criteria used to select a superdonor have been based on the factors known to affect the intestinal microbiota (Table 2). Aging (> 50 years), smoking/smoking cessation, being born by cesarean section, consuming formula as a baby, frequent treatment with antibiotics, and regular intake of nonantibiotic drugs are known to reduce the bacterial diversity[33-42], whereas regular exercise combined with consuming a sport-specific diet are associated with a favorable intestinal microbiota[43-45]. The genetic composition also affects the intestinal microbiota, and hence the superdonor should not be a first-degree relative of any recipient[46,47]. Thus, the superdonor selected in the RCT by El-Salhy *et al*[11] was a healthy young male with a normal body mass index, born *via* a vaginal delivery, breastfed, and a nonsmoker, and did take any medication, had been treated only a few times with antibiotics, exercised regularly, and consumed a sport-specific diet that was richer in protein, fiber, minerals, and vitamins than average[48]. Furthermore, the superdonor was not related to any of the recipients[11]. The analysis of the fecal microbiota of this donor showed that he had a high microbial diversity (normobiotic), and his fecal bacterial composition deviated from the normal abundance of 165 healthy subjects in 14 of 39 tested bacteria markers (Figure 2)[11]. Twelve of these deviated bacteria were in the Firmicutes phylum, with one each in the Proteobacteria and Verrucomicrobia phyla[11]. This deviation included an increased abundance of favorable bacteria such as *Streptococcus*, *Dorea*, *Lactobacillus*, and *Ruminococcaceae* spp.[10,49-51]. The fecal bacterial composition of the superdonor was stable over the 18-mo period during which he donated his feces (Figure 3)[11].

Like in IBD, pooling the feces from several donors to ensure the presence of superdonor feces resulted in no response or only a transient improvement[22,23].

Patient inclusion

The patients included in RCTs of FMT for IBS have included subsets of IBS patients who do not represent the entire IBS population, and so caution should be exercised when generalizing the outcomes of these RCTs[11,22-27]. Four of the RCTs only included patients with the diarrhea-predominant IBS (IBS-D) and mixed-diarrhea-and-constipation IBS (IBS-M)[22,24,26,27], while the other three RCTs included three IBS subtypes: IBS-D, constipation-predominant IBS (IBS-C), and IBS-M[11,23,25]. Moreover, the patients included in the RCT of El-Salhy *et al*[11] had participated in a 2-d course related to living with IBS, and for at least the past 3 mo they had moderate-to-severe IBS symptoms despite adhering to a diet consistent with the National Institute for Health and Care Excellence (NICE)-modified diet. The RCT of Holster *et al*[25] included patients with low amounts of fecal butyrate-producing bacteria. Holvoet *et al*[27] only included refractory patients with severe bloating who had failed to respond to at least three conventional therapies for IBS.

Table 1 Summary of seven randomized controlled trials of fecal microbiota transplantation for irritable bowel syndrome

Study	IBS subtypes	Number of patients (% female)	Intervention (number of patients)	Route of administration	Primary endpoint	Results	Microbiota assessment
Johnsen *et al*[22], 2018	53.0% IBS-D, 47.0% IBS-M	83 (66.3%)	50-80 g of single FMT (2 donors) (55) *vs* autologous stool (28)	Colon *via* a colonoscope	Decrease in IBS severity scoring system of ≥ 75 points at 12 wk and 12 mo	Response rate: 12-wk FMT 65% *vs* placebo 43% ($P < 0.05$); 12-mo 56% in FMT *vs* placebo 36% ($P > 0.05$)	Unkown
Halkjær *et al*[23], 2018	37.3% IBS-M, 33.3% IBS-C, 29.4% IBS-D	52 (68.6)	50 g ofdonor stool per dayfor 12 d (4 donors) (26) *vs* placebo capsules (26)	Oral capsules	Decrease in IBS severity scoring system of ≥ 50 points at 12 wk	Response rate: FMT 36.4% *vs* placebo 79.2%, $P < 0.05$	FMT increased microbiota biodiversity shift to the donor
Aroniadis *et al*[24], 2019	100% IBS-D	24 (37.5) Crossover after 12 wk	9.5 g of donor stool per day for 3 d (4 donors involved but each participant received from 1 donor) (24) *vs* placebo capsules (24)	Oral capsules	Decrease in IBS severity scoring system of ≥ 50 points at 12 wk	FMT-first 50% *vs* placebo-first 61%, ($P = 0.46$)	Prevotella abundance did not change after FMT; Similar diversity between responders and non-responders
Holster *et al*[25], 2019	56% IBS-D, 25.0% IBS-C, 19% IBS-M with a low amount of butyrate producing bacteria in their fecal samples	17 (50.0%)	30 g of single FMT (1 donor) (8) *vs* autologous stool (8)	Colon *via* a colonoscope	Decrease in gastrointestinal symptom rating scale-IBS of ≥ 30%	Symptom and QoL improved from baseline only in FMT arm ($P < 0.05$) but not different between arms	Trend of fecal and mucosal microbiota shift to the donor. No significant change in diversity and butyrate-producing bacteria
Lahtinen *et al*[26], 2020	51% IBS-D, 28.6% IBS-U. 14% IBS-M, 6% in remission	49 (59.2%)	30 g of single FMT (1 donor) (23) *vs* 30 g of autologous stool (26)	Colon *via* a colonoscope	decline in the IBS-SSS score of ≥ 50 points throughout 52 wk	Primary endpoint was not achieved in both arms. Only transient improved in FMT arm *vs* baseline at week 12	Significant shift in the microbiota profile and richness increased in FMT group
El-Salhy *et al*[11], 2020	38.4% IBS-D, 37.8% IBS-C, 23.8% IBS-M	164 (81%)	single 30 g FMT (54) *vs* 60 g (55) *vs* autologous stool (55)	Distal duodenum *via* a gastroscope	total IBS-SSS score decreased by ≥ 50 points at 3 mo	Response rate: 30 g FMT 76.9%, 60 g FMT 89.1%, control 23.6% $P < 0.05$; response independent of gender, IBS subtype	At 1 mo: Dysbiosis index/prevalence were not different at baseline *vs* post FMT ($P > 0.05$), Responder had higher signals for*Eubacterium biforme*, *Lactobacillus* spp. and *Alistipes* spp. After FMT, and lower signals for *Bacteroides* spp
Holvoet *et al*[27], 2021	100% IBS-D or IBS-M with predominant bloating	62 (64.7)	Single FMT (2 male donors, each participant received from 1 donor) (43) *vs* autologous stool (19)	Small intestine *via* a nasojejunal tube	Self-reported adequate relief of symptoms at 12 wk and 1 yr	Response rate: FMT 56% *vs* control 26% ($P < 0.05$); Good response predictor: women (69% *vs* 29%), patients with high baseline diversity, distinct composition ($P < 0.05$) 1 yr response FMT 21% *vs* control 6%; Second FMT restored response in 67% of patients with prior response	Unknown

IBS-D: Diarrhea-predominant irritable bowel syndrome; IBS-M: Mixed-diarrhea-and-constipation irritable bowel syndrome; IBS-C: constipation-predominant irritable bowel syndrome; FMT: Fecal microbiota transplantation; IBS-SSS: Irritable bowel syndrome severity scoring system.

Route of administration and dose of the fecal transplant

The fecal transplant can be administered either to the small intestine *via* the working channel of a gastroscope or a nasojejunal probe, or to the large intestine *via* the working channel of a colonoscope (Figures 4 and 5)[11,22,25-27]. Administering the

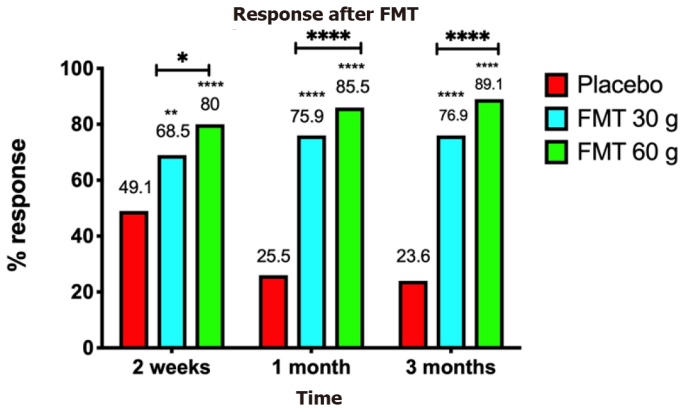


Figure 1 Responses of irritable bowel syndrome patients to placebo, 30-g fecal microbiota transplantation, and 60-g fecal microbiota transplantation at different intervals after transplantation. **P < 0.001; ****P < 0.0001 compared with placebo. **P < 0.001; ****P < 0.0001 for 30-g fecal microbiota transplantation (FMT) compared with 60-g FMT. Citation: El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; 69: 859-867. Copyright @BMJ Publishing Group Ltd 2020. Published by BMJ Publishing Group Ltd[11].

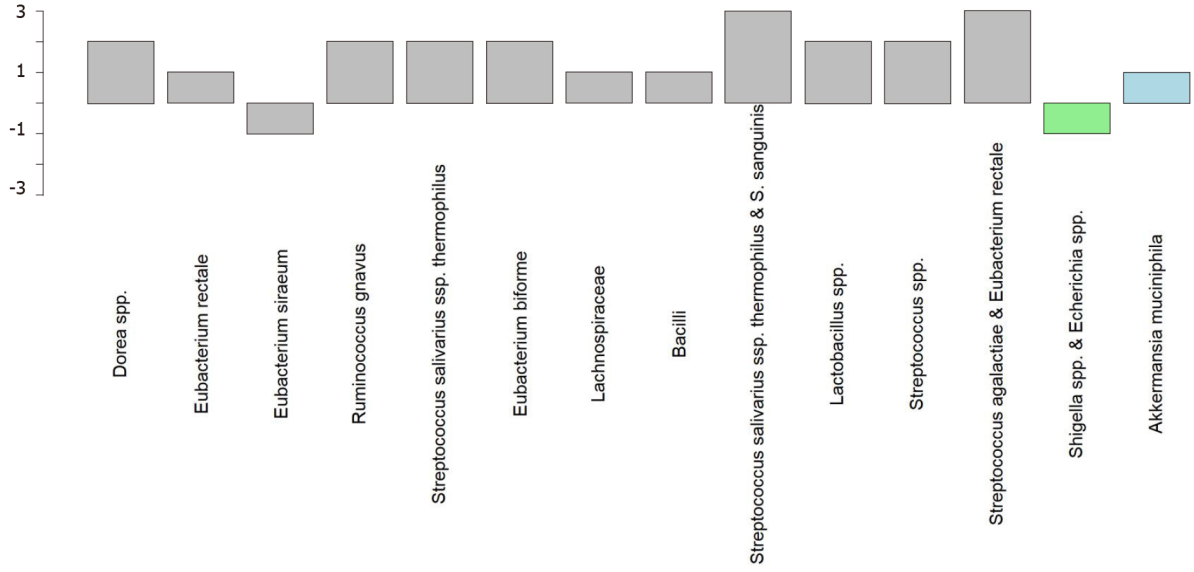


Figure 2 Superdonor bacterial profile deviated from the expected normal abundance in 14 of the 39 bacteria markers. The deviating bacteria belong to the typical commensal bacteria species that do not contribute to dysbiosis. Twelve of these bacteria belong to the *Firmicutes* phylum (gray), one to the *Proteobacteria* phylum (light green), and one to the *Verrucomicrobia* phylum (light blue). Citation: El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; 69: 859-867. Copyright @BMJ Publishing Group Ltd 2020. Published by BMJ Publishing Group Ltd[11].

fecal transplant to either the small or large intestine seems to be effective[11,22,25-27]. However, a placebo effect was evident in 43%-44% and 23.6%-26% of the patients that received the fecal transplant into the large and small intestine, respect-ively[11,22,25-27]. This placebo effect might have been greater in patients that received fecal transplant into the colon due to the favorable effect of the bowel preparation needed for a colonoscopy on IBS symptoms[52].

While administering fecal transplants *via* capsule ingestion has been effective in CDI, it was unsuccessful in IBS[23,24,53]. This is could be due to the selection of the donor, a low transplant dose, or pooling of the donors rather than the mode of administration[23,24].

The dose of the fecal transplant seems to significantly affect the outcome of FMT, with a dose–dependent response appearing to be present (Figure 1)[11]. This was confirmed by 70% of patients that did not respond to 30-g FMT responding to 60-g FMT (Figure 6)[54]. All but two of the RCTs of FMT for IBS used a dose of at least 30 g [11,22,23,25,26]; in the remaining two studies the dose was either lower than 30 g or not specified[24,27]. Further investigations are needed to evaluate the efficacy single *vs*

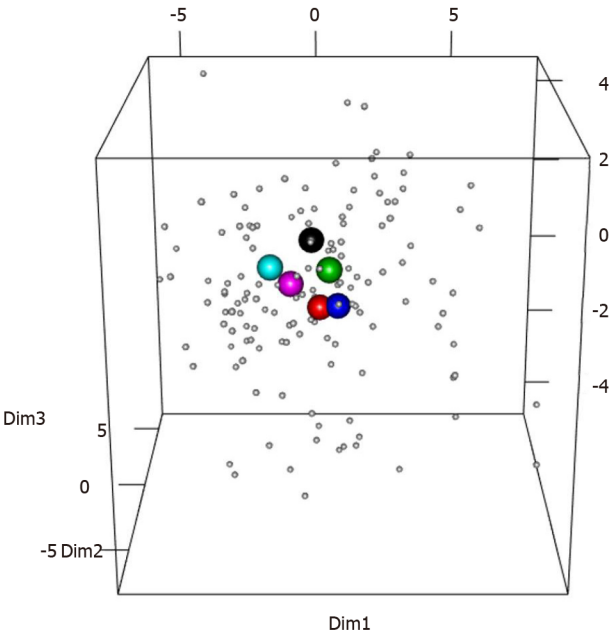


Figure 3 Scaled principal component analysis plot of fecal samples from the superdonor and patients before transplantation. The patient samples are indicated by small gray circles. The superdonor samples are indicated by the larger circles of different colors reflecting the sampling times: black, 3 mo; red, 6 mo; green, 9 mo; blue, 12 mo; light blue, 15 mo; and pink, 18 mo. All of the superdonor samples are grouped closely together and remain in very similar positions over time. Citation: El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; 69: 859-867. Copyright @BMJ Publishing Group Ltd 2020. Published by BMJ Publishing Group Ltd[11].

repeated transplantation.

CHANGES IN BACTERIAL AND FERMENTATION PROFILES AFTER FMT

In the RCT of El-Salhy *et al*[11,55] in which FMT was most effective, the severity of dysbiosis did not differ significantly between the placebo and active treated groups after 1 mo, but it was reduced significantly after 1 year. One month after FMT, the bacterial profile changed significantly in the active treated groups compared with the placebo group (Figure 7)[11]. Higher signals for *Eubacterium biforme*, *Lactobacillus* spp., and *Alistipes* spp., and lower signals for *Bacteroides* spp. were observed at 1 mo after FMT in the active treated groups (Figure 8) but not in the placebo group (Figures 9–12)[11]. The IBS severity scoring system (IBS-SSS) score was significantly correlated with the signals of *Lactobacillus* spp. (P = 0.002, r = –0.3) and *Alistipes* spp. (P = 0.001, r = –0.3) but not with those of *Eubacterium biforme*(P = 0.754, r = 0.03) or *Bacteroides* spp. (P = 0.458, r = 0.06)[11]. The signals of nine bacteria had changed at 1 year after FMT (Figure 13)(unpublished data): Whereas the signals of *Bacteroides* spp., *Prevotella* spp., *Alistipes* spp., *Bacteroides stercoris*, *Parabacteroides* spp., and *Bacteroides zoogleoformans* were significantly higher than those at the baseline, the signals of *Streptococcus salivarius* ssp. *thermophilus, Shigella* spp., *Escherichia* spp., *Eubacterium hallii*, and *Dorea* spp. were significantly lower than those at the baseline. The signals of the following six of these bacteria were correlated with IBS-SSS total scores: *Bacteroides* spp. And *Prevotella* spp. (P < 0.0001, r = -0.4), *Alistipes* spp. (P < 0.001, r = -0.3), *Bacteroides stercoris* and *Streptococcus salivarius* ssp. *thermophilus* (P < 0.0001, r = 0.3), *Bacteroides zoogleoformans* (P < 0.002, r = -0.3), and *Eubacterium hallii* (P < 0.04, r = -0.2) (unpublished data). Changes in the bacterial profiles in IBS patients that received FMT have been recorded, but which bacteria had changed was not described in detail[23,25-27].

The main products of the bacterial fermentation of intestinal undigested and unabsorbed carbohydrates are short-chain fatty acids (SCFAs)[55]. It has been reported that the fecal level of propionic acid was significantly higher in IBS patients[13]. Moreover, the level of butyric acid has been found to be lower in IBS-C patients and higher in IBS-D patients than in healthy subjects[13]. The levels of total SCFAs at 1 mo after FMT were significantly higher in IBS patients that received 60-g FMT but not in those that received 30-g FMT[55]. The levels of butyric acid at 1 mo after FMT were

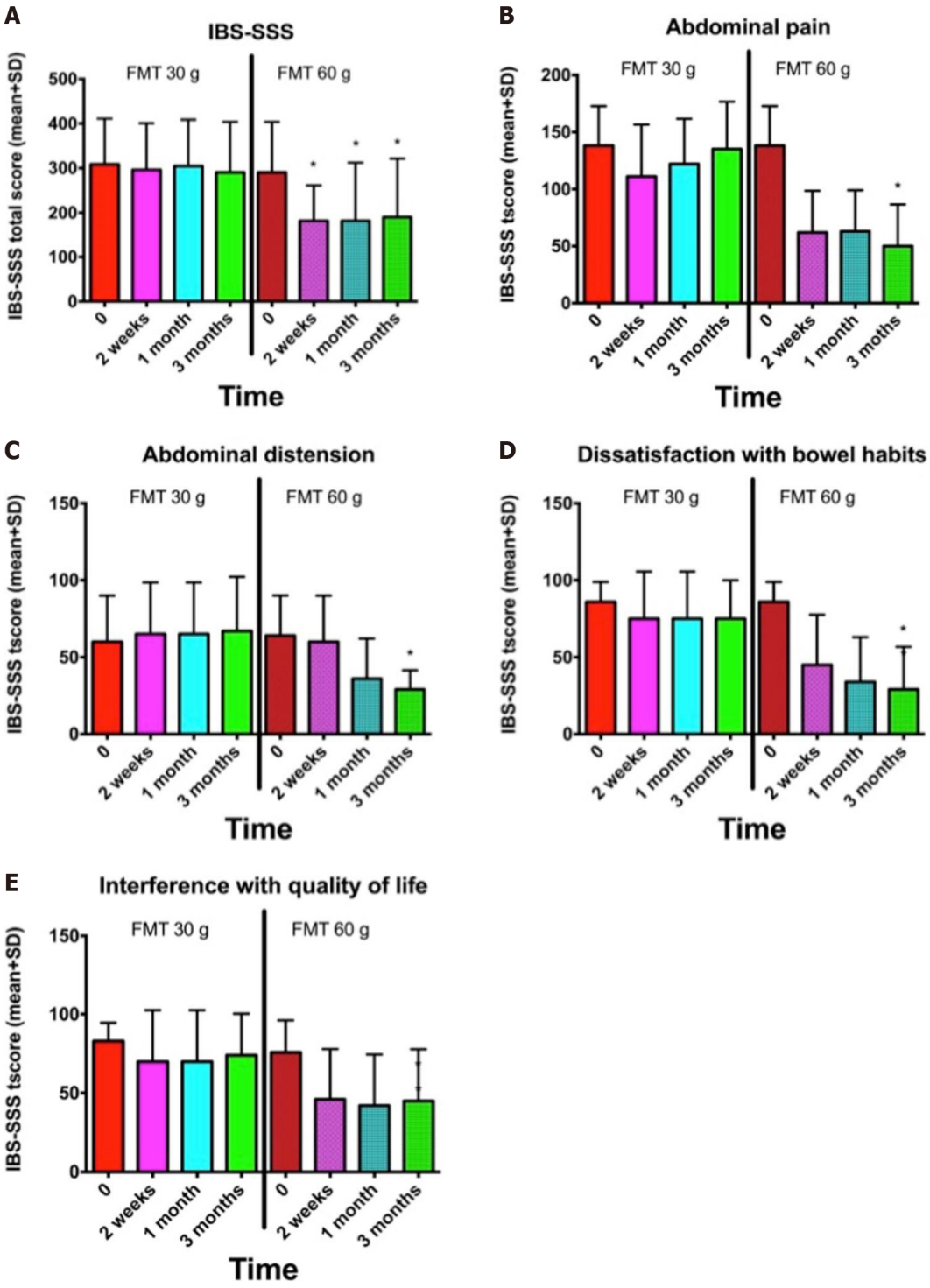


Figure 4 Nonresponders to 30-g donor`s fecal transplant were retransplanted with 60-g donor´s transplant. A-E: Irritable bowel syndrome-severity scoring system (SSS) total score (A) and IBS-SSS scores for abdominal pain (B), abdominal distension (C), dissatisfaction with bowel habits (D), and interference with quality of life (E) at different intervals after receiving 30-g and 60-g fecal microbiota transplantation. *$P < 0.05$ compared with baseline. Citation: El-Salhy M, Hausken T, Hatlebakk JG. Increasing the Dose and/or Repeating Faecal Microbiota Transplantation (FMT) Increases the Response in Patients with Irritable Bowel Syndrome (IBS). *Nutrients* 2019; **11** [PMID: 31238507 DOI: 10.3390/nu11061415]54].

also significantly higher in IBS patients that received either 30-g or 60-g FMT[55]. There were no significant differences in the levels of total SCFAs and in level of individual SCFAs in the placebo group at 1 mo after FMT[55]. The butyric acid level was inversely correlated with the scores on the IBS-SSS and fatigue assessment scale (Figure 14)[55].

The fecal levels of total SCFAs and butyric acid remained elevated in the active treated group with FMT at 1 year after FMT (unpublished data). Furthermore, the fecal levels of isobutyric and isovaleric acids were significantly higher while that of acetic

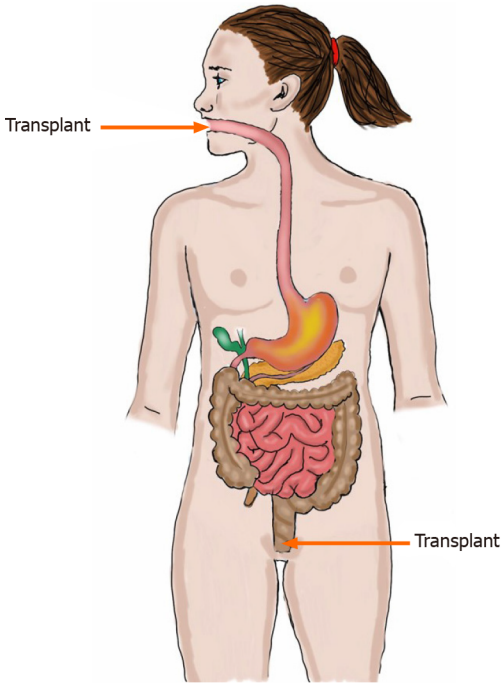


Figure 5 Fecal transplant can be administered to the small intestine (distal duodenum) or to the large intestine (colon).



Figure 6 Although the fecal transplant can be administered to the small intestine *via* a nasojejunal probe and to the colon *via* an enema, administering a fecal transplant *via* the working channel of a gastroscope or colonoscope is faster and more acceptable to patients.

acid was significantly lower (unpublished data).

DIFFERENCES IN RESPONSES BETWEEN IBS SUBTYPES AND SEX

The response to FMT did not differ between the IBS-D, IBS-C, and IBS-M subtypes of IBS[11], whereas it was not clear whether there was a difference between females and males. In the subgroup of IBS-D and IBS-M patients with refractory IBS and severe bloating who had not responded to at least three conventional therapies for IBS, the response to FMT was better for females than for males[27]. On the other hand, the response to FMT did not differ between female and male IBS patients with moderate-to-severe symptoms belonging to the IBS-D, IBS-C, and IBS-M subtypes who had

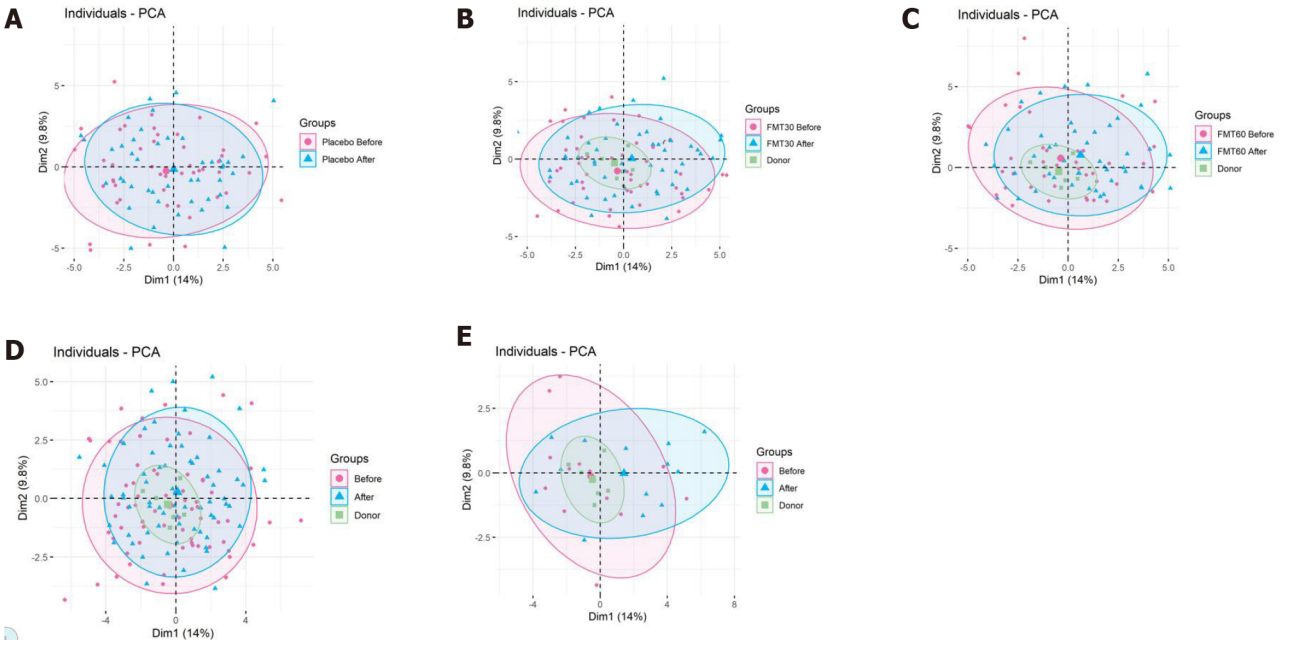


Figure 7 Scaled principal component analysis plot of fecal samples before and 1 mo after transplantation for placebo, 30-g and 60-g fecal microbiota transplantation, responders and nonresponders. A: Placebo; B: 30-g fecal microbiota transplantation (FMT); C: 60-g FMT; D: Responders; E: Nonresponders. Fecal samples before and after transplantation are indicated by pink circles and blue triangles, respectively. The ellipses cover 80% of the samples within a group. Citation: El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; 69: 859-867. Copyright @BMJ Publishing Group Ltd 2020. Published by BMJ Publishing Group Ltd[11].

previously not responded to the NICE-modified diet[56].

SAFETY OF FMT

A recently published consensus report from a multidisciplinary United European Gastroenterology working group did not classify voluntarily donated material (feces) as a drug. Instead, it was recommended to be collected, handled, and used according to the standards defined by the EU Commission in EU Tissue and Cells Directive 2004/23/EC[21,57].

FMT appears to be safe, with limited side effects[58,59]. The short-term adverse events for FMT in IBS patients have been reported to be mild, self-limiting, and occurring during the first few days following transplantation: Abdominal pain, cramping, tenderness, diarrhea, and constipation[11,22-27]. However, infectious complications have been described in two patients that received FMT for diseases other than IBS, which resulted in one fatality[60,61]. While these patients were old, severely ill, and immunocompromised, such events raised questions about safety issues regarding FMT for IBS, especially given that IBS is considered to be a benign gastrointestinal condition[3,62,63]. It has therefore been suggested that the screening of FMT donors should include testing of their feces for extended-spectrum-beta-lactase-producing *E. coli* and severe acute respiratory syndrome coronavirus 2. This would reduce the risks of infection and restrict the selection of IBS patients for FMT to those who are not immunocompromised and do not have systemic disease or severe illness [3].

No adverse events were observed 1-year after FMT for IBS patients (unpublished data). However, long-term follow-ups of patients that received FMT because of CDI raised some concerns about weight gain, the development or worsening of IBD, cancer, autoimmune diseases, allergies, and neurological diseases[64].

POSSIBLE MECHANISMS UNDERLYING THE EFFECTS OF FMT

The enteroendocrine cells and fecal SCFAs in IBS patients differ from those of healthy subjects, and these differences are believed to play a central role in the patho-

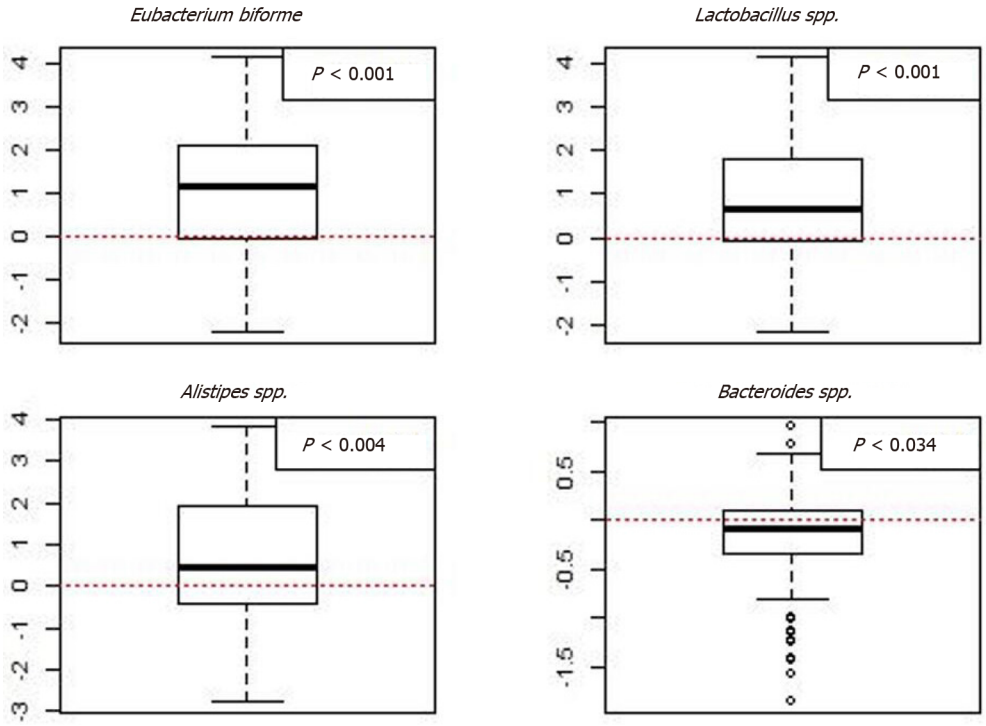


Figure 8 Box plots of the logarithm of the difference in signals between baseline and 1 mo after fecal microbiota transplantation for the responders in both the 30-g and 60-g fecal microbiota transplantation groups. Red stippled line indicates 0, where no change occurred between before and after fecal microbiota transplantation (FMT). Signals after FMT in responders were higher for *Eubacterium biforme*, *Lactobacillus* spp., and *Alistipes* spp., and lower for *Bacteroides* spp. Citation: El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; 69: 859-867. Copyright @BMJ Publishing Group Ltd 2020. Published by BMJ Publishing Group Ltd[11].

physiology of IBS[2,65-70]. Although it is early to speculate about the mechanisms underlying the effects of FMT, there are data suggesting that the improvement in IBS symptoms by FMT is caused by changes in the enteroendocrine cells and in SCFAs.

Most of the body serotonin (95%) is localized to the gastrointestinal tract, of which only 10% is present in the neurons of the enteric nervous system and the rest is in the serotonin-containing enterochromaffin (EC) cells that are scattered between the epithelial cells lining the gastrointestinal lumen[71-74]. Serotonin plays an important role in gastrointestinal motility by inhibiting gastric emptying and stimulating colonic motility, and accelerating transit through the small and large intestines. Serotonin also activates the submucosal sensory branch of the enteric nervous system that conveys sensation from the gut to the central nervous system[70]. When serotonin exerts its effects at serotonin receptors, it is transported by the serotoninselective reuptake transporter (SERT) into intestinal epithelial cells, where it is degraded[75,76]. The epithelial cells lining the luminal surface of the intestine express SERT[70,77], and a reduction in SERT results in impaired intracellular uptake of serotonin and its degradation in the intestinal epithelial cells[78,79]. In IBS patients, the density of EC cells in the colon and the SERT immune-intensity in the large intestine have been reported to be lower than those in healthy subjects (Figures 15 and 16)[80-82]. Several bacteria including *Corynebacterium*, *Streptococcus*, and *Enterococcus* spp. produce serotonin and indigenous spore-forming bacteria from the microbiota of mice and humans that promote serotonin biosynthesis by colonic EC cells[83,84]. Furthermore, *Clostridium ramosum* regulates the release of serotonin from EC cells[85]. It is possible that the changes in the intestinal bacterial composition induced by FMT affect the serotonin-regulating system.

Peptide YY (PYY) occurs in enteroendocrine cells of the ileum, colon, and rectum[86,87]. PYY binds to and activates receptors Y_1 and Y_2, which are localized to the epithelial cells lining the intestinal lumen and in the submucosal and myenteric plexus neurons of the small intestine and colon[88-100]. PYY acts as a mediator of the ileal brake and stimulates the absorption of water and electrolytes in the large intestine[87,101,102]. The density of colonic PYY cells is reduced in IBS patients (Figure 17)[80-82]. The total levels of fecal SCFAs increased significantly in IBS patients at 1 mo after FMT and remained higher than those at the baseline at 1 year after transplanta-

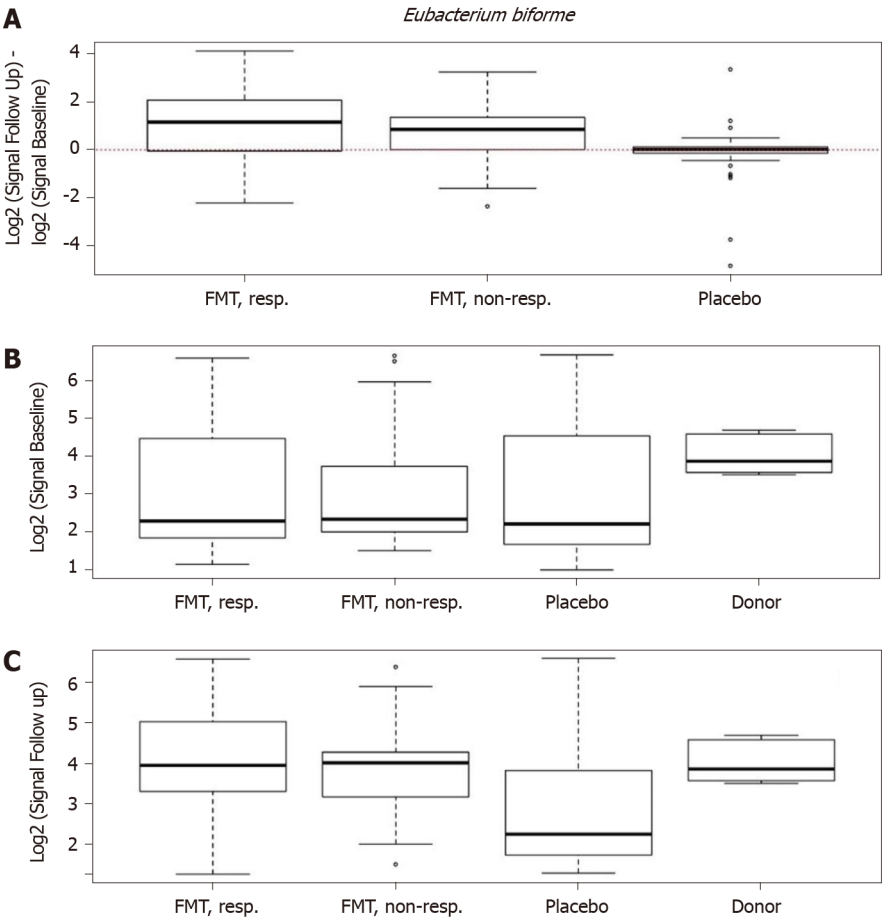


Figure 9 Differences in signals for *Eubacterium biforme* in the responder, nonresponder, and placebo groups between before and 1 mo after transplantation. There were significant changes in the signal levels in responders ($P < 0.001$). The nonresponders also exhibited increased signal levels, though the changes were smaller than those in the responders. A: The signal level did not change in the placebo group; B: Signal levels at baseline were similar in the responder, nonresponder, and placebo groups, but they were higher for the superdonor; C: The signal levels in the responders and nonresponders became as high as that for the superdonor after fecal microbiota transplantation, while that in the placebo group did not change.

tion(unpublished data)[55]. Intestinal SCFAs increase the secretion of peptide PYY by up-regulating the gene expression of PYY[102-105]. It is likely that the improvement in IBS symptoms following FMT is due to the effect of SCFAs on PYY secretion.

In the patients that received FMT, the fecal levels of butyric acid increased at 1 mo after FMT and remained elevated at 1 year after transplantation(unpublished data)[56]. Butyrate provides colonic epithelial cells with energy[105], modulates the immune response and oxidative stress, and decreases intestinal motility and the permeability of intestinal cells[87]. Butyrate also modulates colonic hypersensitivity, and an intake of butyrate was found to reduce the abdominal pain in patients with IBS[106-108]. An increase in butyric acid could be one of the factors underlying the improvement seen after FMT.

The fecal level of the straight SCFA acetic acid was reduced at 1 year after FMT(unpublished data). This reduction could be significant since acetic acid has been found to induce visceral hypersensitivity in rodents[109]. The levels of the branched SCFAs isobutyric and isovaleric acids were significantly increased at 1 year after FMT(unpublished data). These increases indicate a shift in the microbial metabolism from a saccharolytic to a proteolytic fermentation pattern, which could be of pathophysiological relevance[110]. It is particularly interesting that similar changes in the profiles of isobutyric and isovaleric acids have been found in patients with IBS following adherence to a low-FODMAPs (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) diet[111].

CONCLUSION

FMT is a longstanding Chinese treatment applied to both gastrointestinal and

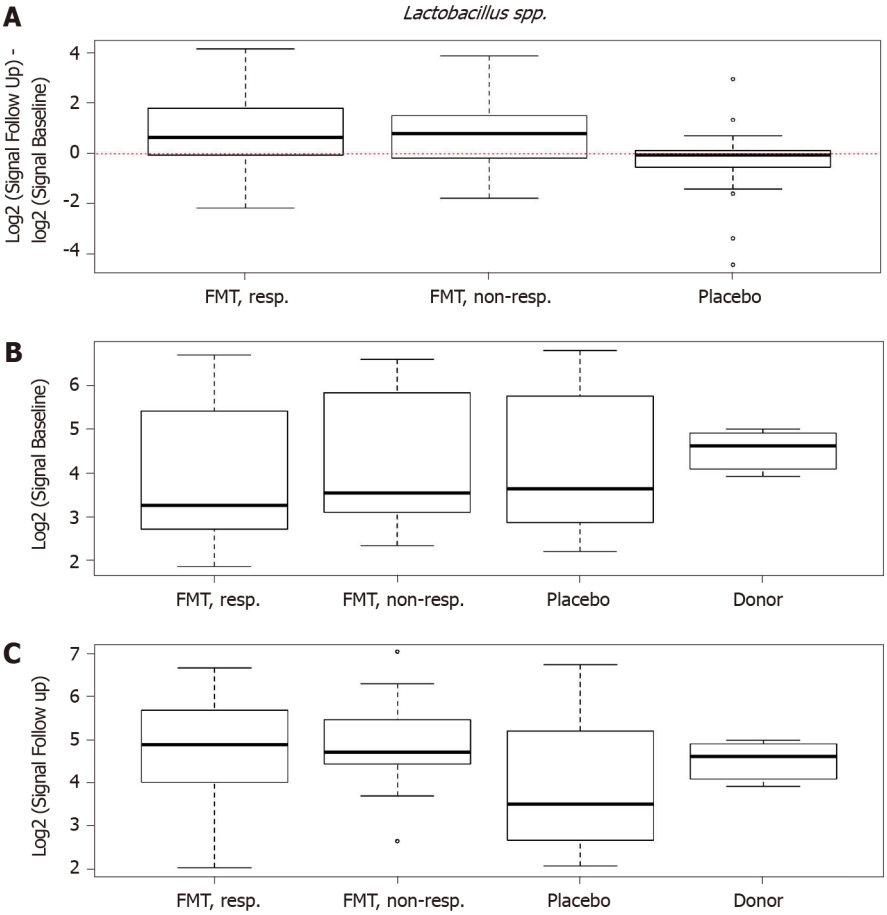


Figure 10 Differences in signals for *Lactobacillus* spp. in the responder, nonresponder, and placebo groups between before and 1 mo after transplantation. There were significant changes in the signal levels in responders ($P < 0.001$). A: The nonresponders exhibited similar signal levels to those in the responders, while there was no change in the placebo group; B: The signal levels in the responder, nonresponder, and placebo groups were similar, but it was higher for the superdonor; C: After transplantation, the signal levels in responders and nonresponders became similar to that for the superdonor, while that in the placebo group did not differ from the baselin. Citation: El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; 69: 859-867. Copyright @BMJ Publishing Group Ltd 2020. Published by BMJ Publishing Group Ltd[11].

nongastrointestinal diseases[18], and is now also a promising treatment for IBS patients since it improves the IBS symptoms, fatigue, and quality of life in about 90% of patients in a RCT[11]. However, several questions remain to be answered, and further investigations are needed before FMT can be applied in everyday clinical practice. The criteria to apply when selecting an effective (superdonor) for FMT remain unclear, as do the optimal dose, administration route, and frequency of treatment. Moreover, it is not clear whether FMT is effective for all IBS patients, or whether it should be restricted to certain subsets of IBS patients. There is also some concern regarding the long-term side effects of FMT[21].

There is an exciting task in front of us to modernize an effective treatment for IBS that was first used more than a thousand years ago.

Table 2 Criteria of a super-donor and how the 7 published randomized clinical trials met them

Study	BMI sex	Age < 50 yr	Smoking	Vaginal delivery	Formula-fed	frequent treatment with antibiotics	regular intake of non-antibiotic drugs	regular exercise	consuming a sport-specific diet	first-degree relative	bacterial signature	stability of the bacterial composition over time
El-Salhy *et al* [11], 2020	Yes/male	Yes	No	Yes	No	No	No	Yes	Yes	No	YES	Yes
Johnsen *et al* [22], 2018	Unknown/unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Halkjær *et al* [23], 2018	Yes/unknown	Yes	Unknown	Unknown	Unknown	Unknown	No	Unknown	Unknown	Unknown	Unknown	Unknown
Aroniadis *et al* [24], 2019	Yes/unknown	Yes	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Holster *et al* [25], 2019	Unknown/unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Lahtinen *et al* [26], 2020	Yes/male	Yes	Unknown	Yes	Unknown	No	No	Unknown	No	Unknown	Unknown	Unknown
Holvoet *et al* [27], 2021	Yes/male	Yes	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Yes

BMI: Body mass index.

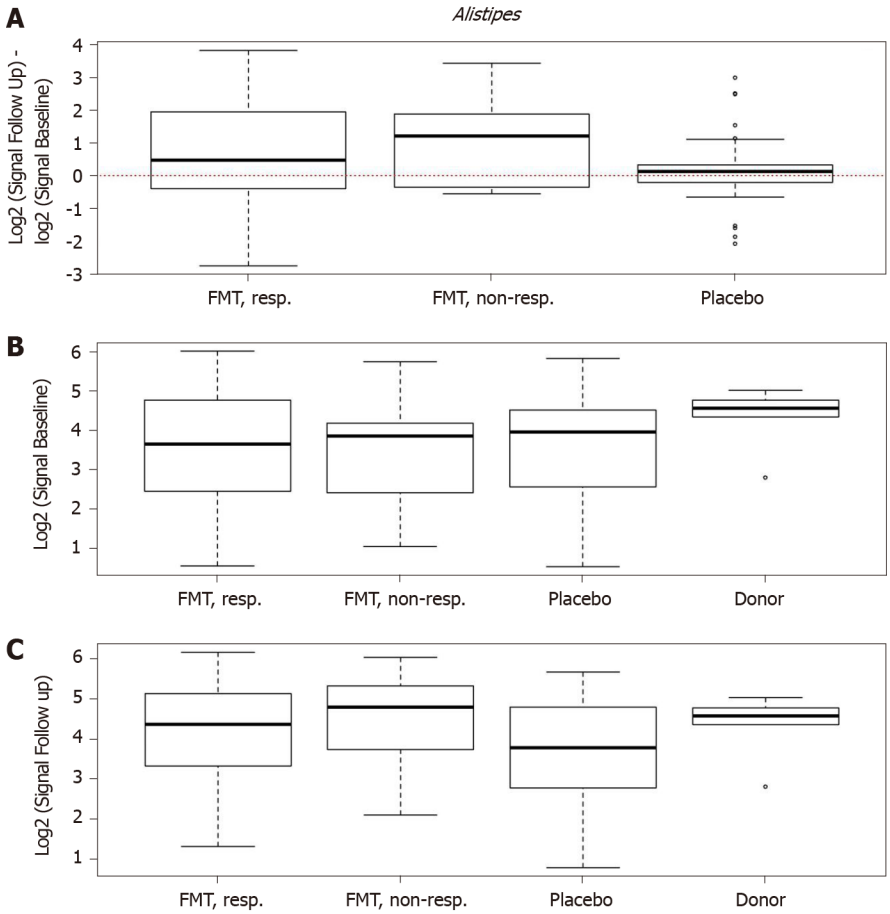


Figure 11 Changes in signals for *Alistipes* spp. in responders, nonresponders, and placebo groups as well as in the donor. A: Differences in signals for *Alistipes* spp. between before and 1 mo after transplantation in the responder, nonresponder, and placebo groups; B: There were significant changes in the signals level in responders (P = 0.004). The signal levels also changed in nonresponders, but they did not change in the placebo group. Signal levels at baseline in the responder, nonresponder, and placebo groups as well as the superdonor; C: The baseline signal levels were similar in the responder, nonresponder, and placebo groups, while that for the superdonor was higher. Signal levels in the responder, nonresponder, and placebo groups and the superdonor after transplantation. The signal levels were similar in responders, nonresponders and the superdonor, while it was lower in the placebo group. Citation: El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; 69: 859-867. Copyright @BMJ Publishing Group Ltd 2020. Published by BMJ Publishing Group Ltd[11].

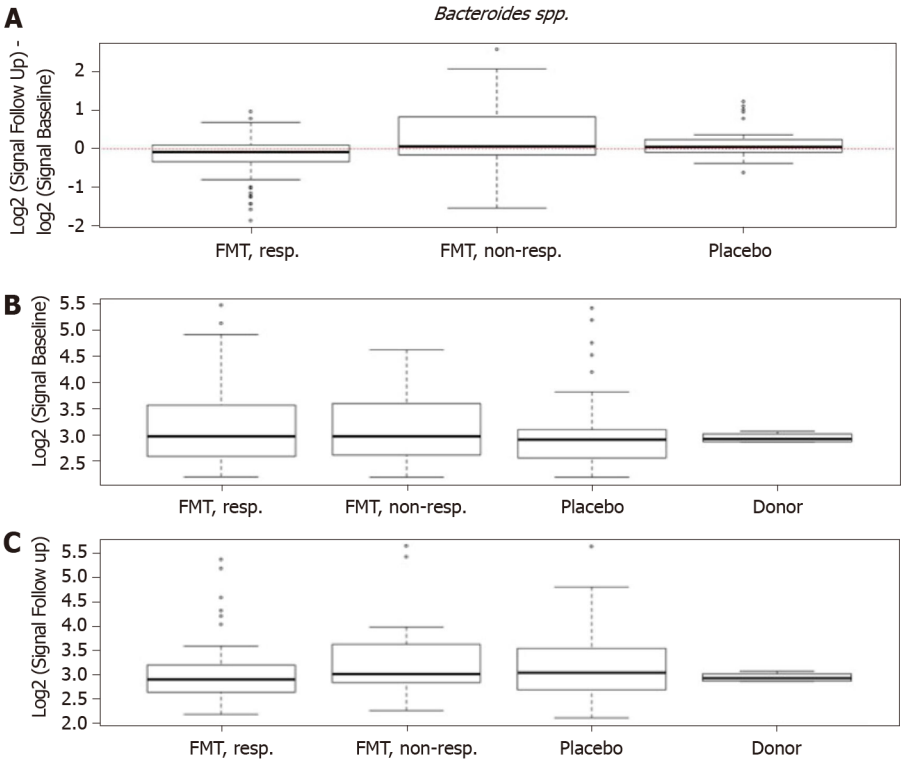


Figure 12 Changes in signals for *Bacteroides* spp. in responders, nonresponders, and placebo groups as well as in the donor. A: Differences in signals for *Bacteroides* spp. between before and 1 mo after fecal microbiota transplantation in the responder, nonresponder, and placebo groups; B: There were significant changes in the signal levels in responders ($P < 0.034$). The nonresponders showed similar changes, while there were no changes observed in the placebo group. Signal levels at baseline in the responder, nonresponder, and placebo groups and for the superdonor; C: The baseline signal levels were similar in the responder, nonresponder, and placebo groups, and both higher and lower than those for the superdonor. Signal levels in the same groups after transplantation. Citation: El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; 69: 859-867. Copyright @BMJ Publishing Group Ltd 2020. Published by BMJ Publishing Group Ltd[11].

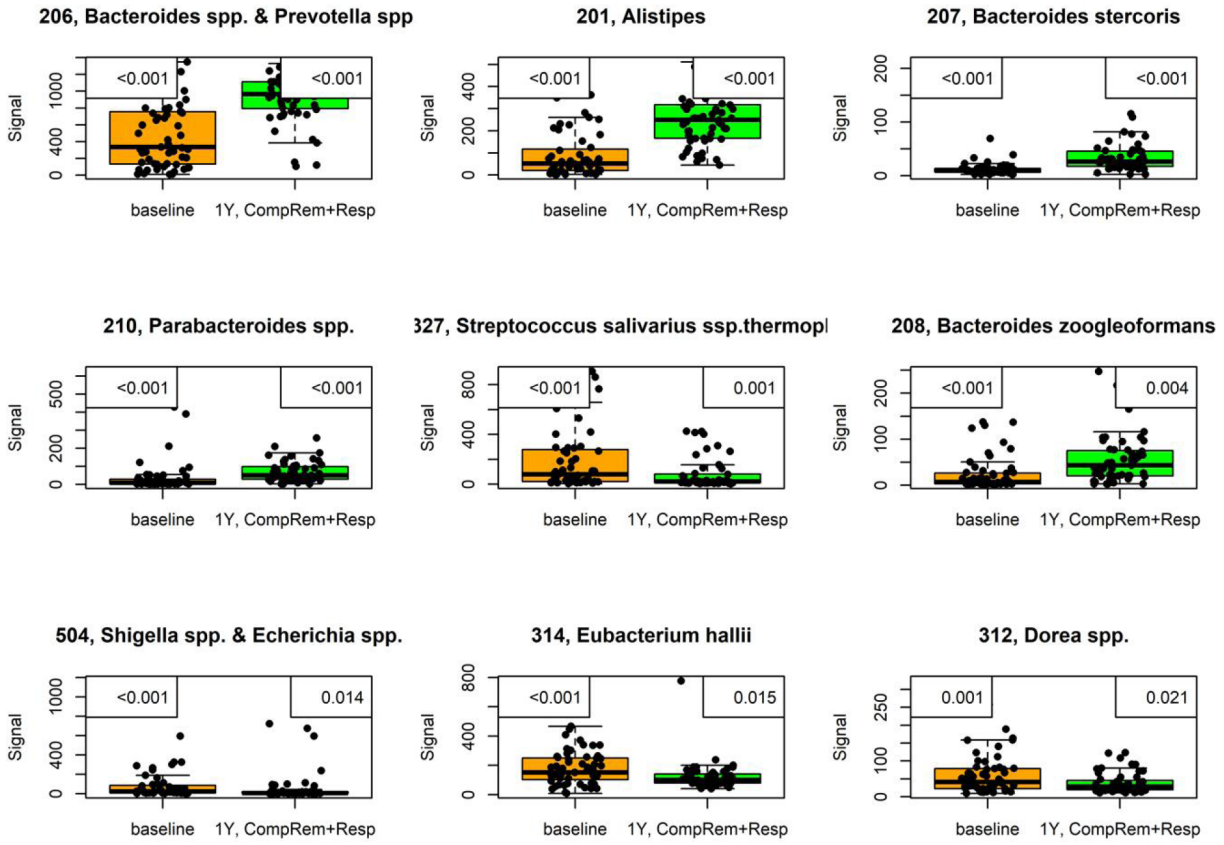


Figure 13 Signals of nine bacteria had changed at 1 year after fecal microbiota transplantation in irritable bowel syndrome patients that either maintained a response or experienced complete remission. *P* values are in the upper-left corner, with corrected *P* values in the upper-right corner. This figure is based on unpublished data from El-Salhy *et al*.

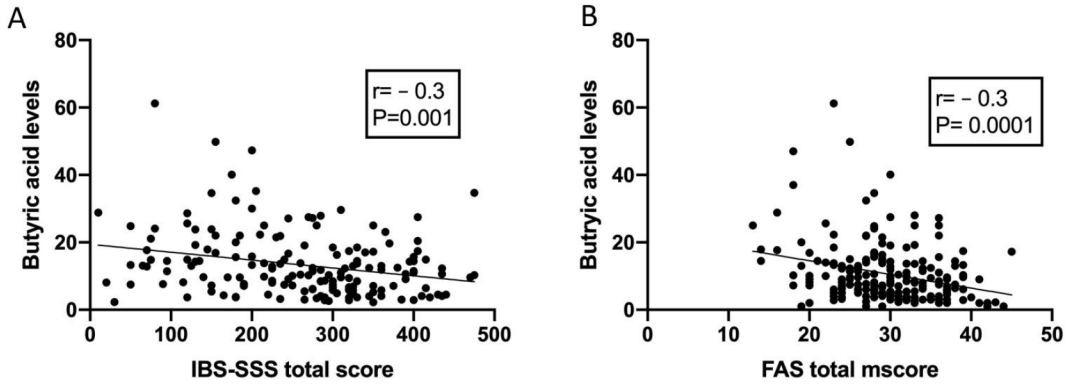


Figure 14 The correlation between butyric acid levels and IBS symptoms and fatigue 1 mo after fecal transplation. A and B: Correlations between butyric acid levels and irritable bowel syndrome-severity scoring system total scores (A) and fatigue assessment total score (B). Citation: El-Salhy M, Hausken T, Hatlebakk JG. Increasing the Dose and/or Repeating Faecal Microbiota Transplantation (FMT) Increases the Response in Patients with Irritable Bowel Syndrome (IBS). El-Salhy M, Valeur J, Hausken T, Gunnar Hatlebakk J. Changes in fecal short-chain fatty acids following fecal microbiota transplantation in patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2021; **33**: e13983 [PMID: 32945066 DOI: 10.1111/nmo.13983][55].

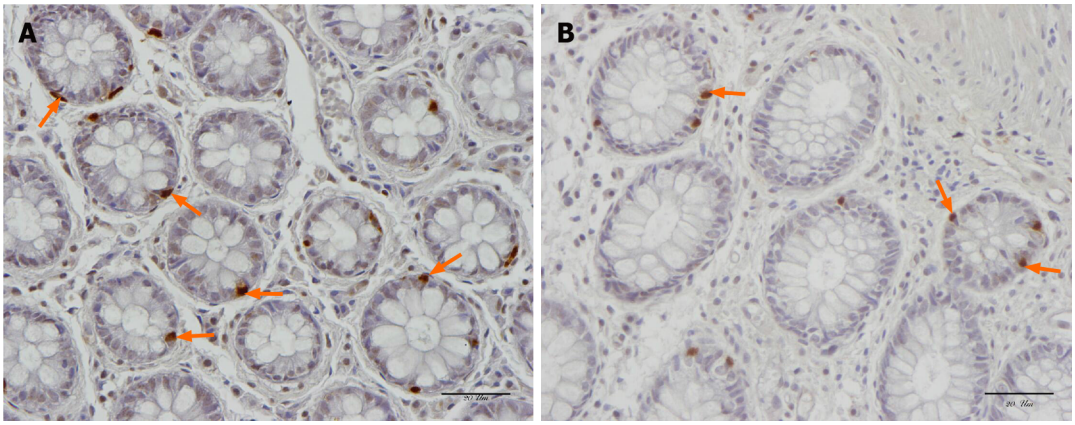


Figure 15 Serotonin-immunoreactive cells (arrows) in the colon of a healthy subject and in a patient with irritable bowel syndrome. A: Healthy subject; B: Patient with irritable bowel syndrome.

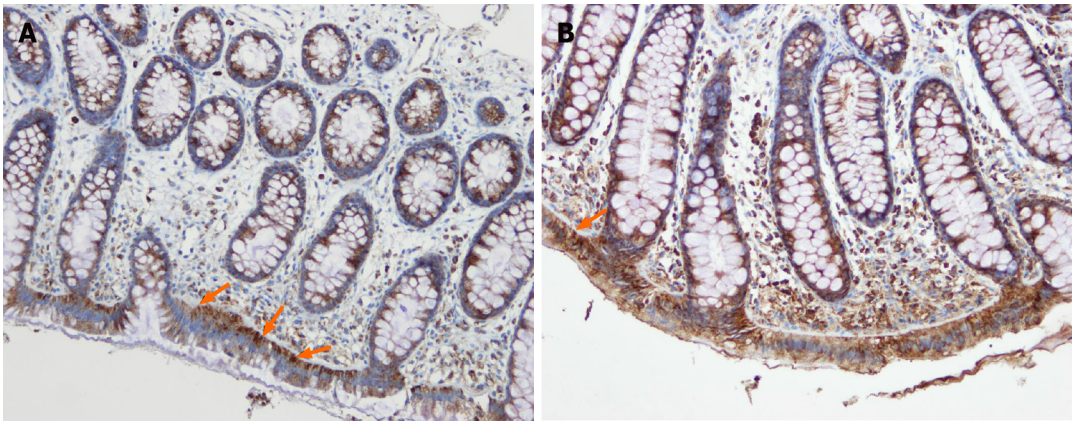


Figure 16 Difference in serotonin selective reuptake transporter immunoreactivity between a healthy subject and a patient with irritable bowel syndrome. A and B: Serotonin selective reuptake transporter immunoreactivity in the cytoplasm of the epithelial cells lining the rectal lumen (arrows) of a healthy subject (A) and of an irritable bowel syndrome patient (B).

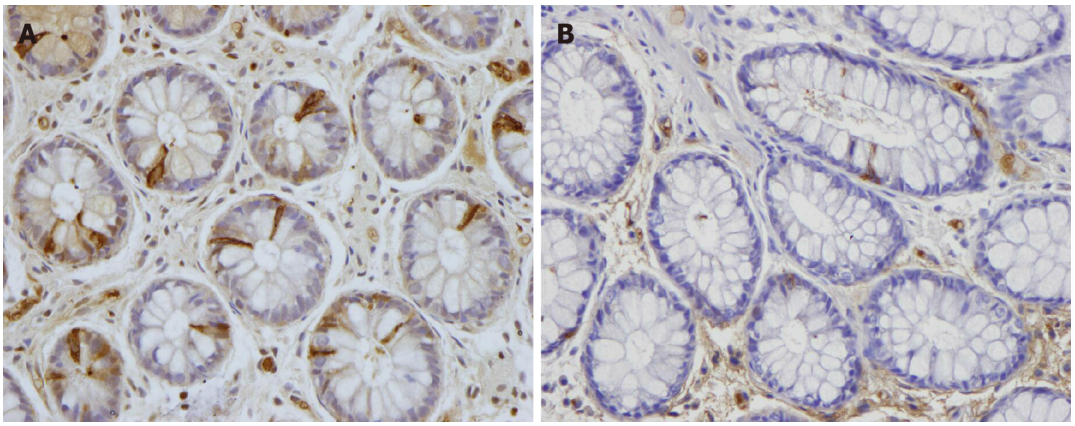


Figure 17 Peptide YY immunoreactivity (arrows) in the colon of a healthy subject and in a patient with irritable bowel syndrome. A: Healthy subject; B: Patient with irritable bowel syndrome.

REFERENCES

1 **Canavan C**, West J, Card T. The epidemiology of irritable bowel syndrome. *Clin Epidemiol* 2014; **6**: 71-80 [PMID: 24523597 DOI: 10.2147/CLEP.S40245]
2 **El-Salhy M**. Recent developments in the pathophysiology of irritable bowel syndrome. *World J Gastroenterol* 2015; **21**: 7621-7636 [PMID: 26167065 DOI: 10.3748/wjg.v21.i25.7621]

3 **El-Salhy M**. FMT in IBS: how cautious should we be? *Gut* 2021; **70**: 626-628 [PMID: 32665338 DOI: 10.1136/gutjnl-2020-322038]
4 **Konturek PC**, Brzozowski T, Konturek SJ. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. *J Physiol Pharmacol* 2011; **62**: 591-599 [PMID: 22314561]
5 **El-Salhy M**, Gilja OH, Hatlebakk JG. Overlapping of irritable bowel syndrome with erosive esophagitis and the performance of Rome criteria in diagnosing IBS in a clinical setting. *Mol Med Rep* 2019; **20**: 787-794 [PMID: 31180516 DOI: 10.3892/mmr.2019.10284]
6 **Thursby E**, Juge N. Introduction to the human gut microbiota. *Biochem J* 2017; **474**: 1823-1836 [PMID: 28512250 DOI: 10.1042/BCJ20160510]
7 **Chong PP**, Chin VK, Looi CY, Wong WF, Madhavan P, Yong VC. The Microbiome and Irritable Bowel Syndrome - A Review on the Pathophysiology, Current Research and Future Therapy. *Front Microbiol* 2019; **10**: 1136 [PMID: 31244784 DOI: 10.3389/fmicb.2019.01136]
8 **Hugon P**, Dufour JC, Colson P, Fournier PE, Sallah K, Raoult D. A comprehensive repertoire of prokaryotic species identified in human beings. *Lancet Infect Dis* 2015; **15**: 1211-1219 [PMID: 26311042 DOI: 10.1016/S1473-3099(15)00293-5]
9 **Ley RE**, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI. Evolution of mammals and their gut microbes. *Science* 2008; **320**: 1647-1651 [PMID: 18497261 DOI: 10.1126/science.1155725]
10 **Wilson BC**, Vatanen T, Cutfield WS, O'Sullivan JM. The Super-Donor Phenomenon in Fecal Microbiota Transplantation. *Front Cell Infect Microbiol* 2019; **9**: 2 [PMID: 30719428 DOI: 10.3389/fcimb.2019.00002]
11 **El-Salhy M**, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 2020; **69**: 859-867 [PMID: 31852769 DOI: 10.1136/gutjnl-2019-319630]
12 **Kriss M**, Hazleton KZ, Nusbacher NM, Martin CG, Lozupone CA. Low diversity gut microbiota dysbiosis: drivers, functional implications and recovery. *Curr Opin Microbiol* 2018; **44**: 34-40 [PMID: 30036705 DOI: 10.1016/j.mib.2018.07.003]
13 **El-Salhy M**, Mazzawi T. Fecal microbiota transplantation for managing irritable bowel syndrome. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 439-445 [PMID: 29493330 DOI: 10.1080/17474124.2018.1447380]
14 **Casén C**, Vebø HC, Sekelja M, Hegge FT, Karlsson MK, Ciemniejewska E, Dzankovic S, Frøyland C, Nestestog R, Engstrand L, Munkholm P, Nielsen OH, Rogler G, Simrén M, Öhman L, Vatn MH, Rudi K. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment Pharmacol Ther* 2015; **42**: 71-83 [PMID: 25973666 DOI: 10.1111/apt.13236]
15 **Enck P**, Mazurak N. Dysbiosis in Functional Bowel Disorders. *Ann Nutr Metab* 2018; **72**: 296-306 [PMID: 29694952 DOI: 10.1159/000488773]
16 **Pozuelo M**, Panda S, Santiago A, Mendez S, Accarino A, Santos J, Guarner F, Azpiroz F, Manichanh C. Reduction of butyrate- and methane-producing microorganisms in patients with Irritable Bowel Syndrome. *Sci Rep* 2015; **5**: 12693 [PMID: 26239401 DOI: 10.1038/srep12693]
17 **Liu HN**, Wu H, Chen YZ, Chen YJ, Shen XZ, Liu TT. Altered molecular signature of intestinal microbiota in irritable bowel syndrome patients compared with healthy controls: A systematic review and meta-analysis. *Dig Liver Dis* 2017; **49**: 331-337 [PMID: 28179092 DOI: 10.1016/j.dld.2017.01.142]
18 **Zhang F**, Luo W, Shi Y, Fan Z, Ji G. Should we standardize the 1,700-year-old fecal microbiota transplantation? *Am J Gastroenterol* 2012; **107**: 1755; author reply p.1755-1755; author reply p.1756 [PMID: 23160295 DOI: 10.1038/ajg.2012.251]
19 **Liubakka A**, Vaughn BP. Clostridium difficile Infection and Fecal Microbiota Transplant. *AACN Adv Crit Care* 2016; **27**: 324-337 [PMID: 27959316 DOI: 10.4037/aacnacc2016703]
20 **Green JE**, Davis JA, Berk M, Hair C, Loughman A, Castle D, Athan E, Nierenberg AA, Cryan JF, Jacka F, Marx W. Efficacy and safety of fecal microbiota transplantation for the treatment of diseases other than *Clostridium difficile* infection: a systematic review and meta-analysis. *Gut Microbes* 2020; **12**: 1-25 [PMID: 33345703 DOI: 10.1080/19490976.2020.1854640]
21 **Keller JJ**, Ooijevaar RE, Hvas CL, Terveer EM, Lieberknecht SC, Högenauer C, Arkkila P, Sokol H, Gridnyev O, Mégraud F, Kump PK, Nakov R, Goldenberg SD, Satokari R, Tkatch S, Sanguinetti M, Cammarota G, Dorofeev A, Gubska O, Laniro G, Mattila E, Arasaradnam RP, Sarin SK, Sood A, Putignani L, Alric L, Baunwall SMD, Kupcinskas J, Link A, Goorhuis AG, Verspaget HW, Ponsioen C, Hold GL, Tilg H, Kassam Z, Kuijper EJ, Gasbarrini A, Mulder CJJ, Williams HRT, Vehreschild MJGT. A standardised model for stool banking for faecal microbiota transplantation: a consensus report from a multidisciplinary UEG working group. *United European Gastroenterol J* 2021; **9**: 229-247 [PMID: 33151137 DOI: 10.1177/2050640620967898]
22 **Johnsen PH**, Hilpüsch F, Cavanagh JP, Leikanger IS, Kolstad C, Valle PC, Goll R. Faecal microbiota transplantation versus placebo for moderate-to-severe irritable bowel syndrome: a double-blind, randomised, placebo-controlled, parallel-group, single-centre trial. *Lancet Gastroenterol Hepatol* 2018; **3**: 17-24 [PMID: 29100842 DOI: 10.1016/S2468-1253(17)30338-2]
23 **Halkjær SI**, Christensen AH, Lo BZS, Browne PD, Günther S, Hansen LH, Petersen AM. Faecal microbiota transplantation alters gut microbiota in patients with irritable bowel syndrome: results

from a randomised, double-blind placebo-controlled study. *Gut* 2018; **67**: 2107-2115 [PMID: 29980607 DOI: 10.1136/gutjnl-2018-316434]

24 **Aroniadis OC**, Brandt LJ, Oneto C, Feuerstadt P, Sherman A, Wolkoff AW, Kassam Z, Sadovsky RG, Elliott RJ, Budree S, Kim M, Keller MJ. Faecal microbiota transplantation for diarrhoea-predominant irritable bowel syndrome: a double-blind, randomised, placebo-controlled trial. *Lancet Gastroenterol Hepatol* 2019; **4**: 675-685 [PMID: 31326345 DOI: 10.1016/S2468-1253(19)30198-0]

25 **Holster S**, Lindqvist CM, Repsilber D, Salonen A, de Vos WM, König J, Brummer RJ. The Effect of Allogenic Versus Autologous Fecal Microbiota Transfer on Symptoms, Visceral Perception and Fecal and Mucosal Microbiota in Irritable Bowel Syndrome: A Randomized Controlled Study. *Clin Transl Gastroenterol* 2019; **10**: e00034 [PMID: 31009405 DOI: 10.14309/ctg.0000000000000034]

26 **Lahtinen P**, Jalanka J, Hartikainen A, Mattila E, Hillilä M, Punkkinen J, Koskenpato J, Anttila VJ, Tillonen J, Satokari R, Arkkila P. Randomised clinical trial: faecal microbiota transplantation versus autologous placebo administered *via* colonoscopy in irritable bowel syndrome. *Aliment Pharmacol Ther* 2020; **51**: 1321-1331 [PMID: 32343000 DOI: 10.1111/apt.15740]

27 **Holvoet T**, Joossens M, Vázquez-Castellanos JF, Christiaens E, Heyerick L, Boelens J, Verhasselt B, van Vlierberghe H, De Vos M, Raes J, De Looze D. Fecal Microbiota Transplantation Reduces Symptoms in Some Patients With Irritable Bowel Syndrome With Predominant Abdominal Bloating: Short- and Long-term Results From a Placebo-Controlled Randomized Trial. *Gastroenterology* 2021; **160**: 145-157. e8 [PMID: 32681922 DOI: 10.1053/j.gastro.2020.07.013]

28 **Segal JP**, Mullish BH, Quraishi MN, Iqbal TH. Letter: faecal microbiota transplantation for IBS. *Aliment Pharmacol Ther* 2020; **52**: 556-557 [PMID: 32656836 DOI: 10.1111/apt.15824]

29 **Ianiro G**, Porcari S, Ford AC, Gasbarrini A, Cammarota G. Letter: faecal microbiota transplantation for irritable bowel syndrome-room for improvement. *Aliment Pharmacol Ther* 2020; **52**: 923-924 [PMID: 32852834 DOI: 10.1111/apt.15923]

30 **Moayyedi P**, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, Armstrong D, Marshall JK, Kassam Z, Reinisch W, Lee CH. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. *Gastroenterology* 2015; **149**: 102-109. e6 [PMID: 25857665 DOI: 10.1053/j.gastro.2015.04.001]

31 **Kazerouni A**, Wein LM. Exploring the Efficacy of Pooled Stools in Fecal Microbiota Transplantation for Microbiota-Associated Chronic Diseases. *PLoS One* 2017; **12**: e0163956 [PMID: 28068341 DOI: 10.1371/journal.pone.0163956]

32 **Paramsothy S**, Kamm MA, Kaakoush NO, Walsh AJ, van den Bogaerde J, Samuel D, Leong RWL, Connor S, Ng W, Paramsothy R, Xuan W, Lin E, Mitchell HM, Borody TJ. Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet* 2017; **389**: 1218-1228 [PMID: 28214091 DOI: 10.1016/S0140-6736(17)30182-4]

33 **Capurso G**, Lahner E. The interaction between smoking, alcohol and the gut microbiome. *Best Pract Res Clin Gastroenterol* 2017; **31**: 579-588 [PMID: 29195678 DOI: 10.1016/j.bpg.2017.10.006]

34 **Biedermann L**, Brülisauer K, Zeitz J, Frei P, Scharl M, Vavricka SR, Fried M, Loessner MJ, Rogler G, Schuppler M. Smoking cessation alters intestinal microbiota: insights from quantitative investigations on human fecal samples using FISH. *Inflamm Bowel Dis* 2014; **20**: 1496-1501 [PMID: 25072500 DOI: 10.1097/MIB.0000000000000129]

35 **Korpela K**, Dikareva E, Hanski E, Kolho KL, de Vos WM, Salonen A. Cohort profile: Finnish Health and Early Life Microbiota (HELMi) longitudinal birth cohort. *BMJ Open* 2019; **9**: e028500 [PMID: 31253623 DOI: 10.1136/bmjopen-2018-028500]

36 **Yeung OY**, Ng YF, Chiou J, Wong MS. Pilot Study to Determine the Gut Microbiota of Hong Kong Infants Fed with Breast-milk And/or Infant Formula (P11-101-19). *Curr Develop Nutr* 2019; 3 (Suppl 1) [DOI: 10.1093/cdn/nzz048.P11-101-19]

37 **Jakobsson HE**, Abrahamsson TR, Jenmalm MC, Harris K, Quince C, Jernberg C, Björkstén B, Engstrand L, Andersson AF. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. *Gut* 2014; **63**: 559-566 [PMID: 23926244 DOI: 10.1136/gutjnl-2012-303249]

38 **Rutayisire E**, Huang K, Liu Y, Tao F. The mode of delivery affects the diversity and colonization pattern of the gut microbiota during the first year of infants' life: a systematic review. *BMC Gastroenterol* 2016; **16**: 86 [PMID: 27475754 DOI: 10.1186/s12876-016-0498-0]

39 **Ianiro G**, Tilg H, Gasbarrini A. Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut* 2016; **65**: 1906-1915 [PMID: 27531828 DOI: 10.1136/gutjnl-2016-312297]

40 **Modi SR**, Collins JJ, Relman DA. Antibiotics and the gut microbiota. *J Clin Invest* 2014; **124**: 4212-4218 [PMID: 25271726 DOI: 10.1172/JCI72333]

41 **Maier L**, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, Brochado AR, Fernandez KC, Dose H, Mori H, Patil KR, Bork P, Typas A. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* 2018; **555**: 623-628 [PMID: 29555994 DOI: 10.1038/nature25979]

42 **Bibbò S**, Settanni CR, Porcari S, Bocchino E, Ianiro G, Cammarota G, Gasbarrini A. Fecal Microbiota Transplantation: Screening and Selection to Choose the Optimal Donor. *J Clin Med* 2020; **9** [PMID: 32517023 DOI: 10.3390/jcm9061757]

43 **Murtaza N**, Burke LM, Vlahovich N, Charlesson B, O' Neill H, Ross ML, Campbell KL, Krause L, Morrison M. The Effects of Dietary Pattern during Intensified Training on Stool Microbiota of Elite Race Walkers. *Nutrients* 2019; **11** [PMID: 30682843 DOI: 10.3390/nu11020261]

44 **Dalton A**, Mermier C, Zuhl M. Exercise influence on the microbiome-gut-brain axis. *Gut Microbes* 2019; **10**: 555-568 [PMID: 30704343 DOI: 10.1080/19490976.2018.1562268]

45 **Motiani KK**, Collado MC, Eskelinen JJ, Virtanen KA, LÖyttyniemi E, Salminen S, Nuutila P, Kalliokoski KK, Hannukainen JC. Exercise Training Modulates Gut Microbiota Profile and Improves Endotoxemia. *Med Sci Sports Exerc* 2020; **52**: 94-104 [PMID: 31425383 DOI: 10.1249/MSS.0000000000002112]

46 **Pinn DM**, Aroniadis OC, Brandt LJ. Is fecal microbiota transplantation (FMT) an effective treatment for patients with functional gastrointestinal disorders (FGID)? *Neurogastroenterol Motil* 2015; **27**: 19-29 [PMID: 25424663 DOI: 10.1111/nmo.12479]

47 **Pinn DM**, Aroniadis OC, Brandt LJ. Is fecal microbiota transplantation the answer for irritable bowel syndrome? *Am J Gastroenterol* 2014; **109**: 1831-1832 [PMID: 25373585 DOI: 10.1038/ajg.2014.295]

48 **Ostgaard H**, Hausken T, Gundersen D, El-Salhy M. Diet and effects of diet management on quality of life and symptoms in patients with irritable bowel syndrome. *Mol Med Rep* 2012; **5**: 1382-1390 [PMID: 22446969 DOI: 10.3892/mmr.2012.843]

49 **Bull MJ**, Plummer NT. Part 2: Treatments for Chronic Gastrointestinal Disease and Gut Dysbiosis. *Integr Med (Encinitas)* 2015; **14**: 25-33 [PMID: 26770128]

50 **Holvoet T**, Joossens M, Wang J, Boelens J, Verhasselt B, Laukens D, van Vlierberghe H, Hindryckx P, De Vos M, De Looze D, Raes J. Assessment of faecal microbial transfer in irritable bowel syndrome with severe bloating. *Gut* 2017; **66**: 980-982 [PMID: 27511198 DOI: 10.1136/gutjnl-2016-312513]

51 **Chong CYL**, Bloomfield FH, O'Sullivan JM. Factors Affecting Gastrointestinal Microbiome Development in Neonates. *Nutrients* 2018; **10** [PMID: 29495552 DOI: 10.3390/nu10030274]

52 **Chen QY**, Tian HL, Yang B, Lin ZL, Zhao D, Ye C, Zhang XY, Qin HL, Li N. [Effect of intestinal preparation on the efficacy and safety of fecal microbiota transplantation treatment]. *Zhonghua Wei Chang Wai Ke Za Zhi* 2020; **23**: 48-55 [PMID: 32594726 DOI: 10.3760/cma.j.cn.441530-20200418-00225]

53 **Ianiro G**, Maida M, Burisch J, Simonelli C, Hold G, Ventimiglia M, Gasbarrini A, Cammarota G. Efficacy of different faecal microbiota transplantation protocols for *Clostridium difficile* infection: A systematic review and meta-analysis. *United European Gastroenterol J* 2018; **6**: 1232-1244 [PMID: 30288286 DOI: 10.1177/2050640618780762]

54 **El-Salhy M**, Hausken T, Hatlebakk JG. Increasing the Dose and/or Repeating Faecal Microbiota Transplantation (FMT) Increases the Response in Patients with Irritable Bowel Syndrome (IBS). *Nutrients* 2019; **11** [PMID: 31238507 DOI: 10.3390/nu11061415]

55 **El-Salhy M**, Valeur J, Hausken T, Gunnar Hatlebakk J. Changes in fecal short-chain fatty acids following fecal microbiota transplantation in patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2021; **33**: e13983 [PMID: 32945066 DOI: 10.1111/nmo.13983]

56 **El-Salhy M**, Casen C, Valeur J, Hausken T, Hatlebakk JG. Responses to faecal microbiota transplantation in female and male patients with irritable bowel syndrome. *World J Gastroenterol* 2021; **27**: 2219-2237 [DOI: 10.3748/wjg.v27.i18.2219]

57 **Keller JJ**, Vehreschild MJ, Hvas CL, Jørgensen SM, Kupciskas J, Link A, Mulder CJ, Goldenberg SD, Arasaradnam R, Sokol H, Gasbarrini A, Hoegenauer C, Terveer EM, Kuijper EJ, Arkkila P; UEG working group of the Standards and Guidelines initiative Stool banking for FMT. Stool for fecal microbiota transplantation should be classified as a transplant product and not as a drug. *United European Gastroenterol J* 2019; **7**: 1408-1410 [PMID: 31839966 DOI: 10.1177/2050640619887579]

58 **Wang S**, Xu M, Wang W, Cao X, Piao M, Khan S, Yan F, Cao H, Wang B. Systematic Review: Adverse Events of Fecal Microbiota Transplantation. *PLoS One* 2016; **11**: e0161174 [PMID: 27529553 DOI: 10.1371/journal.pone.0161174]

59 **Dailey FE**, Turse EP, Daglilar E, Tahan V. The dirty aspects of fecal microbiota transplantation: a review of its adverse effects and complications. *Curr Opin Pharmacol* 2019; **49**: 29-33 [PMID: 31103793 DOI: 10.1016/j.coph.2019.04.008]

60 **DeFilipp Z**, Bloom PP, Torres Soto M, Mansour MK, Sater MRA, Huntley MH, Turbett S, Chung RT, Chen YB, Hohmann EL. Drug-Resistant *E. coli* Bacteremia Transmitted by Fecal Microbiota Transplant. *N Engl J Med* 2019; **381**: 2043-2050 [PMID: 31665575 DOI: 10.1056/NEJMoa1910437]

61 **Blaser MJ**. Fecal Microbiota Transplantation for Dysbiosis - Predictable Risks. *N Engl J Med* 2019; **381**: 2064-2066 [PMID: 31665573 DOI: 10.1056/NEJMe1913807]

62 **Barbara G**, Ianiro G. Faecal microbial transplantation in IBS: ready for prime time? *Gut* 2020; **69**: 795-796 [PMID: 32102925 DOI: 10.1136/gutjnl-2019-320411]

63 **Camilleri M**. FMT in IBS: a call for caution. *Gut* 2021; **70**: 431 [PMID: 32414814 DOI: 10.1136/gutjnl-2020-321529]

64 **Perler BK**, Chen B, Phelps E, Allegretti JR, Fischer M, Ganapini V, Krajiceck E, Kumar V, Marcus J, Nativ L, Kelly CR. Long-Term Efficacy and Safety of Fecal Microbiota Transplantation for Treatment of Recurrent Clostridioides difficile Infection. *J Clin Gastroenterol* 2020; **54**: 701-706 [PMID: 32011405 DOI: 10.1097/MCG.0000000000001281]

65 **Sun Q**, Jia Q, Song L, Duan L. Alterations in fecal short-chain fatty acids in patients with irritable bowel syndrome: A systematic review and meta-analysis. *Medicine (Baltimore)* 2019; **98**: e14513 [PMID: 30762787 DOI: 10.1097/MD.0000000000014513]

66 **El-Salhy M**, Hatlebakk JG, Hausken T. Diet in Irritable Bowel Syndrome (IBS): Interaction with Gut Microbiota and Gut Hormones. *Nutrients* 2019; **11** [PMID: 31394793 DOI: 10.3390/nu11081824]

67 **El-Salhy M**. Irritable bowel syndrome: diagnosis and pathogenesis. *World J Gastroenterol* 2012; **18**: 5151-5163 [PMID: 23066308 DOI: 10.3748/wjg.v18.i37.5151]

68 **El-Salhy M**, Hatlebakk JG, Gilja OH, Hausken T. Irritable bowel syndrome: recent developments in diagnosis, pathophysiology, and treatment. *Expert Rev Gastroenterol Hepatol* 2014; **8**: 435-443 [PMID: 24580043 DOI: 10.1586/17474124.2014.888952]

69 **El-Salhy M**, Seim I, Chopin L, Gundersen D, Hatlebakk JG, Hausken T. Irritable bowel syndrome: the role of gut neuroendocrine peptides. *Front Biosci (Elite Ed)* 2012; **4**: 2783-2800 [PMID: 22652678 DOI: 10.2741/e583]

70 **El-Salhy M**, Gundersen D, Gilja OH, Hatlebakk JG, Hausken T. Is irritable bowel syndrome an organic disorder? *World J Gastroenterol* 2014; **20**: 384-400 [PMID: 24574708 DOI: 10.3748/wjg.v20.i2.384]

71 **Khan WI**, Ghia JE. Gut hormones: emerging role in immune activation and inflammation. *Clin Exp Immunol* 2010; **161**: 19-27 [PMID: 20408856 DOI: 10.1111/j.1365-2249.2010.04150.x]

72 **Gershon MD**. 5-Hydroxytryptamine (serotonin) in the gastrointestinal tract. *Curr Opin Endocrinol Diabetes Obes* 2013; **20**: 14-21 [PMID: 23222853 DOI: 10.1097/MED.0b013e32835bc703]

73 **Gershon MD**, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology* 2007; **132**: 397-414 [PMID: 17241888 DOI: 10.1053/j.gastro.2006.11.002]

74 **El-Salhy M**, Solomon T, Hausken T, Gilja OH, Hatlebakk JG. Gastrointestinal neuroendocrine peptides/amines in inflammatory bowel disease. *World J Gastroenterol* 2017; **23**: 5068-5085 [PMID: 28811704 DOI: 10.3748/wjg.v23.i28.5068]

75 **Mawe GM**, Coates MD, Moses PL. Review article: intestinal serotonin signalling in irritable bowel syndrome. *Aliment Pharmacol Ther* 2006; **23**: 1067-1076 [PMID: 16611266 DOI: 10.1111/j.1365-2036.2006.02858.x]

76 **Erspamer V**. Occurrence and distribution of 5-hydroxytryptamine (enteramine) in the living organism. *Z Vitam Horm Fermentforsch* 1957; **9**: 74-96 [PMID: 13531578]

77 **Chen JX**, Pan H, Rothman TP, Wade PR, Gershon MD. Guinea pig 5-HT transporter: cloning, expression, distribution, and function in intestinal sensory reception. *Am J Physiol* 1998; **275**: G433-G448 [PMID: 9724254 DOI: 10.1152/ajpgi.1998.275.3.G433]

78 **Keating C**, Beyak M, Foley S, Singh G, Marsden C, Spiller R, Grundy D. Afferent hypersensitivity in a mouse model of post-inflammatory gut dysfunction: role of altered serotonin metabolism. *J Physiol* 2008; **586**: 4517-4530 [PMID: 18653657 DOI: 10.1113/jphysiol.2008.156984]

79 **Coleman NS**, Foley S, Dunlop SP, Wheatcroft J, Blackshaw E, Perkins AC, Singh G, Marsden CA, Holmes GK, Spiller RC. Abnormalities of serotonin metabolism and their relation to symptoms in untreated celiac disease. *Clin Gastroenterol Hepatol* 2006; **4**: 874-881 [PMID: 16797248 DOI: 10.1016/j.cgh.2006.04.017]

80 **El-Salhy M**, Gundersen D, Ostgaard H, Lomholt-Beck B, Hatlebakk JG, Hausken T. Low densities of serotonin and peptide YY cells in the colon of patients with irritable bowel syndrome. *Dig Dis Sci* 2012; **57**: 873-878 [PMID: 22057239 DOI: 10.1007/s10620-011-1948-8]

81 **El-Salhy M**, Patcharatrakul T, Hatlebakk JG, Hausken T, Gilja OH, Gonlachanvit S. Enteroendocrine, Musashi 1 and neurogenin 3 cells in the large intestine of Thai and Norwegian patients with irritable bowel syndrome. *Scand J Gastroenterol* 2017; **52**: 1331-1339 [PMID: 28853300 DOI: 10.1080/00365521.2017.1371793]

82 **El-Salhy M**, Wendelbo I, Gundersen D. Serotonin and serotonin transporter in the rectum of patients with irritable bowel disease. *Mol Med Rep* 2013; **8**: 451-455 [PMID: 23778763 DOI: 10.3892/mmr.2013.1525]

83 **Yano JM**, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, Nagler CR, Ismagilov RF, Mazmanian SK, Hsiao EY. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell* 2015; **161**: 264-276 [PMID: 25860609 DOI: 10.1016/j.cell.2015.02.047]

84 **Mishima Y**, Ishihara S. Molecular Mechanisms of Microbiota-Mediated Pathology in Irritable Bowel Syndrome. *Int J Mol Sci* 2020; **21** [PMID: 33212919 DOI: 10.3390/ijms21228664]

85 **Mandić AD**, Woting A, Jaenicke T, Sander A, Sabrowski W, Rolle-Kampcyk U, von Bergen M, Blaut M. Clostridium ramosum regulates enterochromaffin cell development and serotonin release. *Sci Rep* 2019; **9**: 1177 [PMID: 30718836 DOI: 10.1038/s41598-018-38018-z]

86 **El-Salhy M**, Grimelius L, Wilander E, Ryberg B, Terenius L, Lundberg JM, Tatemoto K. Immunocytochemical identification of polypeptide YY (PYY) cells in the human gastrointestinal tract. *Histochemistry* 1983; **77**: 15-23 [PMID: 6341321 DOI: 10.1007/BF00496632]

87 **El-Salhy M**, Hatlebakk JG, Hausken T. Possible role of peptide YY (PYY) in the pathophysiology of irritable bowel syndrome (IBS). *Neuropeptides* 2020; **79**: 101973 [PMID: 31727345 DOI: 10.1016/j.npep.2019.101973]

88 **Cox HM**, Pollock EL, Tough IR, Herzog H. Multiple Y receptors mediate pancreatic polypeptide responses in mouse colon mucosa. *Peptides* 2001; **22**: 445-452 [PMID: 11287100 DOI: 10.1016/s0196-9781(01)00355-2]

89 **Cox HM**, Tough IR. Neuropeptide Y, Y1, Y2 and Y4 receptors mediate Y agonist responses in isolated human colon mucosa. *Br J Pharmacol* 2002; **135**: 1505-1512 [PMID: 11906964 DOI: 10.1038/sj.bjp.0704604]

90 **Sheikh SP**, Williams JA. Structural characterization of Y1 and Y2 receptors for neuropeptide Y and peptide YY by affinity cross-linking. *J Biol Chem* 1990; **265**: 8304-8310 [PMID: 2159475]

91 **Inui A**, Sano K, Miura M, Hirosue Y, Nakajima M, Okita M, Baba S, Kasuga M. Evidence for further heterogeneity of the receptors for neuropeptide-Y and peptide-YY in tumor cell lines derived from neural crest. *Endocrinology* 1992; **131**: 2090-2096 [PMID: 1330489 DOI: 10.1210/endo.131.5.1330489]

92 **Mao YK**, Wang YF, Ward G, Cipris S, Daniel EE, McDonald TJ. Peptide YY receptor in submucosal and myenteric plexus synaptosomes of canine small intestine. *Am J Physiol* 1996; **271**: G36-G41 [PMID: 8760104 DOI: 10.1152/ajpgi.1996.271.1.G36]

93 **Walsh DA**, Wharton J, Blake DR, Polak JM. Localization and characterization of neuropeptide Y binding sites in porcine and human colon. *Br J Pharmacol* 1993; **108**: 304-311 [PMID: 8448582 DOI: 10.1111/j.1476-5381.1993.tb12800.x]

94 **Wharton J**, Gordon L, Byrne J, Herzog H, Selbie LA, Moore K, Sullivan MH, Elder MG, Moscoso G, Taylor KM. Expression of the human neuropeptide tyrosine Y1 receptor. *Proc Natl Acad Sci USA* 1993; **90**: 687-691 [PMID: 8421707 DOI: 10.1073/pnas.90.2.687]

95 **Gregor P**, Feng Y, DeCarr LB, Cornfield LJ, McCaleb ML. Molecular characterization of a second mouse pancreatic polypeptide receptor and its inactivated human homologue. *J Biol Chem* 1996; **271**: 27776-27781 [PMID: 8910373 DOI: 10.1074/jbc.271.44.27776]

96 **Gregor P**, Millham ML, Feng Y, DeCarr LB, McCaleb ML, Cornfield LJ. Cloning and characterization of a novel receptor to pancreatic polypeptide, a member of the neuropeptide Y receptor family. *FEBS Lett* 1996; **381**: 58-62 [PMID: 8641440 DOI: 10.1016/0014-5793(96)00067-1]

97 **Yan H**, Yang J, Marasco J, Yamaguchi K, Brenner S, Collins F, Karbon W. Cloning and functional expression of cDNAs encoding human and rat pancreatic polypeptide receptors. *Proc Natl Acad Sci USA* 1996; **93**: 4661-4665 [PMID: 8643460 DOI: 10.1073/pnas.93.10.4661]

98 **Gué M**, Junien JL, Reeve JR Jr, Rivier J, Grandt D, Taché Y. Reversal by NPY, PYY and 3-36 molecular forms of NPY and PYY of intracisternal CRF-induced inhibition of gastric acid secretion in rats. *Br J Pharmacol* 1996; **118**: 237-242 [PMID: 8735621 DOI: 10.1111/j.1476-5381.1996.tb15393.x]

99 **Hyland NP**, Cox HM. The regulation of veratridine-stimulated electrogenic ion transport in mouse colon by neuropeptide Y (NPY), Y1 and Y2 receptors. *Br J Pharmacol* 2005; **146**: 712-722 [PMID: 16100526 DOI: 10.1038/sj.bjp.0706368]

100 **Hyland NP**, Sjöberg F, Tough IR, Herzog H, Cox HM. Functional consequences of neuropeptide Y Y 2 receptor knockout and Y2 antagonism in mouse and human colonic tissues. *Br J Pharmacol* 2003; **139**: 863-871 [PMID: 12813010 DOI: 10.1038/sj.bjp.0705298]

101 **Hamer HM**, Jonkers DM, Bast A, Vanhoutvin SA, Fischer MA, Kodde A, Troost FJ, Venema K, Brummer RJ. Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. *Clin Nutr* 2009; **28**: 88-93 [PMID: 19108937 DOI: 10.1016/j.clnu.2008.11.002]

102 **Soret R**, Chevalier J, De Coppet P, Poupeau G, Derkinderen P, Segain JP, Neunlist M. Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. *Gastroenterology* 2010; **138**: 1772-1782 [PMID: 20152836 DOI: 10.1053/j.gastro.2010.01.053]

103 **Zhou J**, Martin RJ, Tulley RT, Raggio AM, McCutcheon KL, Shen L, Danna SC, Tripathy S, Hegsted M, Keenan MJ. Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. *Am J Physiol Endocrinol Metab* 2008; **295**: E1160-E1166 [PMID: 18796545 DOI: 10.1152/ajpendo.90637.2008]

104 **Karaki S**, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, Furness JB, Kuwahara A. Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. *Cell Tissue Res* 2006; **324**: 353-360 [PMID: 16453106 DOI: 10.1007/s00441-005-0140-x]

105 **Hamer HM**, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* 2008; **27**: 104-119 [PMID: 17973645 DOI: 10.1111/j.1365-2036.2007.03562.x]

106 **Zhang J**, Song L, Wang Y, Liu C, Zhang L, Zhu S, Liu S, Duan L. Beneficial effect of butyrate-producing Lachnospiraceae on stress-induced visceral hypersensitivity in rats. *J Gastroenterol Hepatol* 2019; **34**: 1368-1376 [PMID: 30402954 DOI: 10.1111/jgh.14536]

107 **Long X**, Li M, Li LX, Sun YY, Zhang WX, Zhao DY, Li YQ. Butyrate promotes visceral hypersensitivity in an IBS-like model *via* enteric glial cell-derived nerve growth factor. *Neurogastroenterol Motil* 2018; **30**: e13227 [PMID: 29052293 DOI: 10.1111/nmo.13227]

108 **Banasiewicz T**, Krokowicz Ł, Stojcev Z, Kaczmarek BF, Kaczmarek E, Maik J, Marciniak R, Krokowicz P, Walkowiak J, Drews M. Microencapsulated sodium butyrate reduces the frequency of abdominal pain in patients with irritable bowel syndrome. *Colorectal Dis* 2013; **15**: 204-209 [PMID: 22738315 DOI: 10.1111/j.1463-1318.2012.03152.x]

109 **Winston J**, Shenoy M, Medley D, Naniwadekar A, Pasricha PJ. The vanilloid receptor initiates and maintains colonic hypersensitivity induced by neonatal colon irritation in rats. *Gastroenterology* 2007; **132**: 615-627 [PMID: 17258716 DOI: 10.1053/j.gastro.2006.11.014]

110 **Tana C**, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. *Neurogastroenterol Motil* 2010; **22**: 512-519, e114 [PMID: 19903265 DOI: 10.1111/j.1365-2982.2009.01427.x]

111 **Valeur J**, Røseth AG, Knudsen T, Malmstrøm GH, Fiennes JT, Midtvedt T, Berstad A. Fecal

Fermentation in Irritable Bowel Syndrome: Influence of Dietary Restriction of Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols. *Digestion* 2016; **94**: 50-56 [PMID: 27487397 DOI: 10.1159/000448280]

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REVIEW

Interplay between nuclear factor erythroid 2-related factor 2 and inflammatory mediators in COVID-19-related liver injury

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Abstract

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 is a global pandemic and poses a major threat to human health worldwide. In addition to respiratory symptoms, COVID-19 is usually accompanied by systemic inflammation and liver damage in moderate and severe cases. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor that regulates the expression of antioxidant proteins, participating in COVID-19-mediated inflammation and liver injury. Here, we show the novel reciprocal regulation between NRF2 and inflammatory mediators associated with COVID-19-related liver injury. Additionally, we describe some mechanisms and treatment strategies.

Key Words: COVID-19-related liver injury; Nuclear factor erythroid 2-related factor 2; Inflammatory mediator; Oxidative stress; Therapeutic targets

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Core Tip: Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 is a global pandemic and poses a major threat to human health worldwide. In this review, we provide the most comprehensive guide to nuclear factor erythroid 2-related factor 2 and inflammatory mediators in COVID-19-related liver injury, emphasizing their potential as targets for COVID-19 therapy.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), a disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared to be a global pandemic by the World Health Organization in 2020. COVID-19 is caused by infection with SARS-CoV-2, which has a single-stranded RNA approximately 26-32 kb in length and belongs to the same coronavirus family as Middle East respiratory syndrome coronavirus and SARS-CoV-1[1,2].

Viral toxicity, body immunity, and the induced inflammatory response all affect the occurrence and progression of COVID-19[3]. The commonly accepted method of transmission is that SARS-CoV-2 binds to the receptor angiotensin-converting enzyme 2 (ACE2) on cells through its spike (S) glycoprotein protein, which has two domains, S1 and S2, with the former binding the peptidase domain of ACE2, called the receptor-binding domain, and the latter catalyzing membrane fusion to release genetic material into the cell[4,5]. After entry, SARS-CoV-2 interferes with the host's immune defenses, evades host immune surveillance, and rapidly replicates. The replication of the virus activates monocytes, macrophages and granulocytes, accompanied by the release of many reactive oxygen species (ROS) and inflammatory cytokine storms, which eventually lead to tissue inflammatory cell infiltration, necrosis, and fibrosis[3,6].

The pathological mechanism of SARS-CoV-2 infection is very complex and unclear. It is now widely believed to be associated with the host's immune response, the inflammatory response, and oxidative stress[3]. It manifests with moderate to severe respiratory symptoms and is usually accompanied by multiple organ dysfunction syndrome (MODS)[7]. Based on the pathogenesis of COVID-19, it has been hypothesized that SARS-CoV-2 might directly bind to the receptor ACE2, which is widely distributed among body tissues, such as liver tissue, and is considered a target for SARS-CoV-2 entry, which causes inflammation, oxidative stress, and proapoptotic reactions, ultimately leading to liver injury[5]. SARS-CoV-2 can directly cause bile duct epithelial cell dysfunction and affect hepatocyte regeneration and the immune response[8]. Liver dysfunction is a common complication and mainly manifests as elevated transaminase and bilirubin levels[9-12]. According to statistics, 5.1%-69.8% of patients with COVID-19 suffer from liver dysfunction, occurring more commonly in patients with other severe symptoms[13,14], and 2.1%-4.1% of patients with COVID-19 undergo pre-existing liver disease[14]. Interestingly, liver dysfunction appears earlier in COVID-19-related MODS, most likely because of the number of Kupffer cells, natural killer (NK) cells and NK T cells. The expression of endothelial adhesion molecules is higher in the liver than in other organs[15], which demonstrates that the inflammatory response, oxidative stress, and immune response may play important roles in COVID-19-related liver injury. Moreover, SARS-CoV-2-induced cytotoxicity, ischemia, drug toxicity, and triggering of pre-existing liver diseases are also possible contributors to the pathogenesis of COVID-19-related liver injury[16,17]. However, the exact mechanism of COVID-19-related liver injury remains unclear.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a nuclear transcription factor that is activated by oxidative stress and belongs to the family of Cap'n'Collar family of basic leucine zippers[18,19]. Kelch-like ECH-associated protein-1 (KEAP1), a redox-regulated substrate adaptor protein, which interacts with the Cul3-dependent ubiquitin ligase complex[20]. The antioxidant response element (ARE), an electrophilic

response element, is a cis-regulatory element[21]. NRF2 is recognized as a regulator of oxidative stress, and KEAP1-NRF2-ARE is one of the most important pathways of antioxidant and anti-inflammatory element signaling[22]. Under homeostasis, NRF2 binds to KEAP1 and is anchored to the cytoplasm[23]. When cells are attacked by ROS or other stimuli, NRF2 dissociates from KEAP1 and is quickly translocated to the nucleus, forming a heterodimer with the transcriptional regulator and binding to an ARE[24]. The complex ultimately activates the expression of antioxidant enzymes and thus leads to the removal of excessive ROS in the body, thereby exerting antioxidant effects[25]. Then, NRF2 is degraded in the nucleus through the β-transducin repeat-containing protein-glycogen synthase kinase 3 (β-TrCP-GSK3) axis or by translocating to the cytoplasm and becoming rapidly degraded by KEAP1[26]. Since the outbreak of COVID-19, continuous studies have shown that enhancing immunity, establishing resistance to the inflammatory response, and reducing oxidative stress are keys to COVID-19 treatment, while NRF2 may be an important target for COVID-19 treatment and plays a protective role because of its dual anti-viral and anti-inflammatory properties [27]. Currently, NRF2 activators, such as 4-octyl-itaconate (4-OI) and dimethyl fumarate (DMF), can be used for the treatment of COVID-19[28]. As most liver injury is associated with increased oxidative stress and an overloaded antioxidant defense system, NRF2 may also be an ideal therapeutic target for treating chronic liver disease. Indeed, NRF2 has previously been shown to protect liver cells in viral hepatitis[29,30], drug-induced liver injury[31-33], cholestasis liver injury[34-36], fatty liver[37,38], and other liver diseases by reversing insulin resistance, inhibiting gluconeogenesis and lipid accumulation, and enhancing the anti-inflammatory and antioxidative effects[39]. We speculate that NRF2 may also play a regulatory role in COVID-19-related liver injury. In this review, we systematically describe the reciprocal regulation between NRF2 and inflammatory mediators in COVID-19-related liver injury.

NRF2 AND INFLAMMATORY MEDIATORS

Cytokines are small-molecule messengers secreted by cells, and they can exert effects on the same type of cell in the organ periphery and cells in other organs[40]. Most cytokines are in a lytic state, and some exist in membrane form. Cytokines can be secreted by most cells and possess a wide range of functions. Interleukins (ILs) are a group of cytokines that were first seen to be expressed by white blood cells. Chemically attractive cytokines are called chemokines. Cytokines that interfere with viral replication are called interferons (IFNs).

When the body is stimulated by endogenous or exogenous stimuli such as viruses, it usually triggers the immune defense system and oxidative stress response to maintain homeostasis. This process is often accompanied by the production of many cytokines [41], which interfere with mitochondrial function and lead to the production of a large amount of ROS, thereby inducing the expression of IL-1, IL-6, tumor necrosis factor alpha (TNF-α), IL-8 receptor-β (CXCR2), monocyte chemotactic protein-1, and cell adhesion molecule 1 and promoting the aggregation of macrophages and chemotactic neutrophils[42-44]. Then neutrophils produce myeloperoxidase and catalyze the production of large amounts of hypochlorous acid, which kills cells through strong oxidation reactions[45]. Cell injury induces the expression of high mobility group box 1, osteopontin, and Toll-like receptor 4, recruiting more neutrophils, amplifying the inflammatory response, and further aggravating tissue injury[46].

It is thought that COVID-19 patients suffer from cytokine storm symptoms and an uncontrolled release of proinflammatory cytokines. The levels of proinflammatory factors such as IL-1β, IL-7, IL-8, IL-9, IL-10, TNF-α, and other inflammatory factors are higher in COVID-19 patients than in healthy adults[47,48]. Serum inflammatory cytokine levels are positively correlated with indicators of liver dysfunction in patients, suggesting potential mechanisms between liver injury and the inflammatory response[49]. NRF2 negatively regulates the expression of these inflammatory cytokines. For example, in human macrophages, NRF2 can inhibit the expression of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, by blocking the recruitment of RNA polymerase II, thus reducing the inflammatory response. Moreover, an agonist of NRF2 represses the replication of SARS-CoV-2 and the inflammatory response[50].

The NRF2-activating compound resveratrol is a growth suppressor and apoptosis promoter in liver cancer cells[51]. Resveratrol can reduce the expression of IL-1β. Another NRF2-activating compound, PB125, which contains the active ingredients carnosol, withaferin A, and luteolin, significantly downregulates IL-1β, IL6, TNF-α,

intercellular adhesion molecule 1, vascular cell adhesion molecule 1 (VCAM1), E-selectin, and IFN-γ-induced gene expression[52]. The inflammatory response and oxidative stress are the main pathological features of COVID-19, and disease progression has been related to redox imbalance. The NRF2 antioxidant pathway is suppressed in COVID-19 patients and cells infected with SARS-CoV-2 show activated GSK-3, which is a conserved serine/threonine kinase that degrades NRF2[53]. The NRF2 agonists 4-OI and DMF can inhibit the replication of SARS-CoV-2[28]. All of these findings support the idea that NRF2 has dual antiviral and anti-inflammatory properties and may be a potential therapeutic target for the treatment of COVID-19.

Pre-existing liver disease may play an important role in COVID-19-related liver injury, since an investigation found that a small proportion of patients with COVID-19 also have pre-existing liver diseases[54-56]. This effect may be due to the significant decrease in the number of lymphocytes, CD4+ T cells, CD8+ T cells, B cells, and NK cells in patients with COVID-19, which prevents a proper immune response[57], ultimately leading to the recurrence of pre-existing liver disease. Glucocorticoids effectively reduce mortality in patients with severe COVID-19[58]. In addition to its anti-inflammatory function, glucocorticoids also have immunosuppressive effects, which can also participate in the reactivation of pre-existing liver diseases.

As shown in Figure 1, inflammatory molecules are involved in the interactions with NRF2 in viral infection, as listed in a dataset generated by ingenuity pathway analysis (IPA). They include IL1A, IL1B, IL4, IL5, IL6, IL10, IL13, IL19, IL36G, IL1RN, IFNGR2, IFNB1, CCL2, CYSLTR1, EXCR3, CXCL8, CXCL6, CXCL3, CXCL2, CXCL10, CCL5, CCL4, CCL20 CCL11, TGF-β, TNFRSFA, TNF, and MST1R. Current literature shows that these molecules are related to the COVID-19-mediated inflammatory response [41-48].

INTERPLAY OF NRF2/NF-κB AND INFLAMMATORY MEDIATORS IN COVID-19-RELATED LIVER INJURY

Activated nuclear factor kappa-B (NF-κB) is a key transcription factor in the inflammatory response and oxidative stress. The NF-κB signal transduction pathway is a typical proinflammatory pathway[59]. There are five members in the NF-κB family: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel[60]. Under physiological conditions, NF-κB and inhibitor kappa B (IκB) combine and are maintained in the cytoplasm in an inactive state. Upon viral infection or other stimuli, IκB is phosphorylated under the action of IκB kinase (IKK) and is then ubiquitinated by the β-TrCP-Skp1-Cullin1 pathway, resulting in the release and nuclear translocation of NF-κB, inducing the production of a large number of inflammatory mediators, and leading to oxidative stress and inflammatory cascades, thereby affecting cell survival, mutation, and proliferation[61]. According to current studies, NRF2 plays a beneficial role mainly by regulating redox metabolism through interactions with NF-κB and regulating proinflammatory genes[62-64]. Several studies have shown reciprocal regulation between NRF2 and NF-κB in inflammatory diseases[65]. Moreover, the activation of NF-κB can promote the release of different types of cytokines, such as IL-1, IL-2, IL-6, IL-12, TNF-α and granulocyte-macrophage colony stimulating factor, which can directly participate in acute and chronic inflammation of the liver (Figure 2), causing liver injury[60]. Among these cytokines, TNF-α is a cytokine mainly produced by macrophages/monocytes and is involved in the gene expression of growth factors, cytokines, transcription factors, and receptors[66]. There are two types of TNF-α receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). TNF-α can activate NF-κB through the TNFR1-NF-κB signaling axis[66], and in turn, NF-kB promotes the expression of inflammatory cytokines[60], thereby forming a negative cycle that further feeds the destructive cytokine storm and aggravates damage to the liver tissue [36,67,68]. According to previous studies, lipopolysaccharides can promote NF-κB- and NRF2-mediated responses[69]. The initial proinflammatory response is driven by NF-κB[60], and when the activity of NRF2 reaches its maximum level, NF-κB activation is inhibited. NF-κB subunits p50 and p65 can induce NRF2 transcription by binding specific sites of NRF2, and NRF2 can inhibit the expression of NF-kB in turn upon IκB dephosphorylation[62]. CBP-p300, a transcriptional auxiliary activator, can acetylate nonhistone proteins, such as NRF2 and p65, the lysine residues of which are equipped with acetyl groups, enhancing gene transcription[70-72]. NRF2 has 605 amino acid residues and six conserved domains, namely, Neh1-Neh6[18,19]. CBP can bind to Neh4 and Neh5 of NRF2, resulting in acetylation of the Neh1 domain. In addition, CBP can bind to phosphorylated p65 at Ser276. Overexpressed p65 can

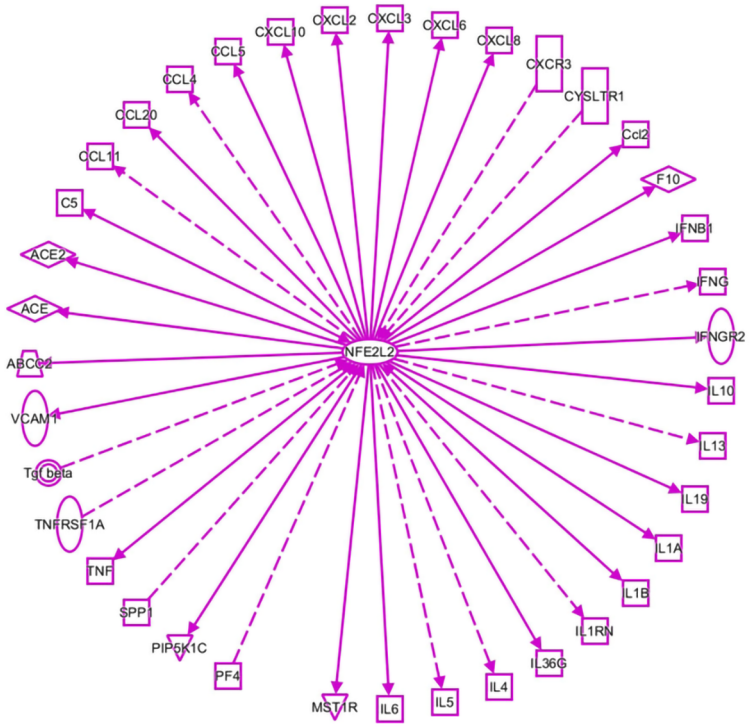


Figure 1 An interaction network analysis (ingenuity pathway analysis) was performed to predict nuclear factor, erythroid 2-like 2-mediated inflammatory cytokine-related genes in viral infections. Solid lines indicate direct regulation, and dotted lines depict indirect interactions. ABCC2: ATP-binding cassette subfamily C member 2; ACE: Angiotensin I-converting enzyme; ACE2: Angiotensin I-converting enzyme 2; C5: Complement C5; Ccl2: Chemokine (C-C motif) ligand 2; CCL: C-C motif chemokine ligand; CXCR3: C-X-C motif chemokine receptor 3; CYSLTR1: Cysteinyl leukotriene receptor 1; F10: Coagulation factor X; IFNB1: Interferon beta 1; IFNG: Interferon gamma; IFNGR2: Interferon gamma receptor 2; IL: Interleukin; IL1A: Interleukin 1 alpha; IL1B: Interleukin 1 beta; IL1RN: Interleukin 1 receptor antagonist; IL36G: Interleukin 36 gamma; MST1R: Macrophage-stimulating 1 receptor; NFE2L2: Nuclear factor, erythroid 2-like 2; PF4: Platelet factor 4; PIP5K1C: Phosphatidylinositol-4-phosphate 5-kinase type 1 gamma; SPP1: Secreted phosphoprotein 1; TGF-beta: Transforming growth factor beta; TNF: Tumor necrosis factor; TNFRSF1A: TNF receptor superfamily member 1A; VCAM1: Vascular cell adhesion molecule 1.

competitively bind to CBP, thereby inhibiting the transcriptional activity of NRF2 but enhancing the transcriptional activity of NF-κB promoter genes[70].

KEAP1 is the intermediate link between NRF2 and NF-κB. ARE is a cis-acting element, and the NRF2-ARE pathway is the most important endogenous antioxidant excitation pathway[73]. On one hand, p65 can increase the nuclear level of KEAP1 to inhibit the NRF2-ARE pathway, thus reducing the expression of cytoprotective enzymes. On the other hand, KEAP1 can initiate the autophagic degradation of inhibitor of NF-κB kinase subunit β (IKKβ) by preventing the binding of heat shock protein 90 (HSP90) to IKKβ. Additionally, KEAP1 can specifically bind to IKKβ and then connect with the ubiquitin connexin CUL3-RBX1 complex, which promotes IKKβ degradation and inhibits the expression of NF-kB[74].

COVID-19 is always accompanied by an increase in inflammatory cells and proinflammatory factors. Oxidative stress is an important mechanism for the occurrence and development of COVID-19, and is closely related to the prognosis of the disease[12]. ROS are the key agents in oxidative stress; some researchers propose monitoring ROS levels as diagnostic criteria for COVID-19[75]. As a key regulator of oxidative stress and inflammation, NF-κB plays an important role in the progression of COVID-19. In patients with COVID-19, a severe systemic inflammatory response is always followed by acute respiratory distress syndrome (ARDS) and sepsis, which leads to tissue ischemia and hypoxia and the generation of ROS, thus activating the NF-κB pathway[76]. In addition, Hirano *et al*[77] proposed that ACE2 is the key factor in the early stage of COVID-19, and the IL-6-signal transducer and activator of transcription 3 (STAT3) axis plays an important role in the inflammatory cytokine storm in the late stage of the disease. ACE2 is necessary for SARS-CoV-2 entry, and it can also inactivate angiotensin 2 (AngII), which can play a proinflammatory role *via* the angiotensin 2 receptor (AT1R). SARS-CoV, has high homology with SARS-CoV-2 and binds to ACE2 on the cell membrane, which results in an increase in AngII in serum[78]. In animal models, AT1R inhibitors can prevent ARDS caused by SARS-CoV infection[78]. The AngII-AT1R axis also activates NF-κB and ADAM metallopeptidase domain 17 (ADAM17), which can promote the production of an epidermal growth

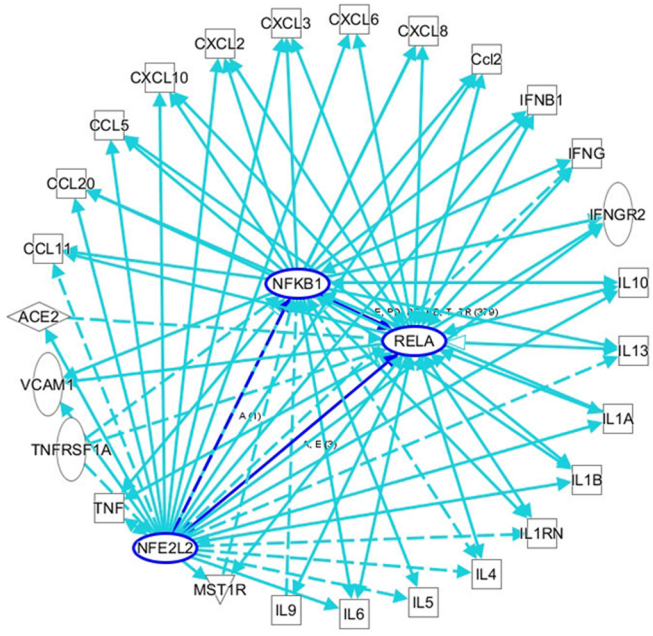


Figure 2 An ingenuity pathway analysis was performed to predict nuclear factor, erythroid 2-like 2/nuclear factor kappa B-mediated inflammatory cytokine-related genes in viral infections. Solid lines indicate direct regulation, and dotted lines depict indirect interactions. ACE: Angiotensin I-converting enzyme; Ccl2: Chemokine (C-C motif) ligand 2; CXCL: C-X-C motif ligand; IL: Interleukin; IL1B: Interleukin 1 beta; IFNG: Interferon gamma; IL1RN: Interleukin 1 receptor antagonist; NFKB1: Nuclear factor kappa B subunit 1 (p50); RELA: RELA proto-oncogene, NF-κB subunit (p65); TNF: Tumor necrosis factor; TNFRSF1A: TNF receptor superfamily member 1A; VCAM1: Vascular cell adhesion molecule 1.

factor ligand (EGFR) ligand and TNF-α, and this EGFR ligand and TNF-α can also stimulate NF-κB[79]. ADAM17 can also activate STAT3 *via* IL-6, thus activating NF-κB [80]. The NF-κB-triggered positive feedback loop for IL-6 signaling in type 1 collagen+ nonimmune cells is termed an IL-6 amplifier. Indeed, it has been proven that both the NF-κB pathway and STAT3 pathway are activated in COVID-19, promoting an increase in proinflammatory factors and inflammatory cells by activating the IL-6 amplifier, thus forming a vicious cycle and eventually leading to a systemic inflammatory response and MODS[80]. Among the various organs affected by MODS, COVID-19-related liver injury has a high incidence, ranging from 5.1% to 69.8% according to recent reports, and manifests mainly as elevated transaminase and bilirubin levels (Table 1). Liver injury greatly affects the progression and prognosis of COVID-19. However, the exact mechanisms of liver injury are unclear due to limited data. In addition to the application of drugs that cause liver injury, direct viral cytotoxicity and reactivity of existing liver disease, systemic inflammatory response, ischemia, and hypoxia may play crucial roles, as increased oxidative stress and an overloaded antioxidant defense system have always been acknowledged as key factors in the occurrence and development of various liver injuries[81]. Oxidative stress can stimulate Kupffer cells to produce cytokines that promote the apoptosis of hepatocytes and can also induce stellate cell activation/proliferation and collagen synthesis to promote fibrosis and cirrhosis[82]. To maintain redox homeostasis, the liver fights oxidative stress through a complex antioxidant system. NRF2 is associated with enhancement of the endogenous antioxidant system and has been identified as a target to prevent liver injury, inhibit liver fibrosis, and promote liver regeneration[74,83-86]. As shown through IPA analyses of the interactions among NRF2, NF-κB, and inflammatory cytokines in viral infections, the interaction and balance between NRF2 and NF-kB may play important roles in COVID-19-related liver injury, and targeting their interactions may be an important therapeutic strategy for COVID-19 treatment.

NRF-HO1 INTERPLAY IS AN IMPORTANT TARGET FOR INFLAMMATORY MEDIATORS IN COVID-19-RELATED LIVER INJURY

Glutathione (GSH) is an important molecule involved in redox homeostasis, and NRF2 promotes its synthesis by regulating glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase regulatory subunit (GCLM), glutamine synthetase

Table 1 Characteristics of liver injury during coronavirus disease

Ref.	Total number of patients	Proportion of liver injury, (%)	Proportions of pre-existing liver diseases, *n* (%)	Others
Guan *et al*[87]	1099	Elevated AST 168/757 (22.2); Elevated ALT 158/741 (21.3); Elevated TB 76/722 (10.5)	23 (2.1)	
Lian *et al* [56]	465	61 (13.1)	19 (4.1)	Liver injury was the most common complication among these patients
Chen *et al*[88]	503	301 (69.8)	NA	Liver damage is an independent prognostic factor of COVID-19
Jin *et al* [55]	651	64 (9.8)	25 (3.8)	
Xie *et al* [89]	79	Elevated ALT (31.6); Elevated AST (35.4); Elevated TB (5.1)	NA	COVID-19 with liver injury in non-ICU hospitalized patients was common
Wang *et al*[90]	293	19 (6.5)	12 (4.1)	Compared with the survival group, the non-survival group was more prone to complications of acute liver injury
Chen *et al*[91]	274	Elevated AST 84 (31)	11 (4)	The frequency of liver injury in the deceased patients was significantly higher than that in the recovered patients
Yu *et al* [54]	1633	Elevated ALT 298/1445 (20.6); Elevated AST 303/1445 (21.0)	38 (2.3)	Severe patients are more likely to have liver injury than mild patients

ALT: Alanine transaminase; AST: Aspartate aminotransferase; ICU: Intensive care unit; NA: Not applicable; TB: Total bilirubin.

(GS or GSr1), GSH reductase (GR), GSH peroxidase (GPx), and GSH S-transferases (GSTs)[92]. The tricarboxylic acid cycle (TCA cycle) is the central link and cross hub of glucose metabolism, lipid metabolism, and protein metabolism. NRF2 participates in the TCA cycle by regulating the expression of glucose 6-phosphate dehydrogenase (G6PD), malic enzyme 1, and isocitrate dehydrogenase (IDH), which are involved in the production of NADPH, a key cofactor in promoting antioxidant reactions[93-95]. NRF2 can also affect intermediate metabolism, increases the availability of substrates for the mitochondrial respiratory chain, and increases nicotinamide adenine dinucleotide phosphate (NAPDH) production through crosstalk between the glycolysis and pentose phosphate pathways[96]. In addition, under oxidative stress or starvation, NRF2 helps maintain mitochondrial homeostasis, clears oxidized or damaged proteins and organelles and removes excessive ROS, providing a favorable environment for cell survival[97]. In conclusion, NRF2 can play roles in antioxidative stress and cell protection by maintaining redox homeostasis in the body.

Many researchers have proposed the hypothesis that COVID-19-related liver damage may be caused by systemic inflammatory response to viral infection. After SARS-CoV-2 infection and the increased production of ROS[98], the inflammation response of the body is activated and the expression of proinflammatory cytokines including IL-1β, IL-6, IL-8, and TNF-α is greatly increased, and immune cells are continuously activated and are accumulating[99,100]. The combined effects of many immune cells and proinflammatory factors lead to a cytokine storm[4,99-102], which may further result in multiple organ function damage, including liver dysfunction, and even the death of the patient[103,104]. Moreover, there seems to be a positive correlation between the severity of COVID-19 and the level of inflammation [57,100,105-107], and the incidence of liver damage is related to the severity of COVID-19[13], which also reveals the close relationship between COVID-19-related liver injury and the inflammatory response. Since many cases of limb[108], gastrointestinal[109], heart [110], and cerebral[111] ischemia have been reported, it is reasonable to suspect that tissue ischemia is among the causes of COVID-19-related liver injury, in which the inflammatory response may cause local blood stasis, vascular endothelial destruction and excessive activation of the coagulation system, leading to local thrombosis and tissue ischemia, ultimately resulting in liver injury. Moreover, severe inflammatory reactions, such as sepsis[76], which cause hemodynamic instability and lead to ischemia, may also be among the causes of liver injury. Strong evidence points to the major causes for liver injury involve the systemic inflammatory response and

oxidative stress following SARS-CoV-2 infection.

Regarding the mechanisms of direct damage and inflammation-induced damage after SARS-CoV-2 infection, heme oxygenase 1 (HO-1) seems to be involved and could represent a therapeutic strategy. HO-1 can inhibit the replication of influenza A virus, human respiratory syncytial virus, and hepatitis B virus, among others. In all cases, viral replication blockade is achieved through the type I IFN response mediated by HO-1[112]. Therefore, it is reasonable to assume that HO-1 inhibits SARS-CoV-2 replication by the same mechanism. HO-1 and its metabolites have significant anti-inflammatory effects *via* NRF2. HO-1 metabolizes free heme into carbon monoxide (CO), biliverdin and iron, exhibiting cell protection and anti-apoptosis and immune regulation properties[113]. In animal models of sepsis and renal ischemia-reperfusion injury with significant systemic inflammatory responses, HO-1 showed great anti-inflammatory effects and its overexpression provided cell protection[112]. Hence, it can be speculated that HO-1 counteracts the cytokine storm produced by COVID-19. Additionally, the CO product of HO-1 can stimulate the release of hydrogen sulfide (H_2S)[114]. H_2S is a natural defense against enveloped RNA virus infections that can activate regulatory T cells, maintain the body's immune homeostasis, and exert anti-viral effects[114]. H_2S can also oversulfate the KATP channel on the white blood cell membrane to keep it open, preventing white blood cells adherence to the endothelium and triggering the inflammatory cascade[114]. On the one hand, H_2S regulates endothelial cell function and attenuates ischemia. On the other hand, H_2S controls the body's inflammatory response and prevents liver injury. Therefore, HO-1 activated by NRF2 can inhibit viral replication to control viral infection and can also balance host defenses through the HO-1/CO/H_2S axis, protecting endothelial structures, improving ischemia, and protecting the liver from damage induced by SARS-CoV-2 infection.

Figure 3 shows reciprocal regulation between NRF2/redox-related genes and inflammatory mediators. In particular, upregulation of NRF2/HO-1 (HMOX1) may be an important strategy for the treatment of the liver damage caused by COVID-19.

NRF2-MYC IS A KEY TARGET FOR INFLAMMATORY MEDIATORS IN COVID-19-RELATED LIVER INJURY

NRF2 consists of seven highly conserved domains, NRF2-ECH homology 1 (Neh1) to Neh7. Each of the Neh domains executes distinct functions[18,19]. Neh1 is a CNC-bZIP domain that allows NRF2 to heterodimerize with small musculoaponeurotic fibrosarcoma (MAF) proteins (MAFF, MAFG, and MAFK)[115]. Under normal physiological conditions, NRF2 is degraded *via* the ubiquitin-proteasome pathway in a KEAP1-dependent manner and maintained at a basal level[36]. Under stress conditions, the cysteine residues in KEAP1 are modified when they are oxidized, causing the loss of its adaptor activity[20]. Then NRF2 is disengaged from the regulatory complex, is phosphorylated, and accumulates in the nucleus, where it heterodimerizes with small MAF proteins and regulates ARE-driven genes[115]. These proteins regulate transcription when they dimerize among themselves[116,117]; that is, they seem to serve as transcriptional activators by dimerizing with other basic zipper proteins such as NFE2L2[115]. The interaction of MAFG and NFE2L2 results in activation of the ARE, which is present at the promoter-proximal region of many genes involved in the antioxidant defense and triggers the expression of certain genes[115]. MAFG expression is induced by oxidative stressors, such as hydrogen peroxide and electrophilic compounds, increasing NRF2 and GSH expression and activating the NRF2-ARE pathway[118]. NRF2 exerts protective effects on various liver diseases [119, 120]. Therefore, NRF2 can also be a therapeutic target and NRF2 activators can also be used for the treatment of COVID-19-related liver injury. MAFG, as a molecular chaperone is required for the function of NRF2, may also be a target for the treatment of COVID-19-related liver injury.

C-MYC is a family of proto-oncogenes and encodes a nuclear phosphoprotein that plays roles in cell cycle progression, apoptosis, and cell transformation[121]. MNT is a MAX network transcriptional repressor[122]. C-MYC and MNT each have a basic helix-loop-helix-zipper domain (bHLHzip) with which they bind the canonical DNA sequence CACGTG, known as an E-box, following heterodimerization with MAX proteins. The protein complexes bind to the E-box DNA sequence and regulate the transcription of specific target genes[122]. The MYC-MAX and MNT-MAX networks comprise transcription factors that regulate gene-specific transcription[122]. The heterodimer MNT-MAX is a transcriptional repressor, and the heterodimer MYC-MAX is a transcriptional activator[123-125]. We also reported that c-MYC can interact

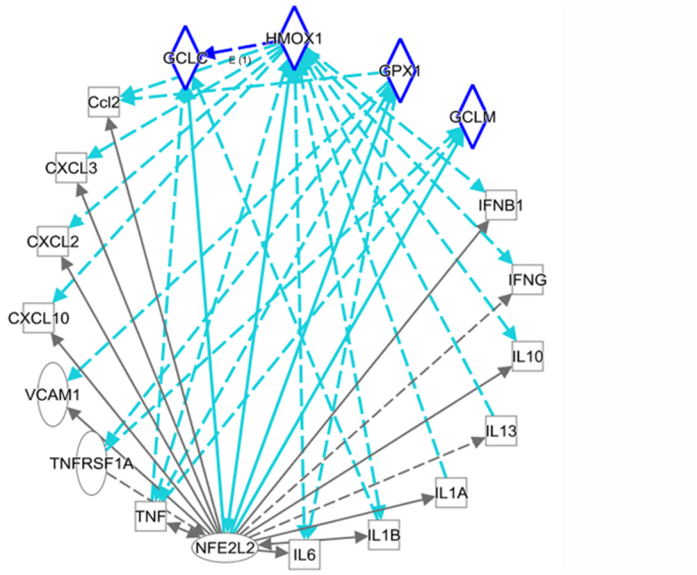


Figure 3 An ingenuity pathway analysis was performed to predict NRF2/redox-related genes and inflammatory cytokine-related gene expression in viral infections. Solid lines indicate direct regulation, and dotted lines depict indirect interactions. Ccl2: Chemokine (C-C motif) ligand 2; CXCL: C-X-C motif ligand; GCLC: Glutamate-cysteine ligase catalytic subunit; GCLM: Glutamate-cysteine ligase modifier subunit; GPX1: Glutathione peroxidase 1; HMOX1: Heme oxygenase 1 (HO-1); IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; TNFRSF1A: TNF receptor superfamily member 1A; VCAM1: Vascular cell adhesion molecule 1.

with NRF2 to repress NRF2-mediated transactivation of the ARE[126]. Their interaction may regulate the oxidative stress pathway. An IPA showed the interactions among NFE2L2/MAF/MYC/MNT and inflammatory mediators. NRF2/MYC/MNT/MAFG are involved with inflammatory mediators IL10, IL1A, IL1B, IL36G, IL4, IL5, IL6, IFNG, IFNG, CXCL8, VCAM1, TNF, TGF-β, SPP1 and MST1R (Figure 4).

Upon analyses of a protein-protein interaction network with 65 intersecting targets, c-MYC was found to be one of the core targets for COVID-19 treatment[127]. MAFG and c-MYC are upregulated and MNT is downregulated in liver injury[125,128]. MAX directly interacts with the c-MYC, MAFG, MNT, and NRF2 proteins, whereas c-MYC and MAFG interact with NRF2. As shown in Figure 5, these complexes bind to the E-box region in the promoters of the c-MYC and MAFG genes, leading to gene activation. The complexes can also bind to AREs, leading to the dysregulation of the following genes: TXN-related genes (TXN, TXNrd1, and Srxn1); ROS and xenobiotic detoxification-related genes (GSTA1/2 and GSTM1/2/3), GSTP1, and Nqo1); heme and ion metabolism-related genes (HMOX1 or HO-1, Ft1, and Fth); NADPH regeneration-related genes (G6pD, PgD, IDH1 and Me1); and GSH production-related genes (XCT, GCLC, GCLM, and GSR1). This gene dysregulation may lead to COVID-19-related cytokine storm and liver injury.

SEVERITY OF LIVER INJURY IN TERMS OF ADVERSE OUTCOMES OF SARS-COV-2 INFECTION

Alanine aminotransferase (ALT) was selected to represent liver injury rather than aspartate aminotransferase (AST) because the sources of extrahepatic AST are very common, including the heart and skeletal muscle during related muscle breakdown. Therefore, in patients infected with SAR-CoV-2, we classified liver damage into normal/mild (< 2 fold the upper limit of normal, upper limit of normal [ULN]), moderate (2- to 5-fold ULN), and severe (> 5 fold ULN) according to the degree of ALT elevation (Table 2)[129].

Normal and mild liver injury account for the largest proportion (60%-90%) of SAR-CoV-2-infected patients. Severe liver injury accounts for less than 20% of these patients. In addition, moderate to severe liver injury is more common in patients requiring intensive care unit (ICU) care. However, in general, the most common liver injury is mild liver injury, and the degree of liver injury is independently related to the occurrence of end events (ICU admission, death, mechanical injury)[129-131]. Only 5% of COVID-19 patients acquire severe liver damage[132]. The Cai *et al*[133] study

Table 2 Classification of the severity of liver injury in terms of the adverse outcome of coronavirus disease

Ref.	Date	Cohort	The severity of liver injury			How the ULN was defined
			Normal/mild (< 2 times ULN)	moderate (2-5 times ULN)	Severe (> 5 times ULN)	
Phipps *et al*[129]	March 8, 2020-April 14, 2020	2273	1784 (78.5)	344 (15.1)	145 (6.4)	ALT = 50 U/L
Mendizabal *et al*[130]	April 15, 2020-July 31, 2020	1611	322 (84.7)	185 (11.5)	61 (3.8)	ALT (the reference value of different institutions)
Hundt *et al*[131]	March 14, 2020-April, 2020	1753	1123 (64.1)	408 (23.3)	222 (12.7)	ALT = 34 U / L
Yip *et al*[132]	January 23, 2020-May 1, 2020	816	635 (77.8)	141 (17.3)	40 (4.9)	ALT = 40 U / L

Data are expressed as *n* (%). ALT: Alanine aminotransferase; ULN: Upper limit of normal.

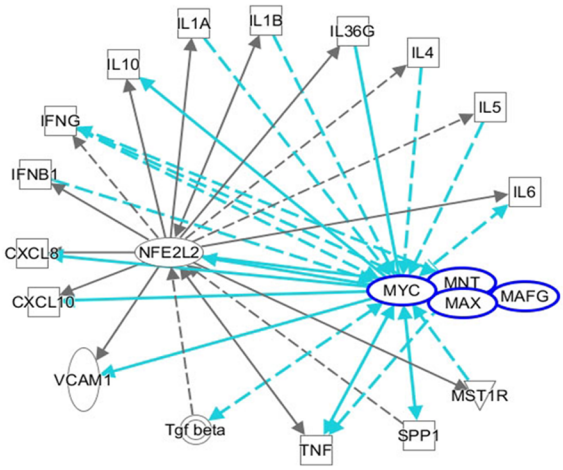


Figure 4 An ingenuity pathway analysis was performed to predict that nuclear factor, erythroid 2-like 2/MYC proto-oncogene, bHLH transcription factor/MAX network transcriptional repressor/MAF bZIP transcription factor G regulates the corresponding inflammatory cytokines. Solid lines indicate direct regulation, and dotted lines depict indirect interactions. CXCL: C-X-C motif ligand; MAFG: MAF bZIP transcription factor G; MAX: MYC-associated factor X; MNT: MAX network transcriptional repressor; MYC: MYC proto-oncogene, bHLH transcription factor; IFN: Interferon; IL: Interleukin; TGF-beta: Transforming growth factor beta; TNF: Tumor necrosis factor; TNFRSF1A: TNF receptor superfamily member 1A; VCAM1: Vascular cell adhesion molecule 1.

showed that the incidence of liver injury in hospitals was higher than that at the time of admission. For these patients, few other factors affected liver test abnormalities, such as potential liver disease or drug use. Therefore, it can be speculated that the occurrence of liver damage is caused by COVID-19. Zhao *et al*[134] compared patients with mild COVID-19 systems with non-infected patients. The absolute value of lymphocyte level differences in the two groups was low, and there was no difference in C-reactive protein or IL-6 level. ALT was not elevated in patients with pneumonia unrelated to COVID-19 when they were admitted to the hospital, but it was elevated in 28% of COVID-19 patients. Compared with the general inflammation caused by other pathogens, the specific inflammation caused by COVID-19 is more likely to cause abnormal liver function. Additionally, severe liver damage caused by SARS-CoV-2 infection is associated with serum inflammatory markers (white blood cell count and neutrophil-to-lymphocyte ratio are significantly higher). Age, hypertension, and the presence of diabetes are negatively correlated with severe liver damage in COVID-19 patients[129]. It can be speculated that young patients may have a stronger immune and inflammatory response to infection, which may lead to more severe liver damage. In other words, the inflammatory response can cause liver damage and determine the degree of liver damage. Therefore, inflammation plays an important role in the development of liver injury induced by COVID-19. Because of the individual response to the virus, different patients may exhibit different degrees of liver injury; therefore, it is necessary to grade the degree of liver injury. We described the anti-inflammatory

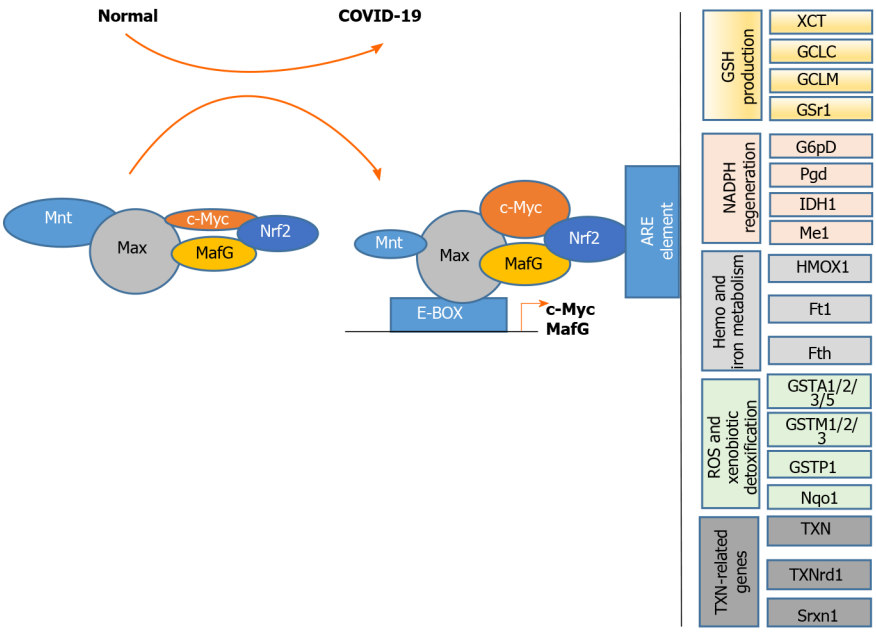


Figure 5 Interplay between c-MYC, MAX network transcriptional repressor, MAF bZIP transcription factor G, and nuclear factor, erythroid 2-like 2 proteins in viral infections. MAX network transcriptional repressor (MAX) directly interacts with c-MYC and MAX network transcriptional repressor proteins, while c-MYC and MAF bZIP transcription factor G (MAFG) interact with nuclear factor, erythroid 2-like 2 (NRF2). These complexes bind to E-box elements in the promoters of c-MYC and *MAFG* genes, regulating their activation. In addition, c-MYC and MAFG interact with NRF2, and their complexes bind to antioxidant response elements, regulating downstream gene expression. COVID-19: Coronavirus disease 2019.

effect of NRF2 above, which is of great benefit to the prevention or treatment of COVID-19-mediated liver injury, and is also crucial for improving the prognosis of patients.

CONCLUSION

There are many reasons for COVID-19-related liver injury including the inflammatory response and oxidative stress, ischemia, drug toxicity, SARS-CoV-2 cytotoxicity, and pre-existing liver disease. Regardless of the etiology, inflammatory cell infiltration, cell necrosis, and liver tissue fibrosis may be the final pathological result. As an important transcription factor of antioxidant stress and anti-inflammation, NRF2 can exert protective effects on COVID-19-related liver injury. The KEAP1-NRF2-ARE, NRF2-HO-1, NRF2-MYC/MNT/MAFG, and NRF2-NF-κB pathways are potential preventive and therapeutic targets in COVID-19-related liver injury.

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REFERENCES

1 **Wang MY**, Zhao R, Gao LJ, Gao XF, Wang DP, Cao JM. SARS-CoV-2: Structure, Biology, and Structure-Based Therapeutics Development. *Front Cell Infect Microbiol* 2020; **10**: 587269 [PMID: 33324574 DOI: 10.3389/fcimb.2020.587269]

2 **Kim D**, Lee JY, Yang JS, Kim JW, Kim VN, Chang H. The Architecture of SARS-CoV-2 Transcriptome. *Cell* 2020; **181**: 914-921. e10 [PMID: 32330414 DOI: 10.1016/j.cell.2020.04.011]

3 **Mrityunjaya M**, Pavithra V, Neelam R, Janhavi P, Halami PM, Ravindra PV. Immune-Boosting, Antioxidant and Anti-inflammatory Food Supplements Targeting Pathogenesis of COVID-19. *Front Immunol* 2020; **11**: 570122 [PMID: 33117359 DOI: 10.3389/fimmu.2020.570122]

4 **Cuadrado A**, Pajares M, Benito C, Jiménez-Villegas J, Escoll M, Fernández-Ginés R, Garcia Yagüe

AJ, Lastra D, Manda G, Rojo AI, Dinkova-Kostova AT. Can Activation of NRF2 Be a Strategy against COVID-19? *Trends Pharmacol Sci* 2020; **41**: 598-610 [PMID: 32711925 DOI: 10.1016/j.tips.2020.07.003]

5 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280. e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]

6 **Channappanavar R**, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol* 2017; **39**: 529-539 [PMID: 28466096 DOI: 10.1007/s00281-017-0629-x]

7 **Rothan HA**, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J Autoimmun* 2020; **109**: 102433 [PMID: 32113704 DOI: 10.1016/j.jaut.2020.102433]

8 **Wang Y**, Liu S, Liu H, Li W, Lin F, Jiang L, Li X, Xu P, Zhang L, Zhao L, Cao Y, Kang J, Yang J, Li L, Liu X, Li Y, Nie R, Mu J, Lu F, Zhao S, Lu J, Zhao J. SARS-CoV-2 infection of the liver directly contributes to hepatic impairment in patients with COVID-19. *J Hepatol* 2020; **73**: 807-816 [PMID: 32437830 DOI: 10.1016/j.jhep.2020.05.002]

9 **Jorgensen SCJ**, Tse CLY, Burry L, Dresser LD. Baricitinib: A Review of Pharmacology, Safety, and Emerging Clinical Experience in COVID-19. *Pharmacotherapy* 2020; **40**: 843-856 [PMID: 32542785 DOI: 10.1002/phar.2438]

10 **Cha MH**, Regueiro M, Sandhu DS. Gastrointestinal and hepatic manifestations of COVID-19: A comprehensive review. *World J Gastroenterol* 2020; **26**: 2323-2332 [PMID: 32476796 DOI: 10.3748/wjg.v26.i19.2323]

11 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]

12 **Xu Z**, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med* 2020; **8**: 420-422 [PMID: 32085846 DOI: 10.1016/S2213-2600(20)30076-X]

13 **Wang Q**, Zhao H, Liu LG, Wang YB, Zhang T, Li MH, Xu YL, Gao GJ, Xiong HF, Fan Y, Cao Y, Ding R, Wang JJ, Cheng C, Xie W. Pattern of liver injury in adult patients with COVID-19: a retrospective analysis of 105 patients. *Mil Med Res* 2020; **7**: 28 [PMID: 32507110 DOI: 10.1186/s40779-020-00256-6]

14 **Jothimani D**, Venugopal R, Abedin MF, Kaliamoorthy I, Rela M. COVID-19 and the liver. *J Hepatol* 2020; **73**: 1231-1240 [PMID: 32553666 DOI: 10.1016/j.jhep.2020.06.006]

15 **Caraballo C**, Jaimes F. Organ Dysfunction in Sepsis: An Ominous Trajectory From Infection To Death. *Yale J Biol Med* 2019; **92**: 629-640 [PMID: 31866778]

16 **Sun J**, Aghemo A, Forner A, Valenti L. COVID-19 and liver disease. *Liver Int* 2020; **40**: 1278-1281 [PMID: 32251539 DOI: 10.1111/liv.14470]

17 **Karakike E**, Giamarellos-Bourboulis EJ. Macrophage Activation-Like Syndrome: A Distinct Entity Leading to Early Death in Sepsis. *Front Immunol* 2019; **10**: 55 [PMID: 30766533 DOI: 10.3389/fimmu.2019.00055]

18 **Tonelli C**, Chio IIC, Tuveson DA. Transcriptional Regulation by Nrf2. *Antioxid Redox Signal* 2018; **29**: 1727-1745 [PMID: 28899199 DOI: 10.1089/ars.2017.7342]

19 **Hayes JD**, Dinkova-Kostova AT. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem Sci* 2014; **39**: 199-218 [PMID: 24647116 DOI: 10.1016/j.tibs.2014.02.002]

20 **Kopacz A**, Kloska D, Forman HJ, Jozkowicz A, Grochot-Przeczek A. Beyond repression of Nrf2: An update on Keap1. *Free Radic Biol Med* 2020; **157**: 63-74 [PMID: 32234331 DOI: 10.1016/j.freeradbiomed.2020.03.023]

21 **Lu MC**, Ji JA, Jiang ZY, You QD. The Keap1-Nrf2-ARE Pathway As a Potential Preventive and Therapeutic Target: An Update. *Med Res Rev* 2016; **36**: 924-963 [PMID: 27192495 DOI: 10.1002/med.21396]

22 **Sajadimajd S**, Khazaei M. Oxidative Stress and Cancer: The Role of Nrf2. *Curr Cancer Drug Targets* 2018; **18**: 538-557 [PMID: 28969555 DOI: 10.2174/1568009617666171002144228]

23 **David JA**, Rifkin WJ, Rabbani PS, Ceradini DJ. The Nrf2/Keap1/ARE Pathway and Oxidative Stress as a Therapeutic Target in Type II Diabetes Mellitus. *J Diabetes Res* 2017; **2017**: 4826724 [PMID: 28913364 DOI: 10.1155/2017/4826724]

24 **Tang W**, Jiang YF, Ponnusamy M, Diallo M. Role of Nrf2 in chronic liver disease. *World J Gastroenterol* 2014; **20**: 13079-13087 [PMID: 25278702 DOI: 10.3748/wjg.v20.i36.13079]

25 **Cui Y**, Xin H, Tao Y, Mei L, Wang Z. Arenaria kansuensis attenuates pulmonary fibrosis in mice *via* the activation of Nrf2 pathway and the inhibition of NF-kB/TGF-beta1/Smad2/3 pathway. *Phytother Res* 2021; **35**: 974-986 [PMID: 32996197 DOI: 10.1002/ptr.6857]

26 **Rada P**, Rojo AI, Chowdhry S, McMahon M, Hayes JD, Cuadrado A. SCF/{beta}-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Mol Cell Biol* 2011; **31**: 1121-1133 [PMID: 21245377 DOI: 10.1128/MCB.01204-10]

27 **Lin CY**, Yao CA. Potential Role of Nrf2 Activators with Dual Antiviral and Anti-Inflammatory Properties in the Management of Viral Pneumonia. *Infect Drug Resist* 2020; **13**: 1735-1741 [PMID: 32606823 DOI: 10.2147/IDR.S256773]

28 **Olagnier D**, Farahani E, Thyrsted J, Blay-Cadanet J, Herengt A, Idorn M, Hait A, Hernaez B, Knudsen A, Iversen MB, Schilling M, Jørgensen SE, Thomsen M, Reinert LS, Lappe M, Hoang HD, Gilchrist VH, Hansen AL, Ottosen R, Nielsen CG, Møller C, van der Horst D, Peri S, Balachandran S, Huang J, Jakobsen M, Svenningsen EB, Poulsen TB, Bartsch L, Thielke AL, Luo Y, Alain T, Rehwinkel J, Alcamí A, Hiscott J, Mogensen TH, Paludan SR, Holm CK. SARS-CoV2-mediated suppression of NRF2-signaling reveals potent antiviral and anti-inflammatory activity of 4-octyl-itaconate and dimethyl fumarate. *Nat Commun* 2020; **11**: 4938 [PMID: 33009401 DOI: 10.1038/s41467-020-18764-3]

29 **Espinoza JA**, González PA, Kalergis AM. Modulation of Antiviral Immunity by Heme Oxygenase-1. *Am J Pathol* 2017; **187**: 487-493 [PMID: 28082120 DOI: 10.1016/j.ajpath.2016.11.011]

30 **Ruan Y**, Jiang Y, Wang W, Ma J, He B, Chen M. HBV down-regulates PTEN expression *via* Nrf2/GSK3β signaling pathway. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 2020; **45**: 1009-1014 [PMID: 33051413 DOI: 10.11817/j.issn.1672-7347.2020.200189]

31 **Goldring CE**, Kitteringham NR, Elsby R, Randle LE, Clement YN, Williams DP, McMahon M, Hayes JD, Itoh K, Yamamoto M, Park BK. Activation of hepatic Nrf2 *in vivo* by acetaminophen in CD-1 mice. *Hepatology* 2004; **39**: 1267-1276 [PMID: 15122755 DOI: 10.1002/hep.20183]

32 **Jadeja RN**, Urrunaga NH, Dash S, Khurana S, Saxena NK. Withaferin-A Reduces Acetaminophen-Induced Liver Injury in Mice. *Biochem Pharmacol* 2015; **97**: 122-132 [PMID: 26212553 DOI: 10.1016/j.bcp.2015.07.024]

33 **Liu J**, Wu KC, Lu YF, Ekuase E, Klaassen CD. Nrf2 protection against liver injury produced by various hepatotoxicants. *Oxid Med Cell Longev* 2013; **2013**: 305861 [PMID: 23766851 DOI: 10.1155/2013/305861]

34 **Tan KP**, Wood GA, Yang M, Ito S. Participation of nuclear factor (erythroid 2-related), factor 2 in ameliorating lithocholic acid-induced cholestatic liver injury in mice. *Br J Pharmacol* 2010; **161**: 1111-1121 [PMID: 20977460 DOI: 10.1111/j.1476-5381.2010.00953.x]

35 **Okada K**, Shoda J, Taguchi K, Maher JM, Ishizaki K, Inoue Y, Ohtsuki M, Goto N, Sugimoto H, Utsunomiya H, Oda K, Warabi E, Ishii T, Yamamoto M. Nrf2 counteracts cholestatic liver injury *via* stimulation of hepatic defense systems. *Biochem Biophys Res Commun* 2009; **389**: 431-436 [PMID: 19732748 DOI: 10.1016/j.bbrc.2009.08.156]

36 **Aleksunes LM**, Slitt AL, Maher JM, Dieter MZ, Knight TR, Goedken M, Cherrington NJ, Chan JY, Klaassen CD, Manautou JE. Nuclear factor-E2-related factor 2 expression in liver is critical for induction of NAD(P)H:quinone oxidoreductase 1 during cholestasis. *Cell Stress Chaperones* 2006; **11**: 356-363 [PMID: 17278884 DOI: 10.1379/csc-217.1]

37 **Sugimoto H**, Okada K, Shoda J, Warabi E, Ishige K, Ueda T, Taguchi K, Yanagawa T, Nakahara A, Hyodo I, Ishii T, Yamamoto M. Deletion of nuclear factor-E2-related factor-2 Leads to rapid onset and progression of nutritional steatohepatitis in mice. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G283-G294 [PMID: 19926817 DOI: 10.1152/ajpgi.00296.2009]

38 **Lamlé J**, Marhenke S, Borlak J, von Wasielewski R, Eriksson CJ, Geffers R, Manns MP, Yamamoto M, Vogel A. Nuclear factor-eythroid 2-related factor 2 prevents alcohol-induced fulminant liver injury. *Gastroenterology* 2008; **134**: 1159-1168 [PMID: 18395094 DOI: 10.1053/j.gastro.2008.01.011]

39 **Cuadrado A**, Rojo AI, Wells G, Hayes JD, Cousin SP, Rumsey WL, Attucks OC, Franklin S, Levonen AL, Kensler TW, Dinkova-Kostova AT. Therapeutic targeting of the NRF2 and KEAP1 partnership in chronic diseases. *Nat Rev Drug Discov* 2019; **18**: 295-317 [PMID: 30610225 DOI: 10.1038/s41573-018-0008-x]

40 **Oppenheim JJ**. Cytokines: past, present, and future. *Int J Hematol* 2001; **74**: 3-8 [PMID: 11530802 DOI: 10.1007/bf02982543]

41 **Dinarello CA**. Historical insights into cytokines. *Eur J Immunol* 2007; **37** Suppl 1: S34-S45 [PMID: 17972343 DOI: 10.1002/eji.200737772]

42 **Konishi T**, Schuster RM, Goetzman HS, Caldwell CC, Lentsch AB. Cell-specific regulatory effects of CXCR2 on cholestatic liver injury. *Am J Physiol Gastrointest Liver Physiol* 2019; **317**: G773-G783 [PMID: 31604030 DOI: 10.1152/ajpgi.00080.2019]

43 **Saito JM**, Maher JJ. Bile duct ligation in rats induces biliary expression of cytokine-induced neutrophil chemoattractant. *Gastroenterology* 2000; **118**: 1157-1168 [PMID: 10833491 DOI: 10.1016/s0016-5085(00)70369-6]

44 **Jones H**, Alpini G, Francis H. Bile acid signaling and biliary functions. *Acta Pharm Sin B* 2015; **5**: 123-128 [PMID: 26579437 DOI: 10.1016/j.apsb.2015.01.009]

45 **Li M**, Cai SY, Boyer JL. Mechanisms of bile acid mediated inflammation in the liver. *Mol Aspects Med* 2017; **56**: 45-53 [PMID: 28606651 DOI: 10.1016/j.mam.2017.06.001]

46 **Wenzel P**, Kossmann S, Münzel T, Daiber A. Redox regulation of cardiovascular inflammation - Immunomodulatory function of mitochondrial and Nox-derived reactive oxygen and nitrogen species. *Free Radic Biol Med* 2017; **109**: 48-60 [PMID: 28108279 DOI: 10.1016/j.freeradbiomed.2017.01.027]

47 **Ruan Q**, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med* 2020; **46**: 846-848 [PMID: 32125452 DOI: 10.1007/s00134-020-05991-x]

48 **Wu C**, Chen X, Cai Y, Xia J, Zhou X, Xu S, Huang H, Zhang L, Du C, Zhang Y, Song J, Wang S, Chao Y, Yang Z, Xu J, Chen D, Xiong W, Xu L, Zhou F, Jiang J, Bai C, Zheng J, Song Y. Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With

Coronavirus Disease 2019 Pneumonia in Wuhan, China. *JAMA Intern Med* 2020; **180**: 934-943 [PMID: 32167524 DOI: 10.1001/jamainternmed.2020.0994]

49 **Yang RX**, Zheng RD, Fan JG. Etiology and management of liver injury in patients with COVID-19. *World J Gastroenterol* 2020; **26**: 4753-4762 [PMID: 32921955 DOI: 10.3748/wjg.v26.i32.4753]

50 **Robledinos-Antón N**, Fernández-Ginés R, Manda G, Cuadrado A. Activators and Inhibitors of NRF2: A Review of Their Potential for Clinical Development. *Oxid Med Cell Longev* 2019; **2019**: 9372182 [PMID: 31396308 DOI: 10.1155/2019/9372182]

51 **Yang H**, Zheng Y, Li TW, Peng H, Fernandez-Ramos D, Martínez-Chantar ML, Rojas AL, Mato JM, Lu SC. Methionine adenosyltransferase 2B, HuR, and sirtuin 1 protein cross-talk impacts on the effect of resveratrol on apoptosis and growth in liver cancer cells. *J Biol Chem* 2013; **288**: 23161-23170 [PMID: 23814050 DOI: 10.1074/jbc.M113.487157]

52 **McCord JM**, Hybertson BM, Cota-Gomez A, Geraci KP, Gao B. Nrf2 Activator PB125® as a Potential Therapeutic Agent against COVID-19. *Antioxidants (Basel)* 2020; **9** [PMID: 32545518 DOI: 10.3390/antiox9060518]

53 **Rana AK**, Rahmatkar SN, Kumar A, Singh D. Glycogen synthase kinase-3: A putative target to combat severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic. *Cytokine Growth Factor Rev* 2021; **58**: 92-101 [PMID: 32948440 DOI: 10.1016/j.cytogfr.2020.08.002]

54 **Yu C**, Lei Q, Li W, Wang X, Liu W. Epidemiological and clinical characteristics of 1663 hospitalized patients infected with COVID-19 in Wuhan, China: a single-center experience. *J Infect Public Health* 2020; **13**: 1202-1209 [PMID: 32718894 DOI: 10.1016/j.jiph.2020.07.002]

55 **Jin X**, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, Hao SR, Jia HY, Cai H, Zhang XL, Yu GD, Xu KJ, Wang XY, Gu JQ, Zhang SY, Ye CY, Jin CL, Lu YF, Yu X, Yu XP, Huang JR, Xu KL, Ni Q, Yu CB, Zhu B, Li YT, Liu J, Zhao H, Zhang X, Yu L, Guo YZ, Su JW, Tao JJ, Lang GJ, Wu XX, Wu WR, Qv TT, Xiang DR, Yi P, Shi D, Chen Y, Ren Y, Qiu YQ, Li LJ, Sheng J, Yang Y. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. *Gut* 2020; **69**: 1002-1009 [PMID: 32213556 DOI: 10.1136/gutjnl-2020-320926]

56 **Lian J**, Jin X, Hao S, Jia H, Cai H, Zhang X, Hu J, Zheng L, Wang X, Zhang S, Ye C, Jin C, Yu G, Gu J, Lu Y, Yu X, Xiang D, Li L, Liang T, Sheng J, Yang Y. Epidemiological, clinical, and virological characteristics of 465 hospitalized cases of coronavirus disease 2019 (COVID-19) from Zhejiang province in China. *Influenza Other Respir Viruses* 2020; **14**: 564-574 [PMID: 32397011 DOI: 10.1111/irv.12758]

57 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

58 **Angus DC**, Derde L, Al-Beidh F, Annane D, Arabi Y, Beane A, van Bentum-Puijk W, Berry L, Bhimani Z, Bonten M, Bradbury C, Brunkhorst F, Buxton M, Buzgau A, Cheng AC, de Jong M, Detry M, Estcourt L, Fitzgerald M, Goossens H, Green C, Haniffa R, Higgins AM, Horvat C, Hullegie SJ, Kruger P, Lamontagne F, Lawler PR, Linstrum K, Litton E, Lorenzi E, Marshall J, McAuley D, McGlothin A, McGuinness S, McVerry B, Montgomery S, Mouncey P, Murthy S, Nichol A, Parke R, Parker J, Rowan K, Sanil A, Santos M, Saunders C, Seymour C, Turner A, van de Veerdonk F, Venkatesh B, Zarychanski R, Berry S, Lewis RJ, McArthur C, Webb SA, Gordon AC; Writing Committee for the REMAP-CAP Investigators, Angus D, Cheng A, De Jong M, Gordon A, Lawler P, Webb S, Campbell L, Forbes A, Gattas D, Heritier S, Higgins L, Peake S, Presneill J, Seppelt I, Trapani T, Young P, Bagshaw S, Daneman N, Ferguson N, Misak C, Hullegie S, Pletz M, Rohde G, Alexander B, Basile K, Girard T, Huang D, Vates J, Beasley R, Fowler R, McGloughlin S, Morpeth S, Paterson D, Uyeki T, Baillie K, Duffy E, Hills T, Orr K, Patanwala A, Tong S, Netea M, Bihari S, Carrier M, Fergusson D, Goligher E, Haidar G, Hunt B, Kumar A, Laffan M, Lawless P, Lother S, McCallum P, Middeldopr S, McQuilten Z, Neal M, Pasi J, Schutgens R, Stanworth S, Turgeon A, Weissman A, Adhikari N, Anstey M, Brant E, de Man A, Lamonagne F, Masse MH, Udy A, Arnold D, Begin P, Charlewood R, Chasse M, Coyne M, Cooper J, Daly J, Gosbell I, Harvala-Simmonds H, MacLennan S, Menon D, McDyer J, Pridee N, Roberts D, Shankar-Hari M, Thomas H, Tinmouth A, Triulzi D, Walsh T, Wood E, Calfee C, O'Kane C, Shyamsundar M, Sinha P, Thompson T, Young I, Hodgson C, Laffey J, Orford N, Neto A, Lewis R, McGlothlin A, Miller E, Singh V, Zammit C, van Bentum Puijk W, Bouwman W, Mangindaan Y, Parker L, Peters S, Rietveld I, Raymakers K, Ganpat R, Brillinger N, Markgraf R, Ainscough K, Brickell K, Anjum A, Lane JB, Richards-Belle A, Saull M, Wiley D, Bion J, Connor J, Gates S, Manax V, van der Poll T, Reynolds J, van Beurden M, Effelaar E, Schotsman J, Boyd C, Harland C, Shearer A, Wren J, Clermont G, Garrard W, Kalchthaler K, King A, Ricketts D, Malakoutis S, Marroquin O, Music E, Quinn K, Cate H, Pearson K, Collins J, Hanson J, Williams P, Jackson S, Asghar A, Dyas S, Sutu M, Murphy S, Williamson D, Mguni N, Potter A, Porter D, Goodwin J, Rook C, Harrison S, Williams H, Campbell H, Lomme K, Williamson J, Sheffield J, van't Hoff W, McCracken P, Young M, Board J, Mart E, Knott C, Smith J, Boschert C, Affleck J, Ramanan M, D'Souza R, Pateman K, Shakih A, Cheung W, Kol M, Wong H, Shah A, Wagh A, Simpson J, Duke G, Chan P, Cartner B, Hunter S, Laver R, Shrestha T, Regli A, Pellicano A, McCullough J, Tallott M, Kumar N, Panwar R, Brinkerhoff G, Koppen C, Cazzola F, Brain M, Mineall S, Fischer R, Biradar V, Soar N, White H, Estensen K, Morrison L, Cooper M, Health M, Shehabi Y, Al-Bassam W, Hulley A, Whitehead C, Lowrey J, Gresha R, Walsham J, Meyer J, Harward M, Venz E,

Kurenda C, Smith K, Smith M, Garcia R, Barge D, Byrne D, Byrne K, Driscoll A, Fortune L, Janin P, Yarad E, Hammond N, Bass F, Ashelford A, Waterson S, Wedd S, McNamara R, Buhr H, Coles J, Schweikert S, Wibrow B, Rauniyar R, Myers E, Fysh E, Dawda A, Mevavala B, Ferrier J, Nair P, Buscher H, Reynolds C, Santamaria J, Barbazza L, Homes J, Smith R, Murray L, Brailsford J, Forbes L, Maguire T, Mariappa V, Simpson S, Maiden M, Bone A, Horton M, Salerno T, Sterba M, Geng W, Depuydt P, De Waele J, De Bus L, Fierens J, Bracke S, Reeve B, Dechert W, Chassé M, Carrier FM, Boumahni D, Benettaib F, Ghamraoui A, Bellemare D, Cloutier È, Francoeur C, D'Aragon F, Carbonneau E, Leblond J, Vazquez-Grande G, Marten N, Wilson M, Albert M, Serri K, Cavayas A, Duplaix M, Williams V, Rochwerg B, Karachi T, Oczkowski S, Centofanti J, Millen T, Duan E, Tsang J, Patterson L, English S, Watpool I, Porteous R, Miezitis S, McIntyre L, Brochard L, Burns K, Sandhu G, Khalid I, Binnie A, Powell E, McMillan A, Luk T, Aref N, Andric Z, Cviljevic S, Đimoti R, Zapalac M, Mirković G, Baršić B, Kutleša M, Kotarski V, Vujaklija Brajković A, Babel J, Sever H, Dragija L, Kušan I, Vaara S, Pettilä L, Heinonen J, Kuitunen A, Karlsson S, Vahtera A, Kiiski H, Ristimäki S, Azaiz A, Charron C, Godement M, Geri G, Vieillard-Baron A, Pourcine F, Monchi M, Luis D, Mercier R, Sagnier A, Verrier N, Caplin C, Siami S, Aparicio C, Vautier S, Jeblaoui A, Fartoukh M, Courtin L, Labbe V, Leparco C, Muller G, Nay MA, Kamel T, Benzekri D, Jacquier S, Mercier E, Chartier D, Salmon C, Dequin P, Schneider F, Morel G, L'Hotellier S, Badie J, Berdaguer FD, Malfroy S, Mezher C, Bourgoin C, Megarbane B, Voicu S, Deye N, Malissin I, Sutterlin L, Guitton C, Darreau C, Landais M, Chudeau N, Robert A, Moine P, Heming N, Maxime V, Bossard I, Nicholier TB, Colin G, Zinzoni V, Maquigneau N, Finn A, Kreß G, Hoff U, Friedrich Hinrichs C, Nee J, Hagel S, Ankert J, Kolanos S, Bloos F, Petros S, Pasieka B, Kunz K, Appelt P, Schütze B, Kluge S, Nierhaus A, Jarczak D, Roedl K, Weismann D, Frey A, Klinikum Neukölln V, Reill L, Distler M, Maselli A, Bélteczki J, Magyar I, Fazekas Á, Kovács S, Szőke V, Szigligeti G, Leszkoven J, Collins D, Breen P, Frohlich S, Whelan R, McNicholas B, Scully M, Casey S, Kernan M, Doran P, O'Dywer M, Smyth M, Hayes L, Hoiting O, Peters M, Rengers E, Evers M, Prinssen A, Bosch Ziekenhuis J, Simons K, Rozendaal W, Polderman F, de Jager P, Moviat M, Paling A, Salet A, Rademaker E, Peters AL, de Jonge E, Wigbers J, Guilder E, Butler M, Cowdrey KA, Newby L, Chen Y, Simmonds C, McConnochie R, Ritzema Carter J, Henderson S, Van Der Heyden K, Mehrtens J, Williams T, Kazemi A, Song R, Lai V, Girijadevi D, Everitt R, Russell R, Hacking D, Buehner U, Williams E, Browne T, Grimwade K, Goodson J, Keet O, Callender O, Martynoga R, Trask K, Butler A, Schischka L, Young C, Lesona E, Olatunji S, Robertson Y, José N, Amaro dos Santos Catorze T, de Lima Pereira TNA, Neves Pessoa LM, Castro Ferreira RM, Pereira Sousa Bastos JM, Aysel Florescu S, Stanciu D, Zaharia MF, Kosa AG, Codreanu D, Marabi Y, Al Qasim E, Moneer Hagazy M, Al Swaidan L, Arishi H, Muñoz-Bermúdez R, Marin-Corral J, Salazar Degracia A, Parrilla Gómez F, Mateo López MI, Rodriguez Fernandez J, Cárcel Fernández S, Carmona Flores R, León López R, de la Fuente Martos C, Allan A, Polgarova P, Farahi N, McWilliam S, Hawcutt D, Rad L, O'Malley L, Whitbread J, Kelsall O, Wild L, Thrush J, Wood H, Austin K, Donnelly A, Kelly M, O'Kane S, McClintock D, Warnock M, Johnston P, Gallagher LJ, Mc Goldrick C, Mc Master M, Strzelecka A, Jha R, Kalogirou M, Ellis C, Krishnamurthy V, Deelchand V, Silversides J, McGuigan P, Ward K, O'Neill A, Finn S, Phillips B, Mullan D, Oritz-Ruiz de Gordoa L, Thomas M, Sweet K, Grimmer L, Johnson R, Pinnell J, Robinson M, Gledhill L, Wood T, Morgan M, Cole J, Hill H, Davies M, Antcliffe D, Templeton M, Rojo R, Coghlan P, Smee J, Mackay E, Cort J, Whileman A, Spencer T, Spittle N, Kasipandian V, Patel A, Allibone S, Genetu RM, Ramali M, Ghosh A, Bamford P, London E, Cawley K, Faulkner M, Jeffrey H, Smith T, Brewer C, Gregory J, Limb J, Cowton A, O'Brien J, Nikitas N, Wells C, Lankester L, Pulletz M, Birch J, Wiseman S, Horton S, Alegria A, Turki S, Elsefi T, Crisp N, Allen L, McCullagh I, Robinson P, Hays C, Babio-Galan M, Stevenson H, Khare D, Pinder M, Selvamoni S, Gopinath A, Pugh R, Menzies D, Mackay C, Allan E, Davies G, Puxty K, McCue C, Cathcart S, Hickey N, Ireland J, Yusuff H, Isgro G, Brightling C, Bourne M, Craner M, Watters M, Prout R, Davies L, Pegler S, Kyeremeh L, Arbane G, Wilson K, Gomm L, Francia F, Brett S, Sousa Arias S, Elin Hall R, Budd J, Small C, Collins E, Henning J, Bonner S, Hugill K, Cirstea E, Wilkinson D, Karlikowski M, Sutherland H, Wilhelmsen E, Woods J, North J, Sundaran D, Hollos L, Coburn S, Walsh J, Turns M, Hopkins P, Noble H, Depante MT, Clarey E, Laha S, Verlander M, Williams A, Huckle A, Hall A, Cooke J, Gardiner-Hill C, Maloney C, Qureshi H, Flint N, Nicholson S, Southin S, Nicholson A, Borgatta B, Turner-Bone I, Reddy A, Wilding L, Chamara Warnapura L, Agno Sathianathan R, Golden D, Hart C, Jones J, Bannard-Smith J, Henry J, Birchall K, Pomeroy F, Quayle R, Makowski A, Misztal B, Ahmed I, KyereDiabour T, Naiker K, Stewart R, Mwaura E, Mew L, Wren L, Willams F, Innes R, Doble P, Hutter J, Shovelton C, Plumb B, Szakmany T, Hamlyn V, Hawkins N, Lewis S, Dell A, Gopal S, Ganguly S, Smallwood A, Harris N, Metherell S, Lazaro JM, Newman T, Fletcher S, Nortje J, Fottrell-Gould D, Randell G, Zaman M, Elmahi E, Jones A, Hall K, Mills G, Ryalls K, Bowler H, Sall J, Bourne R, Borrill Z, Duncan T, Lamb T, Shaw J, Fox C, Moreno Cuesta J, Xavier K, Purohit D, Elhassan M, Bakthavatsalam D, Rowland M, Hutton P, Bashyal A, Davidson N, Hird C, Chhablani M, Phalod G, Kirkby A, Archer S, Netherton K, Reschreiter H, Camsooksai J, Patch S, Jenkins S, Pogson D, Rose S, Daly Z, Brimfield L, Claridge H, Parekh D, Bergin C, Bates M, Dasgin J, McGhee C, Sim M, Hay SK, Phull MK, Zaidi A, Pogreban T, Rosaroso LP, Harvey D, Lowe B, Meredith M, Ryan L, Hormis A, Walker R, Collier D, Kimpton S, Oakley S, Rooney K, Rodden N, Hughes E, Thomson N, McGlynn D, Walden A, Jacques N, Coles H, Tilney E, Vowell E, Schuster-Bruce M, Pitts S, Miln R, Purandare L, Vamplew L, Spivey M, Bean S, Burt K, Moore L, Day C, Gibson C, Gordon E, Zitter L, Keenan S, Baker E,

Cherian S, Cutler S, Roynon-Reed A, Harrington K, Raithatha A, Bauchmuller K, Ahmad N, Grecu I, Trodd D, Martin J, Wrey Brown C, Arias AM, Craven T, Hope D, Singleton J, Clark S, Rae N, Welters I, Hamilton DO, Williams K, Waugh V, Shaw D, Puthucheary Z, Martin T, Santos F, Uddin R, Somerville A, Tatham KC, Jhanji S, Black E, Dela Rosa A, Howle R, Tully R, Drummond A, Dearden J, Philbin J, Munt S, Vuylsteke A, Chan C, Victor S, Matsa R, Gellamucho M, Creagh-Brown B, Tooley J, Montague L, De Beaux F, Bullman L, Kersiake I, Demetriou C, Mitchard S, Ramos L, White K, Donnison P, Johns M, Casey R, Mattocks L, Salisbury S, Dark P, Claxton A, McLachlan D, Slevin K, Lee S, Hulme J, Joseph S, Kinney F, Senya HJ, Oborska A, Kayani A, Hadebe B, Orath Prabakaran R, Nichols L, Worner R, Faulkner B, Gendall E, Hayes K, Hamilton-Davies C, Mfuko C, Abbass H, Mandadapu V, Leaver S, Forton D, Patel K, Paramasivam E, Powell M, Gould R, Wilby E, Howcroft C, Banach D, Fernández de Pinedo Artaraz Z, Cabreros L, White I, Croft M, Holland N, Pereira R, Zaki A, Johnson D, Jackson M, Garrard H, Juhaz V, Roy A, Rostron A, Woods L, Cornell S, Pillai S, Harford R, Rees T, Ivatt H, Sundara Raman A, Davey M, Lee K, Barber R, Chablani M, Brohi F, Jagannathan V, Clark M, Purvis S, Wetherill B, Dushianthan A, Cusack R, de Courcy-Golder K, Smith S, Attwood B, Parsons P, Page V, Zhao XB, Oza D, Rhodes J, Anderson T, Morris S, Xia Le Tai C, Thomas A, Keen A, Digby S, Cowley N, Southern D, Reddy H, Campbell A, Watkins C, Smuts S, Touma O, Barnes N, Alexander P, Felton T, Ferguson S, Sellers K, Bradley-Potts J, Yates D, Birkinshaw I, Kell K, Marshall N, Carr-Knott L, Summers C. Effect of Hydrocortisone on Mortality and Organ Support in Patients With Severe COVID-19: The REMAP-CAP COVID-19 Corticosteroid Domain Randomized Clinical Trial. *JAMA* 2020; **324**: 1317-1329 [PMID: 32876697 DOI: 10.1001/jama.2020.17022]

59 **Tóbon-Velasco JC**, Cuevas E, Torres-Ramos MA. Receptor for AGEs (RAGE) as mediator of NF-kB pathway activation in neuroinflammation and oxidative stress. *CNS Neurol Disord Drug Targets* 2014; **13**: 1615-1626 [PMID: 25106630 DOI: 10.2174/1871527313666140806144831]

60 **Siebenlist U**, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol* 1994; **10**: 405-455 [PMID: 7888182 DOI: 10.1146/annurev.cb.10.110194.002201]

61 **Hariharan A**, Hakeem AR, Radhakrishnan S, Reddy MS, Rela M. The Role and Therapeutic Potential of NF-kappa-B Pathway in Severe COVID-19 Patients. *Inflammopharmacology* 2021; **29**: 91-100 [PMID: 33159646 DOI: 10.1007/s10787-020-00773-9]

62 **Sivandzade F**, Prasad S, Bhalerao A, Cucullo L. NRF2 and NF-κB interplay in cerebrovascular and neurodegenerative disorders: Molecular mechanisms and possible therapeutic approaches. *Redox Biol* 2019; **21**: 101059 [PMID: 30576920 DOI: 10.1016/j.redox.2018.11.017]

63 **Bellezza I**, Giambanco I, Minelli A, Donato R. Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochim Biophys Acta Mol Cell Res* 2018; **1865**: 721-733 [PMID: 29499228 DOI: 10.1016/j.bbamcr.2018.02.010]

64 **Sies H**, Berndt C, Jones DP. Oxidative Stress. *Annu Rev Biochem* 2017; **86**: 715-748 [PMID: 28441057 DOI: 10.1146/annurev-biochem-061516-045037]

65 **Wardyn JD**, Ponsford AH, Sanderson CM. Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochem Soc Trans* 2015; **43**: 621-626 [PMID: 26551702 DOI: 10.1042/BST20150014]

66 **Zelová H**, Hošek J. TNF-α signalling and inflammation: interactions between old acquaintances. *Inflamm Res* 2013; **62**: 641-651 [PMID: 23685857 DOI: 10.1007/s00011-013-0633-0]

67 **Leist M**, Gantner F, Bohlinger I, Tiegs G, Germann PG, Wendel A. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. *Am J Pathol* 1995; **146**: 1220-1234 [PMID: 7538266]

68 **Ogasawara J**, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S. Lethal effect of the anti-Fas antibody in mice. *Nature* 1993; **364**: 806-809 [PMID: 7689176 DOI: 10.1038/364806a0]

69 **Ren J**, Su D, Li L, Cai H, Zhang M, Zhai J, Li M, Wu X, Hu K. Anti-inflammatory effects of Aureusidin in LPS-stimulated RAW264.7 macrophages *via* suppressing NF-κB and activating ROS- and MAPKs-dependent Nrf2/HO-1 signaling pathways. *Toxicol Appl Pharmacol* 2020; **387**: 114846 [PMID: 31790703 DOI: 10.1016/j.taap.2019.114846]

70 **Liu GH**, Qu J, Shen X. NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim Biophys Acta* 2008; **1783**: 713-727 [PMID: 18241676 DOI: 10.1016/j.bbamcr.2008.01.002]

71 **Sun Z**, Chin YE, Zhang DD. Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Mol Cell Biol* 2009; **29**: 2658-2672 [PMID: 19273602 DOI: 10.1128/MCB.01639-08]

72 **Wang B**, Zhu X, Kim Y, Li J, Huang S, Saleem S, Li RC, Xu Y, Dore S, Cao W. Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. *Free Radic Biol Med* 2012; **52**: 928-936 [PMID: 22226832 DOI: 10.1016/j.freeradbiomed.2011.12.006]

73 **Yu M**, Li H, Liu Q, Liu F, Tang L, Li C, Yuan Y, Zhan Y, Xu W, Li W, Chen H, Ge C, Wang J, Yang X. Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. *Cell Signal* 2011; **23**: 883-892 [PMID: 21262351 DOI: 10.1016/j.cellsig.2011.01.014]

74 **Probst BL**, McCauley L, Trevino I, Wigley WC, Ferguson DA. Cancer Cell Growth Is Differentially Affected by Constitutive Activation of NRF2 by KEAP1 Deletion and Pharmacological Activation of NRF2 by the Synthetic Triterpenoid, RTA 405. *PLoS One* 2015; **10**: e0135257 [PMID: 26301506 DOI: 10.1371/journal.pone.0135257]

75 **Miripour ZS**, Sarrami-Forooshani R, Sanati H, Makarem J, Taheri MS, Shojaeian F, Eskafi AH, Abbasvandi F, Namdar N, Ghafari H, Aghaee P, Zandi A, Faramarzpour M, Hoseinyazdi M, Tayebi M, Abdolahad M. Real-time diagnosis of reactive oxygen species (ROS) in fresh sputum by electrochemical tracing; correlation between COVID-19 and viral-induced ROS in lung/respiratory epithelium during this pandemic. *Biosens Bioelectron* 2020; **165**: 112435 [PMID: 32729548 DOI: 10.1016/j.bios.2020.112435]

76 **Cecchini R**, Cecchini AL. SARS-CoV-2 infection pathogenesis is related to oxidative stress as a response to aggression. *Med Hypotheses* 2020; **143**: 110102 [PMID: 32721799 DOI: 10.1016/j.mehy.2020.110102]

77 **Hirano T**, Murakami M. COVID-19: A New Virus, but a Familiar Receptor and Cytokine Release Syndrome. *Immunity* 2020; **52**: 731-733 [PMID: 32325025 DOI: 10.1016/j.immuni.2020.04.003]

78 **Kuba K**, Imai Y, Rao S, Gao H, Guo F, Guan B, Huan Y, Yang P, Zhang Y, Deng W, Bao L, Zhang B, Liu G, Wang Z, Chappell M, Liu Y, Zheng D, Leibbrandt A, Wada T, Slutsky AS, Liu D, Qin C, Jiang C, Penninger JM. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med* 2005; **11**: 875-879 [PMID: 16007097 DOI: 10.1038/nm1267]

79 **Eguchi S**, Kawai T, Scalia R, Rizzo V. Understanding Angiotensin II Type 1 Receptor Signaling in Vascular Pathophysiology. *Hypertension* 2018; **71**: 804-810 [PMID: 29581215 DOI: 10.1161/HYPERTENSIONAHA.118.10266]

80 **Murakami M**, Kamimura D, Hirano T. Pleiotropy and Specificity: Insights from the Interleukin 6 Family of Cytokines. *Immunity* 2019; **50**: 812-831 [PMID: 30995501 DOI: 10.1016/j.immuni.2019.03.027]

81 **Cichoż-Lach H**, Michalak A. Oxidative stress as a crucial factor in liver diseases. *World J Gastroenterol* 2014; **20**: 8082-8091 [PMID: 25009380 DOI: 10.3748/wjg.v20.i25.8082]

82 **Yu HH**, Qiu YX, Li B, Peng CY, Zeng R, Wang W. Kadsura heteroclita stem ethanol extract protects against carbon tetrachloride-induced liver injury in mice *via* suppression of oxidative stress, inflammation, and apoptosis. *J Ethnopharmacol* 2021; **267**: 113496 [PMID: 33091494 DOI: 10.1016/j.jep.2020.113496]

83 **Wakabayashi N**, Shin S, Slocum SL, Agoston ES, Wakabayashi J, Kwak MK, Misra V, Biswal S, Yamamoto M, Kensler TW. Regulation of notch1 signaling by nrf2: implications for tissue regeneration. *Sci Signal* 2010; **3**: ra52 [PMID: 20628156 DOI: 10.1126/scisignal.2000762]

84 **Duarte TL**, Caldas C, Santos AG, Silva-Gomes S, Santos-Gonçalves A, Martins MJ, Porto G, Lopes JM. Genetic disruption of NRF2 promotes the development of necroinflammation and liver fibrosis in a mouse model of HFE-hereditary hemochromatosis. *Redox Biol* 2017; **11**: 157-169 [PMID: 27936457 DOI: 10.1016/j.redox.2016.11.013]

85 **Wu KC**, Liu JJ, Klaassen CD. Nrf2 activation prevents cadmium-induced acute liver injury. *Toxicol Appl Pharmacol* 2012; **263**: 14-20 [PMID: 22677785 DOI: 10.1016/j.taap.2012.05.017]

86 **Luedde T**, Schwabe RF. NF-κB in the liver--linking injury, fibrosis and hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 108-118 [PMID: 21293511 DOI: 10.1038/nrgastro.2010.213]

87 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

88 **Chen LY**, Chu HK, Bai T, Tu SJ, Wei Y, Li ZL, Hu LL, Zhu R, Zhang L, Han CQ, Xiao L, He Q, Song J, Liu WH, Zhu QJ, Chen H, Yang L, Hou XH. Liver damage at admission is an independent prognostic factor for COVID-19. *J Dig Dis* 2020; **21**: 512-518 [PMID: 32713118 DOI: 10.1111/1751-2980.12925]

89 **Xie H**, Zhao J, Lian N, Lin S, Xie Q, Zhuo H. Clinical characteristics of non-ICU hospitalized patients with coronavirus disease 2019 and liver injury: A retrospective study. *Liver Int* 2020; **40**: 1321-1326 [PMID: 32239591 DOI: 10.1111/liv.14449]

90 **Wang Z**, Ye D, Wang M, Zhao M, Li D, Ye J, Liu J, Xu Y, Zhang J, Pan W, Liu M, Luo Z, Wan J. Clinical Features of COVID-19 Patients with Different Outcomes in Wuhan: A Retrospective Observational Study. *Biomed Res Int* 2020; **2020**: 2138387 [PMID: 33029494 DOI: 10.1155/2020/2138387]

91 **Chen N**, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020; **395**: 507-513 [PMID: 32007143 DOI: 10.1016/S0140-6736(20)30211-7]

92 **Lu SC**. Regulation of glutathione synthesis. *Mol Aspects Med* 2009; **30**: 42-59 [PMID: 18601945 DOI: 10.1016/j.mam.2008.05.005]

93 **Thimmulappa RK**, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res* 2002; **62**: 5196-5203 [PMID: 12234984]

94 **Mitsuishi Y**, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, Yamamoto M, Motohashi H. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* 2012; **22**: 66-79 [PMID: 22789539 DOI: 10.1016/j.ccr.2012.05.016]

95 **Wu KC**, Cui JY, Klaassen CD. Beneficial role of Nrf2 in regulating NADPH generation and consumption. *Toxicol Sci* 2011; **123**: 590-600 [PMID: 21775727 DOI: 10.1093/toxsci/kfr183]

96 **Holmström KM**, Kostov RV, Dinkova-Kostova AT. The multifaceted role of Nrf2 in mitochondrial function. *Curr Opin Toxicol* 2016; **1**: 80-91 [PMID: 28066829 DOI: 10.1016/j.cotox.2016.10.002]

97 **Hu Q**, Ren J, Li G, Wu J, Wu X, Wang G, Gu G, Ren H, Hong Z, Li J. The mitochondrially targeted antioxidant MitoQ protects the intestinal barrier by ameliorating mitochondrial DNA damage *via* the Nrf2/ARE signaling pathway. *Cell Death Dis* 2018; **9**: 403 [PMID: 29540694 DOI: 10.1038/s41419-018-0436-x]

98 **Codo AC**, Davanzo GG, Monteiro LB, de Souza GF, Muraro SP, Virgilio-da-Silva JV, Prodonoff JS, Carregari VC, de Biagi Junior CAO, Crunfli F, Jimenez Restrepo JL, Vendramini PH, Reis-de-Oliveira G, Bispo Dos Santos K, Toledo-Teixeira DA, Parise PL, Martini MC, Marques RE, Carmo HR, Borin A, Coimbra LD, Boldrini VO, Brunetti NS, Vieira AS, Mansour E, Ulaf RG, Bernardes AF, Nunes TA, Ribeiro LC, Palma AC, Agrela MV, Moretti ML, Sposito AC, Pereira FB, Velloso LA, Vinolo MAR, Damasio A, Proença-Módena JL, Carvalho RF, Mori MA, Martins-de-Souza D, Nakaya HI, Farias AS, Moraes-Vieira PM. Elevated Glucose Levels Favor SARS-CoV-2 Infection and Monocyte Response through a HIF-1α/Glycolysis-Dependent Axis. *Cell Metab* 2020; **32**: 437-446. e5 [PMID: 32697943 DOI: 10.1016/j.cmet.2020.07.007]

99 **Martínez-Sánchez G**, Schwartz A, Donna VD. Potential Cytoprotective Activity of Ozone Therapy in SARS-CoV-2/COVID-19. *Antioxidants (Basel)* 2020; **9** [PMID: 32384798 DOI: 10.3390/antiox9050389]

100 **Chen G**, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang M, Wu S, Song J, Chen T, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest* 2020; **130**: 2620-2629 [PMID: 32217835 DOI: 10.1172/JCI137244]

101 **Iddir M**, Brito A, Dingeo G, Fernandez Del Campo SS, Samouda H, La Frano MR, Bohn T. Strengthening the Immune System and Reducing Inflammation and Oxidative Stress through Diet and Nutrition: Considerations during the COVID-19 Crisis. *Nutrients* 2020; **12** [PMID: 32471251 DOI: 10.3390/nu12061562]

102 **Schieber M**, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol* 2014; **24**: R453-R462 [PMID: 24845678 DOI: 10.1016/j.cub.2014.03.034]

103 **Fu Y**, Cheng Y, Wu Y. Understanding SARS-CoV-2-Mediated Inflammatory Responses: From Mechanisms to Potential Therapeutic Tools. *Virol Sin* 2020; **35**: 266-271 [PMID: 32125642 DOI: 10.1007/s12250-020-00207-4]

104 **Prompetchara E**, Ketloy C, Palaga T. Immune responses in COVID-19 and potential vaccines: Lessons learned from SARS and MERS epidemic. *Asian Pac J Allergy Immunol* 2020; **38**: 1-9 [PMID: 32105090 DOI: 10.12932/AP-200220-0772]

105 **Qin C**, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, Xie C, Ma K, Shang K, Wang W, Tian DS. Dysregulation of Immune Response in Patients With Coronavirus 2019 (COVID-19) in Wuhan, China. *Clin Infect Dis* 2020; **71**: 762-768 [PMID: 32161940 DOI: 10.1093/cid/ciaa248]

106 **Zhang W**, Zhao Y, Zhang F, Wang Q, Li T, Liu Z, Wang J, Qin Y, Zhang X, Yan X, Zeng X, Zhang S. The use of anti-inflammatory drugs in the treatment of people with severe coronavirus disease 2019 (COVID-19): The Perspectives of clinical immunologists from China. *Clin Immunol* 2020; **214**: 108393 [PMID: 32222466 DOI: 10.1016/j.clim.2020.108393]

107 **Li X**, Geng M, Peng Y, Meng L, Lu S. Molecular immune pathogenesis and diagnosis of COVID-19. *J Pharm Anal* 2020; **10**: 102-108 [PMID: 32282863 DOI: 10.1016/j.jpha.2020.03.001]

108 **Bellosta R**, Luzzani L, Natalini G, Pegorer MA, Attisani L, Cossu LG, Ferrandina C, Fossati A, Conti E, Bush RL, Piffaretti G. Acute limb ischemia in patients with COVID-19 pneumonia. *J Vasc Surg* 2020; **72**: 1864-1872 [PMID: 32360679 DOI: 10.1016/j.jvs.2020.04.483]

109 **Low SW**, Swanson KL, McCain JD, Sen A, Kawashima A, Pasha SF. Gastric ischemia and portal vein thrombosis in a COVID-19-infected patient. *Endoscopy* 2020; **52**: E465-E466 [PMID: 32877937 DOI: 10.1055/a-1230-3357]

110 **Shafi AMA**, Shaikh SA, Shirke MM, Iddawela S, Harky A. Cardiac manifestations in COVID-19 patients-A systematic review. *J Card Surg* 2020; **35**: 1988-2008 [PMID: 32652713 DOI: 10.1111/jocs.14808]

111 **Garg RK**. Spectrum of Neurological Manifestations in Covid-19: A Review. *Neurol India* 2020; **68**: 560-572 [PMID: 32643664 DOI: 10.4103/0028-3886.289000]

112 **Rossi M**, Piagnerelli M, Van Meerhaeghe A, Zouaoui Boudjeltia K. Heme oxygenase-1 (HO-1) cytoprotective pathway: A potential treatment strategy against coronavirus disease 2019 (COVID-19)-induced cytokine storm syndrome. *Med Hypotheses* 2020; **144**: 110242 [PMID: 33254548 DOI: 10.1016/j.mehy.2020.110242]

113 **Calay D**, Mason JC. The multifunctional role and therapeutic potential of HO-1 in the vascular endothelium. *Antioxid Redox Signal* 2014; **20**: 1789-1809 [PMID: 24131232 DOI: 10.1089/ars.2013.5659]

114 **Dattilo M**. The role of host defences in Covid 19 and treatments thereof. *Mol Med* 2020; **26**: 90 [PMID: 32993497 DOI: 10.1186/s10020-020-00216-9]

115 **Raghunath A**, Sundarraj K, Nagarajan R, Arfuso F, Bian J, Kumar AP, Sethi G, Perumal E. Antioxidant response elements: Discovery, classes, regulation and potential applications. *Redox Biol* 2018; **17**: 297-314 [PMID: 29775961 DOI: 10.1016/j.redox.2018.05.002]

116 **Motohashi H**, Katsuoka F, Miyoshi C, Uchimura Y, Saitoh H, Francastel C, Engel JD, Yamamoto

M. MafG sumoylation is required for active transcriptional repression. *Mol Cell Biol* 2006; **26**: 4652-4663 [PMID: 16738329 DOI: 10.1128/mcb.02193-05]

117 **Shavit JA**, Motohashi H, Onodera K, Akasaka J, Yamamoto M, Engel JD. Impaired megakaryopoiesis and behavioral defects in mafG-null mutant mice. *Genes Dev* 1998; **12**: 2164-2174 [PMID: 9679061 DOI: 10.1101/gad.12.14.2164]

118 **Katsuoka F**, Otsuki A, Takahashi M, Ito S, Yamamoto M. Direct and Specific Functional Evaluation of the Nrf2 and MafG Heterodimer by Introducing a Tethered Dimer into Small Maf-Deficient Cells. *Mol Cell Biol* 2019; **39** [PMID: 31383749 DOI: 10.1128/mcb.00273-19]

119 **Xu D**, Xu M, Jeong S, Qian Y, Wu H, Xia Q, Kong X. The Role of Nrf2 in Liver Disease: Novel Molecular Mechanisms and Therapeutic Approaches. *Front Pharmacol* 2018; **9**: 1428 [PMID: 30670963 DOI: 10.3389/fphar.2018.01428]

120 **Sun J**, Fu J, Li L, Chen C, Wang H, Hou Y, Xu Y, Pi J. Nrf2 in alcoholic liver disease. *Toxicol Appl Pharmacol* 2018; **357**: 62-69 [PMID: 30165058 DOI: 10.1016/j.taap.2018.08.019]

121 **Qin SJ**, Wang BY, Li BR, Zheng K, Cai Y, Li RB, Zeng M, Xiao F, Xu XY. [Effect of c-myc gene silence on the expression of oncogenes and apoptotic genes in hepatocytes treated with PM(2).5]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* 2020; **38**: 657-663 [PMID: 33036527 DOI: 10.3760/cma.j.cn121094-20200326-00056]

122 **Wahlström T**, Henriksson M. Mnt takes control as key regulator of the myc/max/mxd network. *Adv Cancer Res* 2007; **97**: 61-80 [PMID: 17419941 DOI: 10.1016/s0065-230x(06)97003-1]

123 **Yang H**, Li TW, Peng J, Tang X, Ko KS, Xia M, Aller MA. A mouse model of cholestasis-associated cholangiocarcinoma and transcription factors involved in progression. *Gastroenterology* 2011; **141**: 378-388, 388.e1-388. e4 [PMID: 21440549 DOI: 10.1053/j.gastro.2011.03.044]

124 **Yang H**, Li TW, Ko KS, Xia M, Lu SC. Switch from Mnt-Max to Myc-Max induces p53 and cyclin D1 expression and apoptosis during cholestasis in mouse and human hepatocytes. *Hepatology* 2009; **49**: 860-870 [PMID: 19086036 DOI: 10.1002/hep.22720]

125 **Yang H**, Liu T, Wang J, Li TW, Fan W, Peng H, Krishnan A, Gores GJ, Mato JM, Lu SC. Deregulated methionine adenosyltransferase α1, c-Myc, and Maf proteins together promote cholangiocarcinoma growth in mice and humans(‡). *Hepatology* 2016; **64**: 439-455 [PMID: 26969892 DOI: 10.1002/hep.28541]

126 **Yang H**, Li TW, Zhou Y, Peng H, Liu T, Zandi E, Martínez-Chantar ML, Mato JM, Lu SC. Activation of a novel c-Myc-miR27-prohibitin 1 circuitry in cholestatic liver injury inhibits glutathione synthesis in mice. *Antioxid Redox Signal* 2015; **22**: 259-274 [PMID: 25226451 DOI: 10.1089/ars.2014.6027]

127 **Zeng Y**, Lou G, Ren Y, Li T, Zhang X, Wang J, Huang Q. Network pharmacology-based analysis of Zukamu granules for the treatment of COVID-19. *Eur J Integr Med* 2021; **42**: 101282 [PMID: 33425074 DOI: 10.1016/j.eujim.2020.101282]

128 **Li Y**, Lu L, Tu J, Zhang J, Xiong T, Fan W, Wang J, Li M, Chen Y, Steggerda J, Peng H, Li TWH, Zhou ZG, Mato JM, Seki E, Liu T, Yang H, Lu SC. Reciprocal Regulation Between Forkhead Box M1/NF-κB and Methionine Adenosyltransferase 1A Drives Liver Cancer. *Hepatology* 2020; **72**: 1682-1700 [PMID: 32080887 DOI: 10.1002/hep.31196]

129 **Phipps MM**, Barraza LH, LaSota ED, Sobieszczyk ME, Pereira MR, Zheng EX, Fox AN, Zucker J, Verna EC. Acute Liver Injury in COVID-19: Prevalence and Association with Clinical Outcomes in a Large U.S. Cohort. *Hepatology* 2020; **72**: 807-817 [PMID: 32473607 DOI: 10.1002/hep.31404]

130 **Mendizabal M**, Piñero F, Ridruejo E, Anders M, Silveyra MD, Torre A, Montes P, Urzúa A, Pages J, Toro LG, Díaz J, Gonzalez Ballerga E, Miranda-Zazueta G, Peralta M, Gutiérrez I, Michelato D, Venturelli MG, Varón A, Vera-Pozo E, Tagle M, García M, Tassara A, Brutti J, Ruiz García S, Bustios C, Escajadillo N, Macias Y, Higuera-de la Tijera F, Gómez AJ, Dominguez A, Castillo-Barradas M, Contreras F, Scarpin A, Schinoni MI, Toledo C, Girala M, Mainardi V, Sanchez A, Bessone F, Rubinstein F, Silva MO. Prospective Latin American cohort evaluating outcomes of patients with COVID-19 and abnormal liver tests on admission. *Ann Hepatol* 2021; **21**: 100298 [PMID: 33359234 DOI: 10.1016/j.aohep.2020.100298]

131 **Hundt MA**, Deng Y, Ciarleglio MM, Nathanson MH, Lim JK. Abnormal Liver Tests in COVID-19: A Retrospective Observational Cohort Study of 1,827 Patients in a Major U.S. Hospital Network. *Hepatology* 2020; **72**: 1169-1176 [PMID: 32725890 DOI: 10.1002/hep.31487]

132 **Yip TC**, Lui GC, Wong VW, Chow VC, Ho TH, Li TC, Tse YK, Hui DS, Chan HL, Wong GL. Liver injury is independently associated with adverse clinical outcomes in patients with COVID-19. *Gut* 2021; **70**: 733-742 [PMID: 32641471 DOI: 10.1136/gutjnl-2020-321726]

133 **Cai Q**, Huang D, Yu H, Zhu Z, Xia Z, Su Y, Li Z, Zhou G, Gou J, Qu J, Sun Y, Liu Y, He Q, Chen J, Liu L, Xu L. COVID-19: Abnormal liver function tests. *J Hepatol* 2020; **73**: 566-574 [PMID: 32298767 DOI: 10.1016/j.jhep.2020.04.006]

134 **Zhao D**, Yao F, Wang L, Zheng L, Gao Y, Ye J, Guo F, Zhao H, Gao R. A Comparative Study on the Clinical Features of Coronavirus 2019 (COVID-19) Pneumonia With Other Pneumonias. *Clin Infect Dis* 2020; **71**: 756-761 [PMID: 32161968 DOI: 10.1093/cid/ciaa247]

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REVIEW

Mucosal lesions of the upper gastrointestinal tract in patients with ulcerative colitis: A review

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Abstract

Ulcerative colitis (UC) is a chronic, nonspecific, relapsing inflammatory bowel disease. The colorectum is considered the chief target organ of UC, whereas upper gastrointestinal (UGI) tract manifestations are infrequent. Recently, emerging evidence has suggested that UC presents complications in esophageal, stomachic, and duodenal mucosal injuries. However, UC-related UGI tract manifestations are varied and frequently silenced or concealed. Moreover, the endoscopic and microscopic characteristics of UGI tract complicated with UC are nonspecific. Therefore, UGI involvement may be ignored by many clinicians. In addition, no standard criteria have been established for patients with UC who should undergo fibrogastroduodenoscopy. Furthermore, specific treatment recommendations may be needed for patients with UC-associated UGI lesions. Herein, we review the esophageal, gastric, and duodenal mucosal lesions of the UC-associated UGI tract, as well as the potential pathogenesis and therapy.

Key Words: Ulcerative colitis; Upper gastrointestinal tract; Inflammatory bowel disease; Endoscopic and microscopic manifestations

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Core Tip: This is a minireview to summarize the esophageal, gastric, and duodenal mucosal lesions of the ulcerative colitis (UC)-associated upper gastrointestinal (UGI) tract, together with the potential pathogenesis and treatment recommendations. UC-related UGI manifestations are diverse and normally quite or covered by the manifestations of the lower gastrointestinal tract. In addition, the endoscopic and microscopic characteristics of UGI tract complicated with UC are typically unspecified. However, it is very important to identify UC-associated UGI tract diseases. Our research may assist in the diagnosis and monitoring of UC-associated UGI tract diseases.

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INTRODUCTION

Ulcerative colitis (UC) is an idiopathic, chronic, relapsing inflammatory bowel disease (IBD) occurring in the colon and rectum. It has long been agreed that UC starts in the rectum and commonly spreads to a part of or the entire colon[1,2]. The main typical symptoms of UC include frequent bowel movement, mucus pus, bloody diarrhea, abdominal pain and discomfort, urgency, weight loss, and tenesmus[3,4]. Nevertheless, tremendous advances and progress have been made in our understanding of this disease over the last 2 decades. Apart from the above typical symptoms, emerging evidence supports that there are a variety of accompanying symptoms involving the upper gastrointestinal (UGI) tract in patients with UC upon macroscopic and microscopic analyses[5-9], such as eosinophilic esophagitis[10], gastroduodenitis[11], ulcers, or UGI inflammation[12,13]. In addition, the positive rate of UGI endoscopy in asymptomatic individuals was lower than that in symptomatic patients. The reported clinically significant UGI lesions include multiple erosions, granular changes, bamboo joint-like appearance, white spots, friable mucosa, ulcer, and purulent deposits during esophagogastroduodenoscopy[11,14-16], in which multiple erosions are extremely rare (0%-3%) in UC patients[17]. The UC-related UGI endoscopic and microscopic presentations are summarized in Figure 1 and Table 1.

Millions of people worldwide are affected by UC, which has been considered a global disorder[18]. The incidence of UC has been rising since the mid-20th century[19]. Nevertheless, pediatric IBD patients have been generally thought to present more common UGI involvement than adult patients. This discrepancy may be because UGI endoscopy was less routinely performed on asymptomatic adult patients with IBD than on their pediatric counterparts[20]. UC patients receive targeted treatment at various organizations ranging from community hospitals and public hospitals to clinics. However, UGI involvement may be unnoticed by the attending physician due to the lack of the knowledge of gastroduodenal lesions, which should raise concerns during UC treatment[15]. Furthermore, there is no established standard or criteria available for patients with UC who should undergo fibrogastroduodenoscopy (FGDS), because the clinical backgrounds of these patients with UC-associated UGI lesions have not been fully and exactly demonstrated. Specific management may be required for patients with UC-associated UGI lesions.

Henceforth, we conducted this review with the aim of describing various UGI tract presentations in UC and their differential diagnosis and management. Our study may help physicians and other clinicians make a correct and timely diagnosis of UC while avoiding needless examinations.

ESOPHAGEAL MUCOSAL LESIONS

Traditionally, UC is considered a purely colon disease other than limited terminal ileal inflammation. In recent years, however, the prevalence at initial presentation of UC-associated esophageal disease has been 12%-50%[10], while it is as high as 70% to 90% in Crohn's disease (CD) individuals[21,22]. These numbers may underestimate the prevalence of the disease due to the difficulty of precise diagnosis and the lack of standard upper endoscopy in asymptomatic individuals with UC. It is very challenging to diagnose esophageal complications of UC because these complications can present as diverse esophageal symptoms, such as erosive-ulcerative esophagitis or esophageal stricture, which share many characteristics with other common esophageal diseases. In addition, the microscopic manifestations and characteristics of esophageal complications with UC are typically unspecified. Although the prevalence of esophageal disease complicated with UC is lower than esophageal disease complicated with CD, it is still important to identify UC-associated esophageal disease.

Table 1 Upper gastrointestinal endoscopic and microscopic presentations in ulcerative colitis

Classification	Presentations of UGI in UC	
Endoscopy		Erythema
		Edema
		Inconspicuous vascular morphology
		Granularity
		Friability
		Oral ulcers, superficial ulcers
		Bamboo joint-like appearance
		White spots
		Purulent deposits
Microscopy	Esophagus	Esophageal ulcers
		Esophagitis
		Unspecified/nonspecific
	Stomach	Unspecified chronic gastritis
		Lymphocytic gastritis
		FEG
		Ulcers
	Duodenum	Diffuse chronic duodenitis in the presence of mucosal dilatation
		Structural deformation
		Intraepithelial lymphocytic disease with normal mucosal structure

UGI: Upper gastrointestinal; UC: Ulcerative colitis; FEG: Focally enhanced gastritis.

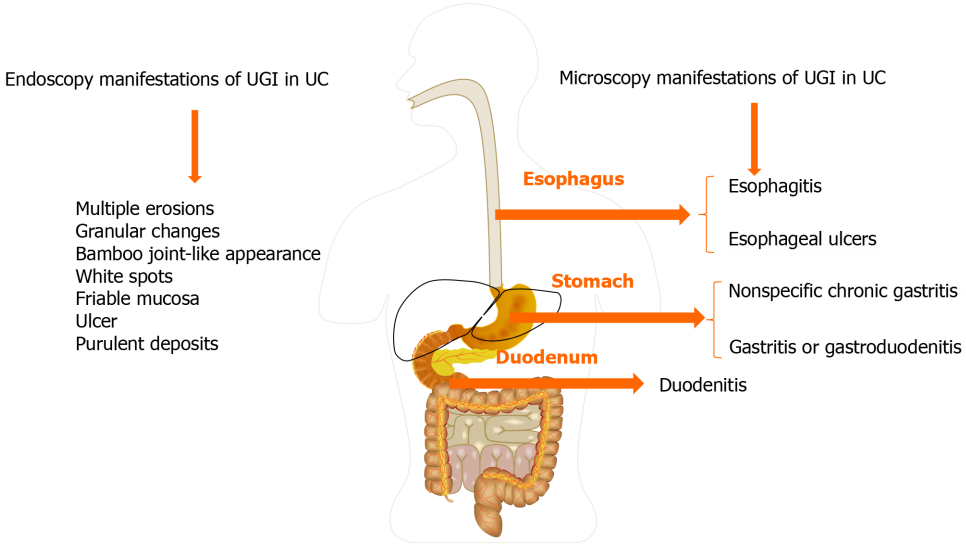


Figure 1 Common upper gastrointestinal endoscopic and microscopic presentations in ulcerative colitis. UGI: Upper gastrointestinal; UC: Ulcerative colitis.

Esophagitis

The first case of esophagitis was reported by Margoles *et al*[23] in 1961. The occurrence of esophagitis has been described more frequently in pediatric patients than in adult individuals[24]. However, the endoscopic and histologic characteristics are unspecified or nonspecific. In addition, it is difficult to evaluate other irrelevant disorders, such as reflux disease, medications, or Candida[13,25,26]. Lymphocytic

esophagitis (LE) is defined as large numbers of peripapillary lymphocytoses (PLs) or intraepithelial lymphocytes (IELs) without significant granulocytes (neutrophils and eosinophils)[27], and LE was originally demonstrated as a UGI tract manifestation in children with IBD[28]. LE has been reported in 7% of pediatric patients with UC and mucosal injury (edema or dyskeratosis) is commonly seen in patients with UC[28,29]; however, a significant difference was not found between IBD and non-IBD control individuals[28]. In contrast, a previous study suggested that PL found in LE might be a marker for disease activity in IBD[27]. The diagnosis of LE in UC patients should be distinguished from candidiasis, lichen planus esophagitis, and lichenoid esopha-gitis [30]. Granulomatous esophagitis has been reported in patients with CD[31-33] but not in patients with UC. The histologic diagnostic criteria for granulomatous esophagitis include chronic granulomatous disease, common variable immunodeficiency, and infection. Moreover, a previous analysis also revealed that there were statistically significant inverse associations between eosinophilic esophagitis and CD or microscopic colitis, but not UC[34].

Esophageal ulcer

There are relatively few reports of esophageal ulcers associated with UC. Until now, there have been only 20 cases of esophageal ulcers (including ours) accompanied by UC[23,24,35-45]. The clinical characteristics of these patients are listed in Table 2. It is a punched-out ulcer and frequently seen in the middle to the lower esophagus[24]. According to the previous findings, esophageal ulcers were more commonly seen in the young individuals than in elderly individuals. Additionally, patients often have a complicated esophageal ulcer at the onset of colitis, or when it recurs. However, the endoscopic manifestations of UC patients with esophageal ulcers were unlike gastroduodenitis associated with UC (GDUC). GDUC is characterized as friable and granular mucosa[11,46], but in contrast, the esophageal ulcers complicated by UC are punched-out ulcers and frequently occur in the middle to lower esophagus (Figure 2A). Pathologically, only nonspecific inflammatory cell infiltration was demonstrated in all reported cases, without any UC-specific characteristics (Figure 2B). Nevertheless, regarding histology, the association between esophageal ulcers and UC was not established; esophageal lesions would still be effective in the treatment of UC. In addition, most UC patients with esophageal ulcers present other extraintestinal manifestations, including ocular injuries, skin disorders, and peripheral arthritis[47]. Nonetheless, the underlying pathological mechanisms are currently unidentified. Therefore, UC-complicated esophageal ulcers are particularly uncommon. They may occur as an extraintestinal manifestation in most cases of UC. The pathological discoveries of esophageal lesions are unspecified for UC. Consequently, a comprehensive diagnosis is needed to exclude diseases related to infection and drugs.

GASTRODUODENAL AND DUODENAL MUCOSAL LESIONS

UC complicated by gastroduodenal lesions was first reported by Sasaki *et al*[14]. Multiple erosions and granular changes in the greater curvature of the gastric antrum and descending part of the duodenum (Figure 3A) have been demonstrated. Histologically, they confirmed obvious inflammatory cell infiltration and crypt abscesses in the gastric and duodenum. Subsequently, an increasing body of evidence suggests gastroduodenal mucosal lesions in UC patients[13,46,48-52]. More recently, the concept of ulcerative gastroduodenal lesion (UGDL) or GDUC was proposed[46,53]. The standard of diagnosis of UGDL includes: The lesions could be improved after UC treatment and the pathological examination is similar to the UC. Nevertheless, the diagnosis criteria are not rigorous enough because of the rare reports. It has been demonstrated that several factors are the major risk factors for GDUC, including more extensive colitis, administration of a lower dose of prednisolone, occurrence of pouchitis, post-colectomy status, and a longer postoperative period[54-56].

Gastritis or gastroduodenitis

The occurrence of gastritis or gastroduodenitis has been estimated in 5%-19% of patients with UC[11,46,48,57-61]. Most UC patients present with mild local symptoms or no symptoms at all. Severe gastritis or gastroduodenitis is usually seen in subjects with long postoperative intervals after colectomy[60] or in individuals with extensive colitis, ileoanal pouchitis, or pancolitis[46,48,58]. Focal enhanced gastritis (FEG) has been considered as the most frequent UGI inflammatory form in patients with UC, followed by gastric basal mixed inflammation and superficial plasmacytosis[58,62-64].

Table 2 Clinical characteristics of esophageal ulcer complicated with ulcerative colitis

Case No.	Ref.	Age	Gender	Duration of UC	Esophageal ulcer	Location	Symptoms	Treatment
1	Margoles *et al* [23], 1961	28	M	17 yr	Web formation	Middle esophagus	No	NA
2	Knudsen *et al* [36], 1967	15	M	2 yr	Severe esophagitis	Lower esophagus	Parasternal chest pain and dysphagia	PSL
3	Rosendorff *et al* [35], 1967	23	M	5 yr	Shallow ulceration with gross diffuse irregularity	Middle and lower esophagus	Dysphagia	PSL
4	Christopher *et al* [37], 1969	21	F	0	Active ulcerative esophagitis	NA	NA	NA
5	Christopher *et al* [37], 1969	19	M	0	Marked ulcerative esophagitis	NA	Substenal pain and dysphagia	NA
6	Christopher *et al* [37], 1969	26	M	2 mo	Esophageal ulcerations with perforaton into theanterior mediastinum	NA	Dysphagia, fever, hypotension	NA
7	Christopher *et al* [37], 1969	14	F	5 yr	Necrotizing fibrinopurulent ulceration	NA	NA	NA
8	Christopher *et al* [37], 1969	24	M	9 yr	Ulcerative and membranous esophagitis	NA	Nausea, vomiting and hematemesis	NA
9	Zimmerman *et al* [38], 1984	21	M	3 yr	Multiple friable ulcerations	Whole esophagus	Severe odynophagia and dysphagia	PSL, tetracycline
10	Konishi *et al*[40], 1998	47	F	0	Multiple irregular esophageal ulcers	Whole esophagus	Anterior chest pain and dysphagia	5ASA, H2blocker
11	Asakawa *et al* [44], 2000	18	F	7 yr	Longitudinal esophageal ulcer with hemorrhage	Middle and lower esophagus	Sore throat and pain on swallowing	PSL
12	Ikeda *et al*[42], 2000	18	M	1 yr	Punched-out esophageal ulcer	Middle esophagus	Sore throat and anterior chest pain	PSL
13	Higashi *et al*[41], 2004	19	M	0	Punched-out esophageal ulcer	Middle esophagus	General fatigue	PSL, SASP
14	Sato *et al*[45], 2005	33	M	0	Punched-out esophageal ulcer	Middle and lower esophagus	Anterior chest pain and dysphagia	PPI
15	Izawa *et al*[39], 2015	52	F	3 yr	Punched-out esophageal ulcer	Middle esophagus	Anterior chest pain on swallowing	PSL
16	Kuroki *et al*[43], 2019	47	M	3 yr	Necrosis	Lower esophagus	Epigastric pain	PPI
17	Kuroki *et al*[43], 2019	53	M	NA	Necrosis	Middle and lower esophagus	Hematemesis	PPI
18	Tominaga *et al* [24], 2020	16	F	0	An esophageal ulcer with aphthae	Middle esophagus	Chest pain	PSL
19	Tominaga *et al* [24], 2020	19	F	0	Longitudinal esophageal ulcer	Low esophagus	Chest pain on swallowing	PSL
20	Ours	24	M	1 yr	Multiple ulcerative lesions	Multiple lesions	Burning sensation	Remicade

UC: Ulcerative colitis; NA: Not available; PSL: Prednisolone; SASP: Salazosulfapyridine; 5-ASA: 5-aminosalicylic acid; PPI: Proton pump inhibitor.

FEG is characterized by localized accumulation of lymphocytes, neutrophils, and macrophages in at least one pit, neck, or gland of the adjacent lamina propria[58]. This focal inflammation pattern can be observed anywhere in the mucosa (Figure 3B), from the basal paramucosa of the muscularis to the superficial subepithelial layers, with the occurrence of a single focal point or as multiple focal points. Previously, FEG was described as a specific diagnostic marker for CD patients[65-67]. However, different opinions have also been proposed[68,69]. Scholars have also found that FEG could be seen in up to 20.8% of children with UC[65]. Although it is still uncertain whether the utility of FEG could distinguish CD from UC in adults, it is unreliable in children[65, 70-72]. Basal and patchy inflammation is the second pattern of gastric inflammation, which includes a loose mixture of lymphocytes, eosinophils, mast cells, and plasma cells. These cells were found in the lamina propria between the deepest glands and the

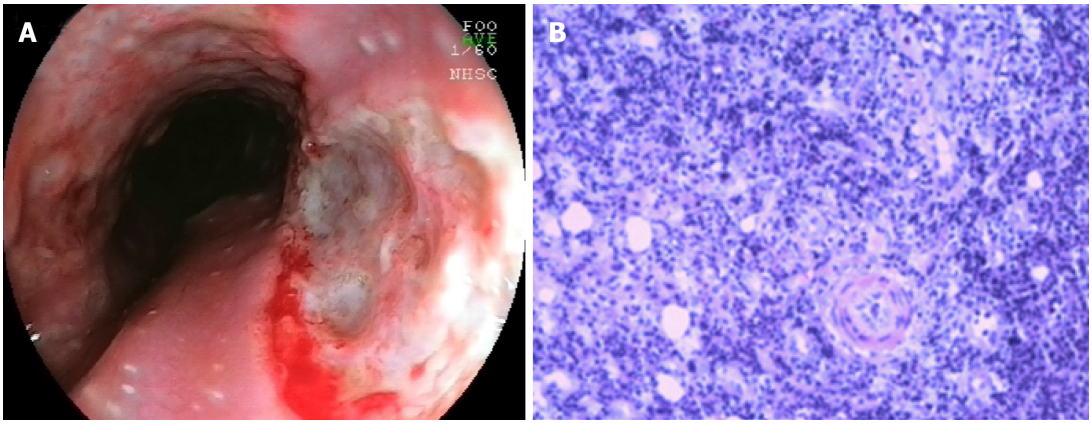


Figure 2 Endoscopic and microscopic presentations of esophageal ulcers associated with ulcerative colitis. A: Endoscopic view showing multiple ulcerative lesions of the esophagus; B: Microscopic view showing squamous epithelial hyperplasia, interstitial fibrous tissue proliferation, inflammatory cell infiltration, and visible neutrophil aggregation.

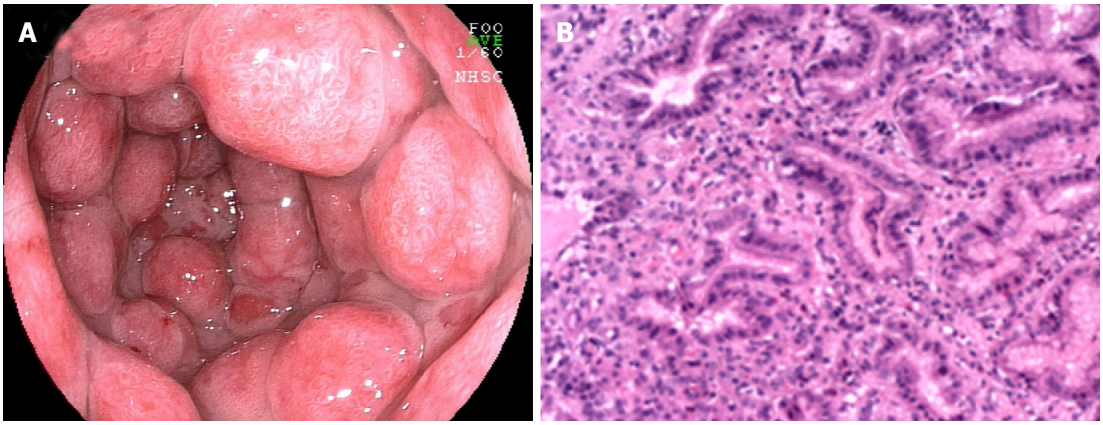


Figure 3 Endoscopic and microscopic manifestations of gastritis-associated ulcerative colitis. A: Endoscopic image displaying multiple protrusion lesions of the gastric mucosa; B: Microscopic image showing mucosal inflammation with hyperplastic polypoid changes.

muscularis mucosae[58]. Moreover, superficial plasmacytosis is the third most common pattern of gastric inflammation. It is regarded as a diffuse band of plasma cells in the superficial lamina propria adjacent to the pits and necks. Furthermore, chronic, diffuse gastritis also seems to be a frequent feature in the gastric biopsy specimens from UC individuals[73]. Moreover, a recent study suggested that the active inflammation of the stomach and/or duodenum might be related to drug-refractory UC[61]. Notably, erosions or ulcers complicated with UC are infrequent and granulomas are always absent. Regarding to the gross and endoscopic features, UC-related gastritis is characterized by diffuse granular or brittle mucosa, as well as aphthous lesions[11,48]. For the differential diagnosis, patients with UC present with mild chronic sinusitis in the presence or absence of neutrophils and have a questionable specificity, which may be correlated with dietary and environmental factors. The infiltration of inflammatory cells observed in *Helicobacter pylori*-relevant gastritis and gastric CD-relevant chronic superficial gastritis is denser and more diffuse than that in UC.

Duodenitis

The duodenum is not usually regarded as a target organ of UC. However, as early as the 1960s, chronic duodenitis associated with UC was first demonstrated[74]. Subsequently, emerging evidence implies the occurrence of chronic duodenitis associated with UC[11,17,46,48,51,53,75-83]. Nevertheless, it remains infrequent. Dyspepsia is the most common symptom of chronic duodenitis associated with UC, but it is often covered by the symptoms of UC[59]. Notably, most symptomatic duodenitis individuals present with severe colitis, which in some cases requires a colectomy. More interestingly, some individuals with a continental ileoanal pouch also have pouchitis[48]. A unique type of UGI inflammation in UC individuals is diffuse

chronic duodenitis, which occurs in 10% of duodenal biopsy patients and combined resection occurs in 40% of UC patients who have had pouchitis. The reported endoscopic findings in symptomatic patients are diverse and include diffuse edema, granular, fragile, and ulcers, *etc*. Furthermore, the microscopic characteristics of duodenitis associated with UC include a dilated mucosa with diffuse inflammatory infiltration of monocytes and neutrophilic inflammation, glandular deformation, and erosion or ulceration[78]. Moreover, duodenal CD3 (+) and CD8 (+) IELs or lamina propria mononuclear cells may be present in a series of UC patients[84,85]. In summary, duodenitis complicated with UC is infrequently identified due to its aspecific capillary and microscopic features as well as its overlap with celiac disease, peptic duodenitis, and drug-related duodenitis. Nevertheless, clinical consciousness should be considered in diagnosing UC, especially if the patients have accomplished previous colectomy for widespread and severe colitis or if the patients also experience concomitant pouchitis or enterocolitis.

PATHOGENESIS OF UC

It is believed that UC may be mainly determined by a complex combination of environmental and host factors, genetic variations, immune response, and gut microbiota. The onset of this disease is activated by disturbance of the mucosal barrier, gut microbiota, and abnormal immune response. Many scholars support that the abnormal immune response (innate and adaptive) is a key direct pathogenesis, in which gut microbiota is an important stimulus for this immune damage process and the environmental and host factors may be the causative factors of the disease.

Environmental and host factors

It has been well acknowledged that environmental and host factors play critical roles in increasing the susceptibility of developing UC. The increasing incidence of UC worldwide implies the significance of environmental factors in the progression of this disease[86,87]. This is similar to the pattern detected in the Western world in the early 20th century[88]. UC has specifically occurred in urban zones, and its incidence is faster and then slower. Westernization and its accompanying urbanization, sedentary lifestyle, exposure to environmental pollution, dietary changes, antibiotics usage, refrigeration, better sanitation, and fewer infections are regarded as contributing factors[89]. For example, former cigarette smoking has been reported as one of the UC strongest risk factors, whereas compared to the former and non-smokers, active smokers are less probably to suffer from UC and they mainly present with a milder clinical course[90-92]. Furthermore, appendectomy is considered to have a protective impact on future development of UC[93].

Genetic variants

Genetic studies have been predominantly effective in recognizing both common and infrequent genetic variants associated with susceptibility to UC[94-96]. Human leukocyte antigen[97] and adenylate cyclase type 7[98] are the two important UC-specific genes. Moreover, many UC-specific genes are confirmed to be responsible for mediation of epithelial barrier function. However, it has been established that UC and CD share most genetic factors. These shared genetic factors could encode cytokine, innate and adaptive immune signal pathways, and immune sensing, such as interleukin (IL)-10, -12, -23R, and caspase recruitment domain containing protein 9. In addition, it has been demonstrated that about 70% of genetic variants are also commonly seen in some other autoimmune diseases, such as ankylosing spondylitis and psoriasis[86]. Overall, genetic factors deliberate a small but certain increase in susceptibility to UC. Nevertheless, many individuals did not present with genetic susceptibility when all susceptibility loci were evaluated by polygenic risk scores[99]. These findings imply that an abnormal adaptive immune response and epithelial barrier dysfunction may play critical roles in the pathogenesis of UC.

Gut microbiota

A series of animal experiments and clinical trials have confirmed the presence of significant intestinal flora dysbiosis in patients with UC[100,101]. The dysbiosis of intestinal flora is featured by decreased biodiversity, irregular composition of gut microbiota, and changes of spatial distribution, together with interactions between microbiota and the host[101]. There is a significant change in the number of intestinal bacteria in patients with UC, which is reflected in a decrease in probiotic bacteria (*e.g.*,

Bifidobacterium and *lactobacillus, etc.*) and an increase in conditionally pathogenic bacteria (such as *Enterococci* and *Enterobacteria*, *etc.*). Therefore, increasing studies have paid attention to the therapeutic effects of faecal microbial transplantation from healthy donors on patients with UC[100,102-105].

Immune system

UC is a pathogenic inflammatory disease mediated by the immune system including innate immunity and adaptive immunity[106]. The innate immunity is the first line of defense against pathogens. Unlike adaptive immunity, innate immunity is non-specific and persistent. Immune cells in innate immunity, such as dendritic cells (DCs), macrophages, natural killer cells, intestinal epithelial cells, and myofibroblasts, can sense the intestinal microbiota and respond to conserved structural motifs of microorganisms, which can trigger a rapid and effective inflammatory response and prevent bacterial invasion[107]. Among them, DCs are specialized antigen-presenting cells responsible for T-cell activation and induction of adaptive immune responses and they are key players in the interplay between innate and adaptive immunity[107]. For the adaptive immune system, the components of this system cooperate with each other, with the molecules, and with the cells in the innate immune system to mount an effective immune response that eliminates invading pathogens under normal conditions. Unlike innate immunity, adaptive immunity has highly specific long-term immunity and is adaptive because antigen specificity is the result of complex maturation and development of immune cells. T helper (Th) cells are key elements to the adaptive immune response. UC is mainly activated by Th2 cells-induced immune response[108]. Th2 cells are mainly induced by IL-13 and then subsequently secrete IL-4, IL-5, and IL-13, in which IL-13 is considered essential to the pathogenesis of UC [109].

PATHOGENESIS OF UGI MUCOSAL LESIONS

The pathogenesis of UGI mucosal lesions has not been clearly clarified. Nevertheless, many researchers supported that it was similar to that of the colonic lesions of UC. The cellular immune response to certain bacteria may contribute to the potential pathogenesis of UC[110]. Interestingly, similar immune molecules were also demonstrated in both the UGI tissue and the colon tissue (Figure 4). For example, a previous study suggested that the Th2 cells activated by CCR3+ and CD62L in the UGI tract might originate from mucosal lesions of the colon[111]. Moreover, Berrebi *et al* [112] reported that the chemokine receptor CXCR3 was unanimously expressed in the epithelium and lamina propria of perivascular areas of gastric biopsy specimens from the UC patients, while the chemokine receptor CCR5 was found in the lymphocytes surrounding the lamina propria. Therefore, the increased lymphocyte population upon stimulation in the colon of UC patients may result in lymphocyte infiltration in the stomach[73]. Furthermore, the excessive autoimmune response to bacterial antigens caused by genetic susceptibility to abnormal immune responses may result in the enrollment of memory T cells in the colorectal mucosa in the UGI tract[112]. However, there is no related evidence of the correlation between *H. pylori* and UGDL [46,113].

MOLECULAR PATHOLOGICAL EPIDEMIOLOGY

Molecular pathological epidemiology (MPE) has been reported as an integrative transdisciplinary science, which integrates the academic disciplines of molecular pathology and epidemiology[114-117]. Unlike conventional epidemiologic research, such as genome-wide association studies, MPE mainly figures out the underlying heterogeneity of disease processes, whereas traditional molecular epidemiology typically treats a disease as an individual entity[118,119]. Similar to the biology systems and WebMed[120,121], MPE combines the analysis of overall populations and macroenvironments, which is involved in the molecular analysis and microenvironments. In addition, the goal of MPE is to explore the mutual relation between exogenous and endogenous elements, molecular markers, and progression of malignancy[117,122,123]. More importantly, MPE research could cover all human diseases. Therefore, MPE studies can offer insightful aspects on the pathogenesis of disease by validating specific mechanisms in disease development and progression [122].

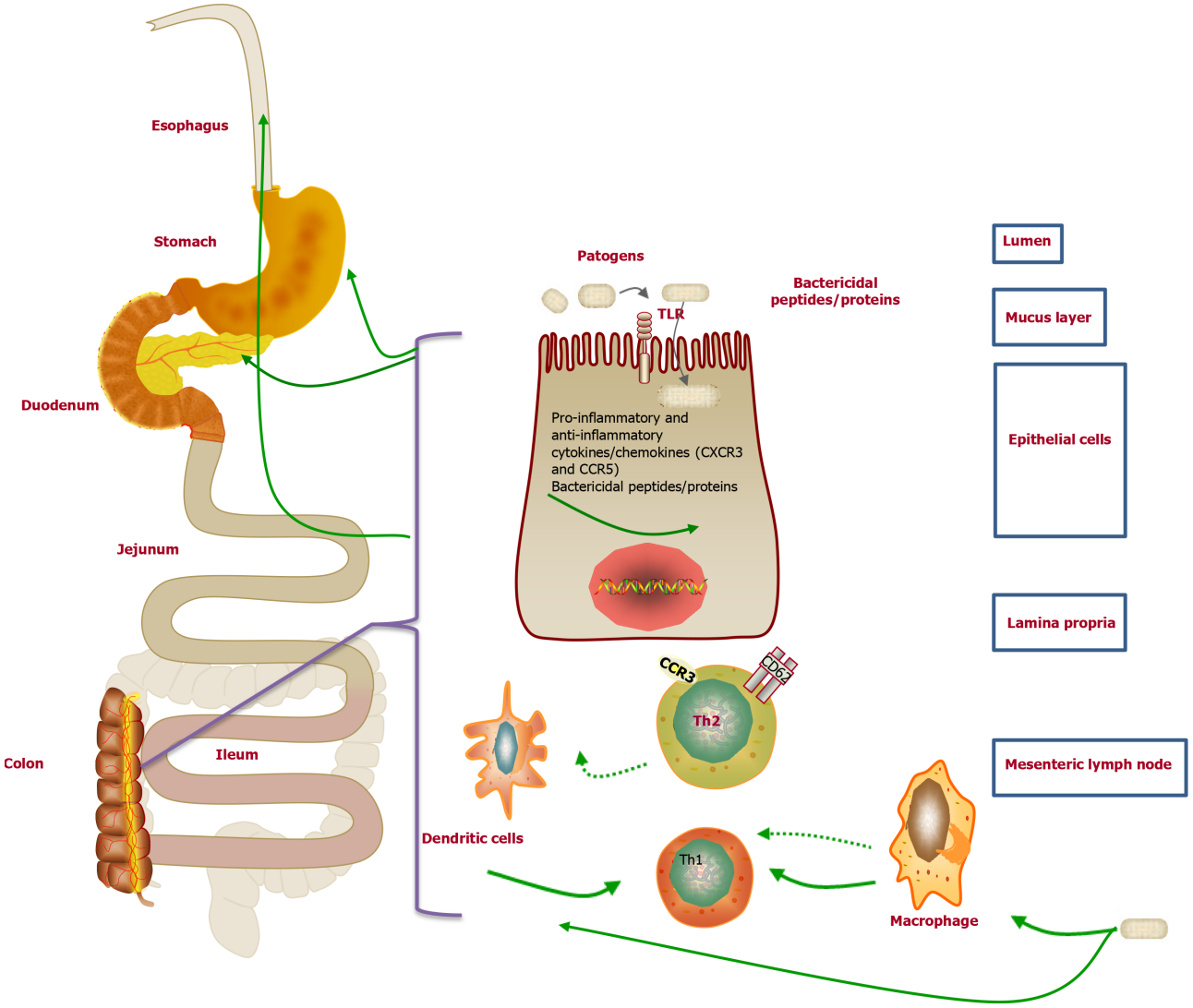


Figure 4 Pathogenesis of upper gastrointestinal mucosal lesions.

It has been well demonstrated that traditional epidemiology investigation reveals many factors, such as lifestyle, dietary, and environmental exposures, could be positively or negatively related with risk of illness[117,124]. Nevertheless, it is still unclear how these exposures impact the disease pathogenesis. Previous studies have confirmed that these above factors probably affect the pathogenic process by changing the local tissue microenvironment, and epigenetics play an important role in cellular response to alterations in the microenvironment[114]. An increasing body of evidence demonstrates application of MPE in colorectal cancer research to find out the possible etiologic factors[125-129]. Nonetheless, little information is available with respect to the research of MPE on UC. UC has been considered a heterogeneous disease, in which smoking, alcohol, diet, obesity, microbiome, inflammation, immunity, germline genetic variations, and gene-by-environment interactions are responsible for the progression of UC[130,131]. Therefore, MPE may be helpful to investigate those factors in relation to molecular pathologies, immunity, and clinical outcomes.

Although the MPE research has many advantages, the challenges should also be taken into consideration. Primarily, the reported challenges in the MPE study comprise the selected sample size, requirement for severe corroboration of molecular examines and study discoveries, and lack of multidisciplinary experts, international forums, as well as the standardized strategies[119]. Furthermore, MPE studies have to confront the problem of various hypothesis challenging and therefore require the formation of a priori hypotheses on the basis of early exploratory discoveries or possible biological mechanisms[117]. Moreover, MPE may also generate more opportunities for false discoveries[119]. In consideration of the pathogenesis of UC, MPE paradigm may be a promising direction and improve prediction of response to pharmacological, dietary, and lifestyle interventions for UC, together with the

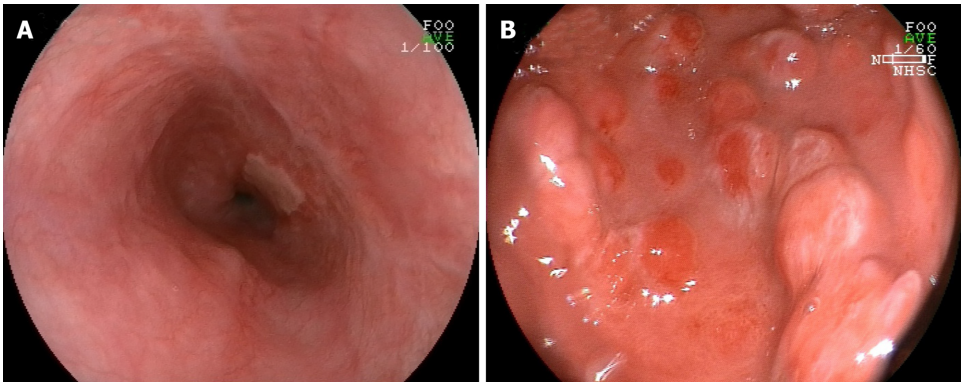


Figure 5 Endoscopic pictures of esophageal ulcer- and gastritis-associated ulcerative colitis after treatment. A: Endoscopic picture revealing a healing scar of multiple esophageal ulcers after administration of remicade (infliximab for injection); B: Endoscopic picture demonstrating multiple protrusion lesions of the gastric mucosa after treatment with remicade and methylprednisolone.

pathomechanism and treatment of mucosal lesions of the UGI tract in patients with UC.

THERAPY OF UGI LESIONS

5-aminosalicylate, infliximab, corticosteroid, antitumor necrosis factor (TNF), or calcium antagonist has been reported to be effective for managing UC[81-83,132]. Nevertheless, currently, standard treatment for UGI lesions in UC has not yet reached agreement. Steroids, leukocytapheresis, and/or peroral mesalazine can be used to treat UGI lesions in UC[46]. Moreover, immunosuppression such as cyclosporine, tacrolimus, or infliximab may be the treatment option for UGI inflamma-tion[77,82,133,134] (Figure 5). For example, for individuals with UC-associated esophageal ulcers, intravenous prednisolone (50 mg/d or 60 mg/d) was administered, and some symptoms including chest pain, bloody stool, pain on swallowing, and diarrhea were improved within a couple of days[24]. For individuals with UGDL, particularly those presenting with mild symptoms, some drugs, such as sulfasalazine or mesalazine, could effectively relieve the symptoms[54], while for some individuals with severe UGDL, TNF antagonists, infliximab, or calcium antagonists might be more effective than sulfasalazine or steroid hormones[82,132]. Therefore, administration of sulfas-alazine or mesalazine is a quick and efficient therapy to relieve the symptoms of UGDL, steroids can be utilized as second-line treatment, and TNF antagonists, infliximab, or calcium antagonists could be applied for patients who are insensitive to hormone therapy or subjects whose symptoms progress rapidly.

CONCLUSION

UC-related UGI manifestations are infrequent and normally nonspecific compared with intestinal manifestations, and hence, an ongoing diagnostic challenge should be extensively considered. Although the occurrence of UGI involvement in UC is rare, FGDS is specifically recommended for patients with UC who show extraintestinal manifestations and have undergone a colectomy or present pancolitis.

REFERENCES

1 **Ordás I**, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. Ulcerative colitis. *Lancet* 2012; **380**: 1606-1619 [PMID: 22914296 DOI: 10.1016/S0140-6736(12)60150-0]
2 **Feuerstein JD**, Moss AC, Farraye FA. Ulcerative Colitis. *Mayo Clin Proc* 2019; **94**: 1357-1373 [PMID: 31272578 DOI: 10.1016/j.mayocp.2019.01.018]
3 **Feuerstein JD**, Cheifetz AS. Ulcerative colitis: epidemiology, diagnosis, and management. *Mayo Clin Proc* 2014; **89**: 1553-1563 [PMID: 25199861 DOI: 10.1016/j.mayocp.2014.07.002]
4 **Gajendran M**, Loganathan P, Jimenez G, Catinella AP, Ng N, Umapathy C, Ziade N, Hashash JG. A comprehensive review and update on ulcerative colitis. *Dis Mon* 2019; **65**: 100851 [PMID:

30837080 DOI: 10.1016/j.disamonth.2019.02.004]
5 **Kovacs M**, Muller KE, Arato A, Lakatos PL, Kovacs JB, Varkonyi A, Solyom E, Polgar M, Nemes E, Guthy I, Tokodi I, Toth G, Horvath A, Tarnok A, Tomsits E, Csoszánszky N, Balogh M, Vass N, Bodi P, Dezsofi A, Gardos L, Micskey E, Papp M, Szucs D, Cseh A, Molnar K, Szabo D, Veres G; Hungarian IBD Registry Group (HUPIR). Diagnostic yield of upper endoscopy in paediatric patients with Crohn's disease and ulcerative colitis. Subanalysis of the HUPIR registry. *J Crohns Colitis* 2012; **6**: 86-94 [PMID: 22261532 DOI: 10.1016/j.crohns.2011.07.008]
6 **Lemberg DA**, Clarkson CM, Bohane TD, Day AS. Role of esophagogastroduodenoscopy in the initial assessment of children with inflammatory bowel disease. *J Gastroenterol Hepatol* 2005; **20**: 1696-1700 [PMID: 16246188 DOI: 10.1111/j.1440-1746.2005.03954.x]
7 **Castellaneta SP**, Afzal NA, Greenberg M, Deere H, Davies S, Murch SH, Walker-Smith JA, Thomson M, Srivistrava A. Diagnostic role of upper gastrointestinal endoscopy in pediatric inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2004; **39**: 257-261 [PMID: 15319625 DOI: 10.1097/00005176-200409000-00006]
8 **Abdullah BA**, Gupta SK, Croffie JM, Pfefferkorn MD, Molleston JP, Corkins MR, Fitzgerald JF. The role of esophagogastroduodenoscopy in the initial evaluation of childhood inflammatory bowel disease: a 7-year study. *J Pediatr Gastroenterol Nutr* 2002; **35**: 636-640 [PMID: 12454578 DOI: 10.1097/00005176-200211000-00009]
9 **Sawczenko A**, Sandhu BK. Presenting features of inflammatory bowel disease in Great Britain and Ireland. *Arch Dis Child* 2003; **88**: 995-1000 [PMID: 14612366 DOI: 10.1136/adc.88.11.995]
10 **Goldstein NS**, Amin M. Upper Gastrointestinal Tract in Inflammatory Bowel Disease. *Surg Pathol Clin* 2010; **3**: 349-359 [PMID: 26839135 DOI: 10.1016/j.path.2010.05.004]
11 **Hori K**, Ikeuchi H, Nakano H, Uchino M, Tomita T, Ohda Y, Hida N, Matsumoto T, Fukuda Y, Miwa H. Gastroduodenitis associated with ulcerative colitis. *J Gastroenterol* 2008; **43**: 193-201 [PMID: 18373161 DOI: 10.1007/s00535-007-2143-8]
12 **Harpaz N**, Polydorides AD. Upper Gastrointestinal Manifestations of Inflammatory Bowel Disease. *Surg Pathol Clin* 2020; **13**: 413-430 [PMID: 32773192 DOI: 10.1016/j.path.2020.05.003]
13 **Tobin JM**, Sinha B, Ramani P, Saleh AR, Murphy MS. Upper gastrointestinal mucosal disease in pediatric Crohn disease and ulcerative colitis: a blinded, controlled study. *J Pediatr Gastroenterol Nutr* 2001; **32**: 443-448 [PMID: 11396811 DOI: 10.1097/00005176-200104000-00010]
14 **Sasaki M**, Okada K, Koyama S, Yoshioka U, Inoue H, Fujiyama Y, Bamba T. Ulcerative colitis complicated by gastroduodenal lesions. *J Gastroenterol* 1996; **31**: 585-589 [PMID: 8844483 DOI: 10.1007/bf02355062]
15 **Kato R**, Iwamuro M, Hiraoka S, Takashima S, Inokuchi T, Takahara M, Kondo Y, Tanaka T, Okada H. Evaluation of the Upper Gastrointestinal Tract in Ulcerative Colitis Patients. *Acta Med Okayama* 2018; **72**: 105-113 [PMID: 29674758 DOI: 10.18926/AMO/55850]
16 **Song J**, Chang J, Kim C, Kim J, Han S, Kim T. The Endoscopic Findings of the Upper Gastrointestinal Tract in Patients with Ulcerative Colitis and Its Correlation with Disease Extent and Upper Gastrointestinal Symptoms. *Gut Liver* 2019; **13**
17 **Rubenstein J**, Sherif A, Appelman H, Chey WD. Ulcerative colitis associated enteritis: is ulcerative colitis always confined to the colon? *J Clin Gastroenterol* 2004; **38**: 46-51 [PMID: 14679327 DOI: 10.1097/00004836-200401000-00011]
18 **Pravda J**. Can ulcerative colitis be cured? *Discov Med* 2019; **27**: 197-200 [PMID: 31361982]
19 **Mak WY**, Zhao M, Ng SC, Burisch J. The epidemiology of inflammatory bowel disease: East meets west. *J Gastroenterol Hepatol* 2020; **35**: 380-389 [PMID: 31596960 DOI: 10.1111/jgh.14872]
20 **Siow VS**, Bhatt R, Mollen KP. Management of acute severe ulcerative colitis in children. *Semin Pediatr Surg* 2017; **26**: 367-372 [PMID: 29126505 DOI: 10.1053/j.sempedsurg.2017.10.006]
21 **Dąbkowski K**, Graca-Pakulska K, Zawada I, Ostrowski J, Starzyńska T. Clinical significance of endoscopic findings in the upper gastrointestinal tract in Crohn's disease. *Scand J Gastroenterol* 2019; **54**: 1075-1080 [PMID: 31456461 DOI: 10.1080/00365521.2019.1656776]
22 **Turner D**, Griffiths AM. Esophageal, gastric, and duodenal manifestations of IBD and the role of upper endoscopy in IBD diagnosis. *Curr Gastroenterol Rep* 2007; **9**: 475-478 [PMID: 18377798 DOI: 10.1007/s11894-007-0062-8]
23 **Margoles JS**, Wenger J. Stomal ulceration associated with pyoderma gangrenosum and chronic ulcerative colitis. Report of two cases. *Gastroenterology* 1961; **41**: 594-598 [PMID: 14469767]
24 **Tominaga K**, Tsuchiya A, Sato H, Mizusawa T, Morita S, Ishii Y, Takeda N, Natsui K, Kawata Y, Kimura N, Arao Y, Takahashi K, Hayashi K, Yokoyama J, Terai S. Esophageal Ulcers Associated with Ulcerative Colitis: A Case Series and Literature Review. *Intern Med* 2020; **59**: 1983-1989 [PMID: 32448835 DOI: 10.2169/internalmedicine.4437-20]
25 **Ruuska T**, Vaajalahti P, Arajärvi P, Mäki M. Prospective evaluation of upper gastrointestinal mucosal lesions in children with ulcerative colitis and Crohn's disease. *J Pediatr Gastroenterol Nutr* 1994; **19**: 181-186 [PMID: 7815240 DOI: 10.1097/00005176-199408000-00007]
26 **Ashton JJ**, Bonduelle Q, Mossotto E, Coelho T, Batra A, Afzal NA, Vadgama B, Ennis S, Beattie RM. Endoscopic and Histological Assessment of Paediatric Inflammatory Bowel Disease Over a 3-Year Follow-up Period. *J Pediatr Gastroenterol Nutr* 2018; **66**: 402-409 [PMID: 28922257 DOI: 10.1097/MPG.0000000000001729]
27 **Basseri B**, Vasiliauskas EA, Chan O, Wang HL, Basseri RJ, Pimentel M, Soffer E, Conklin JL. Evaluation of peripapillary lymphocytosis and lymphocytic esophagitis in adult inflammatory bowel disease. *Gastroenterol Hepatol (N Y)* 2013; **9**: 505-511 [PMID: 24719598]

28 **Ebach DR**, Vanderheyden AD, Ellison JM, Jensen CS. Lymphocytic esophagitis: a possible manifestation of pediatric upper gastrointestinal Crohn's disease. *Inflamm Bowel Dis* 2011; **17**: 45-49 [PMID: 20848529 DOI: 10.1002/ibd.21347]

29 **Sutton LM**, Heintz DD, Patel AS, Weinberg AG. Lymphocytic esophagitis in children. *Inflamm Bowel Dis* 2014; **20**: 1324-1328 [PMID: 24983984 DOI: 10.1097/MIB.0000000000000100]

30 **Abuquteish D**, Putra J. Upper gastrointestinal tract involvement of pediatric inflammatory bowel disease: A pathological review. *World J Gastroenterol* 2019; **25**: 1928-1935 [PMID: 31086461 DOI: 10.3748/wjg.v25.i16.1928]

31 **LiVolsi VA**, Jaretzki A 3rd. Granulomatous esophagitis. A case of Crohn's disease limited to the esophagus. *Gastroenterology* 1973; **64**: 313-319 [PMID: 4686340]

32 **Mahadeva U**, Martin JP, Patel NK, Price AB. Granulomatous ulcerative colitis: a re-appraisal of the mucosal granuloma in the distinction of Crohn's disease from ulcerative colitis. *Histopathology* 2002; **41**: 50-55 [PMID: 12121237 DOI: 10.1046/j.1365-2559.2002.01416.x]

33 **De Matos V**, Russo PA, Cohen AB, Mamula P, Baldassano RN, Piccoli DA. Frequency and clinical correlations of granulomas in children with Crohn disease. *J Pediatr Gastroenterol Nutr* 2008; **46**: 392-398 [PMID: 18367950 DOI: 10.1097/MPG.0b013e31812e95e1]

34 **Sonnenberg A**, Turner KO, Genta RM. Comorbid Occurrence of Eosinophilic Esophagitis and Inflammatory Bowel Disease. *Clin Gastroenterol Hepatol* 2021; **19**: 613-615. e1 [PMID: 32068153 DOI: 10.1016/j.cgh.2020.02.015]

35 **Rosendorff C**, Grieve NW. Ulcerative oesophagitis in association with ulcerative colitis. *Gut* 1967; **8**: 344-347 [PMID: 6039722 DOI: 10.1136/gut.8.4.344]

36 **Knudsen KB**, Sparberg M. Ulcerative esophagitis and ulcerative colitis. *JAMA* 1967; **201**: 140 [PMID: 6072961 DOI: 10.1001/jama.1967.03130020086028]

37 **Christopher NL**, Watson DW, Farber ER. Relationship of chronic ulcerative esophagitis to ulcerative colitis. *Ann Intern Med* 1969; **70**: 971-976 [PMID: 5769629 DOI: 10.7326/0003-4819-70-5-971]

38 **Zimmerman HM**, Rosenblum G, Bank S. Apthous ulcers of the esophagus in a patient with ulcerative colitis. *Gastrointest Endosc* 1984; **30**: 298-299 [PMID: 6489714 DOI: 10.1016/s0016-5107(84)72424-2]

39 **Izawa N**, Sugaya T, Kanamori A, Muraoka R, Hiraishi H. A case of ulcerative colitis with esophageal ulcer. *Prog Digest Endosc* 2015; **87**: 94-95

40 **Konishi H**, Fujino N. A case of right-sided ulcerative colitis associated with unusual esophageal ulcer. *Chiba Med Assoc Mag* 1998; **50**: 143-144

41 **Higashi Y**, Sunayama K. A case of ulcerative colitis combined with esophageal ulcer. *J Japan Surg Assoc* 2004; **65**: 143-146

42 **Ikeda Y**, Suzuki K. A case of ulcerative colitis associated with a hemorrhagic esophageal ulcer. *J Kushiro City Gen* 2000; **12**: 129-133

43 **Kuroki H**, Sugita A, Koganei K, Tatsumi K, Futatsuki R, Arai K. Two cases of esophageal ulcer after surgical treatment for ulcerative colitis. *Clin J Gastroenterol* 2020; **13**: 495-500 [PMID: 31863310 DOI: 10.1007/s12328-019-01082-7]

44 **Asakawa A**, Kojima Y, Fujii E, Ohtaka M, Shimazaki R, Sato T, Nakamura T, Morozumi A, Akahane Y, Fujino MA. Case of ulcerative colitis associated with oesophageal ulcer. *J Int Med Res* 2000; **28**: 191-196 [PMID: 11014327 DOI: 10.1177/147323000002800405]

45 **Sato N**, Kurakata H, Hirano N, Sakai K, Hamatani S. A case of ulcerative colitis combined with esophageal ulcer. *Prog Digest Endosc* 2004; **65**: 90-91

46 **Hisabe T**, Matsui T, Miyaoka M, Ninomiya K, Ishihara H, Nagahama T, Takaki Y, Hirai F, Ikeda K, Iwashita A, Higashi D, Futami K. Diagnosis and clinical course of ulcerative gastroduodenal lesion associated with ulcerative colitis: possible relationship with pouchitis. *Dig Endosc* 2010; **22**: 268-274 [PMID: 21175478 DOI: 10.1111/j.1443-1661.2010.01006.x]

47 **Greenstein AJ**, Janowitz HD, Sachar DB. The extra-intestinal complications of Crohn's disease and ulcerative colitis: a study of 700 patients. *Medicine (Baltimore)* 1976; **55**: 401-412 [PMID: 957999 DOI: 10.1097/00005792-197609000-00004]

48 **Ikeuchi H**, Hori K, Nishigami T, Nakano H, Uchino M, Nakamura M, Kaibe N, Noda M, Yanagi H, Yamamura T. Diffuse gastroduodenitis and pouchitis associated with ulcerative colitis. *World J Gastroenterol* 2006; **12**: 5913-5915 [PMID: 17007066 DOI: 10.3748/wjg.v12.i36.5913]

49 **Maeda K**, Okada M, Seo M, Aoyagi K, Sakisaka S. Evaluation of gastroduodenal mucosal lesions in patients with Crohn's disease and ulcerative colitis. *Digest Endosc* 2010; **16**: 199-203

50 **Uchino M**, Ikeuchi H, Bando T, Matsuoka H, Hirata A, Takahashi Y, Takesue Y, Inoue S, Tomita N. Diffuse gastroduodenitis and enteritis associated with ulcerative colitis and concomitant cytomegalovirus reactivation after total colectomy: report of a case. *Surg Today* 2013; **43**: 321-324 [PMID: 22965486 DOI: 10.1007/s00595-012-0329-z]

51 **Choi YS**, Kim JK, Kim WJ, Kim MJ. Remission of diffuse ulcerative duodenitis in a patient with ulcerative colitis after infliximab therapy: a case study and review of the literature. *Intest Res* 2019; **17**: 273-277 [PMID: 30739436 DOI: 10.5217/ir.2018.00122]

52 **Yang Y**, Li CQ, Chen WJ, Ma ZH, Liu G. Gastroduodenitis associated with ulcerative colitis: A case report. *World J Clin Cases* 2020; **8**: 3847-3852 [PMID: 32953863 DOI: 10.12998/wjcc.v8.i17.3847]

53 **Li M**, Liu Y, Cui J, Qin H, Shi Y, Zhang S, Zhao Y. Ulcerative colitis with mucosal lesions in

duodenum: Two case reports. *Medicine (Baltimore)* 2019; **98**: e15035 [PMID: 30946342 DOI: 10.1097/MD.0000000000015035]

54 **García Gavilán MDC**, López Vega MC, Sánchez IM. Ulcerative colitis with gastric and duodenal involvement. *Rev Esp Enferm Dig* 2017; **109**: 535-536 [PMID: 28530110 DOI: 10.17235/reed.2017.4685/2016]

55 **Kaufman SS**, Vanderhoof JA, Young R, Perry D, Raynor SC, Mack DR. Gastroenteric inflammation in children with ulcerative colitis. *Am J Gastroenterol* 1997; **92**: 1209-1212 [PMID: 9219802]

56 **Annese V**, Caruso N, Bisceglia M, Lombardi G, Clemente R, Modola G, Tardio B, Villani MR, Andriulli A. Fatal ulcerative panenteritis following colectomy in a patient with ulcerative colitis. *Dig Dis Sci* 1999; **44**: 1189-1195 [PMID: 10389695 DOI: 10.1023/a:1026688526551]

57 **Sonnenberg A**, Melton SD, Genta RM. Frequent occurrence of gastritis and duodenitis in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2011; **17**: 39-44 [PMID: 20848539 DOI: 10.1002/ibd.21356]

58 **Lin J**, McKenna BJ, Appelman HD. Morphologic findings in upper gastrointestinal biopsies of patients with ulcerative colitis: a controlled study. *Am J Surg Pathol* 2010; **34**: 1672-1677 [PMID: 20962621 DOI: 10.1097/PAS.0b013e3181f3de93]

59 **Shen B**, Wu H, Remzi F, Lopez R, Shen L, Fazio V. Diagnostic value of esophagogastroduodenoscopy in patients with ileal pouch-anal anastomosis. *Inflamm Bowel Dis* 2009; **15**: 395-401 [PMID: 18972552 DOI: 10.1002/ibd.20771]

60 **Hoentjen F**, Hanauer SB, Hart J, Rubin DT. Long-term treatment of patients with a history of ulcerative colitis who develop gastritis and pan-enteritis after colectomy. *J Clin Gastroenterol* 2013; **47**: 52-57 [PMID: 22858512 DOI: 10.1097/MCG.0b013e3182582c1d]

61 **Sullivan KJ**, Wei M, Chernetsova E, Hallani S, de Nanassy J, Benchimol EI, Mack DR, Nasr A, El Demellawy D. Value of upper endoscopic biopsies in predicting medical refractoriness in pediatric patients with ulcerative colitis. *Hum Pathol* 2017; **66**: 167-176 [PMID: 28652148 DOI: 10.1016/j.humpath.2017.06.006]

62 **Horjus Talabur Horje CS**, Meijer J, Rovers L, van Lochem EG, Groenen MJ, Wahab PJ. Prevalence of Upper Gastrointestinal Lesions at Primary Diagnosis in Adults with Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 1896-1901 [PMID: 27057685 DOI: 10.1097/MIB.0000000000000786]

63 **Putra J**, Ornvold K. Focally enhanced gastritis in children with inflammatory bowel disease: a clinicopathological correlation. *Pathology* 2017; **49**: 808-810 [PMID: 29078999 DOI: 10.1016/j.pathol.2017.06.007]

64 **Parente F**, Cucino C, Bollani S, Imbesi V, Maconi G, Bonetto S, Vago L, Bianchi Porro G. Focal gastric inflammatory infiltrates in inflammatory bowel diseases: prevalence, immunohistochemical characteristics, and diagnostic role. *Am J Gastroenterol* 2000; 95: 705-711 [PMID: 10710061 DOI: 10.1111/j.1572-0241.2000.01851.x]

65 **Sharif F**, McDermott M, Dillon M, Drumm B, Rowland M, Imrie C, Kelleher S, Harty S, Bourke B. Focally enhanced gastritis in children with Crohn's disease and ulcerative colitis. *Am J Gastroenterol* 2002; **97**: 1415-1420 [PMID: 12094859 DOI: 10.1111/j.1572-0241.2002.05785.x]

66 **Hong CH**, Park DI, Choi WH, Park JH, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI, Kim DH, Kim MK, Chae SW, Lee KB, Sohn JH, Oh SJ. [The clinical usefulness of focally enhanced gastritis in Korean patients with Crohn's disease]. *Korean J Gastroenterol* 2009; **53**: 23-28 [PMID: 19158467]

67 **Roka K**, Roma E, Stefanaki K, Panayotou I, Kopsidas G, Chouliaras G. The value of focally enhanced gastritis in the diagnosis of pediatric inflammatory bowel diseases. *J Crohns Colitis* 2013; **7**: 797-802 [PMID: 23207168 DOI: 10.1016/j.crohns.2012.11.003]

68 **Hummel TZ**, Kate FT, Kindermann A, Benninga MA. 547 The Role of Endoscopy of the Upper Gastrointestinal Tract in the Diagnostic Assessment of Childhood Inflammatory Bowel Disease. *Gastroenterology* 2010; **138** [DOI: 10.1016/S0016-5085(10)60342-3]

69 **Choi WT**, Lauwers GY. Patterns of Gastric Injury: Beyond Helicobacter Pylori. *Surg Pathol Clin* 2017; **10**: 801-822 [PMID: 29103534 DOI: 10.1016/j.path.2017.07.003]

70 **McHugh JB**, Gopal P, Greenson JK. The clinical significance of focally enhanced gastritis in children. *Am J Surg Pathol* 2013; **37**: 295-299 [PMID: 23108022 DOI: 10.1097/PAS.0b013e31826b2a94]

71 **Petrolla AA**, Katz JA, Xin W. The clinical significance of focal enhanced gastritis in adults with isolated ileitis of the terminal ileum. *J Gastroenterol* 2008; **43**: 524-530 [PMID: 18648739 DOI: 10.1007/s00535-008-2191-8]

72 **Xin W**, Greenson JK. The clinical significance of focally enhanced gastritis. *Am J Surg Pathol* 2004; **28**: 1347-1351 [PMID: 15371951 DOI: 10.1097/01.pas.0000138182.97366.b4]

73 **Hendrickson BA**. Gastric inflammation as a feature of ulcerative colitis. *J Pediatr Gastroenterol Nutr* 2003; **37**: 228-229 [PMID: 12960641 DOI: 10.1097/00005176-200309000-00005]

74 **Thompson JW 3rd**, Bargen JA. Ulcerative duodenitis and chronic ulcerative colitis: report of two cases. *Gastroenterology* 1960; **38**: 452-455 [PMID: 13838088]

75 **Endo K**, Kuroha M, Shiga H, Kakuta Y, Takahashi S, Kinouchi Y, Shimosegawa T. Two cases of diffuse duodenitis associated with ulcerative colitis. *Case Rep Gastrointest Med* 2012; **2012**: 396521 [PMID: 23119193 DOI: 10.1155/2012/396521]

76 **Mitomi H**, Atari E, Uesugi H, Nishiyama Y, Igarashi M, Arai N, Ihara A, Okayasu I. Distinctive

diffuse duodenitis associated with ulcerative colitis. *Dig Dis Sci* 1997; **42**: 684-693 [PMID: 9073157 DOI: 10.1023/a:1018836218391]

77 **Saito K**, Katsuno T, Nakagawa T, Minemura S, Oyamada A, Kanogawa N, Saito M, Yoshihama S, Maruoka D, Matsumura T, Arai M, Tohma T, Miyauchi H, Matsubara H, Yokosuka O. Severe diffuse duodenitis successfully treated with intravenous tacrolimus after colectomy for ulcerative colitis. *Intern Med* 2014; **53**: 2477-2481 [PMID: 25366006 DOI: 10.2169/internalmedicine.53.1910]

78 **Valdez R**, Appelman HD, Bronner MP, Greenson JK. Diffuse duodenitis associated with ulcerative colitis. *Am J Surg Pathol* 2000; **24**: 1407-1413 [PMID: 11023103 DOI: 10.1097/00000478-200010000-00011]

79 **Terashima S**, Hoshino Y, Kanzaki N, Kogure M, Gotoh M. Ulcerative duodenitis accompanying ulcerative colitis. *J Clin Gastroenterol* 2001; **32**: 172-175 [PMID: 11205658 DOI: 10.1097/00004836-200102000-00018]

80 **Kawai K**, Watanabe T, Nakayama H, Roberts-Thomson I, Nagawa H. Images of interest. Gastrointestinal: small bowel inflammation and ulcerative colitis. *J Gastroenterol Hepatol* 2005; **20**: 1791 [PMID: 16246204 DOI: 10.1111/j.1440-1746.2005.04162.x]

81 **Akitake R**, Nakase H, Tamaoki M, Ueno S, Mikami S, Chiba T. Modulation of Th1/Th2 balance by infliximab rescues postoperative occurrence of small-intestinal inflammation associated with ulcerative colitis. *Dig Dis Sci* 2010; **55**: 1781-1784 [PMID: 19672713 DOI: 10.1007/s10620-009-0910-5]

82 **Chiba M**, Ono I, Wakamatsu H, Wada I, Suzuki K. Diffuse gastroduodenitis associated with ulcerative colitis: treatment by infliximab. *Dig Endosc* 2013; **25**: 622-625 [PMID: 24164601 DOI: 10.1111/j.1443-1661.2012.01398.x]

83 **Willington AJ**, Taylor G, White J, Gearry RB. Gastrointestinal: Ulcerative colitis-associated duodenitis. *J Gastroenterol Hepatol* 2018; **33**: 973 [PMID: 29659081 DOI: 10.1111/jgh.13992]

84 **Patterson ER**, Shmidt E, Oxentenko AS, Enders FT, Smyrk TC. Normal villous architecture with increased intraepithelial lymphocytes: a duodenal manifestation of Crohn disease. *Am J Clin Pathol* 2015; **143**: 445-450 [PMID: 25696804 DOI: 10.1309/AJCPBKQND4SHVX9Q]

85 **Vidali F**, Di Sabatino A, Broglia F, Cazzola P, Biancheri P, Viera FT, Vanoli A, Alvisi C, Perego M, Corazza GR. Increased CD8+ intraepithelial lymphocyte infiltration and reduced surface area to volume ratio in the duodenum of patients with ulcerative colitis. *Scand J Gastroenterol* 2010; **45**: 684-689 [PMID: 20201621 DOI: 10.3109/00365521003663662]

86 **Porter RJ**, Kalla R, Ho GT. Ulcerative colitis: Recent advances in the understanding of disease pathogenesis. *F1000Res* 2020; **9** [PMID: 32399194 DOI: 10.12688/f1000research.20805.1]

87 **Ungaro R**, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis. *Lancet* 2017; **389**: 1756-1770 [PMID: 27914657 DOI: 10.1016/S0140-6736(16)32126-2]

88 **Kirsner JB**. Historical aspects of inflammatory bowel disease. *J Clin Gastroenterol* 1988; **10**: 286-297 [PMID: 2980764 DOI: 10.1097/00004836-198806000-00012]

89 **Kaplan GG**, Ng SC. Understanding and Preventing the Global Increase of Inflammatory Bowel Disease. *Gastroenterology* 2017; **152**: 313-321. e2 [PMID: 27793607 DOI: 10.1053/j.gastro.2016.10.020]

90 **Ananthakrishnan AN**. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 205-217 [PMID: 25732745 DOI: 10.1038/nrgastro.2015.34]

91 **Mahid SS**, Minor KS, Soto RE, Hornung CA, Galandiuk S. Smoking and inflammatory bowel disease: a meta-analysis. *Mayo Clin Proc* 2006; **81**: 1462-1471 [PMID: 17120402 DOI: 10.4065/81.11.1462]

92 **Odes HS**, Fich A, Reif S, Halak A, Lavy A, Keter D, Eliakim R, Paz J, Broide E, Niv Y, Ron Y, Villa Y, Arber N, Gilat T. Effects of current cigarette smoking on clinical course of Crohn's disease and ulcerative colitis. *Dig Dis Sci* 2001; **46**: 1717-1721 [PMID: 11508673 DOI: 10.1023/a:1010609722315]

93 **Sahami S**, Kooij IA, Meijer SL, Van den Brink GR, Buskens CJ, Te Velde AA. The Link between the Appendix and Ulcerative Colitis: Clinical Relevance and Potential Immunological Mechanisms. *Am J Gastroenterol* 2016; **111**: 163-169 [PMID: 26416189 DOI: 10.1038/ajg.2015.301]

94 **Liu JZ**, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, Ripke S, Lee JC, Jostins L, Shah T, Abedian S, Cheon JH, Cho J, Dayani NE, Franke L, Fuyuno Y, Hart A, Juyal RC, Juyal G, Kim WH, Morris AP, Poustchi H, Newman WG, Midha V, Orchard TR, Vahedi H, Sood A, Sung JY, Malekzadeh R, Westra HJ, Yamazaki K, Yang SK; International Multiple Sclerosis Genetics Consortium; International IBD Genetics Consortium, Barrett JC, Alizadeh BZ, Parkes M, Bk T, Daly MJ, Kubo M, Anderson CA, Weersma RK. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet* 2015; **47**: 979-986 [PMID: 26192919 DOI: 10.1038/ng.3359]

95 **Festen EA**, Goyette P, Scott R, Annese V, Zhernakova A, Lian J, Lefèbvre C, Brant SR, Cho JH, Silverberg MS, Taylor KD, de Jong DJ, Stokkers PC, Mcgovern D, Palmieri O, Achkar JP, Xavier RJ, Daly MJ, Duerr RH, Wijmenga C, Weersma RK, Rioux JD. Genetic variants in the region harbouring IL2/IL21 associated with ulcerative colitis. *Gut* 2009; **58**: 799-804 [PMID: 19201773 DOI: 10.1136/gut.2008.166918]

96 **Cleynen I**, Boucher G, Jostins L, Schumm LP, Zeissig S, Ahmad T, Andersen V, Andrews JM, Annese V, Brand S, Brant SR, Cho JH, Daly MJ, Dubinsky M, Duerr RH, Ferguson LR, Franke A, Gearry RB, Goyette P, Hakonarson H, Halfvarson J, Hov JR, Huang H, Kennedy NA, Kupcinskas L,

Lawrance IC, Lee JC, Satsangi J, Schreiber S, Théâtre E, van der Meulen-de Jong AE, Weersma RK, Wilson DC; International Inflammatory Bowel Disease Genetics Consortium, Parkes M, Vermeire S, Rioux JD, Mansfield J, Silverberg MS, Radford-Smith G, McGovern DP, Barrett JC, Lees CW. Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: a genetic association study. *Lancet* 2016; **387**: 156-167 [PMID: 26490195 DOI: 10.1016/S0140-6736(15)00465-1]

97 **Sugimura K**, Asakura H, Mizuki N, Inoue M, Hibi T, Yagita A, Tsuji K, Inoko H. Analysis of genes within the HLA region affecting susceptibility to ulcerative colitis. *Hum Immunol* 1993; **36**: 112-118 [PMID: 8096500 DOI: 10.1016/0198-8859(93)90113-f]

98 **Luo Y**, de Lange KM, Jostins L, Moutsianas L, Randall J, Kennedy NA, Lamb CA, McCarthy S, Ahmad T, Edwards C, Serra EG, Hart A, Hawkey C, Mansfield JC, Mowat C, Newman WG, Nichols S, Pollard M, Satsangi J, Simmons A, Tremelling M, Uhlig H, Wilson DC, Lee JC, Prescott NJ, Lees CW, Mathew CG, Parkes M, Barrett JC, Anderson CA. Exploring the genetic architecture of inflammatory bowel disease by whole-genome sequencing identifies association at ADCY7. *Nat Genet* 2017; **49**: 186-192 [PMID: 28067910 DOI: 10.1038/ng.3761]

99 **Lee HS**, Cleynen I. Molecular Profiling of Inflammatory Bowel Disease: Is It Ready for Use in Clinical Decision-Making? *Cells* 2019; **8** [PMID: 31167397 DOI: 10.3390/cells8060535]

100 **Paramsothy S**, Kamm MA, Kaakoush NO, Walsh AJ, van den Bogaerde J, Samuel D, Leong RWL, Connor S, Ng W, Paramsothy R, Xuan W, Lin E, Mitchell HM, Borody TJ. Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet* 2017; **389**: 1218-1228 [PMID: 28214091 DOI: 10.1016/S0140-6736(17)30182-4]

101 **Guo XY**, Liu XJ, Hao JY. Gut microbiota in ulcerative colitis: insights on pathogenesis and treatment. *J Dig Dis* 2020; **21**: 147-159 [PMID: 32040250 DOI: 10.1111/1751-2980.12849]

102 **Sha S**, Liang J, Chen M, Xu B, Liang C, Wei N, Wu K. Systematic review: faecal microbiota transplantation therapy for digestive and nondigestive disorders in adults and children. *Aliment Pharmacol Ther* 2014; **39**: 1003-1032 [PMID: 24641570 DOI: 10.1111/apt.12699]

103 **Moayyedi P**, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, Armstrong D, Marshall JK, Kassam Z, Reinisch W, Lee CH. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. *Gastroenterology* 2015; **149**: 102-109. e6 [PMID: 25857665 DOI: 10.1053/j.gastro.2015.04.001]

104 **Rossen NG**, Fuentes S, van der Spek MJ, Tijssen JG, Hartman JH, Duflou A, Löwenberg M, van den Brink GR, Mathus-Vliegen EM, de Vos WM, Zoetendal EG, D'Haens GR, Ponsioen CY. Findings From a Randomized Controlled Trial of Fecal Transplantation for Patients With Ulcerative Colitis. *Gastroenterology* 2015; **149**: 110-118. e4 [PMID: 25836986 DOI: 10.1053/j.gastro.2015.03.045]

105 **Costello SP**, Hughes PA, Waters O, Bryant RV, Vincent AD, Blatchford P, Katsikeros R, Makanyanga J, Campaniello MA, Mavrangelos C, Rosewarne CP, Bickley C, Peters C, Schoeman MN, Conlon MA, Roberts-Thomson IC, Andrews JM. Effect of Fecal Microbiota Transplantation on 8-Week Remission in Patients With Ulcerative Colitis: A Randomized Clinical Trial. *JAMA* 2019; **321**: 156-164 [PMID: 30644982 DOI: 10.1001/jama.2018.20046]

106 **Choy MC**, Visvanathan K, De Cruz P. An Overview of the Innate and Adaptive Immune System in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2017; **23**: 2-13 [PMID: 27779499 DOI: 10.1097/MIB.0000000000000955]

107 **Geremia A**, Biancheri P, Allan P, Corazza GR, Di Sabatino A. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmun Rev* 2014; **13**: 3-10 [PMID: 23774107 DOI: 10.1016/j.autrev.2013.06.004]

108 **Buzza MS**, Johnson TA, Conway GD, Martin EW, Mukhopadhyay S, Shea-Donohue T, Antalis TM. Inflammatory cytokines down-regulate the barrier-protective prostasin-matriptase proteolytic cascade early in experimental colitis. *J Biol Chem* 2017; **292**: 10801-10812 [PMID: 28490634 DOI: 10.1074/jbc.M116.771469]

109 **Di Sabatino A**, Biancheri P, Rovedatti L, MacDonald TT, Corazza GR. New pathogenic paradigms in inflammatory bowel disease. *Inflamm Bowel Dis* 2012; **18**: 368-371 [PMID: 21538717 DOI: 10.1002/ibd.21735]

110 **Sands BE**. Inflammatory bowel disease: past, present, and future. *J Gastroenterol* 2007; **42**: 16-25 [PMID: 17322989 DOI: 10.1007/s00535-006-1995-7]

111 **Honma J**, Mitomi H, Murakami K, Igarashi M, Saigenji K, Toyama K. Nodular duodenitis involving CD8+ cell infiltration in patients with ulcerative colitis. *Hepatogastroenterology* 2001; **48**: 1604-1610 [PMID: 11813583]

112 **Berrebi D**, Languepin J, Ferkdadji L, Foussat A, De Lagausie P, Paris R, Emilie D, Mougenot JF, Cezard JP, Navarro J, Peuchmaur M. Cytokines, chemokine receptors, and homing molecule distribution in the rectum and stomach of pediatric patients with ulcerative colitis. *J Pediatr Gastroenterol Nutr* 2003; **37**: 300-308 [PMID: 12960653 DOI: 10.1097/00005176-200309000-00018]

113 **Matsumura M**, Matsui T, Hatakeyama S, Matake H, Uno H, Sakurai T, Yao T, Oishi T, Iwashita A, Fujioka T. Prevalence of Helicobacter pylori infection and correlation between severity of upper gastrointestinal lesions and H. pylori infection in Japanese patients with Crohn's disease. *J Gastroenterol* 2001; **36**: 740-747 [PMID: 11757745 DOI: 10.1007/s005350170015]

114 **Ogino S**, Lochhead P, Chan AT, Nishihara R, Cho E, Wolpin BM, Meyerhardt JA, Meissner A, Schernhammer ES, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of epigenetics: emerging integrative science to analyze environment, host, and disease. *Mod Pathol* 2013; **26**: 465-

484 [PMID: 23307060 DOI: 10.1038/modpathol.2012.214]
115 **Ogino S**, Stampfer M. Lifestyle factors and microsatellite instability in colorectal cancer: the evolving field of molecular pathological epidemiology. *J Natl Cancer Inst* 2010; **102**: 365-367 [PMID: 20208016 DOI: 10.1093/jnci/djq031]
116 **Ogino S**, Galon J, Fuchs CS, Dranoff G. Cancer immunology--analysis of host and tumor factors for personalized medicine. *Nat Rev Clin Oncol* 2011; **8**: 711-719 [PMID: 21826083 DOI: 10.1038/nrclinonc.2011.122]
117 **Ogino S**, Chan AT, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. *Gut* 2011; **60**: 397-411 [PMID: 21036793 DOI: 10.1136/gut.2010.217182]
118 **Ogino S**, King EE, Beck AH, Sherman ME, Milner DA, Giovannucci E. Interdisciplinary education to integrate pathology and epidemiology: towards molecular and population-level health science. *Am J Epidemiol* 2012; **176**: 659-667 [PMID: 22935517 DOI: 10.1093/aje/kws226]
119 **Ogino S**, Nishihara R, VanderWeele TJ, Wang M, Nishi A, Lochhead P, Qian ZR, Zhang X, Wu K, Nan H, Yoshida K, Milner DA Jr, Chan AT, Field AE, Camargo CA Jr, Williams MA, Giovannucci EL. Review Article: The Role of Molecular Pathological Epidemiology in the Study of Neoplastic and Non-neoplastic Diseases in the Era of Precision Medicine. *Epidemiology* 2016; **27**: 602-611 [PMID: 26928707 DOI: 10.1097/EDE.0000000000000471]
120 **Ghosh S**, Matsuoka Y, Asai Y, Hsin KY, Kitano H. Software for systems biology: from tools to integrated platforms. *Nat Rev Genet* 2011; **12**: 821-832 [PMID: 22048662 DOI: 10.1038/nrg3096]
121 **Papp B**, Notebaart RA, Pál C. Systems-biology approaches for predicting genomic evolution. *Nat Rev Genet* 2011; **12**: 591-602 [PMID: 21808261 DOI: 10.1038/nrg3033]
122 **Hamada T**, Keum N, Nishihara R, Ogino S. Molecular pathological epidemiology: new developing frontiers of big data science to study etiologies and pathogenesis. *J Gastroenterol* 2017; **52**: 265-275 [PMID: 27738762 DOI: 10.1007/s00535-016-1272-3]
123 **Ogino S**, Nowak JA, Hamada T, Phipps AI, Peters U, Milner DA Jr, Giovannucci EL, Nishihara R, Giannakis M, Garrett WS, Song M. Integrative analysis of exogenous, endogenous, tumour and immune factors for precision medicine. *Gut* 2018; **67**: 1168-1180 [PMID: 29437869 DOI: 10.1136/gutjnl-2017-315537]
124 **Stein RA**. Epigenetics--the link between infectious diseases and cancer. *JAMA* 2011; **305**: 1484-1485 [PMID: 21486982 DOI: 10.1001/jama.2011.446]
125 **Curtin K**, Slattery ML, Samowitz WS. CpG island methylation in colorectal cancer: past, present and future. *Patholog Res Int* 2011; **2011**: 902674 [PMID: 21559209 DOI: 10.4061/2011/902674]
126 **Kosumi K**, Mima K, Baba H, Ogino S. Dysbiosis of the gut microbiota and colorectal cancer: the key target of molecular pathological epidemiology. *J Lab Precis Med* 2018; **3** [PMID: 30345420 DOI: 10.21037/jlpm.2018.09.05]
127 **Hughes LAE**, Simons CCJM, van den Brandt PA, van Engeland M, Weijenberg MP. Lifestyle, Diet, and Colorectal Cancer Risk According to (Epi)genetic Instability: Current Evidence and Future Directions of Molecular Pathological Epidemiology. *Curr Colorectal Cancer Rep* 2017; **13**: 455-469 [PMID: 29249914 DOI: 10.1007/s11888-017-0395-0]
128 **Lochhead P**, Chan AT, Giovannucci E, Fuchs CS, Wu K, Nishihara R, O'Brien M, Ogino S. Progress and opportunities in molecular pathological epidemiology of colorectal premalignant lesions. *Am J Gastroenterol* 2014; **109**: 1205-1214 [PMID: 24935274 DOI: 10.1038/ajg.2014.153]
129 **Li W**, Qiu T, Ling Y, Guo L, Li L, Ying J. Molecular pathological epidemiology of colorectal cancer in Chinese patients with KRAS and BRAF mutations. *Oncotarget* 2015; **6**: 39607-39613 [PMID: 26530529 DOI: 10.18632/oncotarget.5551]
130 **Yang H**, Rotter JI, Toyoda H, Landers C, Tyran D, McElree CK, Targan SR. Ulcerative colitis: a genetically heterogeneous disorder defined by genetic (HLA class II) and subclinical (antineutrophil cytoplasmic antibodies) markers. *J Clin Invest* 1993; **92**: 1080-1084 [PMID: 8349790 DOI: 10.1172/jci116613]
131 **Targan SR**, Karp LC. Defects in mucosal immunity leading to ulcerative colitis. *Immunol Rev* 2005; **206**: 296-305 [PMID: 16048556 DOI: 10.1111/j.0105-2896.2005.00286.x]
132 **Corporaal S**, Karrenbeld A, van der Linde K, Voskuil JH, Kleibeuker JH, Dijkstra G. Diffuse enteritis after colectomy for ulcerative colitis: two case reports and review of the literature. *Eur J Gastroenterol Hepatol* 2009; **21**: 710-715 [PMID: 19282770 DOI: 10.1097/MEG.0b013e32831bc400]
133 **Queliza K**, Ihekweazu FD, Schady D, Jensen C, Kellermayer R. Granulomatous Upper Gastrointestinal Inflammation in Pediatric Ulcerative Colitis. *J Pediatr Gastroenterol Nutr* 2018; **66**: 620-623 [PMID: 28991840 DOI: 10.1097/MPG.0000000000001771]
134 **Rush B**, Berger L, Rosenfeld G, Bressler B. Tacrolimus Therapy for Ulcerative Colitis-Associated Post-Colectomy Enteritis. *ACG Case Rep J* 2014; **2**: 33-35 [PMID: 26157899 DOI: 10.14309/crj.2014.76]

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REVIEW

Application of artificial intelligence-driven endoscopic screening and diagnosis of gastric cancer

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Abstract

The landscape of gastrointestinal endoscopy continues to evolve as new technologies and techniques become available. The advent of image-enhanced and magnifying endoscopies has highlighted the step toward perfecting endoscopic screening and diagnosis of gastric lesions. Simultaneously, with the development of convolutional neural network, artificial intelligence (AI) has made unprecedented breakthroughs in medical imaging, including the ongoing trials of computer-aided detection of colorectal polyps and gastrointestinal bleeding. In the past demi-decade, applications of AI systems in gastric cancer have also emerged. With AI's efficient computational power and learning capacities, endoscopists can improve their diagnostic accuracies and avoid the missing or mischaracterization of gastric neoplastic changes. So far, several AI systems that incorporated both traditional and novel endoscopy technologies have been developed for various purposes, with most systems achieving an accuracy of more than 80%. However, their feasibility, effectiveness, and safety in clinical practice remain to be seen as there have been no clinical trials yet. Nonetheless, AI-assisted endoscopies shed light on more accurate and sensitive ways for early detection, treatment guidance and prognosis prediction of gastric lesions. This review summarizes the current status of various AI applications in gastric cancer and pinpoints directions for future research and clinical practice implementation from a clinical perspective.

Key Words: Artificial intelligence; Diagnostic; Therapeutic; Endoscopy; Gastric cancer; Gastritis

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Core Tip: Artificial intelligence-assisted endoscopy can assist physicians in the screening and diagnosis of gastric cancer. Most of the systems developed so far, applied in images and videos and using white light imaging and narrow-band imaging endoscopies, have achieved accuracies and sensitivities of at least 80%. However, the efficacy of artificial intelligence applications in gastric cancer depends on its intended role in clinical practice, and there have not been any attempts of clinical trials yet. This review summarizes the existing artificial intelligence applications in gastric cancer and pinpoints future research directions for their clinical practice implementation.

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INTRODUCTION

Diagnostic and therapeutic endoscopies play a major role in the management of gastric cancer (GC). Endoscopy is the mainstay for the diagnosis and treatment of early adenocarcinoma and lesions and the palliation of advanced cancer[1-5]. GC, being the fifth most common cancer and the third leading cause of cancer-related deaths worldwide, affects more than one million people and causes approximately 780000 deaths annually[6-10]. Continued development in endoscopy aims to strengthen its quality indicators. These developments include using higher resolution and magnification endoscopies, chromoendoscopy and optical techniques based on the modulation of the light source, such as narrow-band imaging (NBI), fluorescence endoscopy and elastic scattering spectroscopy[11-13]. New tissue sampling methods to identify the stages of a patient's risk for cancer are also being developed to decrease the burden on patients and clinicians during endoscopy.

Statistically, the relative 5-year survival rate of GC is less than 40%[7,9,14,15], often attributed to the late onset of symptoms and delayed diagnosis[10]. Although early diagnosis is difficult as most patients are asymptomatic in the early stage, the diagnosis point largely determines the patient's prognosis[16,17]. In other words, endoscopic detection of GC at an earlier stage is the only and most effective way to reduce its recurrence and to prolong patient survival. This early diagnosis of GC provides the opportunity for minimally invasive therapy methods such as endoscopic mucosal resection or submucosal dissection[18-20]. The 5-year survival rate was reported to be more than 90% among patients with GC detected at an early stage[21-23]. Yet, the false negative rate of GC detected by esophagogastroduodenoscopy, the current standard diagnostic procedure, was reported to be between 4.6% and 25.8% [24-29]. In terms of the common diagnostic methods, esophagogastroduodenoscopy is the preferred diagnostic modality for patients with suspected GC; the combination of lymph node dissection, endoscopic ultrasonography and computed tomographic scanning is involved in staging the tumor[30,31]. From the differential diagnosis between GC and gastritis, prediction of the horizontal extent of GC to characterizing the depth of invasion of GC, the early abnormal symptoms of GC and its advanced aggressive malignancy as well as the heavy workload of image analysis present ample inevitable challenges for endoscopists[32-34]. With large variations in the diagnostic ability of endoscopists, long-term training and experience may not guarantee their consistency and accuracy of diagnosis[35-37].

In recent years, artificial intelligence (AI) has caught considerable attention in various medical fields, including skin cancer classification[38-41], diagnosis in radiation oncology[42-45] and analysis of brain magnetic resonance imaging[46-49]. Although its applications have shown impressive accuracy and sensitivity identifying and characterizing imaging abnormalities, its improved sensitivity also meant the detection of subtle and indeterminately significant changes[50,51]. For example, in the analysis of brain magnetic resonance imaging, despite the promise of early diagnosis with machine learning, the relationship between subtle parenchymal brain alterations detected by AI and its neurological outcomes is unknown in the absence of a well-defined abnormality[52]. In other words, the use of AI in diagnostic imaging in various medical fields is continuously undergoing extensive evaluation.

In the field of gastroenterology, AI applications in capsule endoscopy[53-56] and in the detection, localization and segmentation of colonic polyps have been reported as well[57-59]. In particular, in the late 2010s, there was an explosion of interest in GC. The use of AI has proven to provide better diagnostic capabilities, although further validation and extensions are necessary to augment their quality and interpretability. An AI system's quality is often described with statistical measures of sensitivity, specificity, positive predictive value and accuracy.

Among the different AI models, the convolutional neural network (CNN) is a method most commonly used in medical imaging[60,61] as it allows the detection, segmentation and classification of image patterns[62] (Figure 1). CNN uses the mathematical operation of convolution to classify the images after recognizing patterns from the raw image pixel. Because the 7-layer Le-Net-5 program was first pioneered by LeCun *et al* in 1998, CNN architectures have been rapidly developing. Today, other widely-used CNNs include AlexNet (2012) with about 15.3% error rate, 22-layer GoogLeNet (2014) with a 6.67% error rate but only 4 million parameters, 19-layer visual geometry group (VGG) Net (2014) with 7.3% error rate and 138 million parameters, and Microsoft's ResNet (2015) with an error rate of 3.6% that can be trained with as many as 152 layers[63-65]. While scholars have lauded AI for the potential and performance it has displayed, some have cast doubts on its generalizability and role in the holistic assessment of gastric abnormalities.

In the beginning of an AI-assisted diagnostic imaging revolution, we have to anticipate and meticulously assess the potential perils, in the context of its capabilities, to ensure effective and safe incorporation into clinical practice[66]. In this paper, we thereby review the current status of AI applications in screening and diagnosing GC. We explore with emphasis on two broad categories: namely, the identification of pathogenic infection and the qualitative diagnosis of GC. Finally, we considered some directions for further research and the future of its introduction into clinical practice.

IDENTIFICATION OF *HELICOBACTER PYLORI* INFECTION

AI applications in identifying pathogenic infections have been widely explored[67] (Table 1). Gastric epithelium *Helicobacter pylori* (*H. pylori*) infection is associated with

Table 1 Summary of artificial intelligence applications in predicting *Helicobacter pylori* infection

Ref.	Endoscopic modality	Training dataset	Validation dataset	Accuracy	Sensitivity	Specificity	PPV
Huang *et al*[78], 2004	WLI	30 patients	74 patients	85.1 (avg)[1]	78.8 (avg)	90.2 (avg)	-
Shichijo *et al*[79], 2017	WLI	32208 images, 1768 patients	11481 images, 397 patients	87.7	88.9	87.4	-
Itoh *et al*[81], 2018	WLI	149 images, 139 patients	30 images, 30 patients	-	86.7	86.7	-
Nakashima *et al* [84], 2018	WLI, BLI and LCI	162 patients	60 patients	-	96.7	-	-
Shichijo *et al*[80], 2019	WLI	98564 images, 4494 patients	23699 images, 847 patients	Infected: 66.0; post-eradication: 86.0	-	-	-
Zheng *et al*[82], 2019	WLI	11729 images, 1507 patients	3755 images, 452 patients	84.5	81.4	90.1	-
Zhu *et al*[100], 2019	WLI	790 images	203 images	89.2	76.5	95.6	89.7
Nakashima *et al* [85], 2020	WLI, BLI and LCI	12887 images, 395 patients	120 patients	80.0 (avg)[2]	61.3 (avg)	89.4 (avg)	74.7 (avg)

[1]Histological characteristics were assessed for the various antrum, body and cardia locations.
[2]White light imaging and linked color imaging-based images were both analyzed, with the linked color imaging obtaining significantly higher accuracy, sensitivity, specificity and positive predictive value. BLI: Blue laser imaging; LCI: Linked color imaging; PPV: Positive predictive value; WLI: White light imaging.

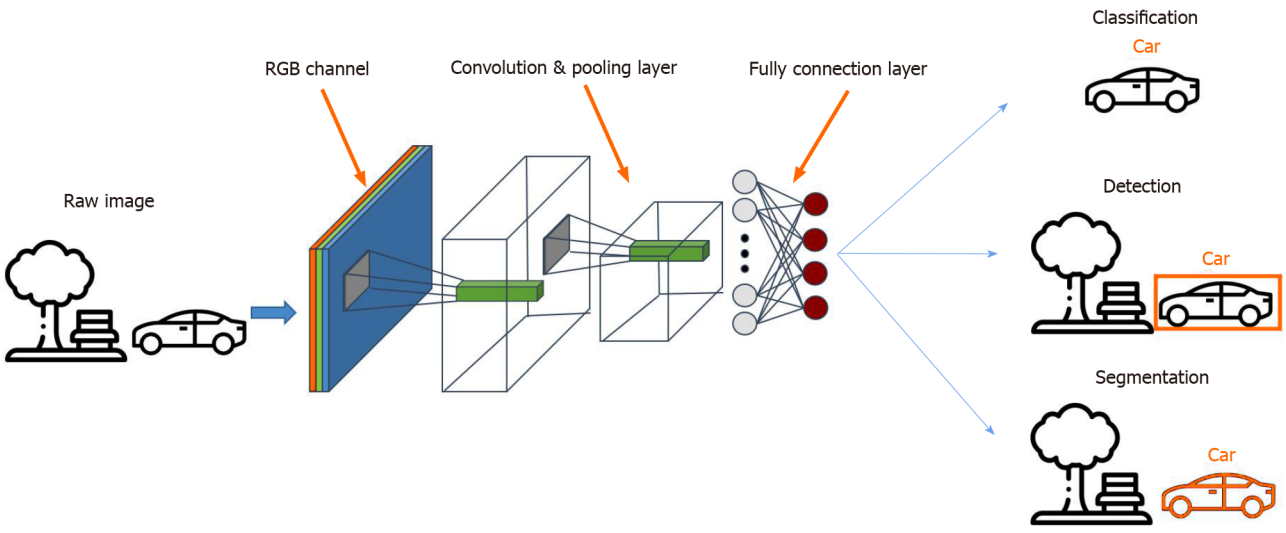


Figure 1 The convolutional neural network model. A convolutional neural network consists of an input layer, a few hidden layers and an output layer. It is commonly applied in medical imaging through the detection, segmentation and classification of image patterns.

functional dyspepsia, peptic ulcers, mucosal atrophy, intestinal metaplasia, atrophic gastritis and GC[68,69]. Because most gastric malignancies correlate with *H. pylori* infection, identifying *H. pylori* infection at its early stage is essential in preventing *H. pylori*-aggravated comorbidities[70-74]. Although physicians usually use the C13 urea breath test to diagnose *H. pylori*, most subclinical *H. pylori* infection cases still rely on the time-consuming and invasive biopsy examination to avoid the risk of a false negative diagnosis. Moreover, the Kyoto Classification, as the current gold standard of *H. pylori* severity classification, requires examiners to measure lesions by their bare eyes. Such a method is a subjective judgment that usually comes with interoperator bias[75-77]. Compelled by such ambiguity, researchers have turned to devising a next-generation semi-automatic standard examination protocol, that is AI.

As early as 2004, before CNN took the lead in machine-assisted image diagnosis, Huang *et al*[78] deployed the refined feature selection neural network to process endoscopic images and return the results of *H. pylori* infection probability and severity. By training AI with 30 patients' endoscopic images including crops of antrum, body and cardia locations of the stomach, they established an algorithm that achieved an average of 78.8% sensitivity, 90.2% specificity and 85.1% accuracy in an independent cohort of 74 patient images. The overall prediction accuracy was better than the one demonstrated by young physicians and fellow doctors, who scored 68.4% and 78.4%, respectively. It was the first model demonstrating the potential of computer-aided diagnosis of *H. pylori* infection by endoscope images.

However, since the introduction of the 7-layer Le-Net-5 program by LeCun *et al* in 1998, the CNNs have gradually taken over in the field of medical image processing. To name a few examples, Shichijo *et al*[79] used 32208 images of 735 *H. pylori*-positive and 1015 *H. pylori*-negative cases to develop an *H. pylori* identifying AI system based on the architecture of 22-layer GoogLeNet. The sensitivity, specificity, accuracy and time consumption were 81.9%, 83.4%, 83.1% and 198 s for the first CNN and 88.9%, 87.4%, 87.7% and 194 s for the secondary CNN developed, respectively, compared with that of 79.0%, 83.2%, 82.4% and 230 min by the endoscopists. Later, still using GoogLeNet, Shichijo *et al*[80] developed another system that further classified the current infection, post-eradication and current noninfection statuses of *H. pylori*, obtaining an accuracy of 48%, 84%, and 80%, respectively. In this system, the CNN was trained with 98564 images from 4494 patients and tested with 23699 images from 847 independent cases. Itoh *et al*[81] also developed a CNN based on GoogLeNet, trained with 149 endoscopic images obtained from 139 patients and tested with 30 images from 30 patients, which could detect and diagnose *H. pylori* with sensitivity and specificity of 86.7%. Additionally, the use of ResNet CNN architecture was reported by Zheng *et al*[82] in 2019, achieving a sensitivity, specificity and accuracy of 81.4%, 90.1% and 84.5%, respectively. In this study, the system was trained with 11729 images from 1507 patients and tested with 3755 images from 452 patients using a 50-layer ResNet-50 (Microsoft) CNN system and PyTorch (Facebook) deep learning framework.

Recently, AI has also been applied to linked color imaging (LCI) and blue laser imaging, two novel image-enhanced endoscopy technologies[83]. It helped diagnose [84] and classify[85] the *H. pylori* infection and has shown greater effectiveness. In 2018, Nakashima *et al*[84] developed a system on a training set of 162 patients and a test set of 60 patients that could diagnose *H. pylori* infection with an area under the curve of 0.96 and 0.95 and sensitivity of 96.7% and 96.7% for blue laser imaging-bright and LCI, respectively. Such performance is superior to systems that use conventional white light imaging (WLI) (with 0.66 area under the curve and sensitivities as mentioned earlier in other studies) as well as that of experienced endoscopists. Another 2020 study by the same team also showed that classifying the *H. pylori* infection status (uninfected, infected and post-eradication) by incorporating deep learning and image-enhanced endoscopies yields more accurate results. The system was trained with 6639 WLI and 6248 LCI images from 395 patients and tested with images from 120 patients[85].

However, there are some limitations of AI in identifying *H. pylori* that remain to be overcome amongst the developed systems and findings. First, the histological time frame, especially for the eradicated infection, was not considered in the AI systems [86]. Second, both the training data sets and test data sets were obtained from a single center for all existing systems. A continued and even more rigorous external validation, which uses more diverse sources of images and endoscopies, is necessary to evaluate each system's generalizability[87,88]. Additionally, the application of CNN algorithms is also still confined to the existing models of CNN algorithms (mostly GoogLeNet and a few ResNet). Further technical refinements may overcome current limitations faced by endoscopists. They also shed light on the possibility of a system that distinguishes between *H. pylori*-infected and *H. pylori*-eradicated patients, determines different parts of the stomach (cardia, body, angle and pylorus) and provides real-time evaluations of *H. pylori*. These will be considerations vital for its implementation in clinical practice in the future.

DETECTION OF GC

Besides *H. pylori* infection, computer-aided pattern recognition with endoscopic images has also been applied to diagnose wall invasion depth (Table 2). An accurate diagnosis of invasion depth and subsequent staging is the basis for determining the

Table 2 Summary of artificial intelligence applications in prediction of invasion depth and differentiation of cancerous areas from noncancerous areas

Ref.	Application	Endoscopic modality	Training dataset	Validation dataset	Accuracy	Sensitivity	Specificity	PPV	NPV
Kubota *et al* [98], 2012	Prediction of invasion depth	WLI	344 patients, 902 images	-	77.2 (T1)	-	-	80.1 (T1)	
Miyaki *et al* [137], 2013	Differentiation of cancerous areas from noncancerous areas	WLI and magnified FICE	493 images	46 images	85.9	84.8	87.0	86.7	85.1
Hirasawa *et al* [99], 2018	Differentiation of cancerous areas from noncancerous areas	WLI	13584 images	2296 images, 69 patients	92.2	92.2	-	30.6	-
Kanesaka *et al* [138], 2018	Detection of EGC	Magnified NBI	126 images	81 images	96.3	96.7	95.0	98.3	-
Horiuchi *et al* [103], 2020	Differentiation of EGC from gastritis	Magnified NBI	2570 images	258 images	85.3	95.4	71.0	82.3	91.7
Yoon *et al* [101], 2019	Detection of EGC and prediction of EGC invasion depth	WLI	11686 images, 800 patients	-		79.2	77.8	79.3	77.7
Horiuchi *et al* [105], 2020	Detection of EGC	Magnified NBI	2570 images	174 videos, 82 patients	85.1	87.4	82.8	83.5	86.7
Li *et al*[139], 2020	Differentiation of EGC from noncancerous lesions	Magnified NBI	2088 images	342 images	90.9	91.2	90.6	90.6	91.2
Nagao *et al* [102], 2020	Prediction of invasion depth	WLI, nonmagnifying NBI and indigo-carmine dye contrast imaging (Indigo)	16557 images, 1084 patients	-	94.4	89.2	98.7	98.3	91.7
Namikawa *et al*[104], 2020	Differentiation of cancerous areas from noncancerous areas	WLI, nonmagnifying NBI and indigo-carmine dye contrast imaging (Indigo)	18410 images	1459 images	95.9	99.0	93.3	92.5	-

EGC: Early gastric cancers; FICE: Flexible spectral imaging color enhancement; NBI: Narrow-band imaging; NPV: Negative predictive value; PPV: Positive predictive value; WLI: White light imaging.

appropriate treatment modality, especially for suspected early GCs (EGC)[89-91]. Classified based on the 7th TNM classification of tumors[92,93], EGC is categorized as tumor invasion of the mucosa (T1a) or invasion of the submucosa (T1b) stages. While endoscopic ultrasonography is useful for T-staging of GC by delineating each gastric wall layer[94,95], conventional endoscopy is still arguably superior to endoscopic ultrasonography for T-staging of EGC[96,97]. However, there remains room for improvement, such as by utilizing AI, to increase its accuracy. In 2012, Kubota *et al*[98] first explored the system with a relatively high sensitivity of 68.9% and 63.6% in T1a and T1b GCs, achieving high accuracy, especially in early tumors. The accuracy for T1 tumors was 77.2% compared to that of 49.1%, 51.0%, and 55.3% for T2, T3 and T4 tumors, respectively. Another system developed by Hirasawa *et al*[99] achieved a high sensitivity of 92.2% of CNN, though at the expense of a low positive predictive value (30.6%). Zhu *et al*[100] later demonstrated a CNN-computer assisted diagnosis system that achieved much higher accuracy (by 17.25%; 95% confidence interval: 11.63-22.59) and specificity (by 32.21%; 95% confidence interval: 26.78-37.44) compared to human endoscopists. These preliminary findings showed that AI is a potentially helpful diagnostic procedure in EGC detection and pointed towards developing an AI system that can differentiate between malignant and benign lesions.

Given that the difference in EGC depth in endoscopic images is subtler and more difficult to discern, Yoon *et al*[101] identified that more sophisticated image classification methods but not merely conventional CNN models are required. The team developed a system that classifies endoscopic images into EGC (T1a or T1b) or non-EGC. This system used the combination of the CNN-based visual geometry group-16 network pretrained on ImageNet and a novel method of the weighted sum of gradient-weighted class activation mapping. This system focused on learning the visual features of EGC regions rather than those of other gastric textures, achieving both high accuracy of 91.0% and high area under the curve of 0.981. In another study in 2020, Nagao *et al*[102] used the state-of-the-art ResNet50 CNN architecture to

develop a system *via* training the images from different angles and distances. This system predicted the invasion depth of GC with an image-based accuracy as high as 94.5%.

However, when using these AI systems for invasion depth diagnosis, distinguishing superficially depressed and differentiated-type intramucosal cancers from gastritis remains a challenge. The diagnostic accuracy of determining invasion depth is largely affected by its histological characteristics. For instance, the system developed by Yoon *et al*[101] achieved an accuracy of 77.1% for differentiated-type tumors in contrast to that of 65.5% for undifferentiated type. Horiuchi *et al*[103] made a substantial effort and developed another system that could differentiate EGC from gastritis using magnifying endoscopy with NBI (M-NBI). The system achieved an accuracy of 85.3% and sensitivity and negative predictive value of 95.4% and 91.7%. Another attempt was made by Namikawa *et al*[104], who developed a system that was trained by gastritis images and tested to classify GC and gastric ulcers. A continued development of AI systems that consider the differentiated type histology will shed light on the future of AI-assisted differentiation of T1a from T1b GC and that of T1a and T1b cancers from the later stages of GC.

To bring AI-assisted systems one step closer to real-time clinical applications, video-based systems have also been explored. In 2020, Horiuchi *et al*[105] used the video-based systems and achieved a comparable accuracy of 85.1% in distinguishing EGC and noncancerous lesions. Based on the CNN-CAD system, their system was trained with 2570 images (1492 cancerous and 1078 noncancerous images) and tested with 174 videos. This preliminary success in the video-based CNN-CAD system pointed out the potential of real-time AI-assisted diagnosis, which could be a promising technique for detecting EGC for clinicians in the future. Early detection of GC means an early treatment of endoscopic dissection in accordance with the works promoted by the Japanese Gastric Cancer Association since 2014[106].

DISCUSSION

Over the past decade, AI has displayed its potential diagnosing GC to amplify human endoscopist capacities. Although the diagnosis of GC requires a holistic set of assessments, AI is applicable and helpful in some parts. A system that detects GC with high sensitivity regardless of its accuracy in determining invasion depth could provide great clinical assistance for physicians to decide if biopsy and endoscopic submucosal dissection are necessary. In the near future, there should be some other diagnostic procedures that can be explored with AI. For example, macroscopic characteristics, namely the "nonextension signs" commonly used to distinguish between SM1 and SM2 invasion depths of GCs[107] have yet to be explored with AI.

Clinically, there are also some distinct markers that endoscopists use to evaluate gastric surface and color changes. Distinguishing the markers such as changes in light reflection and spontaneous bleeding are clinical skills[108,109] that AI could potentially learn and interpret. In clinical practice, antiperistaltic agents are suggested for polyethersulfone preparation, and indigo carmine chromoendoscopy could help diagnose elevated superficial lesions with an irregular surface pattern[110] with which their efficacy could be evaluated by real-time AI endoscopy in the near future.

Although several studies have attempted to apply AI in different types of endoscopies, ranging from WLI to LCI to blue laser imaging, these studies can also continue to extend AI to NBI and other nonconventional endoscopies. For instance, endocytoscopy with NBI has shown higher diagnostic accuracy compared to M-NBI [78.8% (76.4%-83.0%) *vs* 72.2% (69.3%-73.6%), $P < 0.0001$][111]. An AI system that is trained with WLI images and tested with NBI images instead will also have clinical significance[112,113]. Proposed in 2016 was the Magnifying Endoscopy Simple Diagnostic Algorithms for EGC that suggested a systematic approach to WLI magnifying endoscopy. It is recommended that if a suspicious lesion is detected, M-NBI should be performed to distinguish if the lesion is cancer or noncancer[114]. According to this algorithm, changing from WLI to M-NBI endoscopy is therefore critical for diagnosis, and the future development of AI systems can consider accounting for such changes.

In the AI systems developed over the past decade, we summarize the following common limitations faced. First, there seems to be a common lack of high-quality datasets for machine learning development, a problem faced in clinical practice even without AI[115]. Simultaneously, some studies reported that low-quality images result in higher chances of misdiagnosis by the AI system[116], and most studies excluded

large numbers of poor-quality images[99-101]. The call for cross-validation with multicenter observational studies has also been discussed in several studies in hopes of picking out any potential overfitting and spectrum bias that is foreseeable in deep image classification models[117-119]. Some authors have argued that the AI system they have developed is institution-specific and that the validation with the dataset from external sources is necessary[103,105]. In this regard, multicenter studies have been widely used in other medical fields to evaluate deep learning systems[120-122], though there have been no such studies in the field of GC.

Another challenge that remains is seen in the imbalanced class distributions, a common classification problem in which the distribution of samples across the known classes is biased or skewed. For example, in the study reported by Hirasawa *et al*[99] in 2018, there are few samples for the later stages (only 32.5% of samples were T2-T4 cancers) than for early cancer (67.5% of samples). Such imbalanced classifications pose a challenge as machine learning models are primarily designed on the assumption of an equal number of samples for each class[123]. Without sufficient samples for certain classes of the training dataset, their existence might be misperceived as other classes as the AI model becomes more sensitive to classification errors. It may result in poor predictive performance, especially for the minority class and subsequently an overall increased misdiagnosis rate. For example, in the cases of the AI model for GC staging, a misdiagnosis of late-stage cancer for gastritis or nonmalignancy has dangerous implications[124-126] if the AI system was used for its diagnosis alone. However, in most cases, advanced-stage GC might have already metastasized to other parts of the body[127-129], and its diagnosis based only on the AI system alone is unlikely. Nonetheless, in the development of AI systems for such medical applications, these technical problems of imbalanced classification should not be overlooked. It has been discussed by other reviews how modifications can be made to AI models to recognize targets, no matter how frequent or rare they are, to minimize the possibility of misdiagnosis[130,131].

Overall, the potential for AI applications in GC is extensive yet highly specific. In an upcoming era of AI-assisted diagnosis, by combining image information, medical history and laboratory data, endoscopists can look forward to the continued development of new systems for varying purposes (Figure 2). AI systems are specific and unlikely to be generalized[132,133], and it is fallacious to compare a single statistical performance measure across different AI systems. The efficacy of an AI system depends on the intended role it plays in clinical practice. For example, an AI system with a high positive predictive value is desirable in determining which multicancer to send for biopsy, while high sensitivity suffices for a system that helps differentiate cancerous from noncancerous clinical signs, especially for amateur endoscopists. In the foreseeable future, AI can be incorporated in the differential diagnosis of the malignancy and stages of gastric lesions, using various endoscopic technologies and techniques.

CONCLUSION

Overall, the application of AI in gastroenterology is in its infancy. At present, there exist several retrospective models applied in both images and videos and using both WLI and NBI endoscopies that have proven to have better performance for the same tasks carried out by experienced endoscopists. However, there have not been any attempts of clinical trials. In contrast to the ongoing trials for detecting colorectal polyps[134-136], AI applications in GC and its corresponding diagnostic methods are still preliminary. The limitations of existing efforts point towards the importance of continued research in the field that can go a long way in making quicker, more accurate and precise evaluations of GC risk. While we witnessed its rapid and steep growth in the past decade, future studies are needed to streamline the machine learning process and define its role in the computer-aided diagnosis of *H. pylori* infections and GC in real-life clinical scenarios.

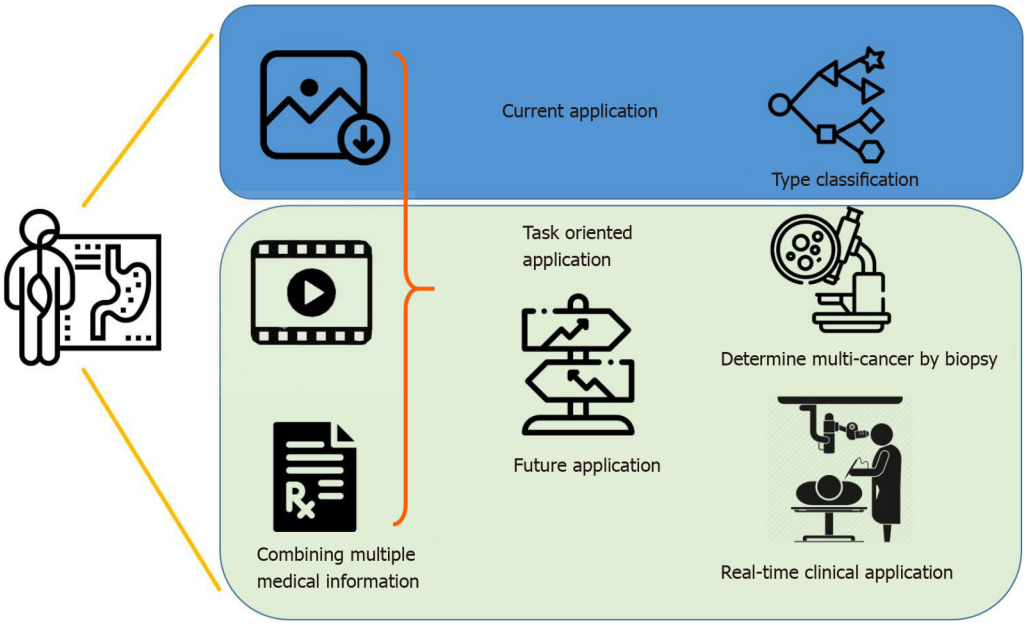


Figure 2 Current status and future research direction for implementation of artificial intelligence-assisted endoscopy in clinical practice. In an upcoming era of artificial intelligence-assisted diagnosis, endoscopists can look forward to the continued development of new artificial intelligence systems for varying purposes. From determining multicancer *via* biopsy to real-time endoscopies, artificial intelligence has the potential of assisting physicians to improve their diagnostic accuracies.

REFERENCES

1 **Drewes AM**, Olesen AE, Farmer AD, Szigethy E, Rebours V, Olesen SS. Gastrointestinal pain. *Nat Rev Dis Primers* 2020; **6**: 1 [PMID: 31907359 DOI: 10.1038/s41572-019-0135-7]

2 **Hoffman A**, Manner H, Rey JW, Kiesslich R. A guide to multimodal endoscopy imaging for gastrointestinal malignancy - an early indicator. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 421-434 [PMID: 28611477 DOI: 10.1038/nrgastro.2017.46]

3 **Mannath J**, Ragunath K. Role of endoscopy in early oesophageal cancer. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 720-730 [PMID: 27807370 DOI: 10.1038/nrgastro.2016.148]

4 **Necula L**, Matei L, Dragu D, Neagu AI, Mambet C, Nedeianu S, Bleotu C, Diaconu CC, Chivu-Economescu M. Recent advances in gastric cancer early diagnosis. *World J Gastroenterol* 2019; **25**: 2029-2044 [PMID: 31114131 DOI: 10.3748/wjg.v25.i17.2029]

5 **Yang D**, Wagh MS, Draganov PV. The status of training in new technologies in advanced endoscopy: from defining competence to credentialing and privileging. *Gastrointest Endosc* 2020; **92**: 1016-1025 [PMID: 32504699 DOI: 10.1016/j.gie.2020.05.047]

6 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

7 **Dicken BJ**, Bigam DL, Cass C, Mackey JR, Joy AA, Hamilton SM. Gastric adenocarcinoma: review and considerations for future directions. *Ann Surg* 2005; **241**: 27-39 [PMID: 15621988 DOI: 10.1097/01.sla.0000149300.28588.23]

8 **Lyons K**, Le LC, Pham YT, Borron C, Park JY, Tran CTD, Tran TV, Tran HT, Vu KT, Do CD, Pelucchi C, La Vecchia C, Zgibor J, Boffetta P, Luu HN. Gastric cancer: epidemiology, biology, and prevention: a mini review. *Eur J Cancer Prev* 2019; **28**: 397-412 [PMID: 31386635 DOI: 10.1097/CEJ.0000000000000480]

9 **Rawla P**, Barsouk A. Epidemiology of gastric cancer: global trends, risk factors and prevention. *Prz Gastroenterol* 2019; **14**: 26-38 [PMID: 30944675 DOI: 10.5114/pg.2018.80001]

10 **Vingeliene S**, Chan DSM, Vieira AR, Polemiti E, Stevens C, Abar L, Navarro Rosenblatt D, Greenwood DC, Norat T. An update of the WCRF/AICR systematic literature review and meta-analysis on dietary and anthropometric factors and esophageal cancer risk. *Ann Oncol* 2017; **28**: 2409-2419 [PMID: 28666313 DOI: 10.1093/annonc/mdx338]

11 **Cummins G**, Cox BF, Ciuti G, Anbarasan T, Desmulliez MPY, Cochran S, Steele R, Plevris JN, Koulaouzidis A. Gastrointestinal diagnosis using non-white light imaging capsule endoscopy. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 429-447 [PMID: 30988520 DOI: 10.1038/s41575-019-0140-z]

12 **Kiesslich R**, Goetz M, Hoffman A, Galle PR. New imaging techniques and opportunities in endoscopy. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 547-553 [PMID: 21894196 DOI: 10.1038/nrgastro.2011.152]

13 **Kuipers EJ**, Haringsma J. Diagnostic and therapeutic endoscopy. *J Surg Oncol* 2005; **92**: 203-209 [PMID: 16299782 DOI: 10.1002/jso.20361]

14 **Cenitagoya GF**, Bergh CK, Klinger-Roitman J. A prospective study of gastric cancer. 'Real' 5-year survival rates and mortality rates in a country with high incidence. *Dig Surg* 1998; **15**: 317-322 [PMID: 9845606 DOI: 10.1159/000018645]

15 **Tarver T**. Cancer Facts & Figures 2012. American Cancer Society (ACS). *J Consum Health Internet* 2012; **16**: 366-367 [DOI: 10.1080/15398285.2012.701177]

16 **Becker KF**, Keller G, Hoefler H. The use of molecular biology in diagnosis and prognosis of gastric cancer. *Surg Oncol* 2000; **9**: 5-11 [PMID: 11525306 DOI: 10.1016/s0960-7404(00)00016-5]

17 **Nakamura K**, Ueyama T, Yao T, Xuan ZX, Ambe K, Adachi Y, Yakeishi Y, Matsukuma A, Enjoji M. Pathology and prognosis of gastric carcinoma. Findings in 10,000 patients who underwent primary gastrectomy. *Cancer* 1992; **70**: 1030-1037 [PMID: 1515980 DOI: 10.1002/1097-0142(19920901)70:5<1030::aid-cncr2820700504>3.0.co;2-c]

18 **Gotoda T**, Jung HY. Endoscopic resection (endoscopic mucosal resection/ endoscopic submucosal dissection) for early gastric cancer. *Dig Endosc* 2013; **25** Suppl 1: 55-63 [PMID: 23362925 DOI: 10.1111/den.12003]

19 **Nishizawa T**, Yahagi N. Endoscopic mucosal resection and endoscopic submucosal dissection: technique and new directions. *Curr Opin Gastroenterol* 2017; **33**: 315-319 [PMID: 28704212 DOI: 10.1097/MOG.0000000000000388]

20 **Uraoka T**, Saito Y, Yamamoto K, Fujii T. Submucosal injection solution for gastrointestinal tract endoscopic mucosal resection and endoscopic submucosal dissection. *Drug Des Devel Ther* 2009; **2**: 131-138 [PMID: 19920900 DOI: 10.2147/dddt.s3219]

21 **Asaka M**, Mabe K. Strategies for eliminating death from gastric cancer in Japan. *Proc Jpn Acad Ser B Phys Biol Sci* 2014; **90**: 251-258 [PMID: 25169671 DOI: 10.2183/pjab.90.251]

22 **Katai H**, Ishikawa T, Akazawa K, Isobe Y, Miyashiro I, Oda I, Tsujitani S, Ono H, Tanabe S, Fukagawa T, Nunobe S, Kakeji Y, Nashimoto A; Registration Committee of the Japanese Gastric Cancer Association. Five-year survival analysis of surgically resected gastric cancer cases in Japan: a retrospective analysis of more than 100,000 patients from the nationwide registry of the Japanese Gastric Cancer Association (2001-2007). *Gastric Cancer* 2018; **21**: 144-154 [PMID: 28417260 DOI: 10.1007/s10120-017-0716-7]

23 **Wu MS**, Lin JT, Lee WJ, Yu SC, Wang TH. [Gastric cancer in Taiwan]. *J Formos Med Assoc* 1994; **93** Suppl 2: S77-S89 [PMID: 7719165]

24 **Amin A**, Gilmour H, Graham L, Paterson-Brown S, Terrace J, Crofts TJ. Gastric adenocarcinoma missed at endoscopy. *J R Coll Surg Edinb* 2002; **47**: 681-684 [PMID: 12463707]

25 **Hosokawa O**, Hattori M, Douden K, Hayashi H, Ohta K, Kaizaki Y. Difference in accuracy between gastroscopy and colonoscopy for detection of cancer. *Hepatogastroenterology* 2007; **54**: 442-444 [PMID: 17523293]

26 **Hosokawa O**, Tsuda S, Kidani E, Watanabe K, Tanigawa Y, Shirasaki S, Hayashi H, Hinoshita T. Diagnosis of gastric cancer up to three years after negative upper gastrointestinal endoscopy. *Endoscopy* 1998; **30**: 669-674 [PMID: 9865554 DOI: 10.1055/s-2007-1001386]

27 **Menon S**, Trudgill N. How commonly is upper gastrointestinal cancer missed at endoscopy? *Endosc Int Open* 2014; **2**: E46-E50 [PMID: 26135259 DOI: 10.1055/s-0034-1365524]

28 **Voutilainen ME**, Juhola MT. Evaluation of the diagnostic accuracy of gastroscopy to detect gastric tumours: clinicopathological features and prognosis of patients with gastric cancer missed on endoscopy. *Eur J Gastroenterol Hepatol* 2005; **17**: 1345-1349 [PMID: 16292088 DOI: 10.1097/00042737-200512000-00013]

29 **Yalamarthi S**, Witherspoon P, McCole D, Auld CD. Missed diagnoses in patients with upper gastrointestinal cancers. *Endoscopy* 2004; **36**: 874-879 [PMID: 15452783 DOI: 10.1055/s-2004-825853]

30 **Park CH**, Kim B, Chung H, Lee H, Park JC, Shin SK, Lee SK, Lee YC. Endoscopic quality indicators for esophagogastroduodenoscopy in gastric cancer screening. *Dig Dis Sci* 2015; **60**: 38-46 [PMID: 25063206 DOI: 10.1007/s10620-014-3288-y]

31 **Willis S**, Truong S, Gribnitz S, Fass J, Schumpelick V. Endoscopic ultrasonography in the preoperative staging of gastric cancer. *Surg Endosc* 2000; **14**: 951-954 [DOI: 10.1007/s004640010040]

32 **Dixon BJ**, Chan H, Daly MJ, Vescan AD, Witterick IJ, Irish JC. The effect of augmented real-time image guidance on task workload during endoscopic sinus surgery. *Int Forum Allergy Rhinol* 2012; **2**: 405-410 [PMID: 22644966 DOI: 10.1002/alr.21049]

33 **Hudler P**. Challenges of deciphering gastric cancer heterogeneity. *World J Gastroenterol* 2015; **21**: 10510-10527 [PMID: 26457012 DOI: 10.3748/wjg.v21.i37.10510]

34 **Reinhart K**, Bannert C, Dunkler D, Salzl P, Trauner M, Renner F, Knoflach P, Ferlitsch A, Weiss W, Ferlitsch M. Prevalence of flat lesions in a large screening population and their role in colonoscopy quality improvement. *Endoscopy* 2013; **45**: 350-356 [PMID: 23616125 DOI: 10.1055/s-0032-1326348]

35 **Anderson ML**, Heigh RI, McCoy GA, Parent K, Muhm JR, McKee GS, Eversman WG, Collins JM. Accuracy of assessment of the extent of examination by experienced colonoscopists. *Gastrointest Endosc* 1992; **38**: 560-563 [PMID: 1397910 DOI: 10.1016/s0016-5107(92)70516-1]

36 **Hawkins SC**, Osborne A, Schofield SJ, Pournaras DJ, Chester JF. Improving the accuracy of self-assessment of practical clinical skills using video feedback--the importance of including benchmarks. *Med Teach* 2012; **34**: 279-284 [PMID: 22455696 DOI: 10.3109/0142159X.2012.658897]

37 **Higashi R**, Uraoka T, Kato J, Kuwaki K, Ishikawa S, Saito Y, Matsuda T, Ikematsu H, Sano Y, Suzuki S, Murakami Y, Yamamoto K. Diagnostic accuracy of narrow-band imaging and pit pattern analysis significantly improved for less-experienced endoscopists after an expanded training program. *Gastrointest Endosc* 2010; **72**: 127-135 [PMID: 20493482 DOI: 10.1016/j.gie.2010.01.054]

38 **Jinnai S**, Yamazaki N, Hirano Y, Sugawara Y, Ohe Y, Hamamoto R. The Development of a Skin Cancer Classification System for Pigmented Skin Lesions Using Deep Learning. *Biomolecules* 2020; **10** [PMID: 32751349 DOI: 10.3390/biom10081123]

39 **Nelson CA**, Pérez-Chada LM, Creadore A, Li SJ, Lo K, Manjaly P, Pournamdari AB, Tkachenko E, Barbieri JS, Ko JM, Menon AV, Hartman RI, Mostaghimi A. Patient Perspectives on the Use of Artificial Intelligence for Skin Cancer Screening: A Qualitative Study. *JAMA Dermatol* 2020; **156**: 501-512 [PMID: 32159733 DOI: 10.1001/jamadermatol.2019.5014]

40 **Tschandl P**, Rinner C, Apalla Z, Argenziano G, Codella N, Halpern A, Janda M, Lallas A, Longo C, Malvehy J, Paoli J, Puig S, Rosendahl C, Soyer HP, Zalaudek I, Kittler H. Human-computer collaboration for skin cancer recognition. *Nat Med* 2020; **26**: 1229-1234 [PMID: 32572267 DOI: 10.1038/s41591-020-0942-0]

41 **Zakhem GA**, Fakhoury JW, Motosko CC, Ho RS. Characterizing the role of dermatologists in developing artificial intelligence for assessment of skin cancer: A systematic review. *J Am Acad Dermatol* 2020 [PMID: 31972254 DOI: 10.1016/j.jaad.2020.01.028]

42 **Hosny A**, Parmar C, Quackenbush J, Schwartz LH, Aerts HJWL. Artificial intelligence in radiology. *Nat Rev Cancer* 2018; **18**: 500-510 [PMID: 29777175 DOI: 10.1038/s41568-018-0016-5]

43 **Huynh E**, Hosny A, Guthier C, Bitterman DS, Petit SF, Haas-Kogan DA, Kann B, Aerts HJWL, Mak RH. Artificial intelligence in radiation oncology. *Nat Rev Clin Oncol* 2020; **17**: 771-781 [PMID: 32843739 DOI: 10.1038/s41571-020-0417-8]

44 **Kocher M**. Artificial intelligence and radiomics for radiation oncology. *Strahlenther Onkol* 2020; **196**: 847 [PMID: 32940764 DOI: 10.1007/s00066-020-01676-y]

45 **Syed K**, Sleeman IV WC, Nalluri JJ, Kapoor R, Hagan M, Palta J, Ghosh P. Artificial intelligence methods in computer-aided diagnostic tools and decision support analytics for clinical informatics. Artificial Intelligence in Precision Health. Elsevier, 2020: 31-59

46 **Bhanumurthy M**, Anne K. An automated detection and segmentation of tumor in brain MRI using artificial intelligence. Proceedings of the 2014 IEEE International Conference on Computational Intelligence and Computing Research; IEEE, 2014: 1-6

47 **Ghafoorian M**, Mehrtash A, Kapur T, Karssemeijer N, Marchiori E, Pesteie M, Guttmann CR, de Leeuw FE, Tempany CM, Van Ginneken B. Transfer learning for domain adaptation in MRI: Application in brain lesion segmentation. Proceedings of the International conference on medical image computing and computer-assisted intervention; New York: Springer: 2017: 516-524

48 **Rauschecker AM**, Rudie JD, Xie L, Wang J, Duong MT, Botzolakis EJ, Kovalovich AM, Egan J, Cook TC, Bryan RN, Nasrallah IM, Mohan S, Gee JC. Artificial Intelligence System Approaching Neuroradiologist-level Differential Diagnosis Accuracy at Brain MRI. *Radiology* 2020; **295**: 626-637 [PMID: 32255417 DOI: 10.1148/radiol.2020190283]

49 **Shahamat H**, Saniee Abadeh M. Brain MRI analysis using a deep learning based evolutionary approach. *Neural Netw* 2020; **126**: 218-234 [PMID: 32259762 DOI: 10.1016/j.neunet.2020.03.017]

50 **Cho J**, Park KS, Karki M, Lee E, Ko S, Kim JK, Lee D, Choe J, Son J, Kim M, Lee S, Lee J, Yoon C, Park S. Improving Sensitivity on Identification and Delineation of Intracranial Hemorrhage Lesion Using Cascaded Deep Learning Models. *J Digit Imaging* 2019; **32**: 450-461 [PMID: 30680471 DOI: 10.1007/s10278-018-00172-1]

51 **Simon R**. Sensitivity, Specificity, PPV, and NPV for Predictive Biomarkers. *J Natl Cancer Inst* 2015; **107** [PMID: 26109105 DOI: 10.1093/jnci/djv153]

52 **Arbabshirani MR**, Fornwalt BK, Mongelluzzo GJ, Suever JD, Geise BD, Patel AA, Moore GJ. Advanced machine learning in action: identification of intracranial hemorrhage on computed tomography scans of the head with clinical workflow integration. *NPJ Digit Med* 2018; **1**: 9 [PMID: 31304294 DOI: 10.1038/s41746-017-0015-z]

53 **Leenhardt R**, Vasseur P, Li C, Saurin JC, Rahmi G, Cholet F, Becq A, Marteau P, Histace A, Dray X; CAD-CAP Database Working Group. A neural network algorithm for detection of GI angiectasia during small-bowel capsule endoscopy. *Gastrointest Endosc* 2019; **89**: 189-194 [PMID: 30017868 DOI: 10.1016/j.gie.2018.06.036]

54 **Soffer S**, Klang E, Shimon O, Nachmias N, Eliakim R, Ben-Horin S, Kopylov U, Barash Y. Deep learning for wireless capsule endoscopy: a systematic review and meta-analysis. *Gastrointest Endosc* 2020; **92**: 831-839. e8 [PMID: 32334015 DOI: 10.1016/j.gie.2020.04.039]

55 **Wang S**, Xing Y, Zhang L, Gao H, Zhang H. A systematic evaluation and optimization of automatic detection of ulcers in wireless capsule endoscopy on a large dataset using deep convolutional neural networks. *Phys Med Biol* 2019; **64**: 235014 [PMID: 31645019 DOI: 10.1088/1361-6560/ab5086]

56 **Xia J**, Xia T, Pan J, Gao F, Wang S, Qian YY, Wang H, Zhao J, Jiang X, Zou WB, Wang YC, Zhou W, Li ZS, Liao Z. Use of artificial intelligence for detection of gastric lesions by magnetically controlled capsule endoscopy. *Gastrointest Endosc* 2021; **93**: 133-139. e4 [PMID: 32470426 DOI: 10.1016/j.gie.2020.05.027]

57 **Sánchez-Peralta LF**, Bote-Curiel L, Picón A, Sánchez-Margallo FM, Pagador JB. Deep learning to find colorectal polyps in colonoscopy: A systematic literature review. *Artif Intell Med* 2020; **108**: 101923 [PMID: 32972656 DOI: 10.1016/j.artmed.2020.101923]

58 **Summers RM**, Jerebko AK, Franaszek M, Malley JD, Johnson CD. Colonic polyps: complementary role of computer-aided detection in CT colonography. *Radiology* 2002; **225**: 391-399 [PMID: 12409571 DOI: 10.1148/radiol.2252011619]

59 **Wang KW**, Dong M. Potential applications of artificial intelligence in colorectal polyps and cancer: Recent advances and prospects. *World J Gastroenterol* 2020; **26**: 5090-5100 [PMID: 32982111 DOI: 10.3748/wjg.v26.i34.5090]

60 **Litjens G**, Kooi T, Bejnordi BE, Setio AAA, Ciompi F, Ghafoorian M, van der Laak JAWM, van Ginneken B, Sánchez CI. A survey on deep learning in medical image analysis. *Med Image Anal* 2017; **42**: 60-88 [PMID: 28778026 DOI: 10.1016/j.media.2017.07.005]

61 **Wieslander H**, Forslid G, Bengtsson E, Wahlby C, Hirsch J-M, Runow Stark C, Kecheril Sadanandan S. Deep convolutional neural networks for detecting cellular changes due to malignancy. Proceedings of the Proceedings of the IEEE International Conference on Computer Vision Workshops; 2017 Oct 22-29; Venice, Italy. IEEE, 2017: 82-89

62 **Ebigbo A**, Palm C, Probst A, Mendel R, Manzeneder J, Prinz F, de Souza LA, Papa JP, Siersema P, Messmann H. A technical review of artificial intelligence as applied to gastrointestinal endoscopy: clarifying the terminology. *Endosc Int Open* 2019; **7**: E1616-E1623 [PMID: 31788542 DOI: 10.1055/a-1010-5705]

63 **Mortazi A**, Bagci U. Automatically designing CNN architectures for medical image segmentation. 2018 Preprint. Available from: arXiv:1807.07663

64 **Lundervold AS**, Lundervold A. An overview of deep learning in medical imaging focusing on MRI. *Z Med Phys* 2019; **29**: 102-127 [PMID: 30553609 DOI: 10.1016/j.zemedi.2018.11.002]

65 **Serte S**, Serener A, Al-Turjman F. Deep learning in medical imaging: A brief review. Transactions on Emerging Telecommunications Technologies. Hoboken: John Wiley & Sons, 2020: e4080

66 **Abe S**, Oda I. How can endoscopists adapt and collaborate with artificial intelligence for early gastric cancer detection? *Dig Endosc* 2021; **33**: 98-99 [PMID: 32455500 DOI: 10.1111/den.13751]

67 **Malfertheiner P**. Diagnostic methods for H. pylori infection: Choices, opportunities and pitfalls. *United European Gastroenterol J* 2015; **3**: 429-431 [PMID: 26535120 DOI: 10.1177/2050640615600968]

68 **Correa P**, Piazuelo MB. The gastric precancerous cascade. *J Dig Dis* 2012; **13**: 2-9 [PMID: 22188910 DOI: 10.1111/j.1751-2980.2011.00550.x]

69 **Parsonnet J**, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131 [PMID: 1891020 DOI: 10.1056/NEJM199110173251603]

70 **Bornschein J**, Malfertheiner P. Gastric carcinogenesis. *Langenbecks Arch Surg* 2011; **396**: 729-742 [PMID: 21611816 DOI: 10.1007/s00423-011-0810-y]

71 **Kato T**, Yagi N, Kamada T, Shimbo T, Watanabe H, Ida K; Study Group for Establishing Endoscopic Diagnosis of Chronic Gastritis. Diagnosis of Helicobacter pylori infection in gastric mucosa by endoscopic features: a multicenter prospective study. *Dig Endosc* 2013; **25**: 508-518 [PMID: 23369058 DOI: 10.1111/den.12031]

72 **Laine L**, Cohen H, Sloane R, Marin-Sorensen M, Weinstein WM. Interobserver agreement and predictive value of endoscopic findings for H. pylori and gastritis in normal volunteers. *Gastrointest Endosc* 1995; **42**: 420-423 [PMID: 8566631 DOI: 10.1016/s0016-5107(95)70043-9]

73 **Goodwin CS**. Helicobacter pylori gastritis, peptic ulcer, and gastric cancer: clinical and molecular aspects. *Clin Infect Dis* 1997; **25**: 1017-1019 [PMID: 9402348 DOI: 10.1086/516077]

74 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789 [PMID: 11556297 DOI: 10.1056/NEJMoa001999]

75 **Christensen AH**, Gjørup T, Hilden J, Fenger C, Henriksen B, Vyberg M, Ostergaard K, Hansen BF. Observer homogeneity in the histologic diagnosis of Helicobacter pylori. Latent class analysis, kappa coefficient, and repeat frequency. *Scand J Gastroenterol* 1992; **27**: 933-939 [PMID: 1455190 DOI: 10.3109/00365529209000166]

76 **de Martel C**, Plummer M, van Doorn LJ, Vivas J, Lopez G, Carillo E, Peraza S, Muñoz N, Franceschi S. Comparison of polymerase chain reaction and histopathology for the detection of Helicobacter pylori in gastric biopsies. *Int J Cancer* 2010; **126**: 1992-1996 [PMID: 19795444 DOI: 10.1002/ijc.24898]

77 **el-Zimaity HM**, Graham DY, al-Assi MT, Malaty H, Karttunen TJ, Graham DP, Huberman RM, Genta RM. Interobserver variation in the histopathological assessment of Helicobacter pylori gastritis. *Hum Pathol* 1996; **27**: 35-41 [PMID: 8543308 DOI: 10.1016/s0046-8177(96)90135-5]

78 **Huang CR**, Sheu BS, Chung PC, Yang HB. Computerized diagnosis of Helicobacter pylori infection and associated gastric inflammation from endoscopic images by refined feature selection using a neural network. *Endoscopy* 2004; **36**: 601-608 [PMID: 15243882 DOI: 10.1055/s-2004-814519]

79 **Shichijo S**, Nomura S, Aoyama K, Nishikawa Y, Miura M, Shinagawa T, Takiyama H, Tanimoto T, Ishihara S, Matsuo K, Tada T. Application of Convolutional Neural Networks in the Diagnosis of Helicobacter pylori Infection Based on Endoscopic Images. *EBioMedicine* 2017; **25**: 106-111 [PMID: 29056541 DOI: 10.1016/j.ebiom.2017.10.014]

80 **Shichijo S**, Endo Y, Aoyama K, Takeuchi Y, Ozawa T, Takiyama H, Matsuo K, Fujishiro M, Ishihara S, Ishihara R, Tada T. Application of convolutional neural networks for evaluating Helicobacter pylori infection status on the basis of endoscopic images. *Scand J Gastroenterol* 2019;

54: 158-163 [PMID: 30879352 DOI: 10.1080/00365521.2019.1577486]
81 **Itoh T**, Kawahira H, Nakashima H, Yata N. Deep learning analyzes Helicobacter pylori infection by upper gastrointestinal endoscopy images. *Endosc Int Open* 2018; **6**: E139-E144 [PMID: 29399610 DOI: 10.1055/s-0043-120830]
82 **Zheng W**, Zhang X, Kim JJ, Zhu X, Ye G, Ye B, Wang J, Luo S, Li J, Yu T, Liu J, Hu W, Si J. High Accuracy of Convolutional Neural Network for Evaluation of Helicobacter pylori Infection Based on Endoscopic Images: Preliminary Experience. *Clin Transl Gastroenterol* 2019; **10**: e00109 [PMID: 31833862 DOI: 10.14309/ctg.0000000000000109]
83 **Kanzaki H**, Takenaka R, Kawahara Y, Kawai D, Obayashi Y, Baba Y, Sakae H, Gotoda T, Kono Y, Miura K, Iwamuro M, Kawano S, Tanaka T, Okada H. Linked color imaging (LCI), a novel image-enhanced endoscopy technology, emphasizes the color of early gastric cancer. *Endosc Int Open* 2017; **5**: E1005-E1013 [PMID: 29159276 DOI: 10.1055/s-0043-117881]
84 **Nakashima H**, Kawahira H, Kawachi H, Sakaki N. Artificial intelligence diagnosis of *Helicobacter pylori* infection using blue laser imaging-bright and linked color imaging: a single-center prospective study. *Ann Gastroenterol* 2018; **31**: 462-468 [PMID: 29991891 DOI: 10.20524/aog.2018.0269]
85 **Nakashima H**, Kawahira H, Kawachi H, Sakaki N. Endoscopic three-categorical diagnosis of Helicobacter pylori infection using linked color imaging and deep learning: a single-center prospective study (with video). *Gastric Cancer* 2020; **23**: 1033-1040 [PMID: 32382973 DOI: 10.1007/s10120-020-01077-1]
86 **Kodama M**, Murakami K, Okimoto T, Sato R, Uchida M, Abe T, Shiota S, Nakagawa Y, Mizukami K, Fujioka T. Ten-year prospective follow-up of histological changes at five points on the gastric mucosa as recommended by the updated Sydney system after Helicobacter pylori eradication. *J Gastroenterol* 2012; **47**: 394-403 [PMID: 22138891 DOI: 10.1007/s00535-011-0504-9]
87 **Kelly CJ**, Karthikesalingam A, Suleyman M, Corrado G, King D. Key challenges for delivering clinical impact with artificial intelligence. *BMC Med* 2019; **17**: 195 [PMID: 31665002 DOI: 10.1186/s12916-019-1426-2]
88 **Luo H**, Xu G, Li C, He L, Luo L, Wang Z, Jing B, Deng Y, Jin Y, Li Y, Li B, Tan W, He C, Seeruttun SR, Wu Q, Huang J, Huang DW, Chen B, Lin SB, Chen QM, Yuan CM, Chen HX, Pu HY, Zhou F, He Y, Xu RH. Real-time artificial intelligence for detection of upper gastrointestinal cancer by endoscopy: a multicentre, case-control, diagnostic study. *Lancet Oncol* 2019; **20**: 1645-1654 [PMID: 31591062 DOI: 10.1016/S1470-2045(19)30637-0]
89 **Goto O**, Fujishiro M, Kodashima S, Ono S, Omata M. Outcomes of endoscopic submucosal dissection for early gastric cancer with special reference to validation for curability criteria. *Endoscopy* 2009; **41**: 118-122 [PMID: 19214889 DOI: 10.1055/s-0028-1119452]
90 **Maruyama K**. The most important factors for gastric cancer patients. A studyusing univariate and multivariate analyses. *Scand J Gastroenterol* 1987; **22**: 63-68 [DOI: 10.3109/00365528709091021]
91 **Wang J**, Yu JC, Kang WM, Ma ZQ. Treatment strategy for early gastric cancer. *Surg Oncol* 2012; **21**: 119-123 [PMID: 21256735 DOI: 10.1016/j.suronc.2010.12.004]
92 **Marrelli D**, Morgagni P, de Manzoni G, Coniglio A, Marchet A, Saragoni L, Tiberio G, Roviello F; Italian Research Group for Gastric Cancer (IRGGC). Prognostic value of the 7th AJCC/UICC TNM classification of noncardia gastric cancer: analysis of a large series from specialized Western centers. *Ann Surg* 2012; **255**: 486-491 [PMID: 22167003 DOI: 10.1097/SLA.0b013e3182389b1a]
93 **Wittekind C**. The development of the TNM classification of gastric cancer. *Pathol Int* 2015; **65**: 399-403 [PMID: 26036980 DOI: 10.1111/pin.12306]
94 **Mocellin S**, Marchet A, Nitti D. EUS for the staging of gastric cancer: a meta-analysis. *Gastrointest Endosc* 2011; **73**: 1122-1134 [PMID: 21444080 DOI: 10.1016/j.gie.2011.01.030]
95 **Yanai H**, Matsumoto Y, Harada T, Nishiaki M, Tokiyama H, Shigemitsu T, Tada M, Okita K. Endoscopic ultrasonography and endoscopy for staging depth of invasion in early gastric cancer: a pilot study. *Gastrointest Endosc* 1997; **46**: 212-216 [PMID: 9378206 DOI: 10.1016/s0016-5107(97)70088-9]
96 **Choi J**, Kim SG, Im JP, Kim JS, Jung HC, Song IS. Comparison of endoscopic ultrasonography and conventional endoscopy for prediction of depth of tumor invasion in early gastric cancer. *Endoscopy* 2010; **42**: 705-713 [PMID: 20652857 DOI: 10.1055/s-0030-1255617]
97 **Pei Q**, Wang L, Pan J, Ling T, Lv Y, Zou X. Endoscopic ultrasonography for staging depth of invasion in early gastric cancer: A meta-analysis. *J Gastroenterol Hepatol* 2015; **30**: 1566-1573 [PMID: 26094975 DOI: 10.1111/jgh.13014]
98 **Kubota K**, Kuroda J, Yoshida M, Ohta K, Kitajima M. Medical image analysis: computer-aided diagnosis of gastric cancer invasion on endoscopic images. *Surg Endosc* 2012; **26**: 1485-1489 [PMID: 22083334 DOI: 10.1007/s00464-011-2036-z]
99 **Hirasawa T**, Aoyama K, Tanimoto T, Ishihara S, Shichijo S, Ozawa T, Ohnishi T, Fujishiro M, Matsuo K, Fujisaki J, Tada T. Application of artificial intelligence using a convolutional neural network for detecting gastric cancer in endoscopic images. *Gastric Cancer* 2018; **21**: 653-660 [PMID: 29335825 DOI: 10.1007/s10120-018-0793-2]
100 **Zhu Y**, Wang QC, Xu MD, Zhang Z, Cheng J, Zhong YS, Zhang YQ, Chen WF, Yao LQ, Zhou PH, Li QL. Application of convolutional neural network in the diagnosis of the invasion depth of gastric cancer based on conventional endoscopy. *Gastrointest Endosc* 2019; **89**: 806-815. e1 [PMID: 30452913 DOI: 10.1016/j.gie.2018.11.011]
101 **Yoon HJ**, Kim S, Kim JH, Keum JS, Oh SI, Jo J, Chun J, Youn YH, Park H, Kwon IG, Choi SH, Noh SH. A Lesion-Based Convolutional Neural Network Improves Endoscopic Detection and Depth

Prediction of Early Gastric Cancer. *J Clin Med* 2019; **8** [PMID: 31454949 DOI: 10.3390/jcm8091310]

102 **Nagao S**, Tsuji Y, Sakaguchi Y, Takahashi Y, Minatsuki C, Niimi K, Yamashita H, Yamamichi N, Seto Y, Tada T, Koike K. Highly accurate artificial intelligence systems to predict the invasion depth of gastric cancer: efficacy of conventional white-light imaging, nonmagnifying narrow-band imaging, and indigo-carmine dye contrast imaging. *Gastrointest Endosc* 2020; **92**: 866-873. e1 [PMID: 32592776 DOI: 10.1016/j.gie.2020.06.047]

103 **Horiuchi Y**, Aoyama K, Tokai Y, Hirasawa T, Yoshimizu S, Ishiyama A, Yoshio T, Tsuchida T, Fujisaki J, Tada T. Convolutional Neural Network for Differentiating Gastric Cancer from Gastritis Using Magnified Endoscopy with Narrow Band Imaging. *Dig Dis Sci* 2020; **65**: 1355-1363 [PMID: 31584138 DOI: 10.1007/s10620-019-05862-6]

104 **Namikawa K**, Hirasawa T, Nakano K, Ikenoyama Y, Ishioka M, Shiroma S, Tokai Y, Yoshimizu S, Horiuchi Y, Ishiyama A, Yoshio T, Tsuchida T, Fujisaki J, Tada T. Artificial intelligence-based diagnostic system classifying gastric cancers and ulcers: comparison between the original and newly developed systems. *Endoscopy* 2020; **52**: 1077-1083 [PMID: 32503056 DOI: 10.1055/a-1194-8771]

105 **Horiuchi Y**, Hirasawa T, Ishizuka N, Tokai Y, Namikawa K, Yoshimizu S, Ishiyama A, Yoshio T, Tsuchida T, Fujisaki J, Tada T. Performance of a computer-aided diagnosis system in diagnosing early gastric cancer using magnifying endoscopy videos with narrow-band imaging (with videos). *Gastrointest Endosc* 2020; **92**: 856-865. e1 [PMID: 32422155 DOI: 10.1016/j.gie.2020.04.079]

106 **Japanese Gastric Cancer Association**. Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer* 2011; **14**: 113-123 [PMID: 21573742 DOI: 10.1007/s10120-011-0042-4]

107 **Nagahama T**, Yao K, Imamura K, Kojima T, Ohtsu K, Chuman K, Tanabe H, Yamaoka R, Iwashita A. Diagnostic performance of conventional endoscopy in the identification of submucosal invasion by early gastric cancer: the "non-extension sign" as a simple diagnostic marker. *Gastric Cancer* 2017; **20**: 304-313 [PMID: 27165641 DOI: 10.1007/s10120-016-0612-6]

108 **Cappellani A**, Zanghi A, Di Vita M, Zanet E, Veroux P, Cacopardo B, Cavallaro A, Piccolo G, Lo Menzo E, Murabito P, Berretta M. Clinical and biological markers in gastric cancer: update and perspectives. *Front Biosci (Schol Ed)* 2010; **2**: 403-412 [PMID: 20036956 DOI: 10.2741/s73]

109 **Li T**, Huang A, Zhang M, Lan F, Zhou D, Wei H, Liu Z, Qin X. Increased Red Blood Cell Volume Distribution Width: Important Clinical Implications in Predicting Gastric Diseases. *Clin Lab* 2017; **63**: 1199-1206 [PMID: 28792691 DOI: 10.7754/Clin.Lab.2017.170115]

110 **Yao K**. The endoscopic diagnosis of early gastric cancer. *Ann Gastroenterol* 2013; **26**: 11-22 [PMID: 24714327]

111 **Horiuchi Y**, Hirasawa T, Ishizuka N, Hatamori H, Ikenoyama Y, Tokura J, Ishioka M, Tokai Y, Namikawa K, Yoshimizu S, Ishiyama A, Yoshio T, Tsuchida T, Fujisaki J. Diagnostic performance in gastric cancer is higher using endocytoscopy with narrow-band imaging than using magnifying endoscopy with narrow-band imaging. *Gastric Cancer* 2021; **24**: 417-427 [PMID: 33011866 DOI: 10.1007/s10120-020-01125-w]

112 **Ang TL**, Pittayanon R, Lau JY, Rerknimitr R, Ho SH, Singh R, Kwek AB, Ang DS, Chiu PW, Luk S, Goh KL, Ong JP, Tan JY, Teo EK, Fock KM. A multicenter randomized comparison between high-definition white light endoscopy and narrow band imaging for detection of gastric lesions. *Eur J Gastroenterol Hepatol* 2015; **27**: 1473-1478 [PMID: 26426836 DOI: 10.1097/MEG.0000000000000478]

113 **Hazewinkel Y**, López-Cerón M, East JE, Rastogi A, Pellisé M, Nakajima T, van Eeden S, Tytgat KM, Fockens P, Dekker E. Endoscopic features of sessile serrated adenomas: validation by international experts using high-resolution white-light endoscopy and narrow-band imaging. *Gastrointest Endosc* 2013; **77**: 916-924 [PMID: 23433877 DOI: 10.1016/j.gie.2012.12.018]

114 **Miyaoka M**, Yao K, Tanabe H, Kanemitsu T, Otsu K, Imamura K, Ono Y, Ishikawa S, Yasaka T, Ueki T, Ota A, Haraoka S, Iwashita A. Diagnosis of early gastric cancer using image enhanced endoscopy: a systematic approach. *Transl Gastroenterol Hepatol* 2020; **5**: 50 [PMID: 33073045 DOI: 10.21037/tgh.2019.12.16]

115 **Yamamoto S**, Nishida T, Kato M, Inoue T, Hayashi Y, Kondo J, Akasaka T, Yamada T, Shinzaki S, Iijima H, Tsujii M, Takehara T. Evaluation of endoscopic ultrasound image quality is necessary in endosonographic assessment of early gastric cancer invasion depth. *Gastroenterol Res Pract* 2012; **2012**: 194530 [PMID: 23024651 DOI: 10.1155/2012/194530]

116 **Ueyama H**, Kato Y, Akazawa Y, Yatagai N, Komori H, Takeda T, Matsumoto K, Ueda K, Hojo M, Yao T, Nagahara A, Tada T. Application of artificial intelligence using a convolutional neural network for diagnosis of early gastric cancer based on magnifying endoscopy with narrow-band imaging. *J Gastroenterol Hepatol* 2021; **36**: 482-489 [PMID: 32681536 DOI: 10.1111/jgh.15190]

117 **Hawkins DM**. The problem of overfitting. *J Chem Inf Comput Sci* 2004; **44**: 1-12 [PMID: 14741005 DOI: 10.1021/ci0342472]

118 **Qian L**, Hu L, Zhao L, Wang T, Jiang R. Sequence-Dropout Block for Reducing Overfitting Problem in Image Classification. *IEEE Access* 2020; **8**: 62830-62840 [DOI: 10.1109/ACCESS.2020.2983774]

119 **Simonyan K**, Zisserman A. Very deep convolutional networks for large-scale image recognition. 2014 Preprint. Available from: arXiv:14091556

120 **Betancur J**, Commandeur F, Motlagh M, Sharir T, Einstein AJ, Bokhari S, Fish MB, Ruddy TD, Kaufmann P, Sinusas AJ, Miller EJ, Bateman TM, Dorbala S, Di Carli M, Germano G, Otaki Y, Tamarappoo BK, Dey D, Berman DS, Slomka PJ. Deep Learning for Prediction of Obstructive

Disease From Fast Myocardial Perfusion SPECT: A Multicenter Study. *JACC Cardiovasc Imaging* 2018; **11**: 1654-1663 [PMID: 29550305 DOI: 10.1016/j.jcmg.2018.01.020]

121 **Commandeur F**, Goeller M, Razipour A, Cadet S, Hell MM, Kwiecinski J, Chen X, Chang HJ, Marwan M, Achenbach S, Berman DS, Slomka PJ, Tamarappoo BK, Dey D. Fully Automated CT Quantification of Epicardial Adipose Tissue by Deep Learning: A Multicenter Study. *Radiol Artif Intell* 2019; **1**: e190045 [PMID: 32090206 DOI: 10.1148/ryai.2019190045]

122 **Nakajima K**, Kudo T, Nakata T, Kiso K, Kasai T, Taniguchi Y, Matsuo S, Momose M, Nakagawa M, Sarai M, Hida S, Tanaka H, Yokoyama K, Okuda K, Edenbrandt L. Diagnostic accuracy of an artificial neural network compared with statistical quantitation of myocardial perfusion images: a Japanese multicenter study. *Eur J Nucl Med Mol Imaging* 2017; **44**: 2280-2289 [PMID: 28948350 DOI: 10.1007/s00259-017-3834-x]

123 **Liu H**, Motoda H. Data reduction via instance selection. In: Liu H, Motoda H. Instance selection and construction for data mining. Boston: Springer, 2001: 3-20

124 **Horiuchi Y**, Fujisaki J, Yamamoto N, Ida S, Yoshimizu S, Ishiyama A, Yoshio T, Hirasawa T, Yamamoto Y, Nagahama M, Takahashi H, Tsuchida T. Pretreatment diagnosis factors associated with overtreatment with surgery in patients with differentiated-type early gastric cancer. *Sci Rep* 2019; **9**: 15356 [PMID: 31653964 DOI: 10.1038/s41598-019-51952-w]

125 **Kuroki K**, Oka S, Tanaka S, Yorita N, Hata K, Kotachi T, Boda T, Arihiro K, Chayama K. Clinical significance of endoscopic ultrasonography in diagnosing invasion depth of early gastric cancer prior to endoscopic submucosal dissection. *Gastric Cancer* 2021; **24**: 145-155 [PMID: 32572791 DOI: 10.1007/s10120-020-01100-5]

126 **Yamada S**, Shimada M, Utsunomiya T, Morine Y, Imura S, Ikemoto T, Mori H, Hanaoka J, Iwahashi S, Saitoh Y, Asanoma M. Hepatic screlosed hemangioma which was misdiagnosed as metastasis of gastric cancer: report of a case. *J Med Invest* 2012; **59**: 270-274 [PMID: 23037199 DOI: 10.2152/jmi.59.270]

127 **Ahn JB**, Ha TK, Kwon SJ. Bone metastasis in gastric cancer patients. *J Gastric Cancer* 2011; **11**: 38-45 [PMID: 22076200 DOI: 10.5230/jgc.2011.11.1.38]

128 **Akagi T**, Shiraishi N, Kitano S. Lymph node metastasis of gastric cancer. *Cancers (Basel)* 2011; **3**: 2141-2159 [PMID: 24212800 DOI: 10.3390/cancers3022141]

129 **Esaki Y**, Hirayama R, Hirokawa K. A comparison of patterns of metastasis in gastric cancer by histologic type and age. *Cancer* 1990; **65**: 2086-2090 [PMID: 2372774 DOI: 10.1002/1097-0142(19900501)65:9<2086::aid-cncr2820650933>3.0.co;2-b]

130 **He H**, Garcia EA. Learning from imbalanced data. *IEEE Trans Knowl Data Eng* 2009; **21**: 1263-1284 [DOI: 10.1109/TKDE.2008.239]

131 **Melillo P**, De Luca N, Bracale M, Pecchia L. Classification tree for risk assessment in patients suffering from congestive heart failure *via* long-term heart rate variability. *IEEE J Biomed Health Inform* 2013; **17**: 727-733 [PMID: 24592473 DOI: 10.1109/jbhi.2013.2244902]

132 **Harari YN**. 21 Lessons for the 21st century. London: Jonathan Cape, 2018

133 **Fjelland R**. Why general artificial intelligence will not be realized. *Humanit Soc Sci Commun* 2020; **7**: 1-9 [DOI: 10.1057/s41599-020-0494-4]

134 **King's College Hospital**. Computer Aided Diagnosis of Colorectal Polyps. [accessed August 25, 2020]. In: ClinicalTrials.gov [Internet]. London: National Library of Medicine. Available from: https://ClinicalTrials.gov/show/NCT04510545 ClinicalTrials.gov Identifier: NCT04510545

135 **Neumann H**. AI for Colorectal Polyp Detection in Endoscopy. [accessed April 28, 2020]. In: ClinicalTrials.gov [Internet]. Mainz: National Library of Medicine. Available from: https://ClinicalTrials.gov/show/NCT04339855 ClinicalTrials.gov Identifier: NCT04339855

136 **Petz Aladar County Teaching Hospital**. Polyp Artificial Intelligence Study. [accessed June 29, 2020]. In: ClinicalTrials.gov [Internet]. Gyor: National Library of Medicine. Available from: https://ClinicalTrials.gov/show/NCT04425941 ClinicalTrials.gov Identifier: NCT04425941

137 **Miyaki R**, Yoshida S, Tanaka S, Kominami Y, Sanomura Y, Matsuo T, Oka S, Raytchev B, Tamaki T, Koide T, Kaneda K, Yoshihara M, Chayama K. Quantitative identification of mucosal gastric cancer under magnifying endoscopy with flexible spectral imaging color enhancement. *J Gastroenterol Hepatol* 2013; **28**: 841-847 [PMID: 23424994 DOI: 10.1111/jgh.12149]

138 **Kanesaka T**, Lee TC, Uedo N, Lin KP, Chen HZ, Lee JY, Wang HP, Chang HT. Computer-aided diagnosis for identifying and delineating early gastric cancers in magnifying narrow-band imaging. *Gastrointest Endosc* 2018; **87**: 1339-1344 [PMID: 29225083 DOI: 10.1016/j.gie.2017.11.029]

139 **Li L**, Chen Y, Shen Z, Zhang X, Sang J, Ding Y, Yang X, Li J, Chen M, Jin C, Chen C, Yu C. Convolutional neural network for the diagnosis of early gastric cancer based on magnifying narrow band imaging. *Gastric Cancer* 2020; **23**: 126-132 [PMID: 31332619 DOI: 10.1007/s10120-019-00992-2]

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REVIEW

Hepatocellular carcinoma in viral and autoimmune liver diseases: Role of CD4+ CD25+ Foxp3+ regulatory T cells in the immune microenvironment

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Abstract

More than 90% of cases of hepatocellular carcinoma (HCC) occurs in patients with cirrhosis, of which hepatitis B virus and hepatitis C virus are the leading causes, while the tumor less frequently arises in autoimmune liver diseases. Advances in understanding tumor immunity have led to a major shift in the treatment of HCC, with the emergence of immunotherapy where therapeutic agents are used to

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target immune cells rather than cancer cells. Regulatory T cells (Tregs) are the most abundant suppressive cells in the tumor microenvironment and their presence has been correlated with tumor progression, invasiveness, as well as metastasis. Tregs are characterized by the expression of the transcription factor Foxp3 and various mechanisms ranging from cell-to-cell contact to secretion of inhibitory molecules have been implicated in their function. Notably, Tregs amply express checkpoint molecules such as cytotoxic T lymphocyte-associated antigen 4 and programmed cell-death 1 receptor and therefore represent a direct target of immune checkpoint inhibitor (ICI) immunotherapy. Taking into consideration the critical role of Tregs in maintenance of immune homeostasis as well as avoidance of autoimmunity, it is plausible that targeting of Tregs by ICI immunotherapy results in the development of immune-related adverse events (irAEs). Since the use of ICI becomes common in oncology, with an increasing number of new ICI currently under clinical trials for cancer treatment, the occurrence of irAEs is expected to dramatically rise. Herein, we review the current literature focusing on the role of Tregs in HCC evolution taking into account their opposite etiological function in viral and autoimmune chronic liver disease, and we discuss their involvement in irAEs due to the new immunotherapies.

Key Words: Autoimmune liver disease; Hepatitis B virus-related chronic hepatitis; Hepatitis C virus-related chronic hepatitis; Hepatocellular carcinoma; Tumor microenvironment

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Core Tip: Hepatocellular carcinoma (HCC) is the fifth most common cancer with poor prognosis despite significant improved diagnostic and therapeutic strategies. Most of HCC occurs in cirrhotic patients, with hepatitis B and C viruses being leading causes, Recent studies showed that HCC development and progression are associated with a unique immune response profile of the liver microenvironment where CD4+CD25+ Foxp3 regulatory T-cells (Tregs) play a crucial role through their immunosuppressive role. We discuss the role of Tregs in chronic liver diseases as well as in HCC initiation and the role of immunotherapy to enhance anti-tumor immune response by blocking Treg activity.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most frequent cause of cancer-related death worldwide, with more than 900000 new cases and more than 800000 deaths in 2020[1].

HCC accounts for nearly 90% of primary liver cancers and is a leading world health problem. The incidence of HCC rises sharply with age in all populations, achieving a peak at age 70 and it is increasing in most countries representing the dominant cause of mortality in cirrhotic patients[2-4].

Worldwide, chronic viral hepatitis has been reported as the leading risk factors for HCC development, although in high-income areas HCC related to non-alcoholic fatty liver disease is increasing due to the rising prevalence of metabolic disorders[5-7].

In contrast, vaccination and treatment for hepatitis B virus (HBV) infection, prevention campaigns for sexual and iatrogenic transmission of hepatitis B (HBV) and hepatitis C virus (HCV), and the introduction of effective HCV antiviral agents are reducing the burden of chronic viral liver disease[8-11].

HCC arises almost exclusively in the setting of chronic liver inflammation and, unlike the elevated risk associated with chronic viral (HBV and HCV) infections, it has been reported that the tumor is less common in liver cirrhosis caused by autoimmune liver diseases[12-15]. However, it is known that, regardless of etiology, cirrhosis *per se* represents a precancerous condition leading to an increased risk of HCC[15,16].

Indeed, irrespective of etiology, a typical sequence of chronic necroinflammation, compensatory liver regeneration, induction of liver fibrosis and subsequent cirrhosis often precedes hepatocarcinogenesis. HCC is a prototypical inflammation-driven tumor arising on the backdrop of liver cirrhosis. The evidence of an immune-rich contexture of the HCC microenvironment has inspired several studies in recent years that have further defined profile and crucial pathogenetic role of immune cells in tumor development[17].

The liver is a central immunomodulator that ensures organ and systemic protection while maintaining immunotolerance. Deregulation of this tightly controlled liver immunological network is a hallmark of chronic liver disease and HCC[18].

Recently, within the cell subset characterizing the HCC immune microenvironment, a key role has been highlighted for CD4+ CD25+ regulatory T cells (Tregs), which are crucially implicated in both pathogenesis of chronic liver diseases and development and spread of HCC[19,20].

In this review we examine the evidence that has recently accumulated on the different role that these cells play in chronic viral liver diseases (HBV and HCV) and autoimmune liver diseases, and their function in the tumor microenvironment (TME) characterizing HCC. We also discuss the possible implications for emerging immunotherapies and the potential risks of immune-mediated liver toxicity from this treatment [21].

HCC RISK IN VIRAL AND AUTOIMMUNE LIVER DISEASES

It is well established that cirrhosis represents the most significant risk factor for the development of HCC[22]. Historically, it has been reported that the risk of HCC in cirrhosis due to viral causes is higher than in other non-viral etiologies, however a precise comparison of the incidence of HCC in various chronic liver diseases, especially in cirrhosis, has only recently been evaluated[23,24].

In a recent meta-analysis it was shown that the annual incidence of HCC in chronic liver diseases and the ratio of HCC incidence in non-cirrhotic/cirrhotic stages has the following etiological hierarchy: HCV-related disease 0.68% to 4.81% (7.07-fold, $P < 0.001$), HBV-related liver disease 0.37% to 3.23% (8.73 fold, $P < 0.001$), primary biliary cholangitis (PBC) (pre-cirrhotic *vs* Scheuer's III-IV stage) 0.26 to 1.79% (6.88-fold, $P < 0.001$), NASH 0.03% to 1.35% (45-fold, $P < 0.001$), autoimmune hepatitis (AIH) 0.19% to 0.53% (2.79-fold, $P = 0.03$), and that the incidence of HCC is markedly increased (2.79-fold to 45-fold) in the cirrhotic stage compared with the non-cirrhotic stage, regardless of etiology[24]. Thus, it is confirmed that there is a significant difference in HCC incidence between viral and autoimmune liver diseases, with the lowest risk in the latter even when in the cirrhotic stage.

Worldwide, about 54% of cases can be ascribed to HBV infection (affecting 400 million people globally) while 31% can be associated with HCV infection (affecting 170 million people), leaving about 15% attributable to other causes[25].

Incidence of HCC in autoimmune liver disease is less definitively established. In a recent systematic review of 25 published cohorts, a total of 6.528 AIH patients with a median follow-up of 8 years were evaluated for the incidence of HCC. The pooled incidence rate was 3.1 per 1.000 person-years in AIH patients that tripled in those with cirrhosis[26,27].

PBC-related cirrhosis has also been reported as a potential HCC risk factor. In a study of 273 PBC-related cirrhotic patients, followed for 3 years, the incidence rate was 5.9%, significantly higher in males with stage III/IV disease than in females[28].

In a systematic review of 17 studies, including 16.368 patients seen between 1984 and 2011, compared with the general population, PBC patients exhibited a significantly higher risk of HCC (pooled risk ratio 18.80; 95% confidence interval: 10.81-26.79)[29].

Factors associated with an increased risk of HCC development in chronic liver disease have been only partially defined. However, chronic inflammation has been reported as a crucial mechanism for the development of HCC[30-32].

In this respect, it has been reported that in HBV and HCV cirrhotic patients, transaminase serum level is one of the predictive factors for the development of HCC[32].

Similar findings have been reported in AIH as persistent elevation of serum transaminases was reported to be associated with development of HCC hence supporting the prominent pathogenic role of chronic inflammation. Other emerged risk factors included cirrhosis ≥ 10 years, portal hypertension, and immunosuppressive therapy ≥ 3 years[33].

The risk factors of HCC usually lead to a unresolving inflammatory response and necrosis resulting in tissue damage which in turn drives the sequential development of regeneration, fibrosis, cirrhosis, and eventually HCC[34,35]. In parallel, immune cells within the premalignant environment produce a wide range of cytokines, growth factors, chemokines, prostaglandins, and proangiogenic factors, contributing to an environment that supports hepatocyte transformation and promotes their survival through activation of anti-apoptotic pathways, neoangiogenesis and inhibition of immune surveillance[36].

It has been recently established that the carcinogenic process is aided by a host of immuno-related factors intrinsically linked to cell infiltrate, chemokines and their receptors that foster cell survival and proliferation[37].

In this regard, it has increasingly gained relevance to fully define the immunological characteristics of liver immune microenvironment.

TREGS IN VIRAL AND AUTOIMMUNE LIVER DISEASES

The liver can be considered as an "immunological" organ, housing a wide range of resident immune cells performing key functions in preserving organ homeostasis[38].

As a result of its intrinsic role in detoxification, the liver is repeatedly exposed to external agents, including dietary products or commensal bacteria derived from the intestine *via* the portal vein, as well as infectious microorganisms arising from the systemic circulation *via* the arterial vein. Therefore, immune surveillance in the organ is extremely dynamic.

Resident innate immune cells comprising macrophages or Kupffer cells, natural killer (NK) cells, NKT cells, and dendritic cells (DCs) are recognized as the most predominant sentinels in the liver[39]. In addition, tissue resident memory T cells, which are normally homing cells without recirculating and which readily attack pathogens at the site of infection, are also implicated[40]. However, a key role appears to be played by resident Tregs that are highly specialized in preserving tissue tolerance [19,41]. Tregs are a subset of T lymphocytes that regulate the immune response by suppressing the proliferation and cytokines production of effector T lymphocytes[42, 43].

In 2003, the forkhead box transcription factor foxp3 was identified as a specific marker of Tregs, and its expression was found crucial for their suppressive activity[44-46].

Tregs arise in the thymus, constitutively express high levels of the interleukin (IL)-2 receptor (IL-2R) α chain (CD25), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and glucocorticoid-induced TNF receptor family-related gene (*GITR*), accounting for 5% to 10% of peripheral CD4+ T cells[47,48].

They play a critical role in mediating immunological self-tolerance by suppressing self-reactive T lymphocytes[44]. Tregs have been proposed to operate both through core mechanisms of suppression, including IL-2 deprivation and CTLA-4–mediated downregulation of costimulatory molecules on antigen presenting cells (APCs), and by diverse context-dependent mechanisms, including the secretion of cytokines[49-52]. Earlier studies have confirmed that Tregs mediate suppressive effects *in vivo* mainly through the production of inhibitory cytokines such as IL-10, IL-35 and transforming growth factor β (TGF-β)[53-55].

With the discovery of Tregs and the understanding of their immunosuppressive role, evidences have been accumulated that this cell population is decisively implicated in the pathogenesis of various conditions such as chronic viral and autoimmune liver diseases as well as HCC[56].

In particular, CD4+CD25+ Tregs are thought to contribute to the impaired immune response during chronic HBV and HCV infection. Patients with chronic HBV infection are characterized by increased percentage of CD4+CD25+ Tregs in their peripheral blood and a significant accumulation of these cells in the liver, with a positive correlation between their frequency and serum HBV DNA load[57-60]. Similarly, in patients with persistent HCV infection it has been reported an increased frequency of CD4+CD25+ Tregs in the blood and in the liver[61-65]. Taken together, these data prove that chronic HBV and HCV infections are immunologically characterized by a host immune response suppression driven by Tregs.

On the contrary, autoimmune liver diseases are related to both numerical and functional defect of CD4+CD25+ Tregs, to the extent that therapeutic interventions aimed at restoring an adequate number and function of these cells are followed by a remission of the autoimmune inflammatory activity[66,67].

These findings have thus outlined a pattern of chronic inflammation characterized by a diametrically opposed liver immune phenotype in chronic viral and autoimmune diseases, the former being characterized by a predominance of Tregs exerting an immunosuppressive effect that hinders the antiviral response and infection eradication, the latter by a significant numerical and functional deficiency of Tregs that do not adequately suppress self-reactive lymphocytes[68-70].

While it has been widely reported that TME characterizing HCC during chronic viral liver disease is dominated by a marked Treg infiltration likely in continuity with the conditions favoring chronic infection, the expression pattern of Tregs in the TME supporting HCC development in autoimmune liver disease is not as well known[71,72].

This finding would be relevant for the assessment of potential adverse events related to emerging immunotherapies that cause a decline in Treg number and function and are therefore associated with the risk of triggering autoimmune disorders, as we discuss below.

IMMUNE TME IN HCC: ROLE OF CD4+CD25+ TREGS

Tumor infiltrating lymphocytes represent the host immune response to cancer and comprise CD8+ cytotoxic T lymphocytes (CTLs) and NK cells as favorable anti-tumor responders, and CD4+ CD25+ Tregs as immunosuppressors.

Recently, many studies have shown that the TME plays a major role in HCC initiation and progression[73]. Lymphocytes contribute to the TME through immunity and inflammation. CD8+ CTLs can directly kill target cells by releasing granules including membrane-lytic materials such as perforin and granzymes (granzyme A and B) in acquired immune responses, thus covering a crucial role in anti-tumor immunity. As a matter of fact, a large presence of CD8+ CTL infiltrating tumor tissue is closely associated with a better prognosis[74,75]. However, despite T cell infiltration, HCC develops and spreads as a result of a depletion of pro-inflammatory T cells and a significant accumulation of Tregs[76-78].

T-cell exhaustion is characterized by reduced responses to stimulation, impaired cytokine production, decreased proliferation and reduced toxicity. Such immune profile is hallmarked by over-expression of co-inhibitory receptors such as CTLA-4 and programmed cell-death 1 receptor (PD-1).

An exhausted state of circulating and intratumoral CD8+ T cells is associated with a worse prognosis in HCC patients[79,80].

Exhaustion inside the TME is dominated by the inhibitory cytokine environment rich in IL-10 and TGF-β released by the Treg, both prohibiting CTLs and TH1 CD4+ T cells activation[81,82].

In addition, tumor associated neutrophils (by secreting CCL17 and CCR4) and M2 tumor-associated macrophages (by secreting IL-10) can induce CD4+ CD25+ Tregs thereby indirectly supporting tumor growth and progression[83,84] (Figure 1).

TARGETING CD4+CD25+ TREGS IN HCC MICROENVIRONMENT WITH IMMUNOTHERAPY

A significant advance in the field of immunotherapy has now reached with the use of immune checkpoint inhibitors (ICIs), which are antagonistic antibodies that inhibit key immune regulatory molecules (checkpoint molecules), such as CTLA-4, PD-1, and its ligand PD-L1, which suppresses T cell effector function under physiological conditions [85].

Tregs are among the most prevalent suppressor cells in TME and their presence has been related to tumor progression, invasiveness, and metastasis. Their regulatory function involves a broad spectrum of immune cells besides T cells, including macrophages, DCs, neutrophils, NK cells, T cells, and innate lymphoid cells[86-88].

There are many factors favoring Treg enrichment in the TME. Experimental evidences implicate the Treg recruitment within the tumor mass through chemokines produced by cancer cells and, specifically, HCC cells have been shown to secrete CCL5, CCL22 and CCL28 chemokines mediating Treg accumulation (Table 1)[89-100].

Table 1 Regulatory T cell function and recruitment in hepatocellular carcinoma

Treg function in HCC		Ref.
Molecule	**Target**	
IL-10	IL-10R	Marra and Tacke[90]
IL-35	IL-12Rβ2	Shen *et al*[91]
TGF-β	TGF-βR	Fu *et al*[92]
CTLA-4	CD80/CD86	Chen *et al*[93]
CD39-CD73	ATP	Chen *et al*[94]
IL-2 Rα (CD 25)	IL-2	Li *et al*[95]
LAG3	MHC class IImolecules	Cabrera *et al*[96]
Treg recruitment in HCC		**Ref.**
Molecule	**Receptor**	
CCL22	CCR4	Li *et al*[97]
CCL5	CCR5	Cheng *et al*[98]
CCL28	CCR3, CCR10	Singh *et al*[99]

CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; LAG3: Lymphocyte activation gene 3 protein; IL: Interleukin; TGF-β: Transforming growth factor β; Treg: Regulatory T cell; HCC: Hepatocellular carcinoma.

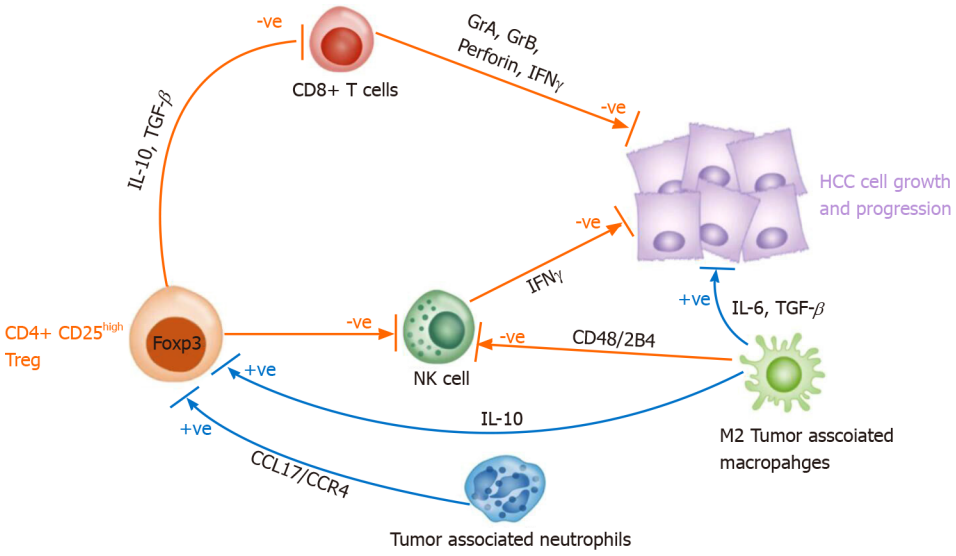


Figure 1 The hepatocellular carcinoma liver microenvironment is characterized by a large population of immune cells. Natural killer and CD8+ T cells exert antitumor effect by secreting interferon-γ, granzyme A, granzyme B and perforin, however they are mostly defective since these cells are suppressed by CD4+ CD25 regulatory T cell (Tregs). Additionally, tumor associated neutrophils (by secreting CCL17 and CCR4) and M2 tumor associated macrophages (by secreting interleukin-10) can induce CD4+ CD25+ Tregs thus supporting tumor growth and progression. NK: Natural killer; Treg: Regulatory T cell; IL: Interleukin; TGF-β: Transforming growth factor β; IFN: Interferon; HCC: Hepatocellular carcinoma.

The mechanisms through which Tregs induce suppression of proliferation, activation and function of immune effector cells have been well studied. Firstly, they modulate the activity of APCs by engaging inhibitory co-stimulatory receptors on their surface and in this way, signaling between APCs and T cells is impaired or abolished [101,102].

On a similar line, they down-regulate the expression of CD40, CD80 and CD86 on DCs[103]. Second, Tregs, through the secretion of inhibitory cytokines (*e.g.*, IL-10, IL-35, TGF-β), repress the activity of immune cells[104].

Of major interest, Tregs express a panel of chemokine receptors and surface molecules such as CTLA4, PD-1 and others, thus potentially making them a very direct target of ICI immunotherapy (Figure 2).

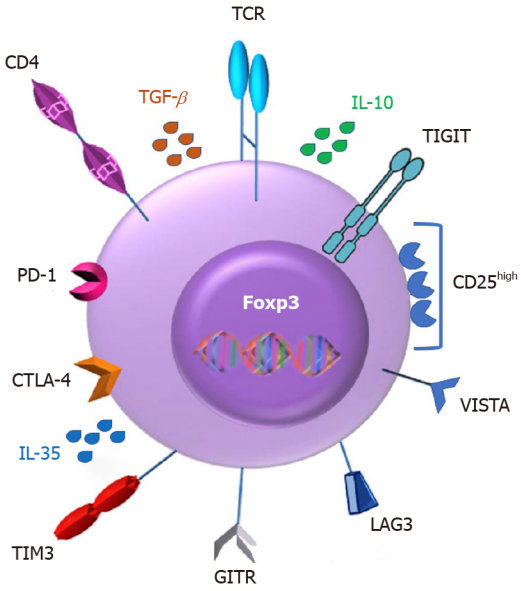


Figure 2 Regulatory T cells are recognized as CD4+CD25high positive and Foxp3+ expressing cells. They feature a range of other phenotypic markers such as T-cell immunoreceptor with Ig and ITIM domains, glucocorticoid-induced tumor necrosis factor receptor-related protein, cytotoxic T lymphocyte-associated antigen 4, programmed cell-death 1 receptor, V-domain Ig suppressor of T cell activation, lymphocyte activation gene-3, T cell immunoglobulin mucin 3. Upon activation, Tregs release the inhibitory cytokines interleukin (IL)-10, transforming growth factor β and IL-35. TCR: T-cell receptor; IL: Interleukin; TGF-β: Transforming growth factor β; TIGIT: T-cell immunoreceptor with Ig and ITIM domains; VISTA: V-domain Ig suppressor of T cell activation; LAG3: Lymphocyte activation gene-3; GITR: Glucocorticoid-induced tumor necrosis factor receptor-related protein; TIM3: T cell immunoglobulin mucin 3; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4; PD-1: Programmed cell-death 1 receptor.

Advances in understanding tumor immunity have resulted in a significant shift in the HCC treatment, with the emergence of immunotherapy where therapeutic interventions are used to target immune cells rather than cancer cells.

Tregs abundantly express both co-inhibitory and co-stimulatory molecules at levels that are likely dependent on the TME. Modulating their function through stimulation of inhibitory receptors and inhibition of activating receptors could therefore result in a decrease of the TME immunosuppressive profile resulting ultimately in an enhanced antitumor immune response (Figure 3).

In support of this therapeutic approach, treatment with blocking antibodies for PD-1 (nivolumab, pembrolizumab, sintilimab, penpulimab, camrelizumab, toripalimab, spartalizumab, tislelizumab), programmed death ligand 1 (durvalumab, avelumab, atezolizumab), and CTLA-4 (tremelimumab, ipilimumab) have reported promising results in HCC treatment[105].

Since CTLA-4 is constitutively expressed on Tregs, its specific deletion is associated with a marked reduction of their suppressive ability potentially resulting in a severe T cell mediated autoimmune disease[106].

Resulting in a break of balance in the immune system, ICI treatments can give rise to a broad spectrum of serious autoimmune manifestations, including liver injury, reported in recent studies as immune-related adverse events (irAEs) and potentially limiting or precluding HCC treatments[107,108].

Unfortunately, clinical studies that include in their objectives the assessment of immune cell populations, particularly Tregs, are still too scarce, thus leaving this potentially relevant issue unresolved. Clinical trials based on ICI agents as treatment for HCC are reported in Table 2.

LIVER TOXICITY DURING TREATMENT WITH ICIS

The development of irAEs, potentially affecting multiple organs, following loss of self-tolerance, has been widely reported[109,110].

Among the possible adverse events, of particular relevance is the development of liver toxicity as it could cause worsening of liver function in patients who almost always have underlying preexisting chronic liver disease[107].

Table 2 Clinical trials assessing immune cell populations as study outcome

Clinical trials identifier	Official title	Phase	Secondary outcome	Intervention/treatment	Status
NCT04518774	The safety assessment of *ex vivo* expanded allogeneic γδT cells in hepatocellular carcinoma patients in phase 1 clinical trial	I	Number and phenotype of γδT cells in peripheral blood	Patients will receive 3 cycles of *ex vivo* expanded allogeneic γδT cells treatments, at four-weeks' intervals, each cycle has 2 infusions. *Ex vivo* expanded γδT cells are transfused to patients in a dosage escalated manner (dose escalation, 1×10^7, 3×10^7, 9×10^7 per kg of body weight)	Recruiting
NCT03841201	IMMUNIB: An open-label, single-arm phase II study of immunotherapy with nivolumab in combination with lenvatinib for advanced stage	II	Immune cell infiltrates FOXP3 expression	Lenvatinib peroral qd (8 mg for patients with body weight < 60 kg and 12 mg for patients with body weight ≥ 60 kg); Nivolumab i.v. q2w (240 mg fixed dose IV) max. 36 cycles	Recruiting
NCT04777708	Pilot feasibility study of intratumoral BO-112 in combination with pembrolizumab for advanced hepatocellular carcinoma	I	Intratumoral CD4+, CD8+ expression and cluster of differentiation 56 (CD56+) expression (natural killer cells)	Patients receive pembrolizumab IV over 30 min on day 1 of odd number cycles. Patients also receive BO-112 by intratumoral injection on day 1, 8, and 15 of cycle 1, and day 15 of subsequent cycles. Treatment repeats every 3 wk for up to 17 cycles in the absence of disease progression or unacceptable toxicity	Not yet recruiting
NCT04721132	An open-label, phase II, pre-operative study of atezolizumab plus bevacizumab for resectable hepatocellular carcinoma	II	To measure baseline and longitudinal changes of immune infiltration including CD8/regulatory T cell ratio and CD68+ density, and fibrosis stage	Patients receive atezolizumab IV over 30-60 min and bevacizumab IV over 30-90 min on day 1. Treatment repeats every 21 d for up to 3 cycles in the absence of disease progression or unacceptable toxicity. Patients then undergo surgery during week 12	Recruiting
NCT00396682	Elimination of CD4+CD25+ regulatory T cells in patients with advanced hepatocellular carcinoma after treatment with cyclophosphamide	I	Function and Phenotype of CD4+CD25+ regulatory T cells	Cyclophosphamide 150 mg to 250 mg to 350 mg	Completed

Clinical trials assessing as secondary study outcomes different immune cell populations, including CD4+CD25+ Tregs (https://www.clinicaltrials.gov/).

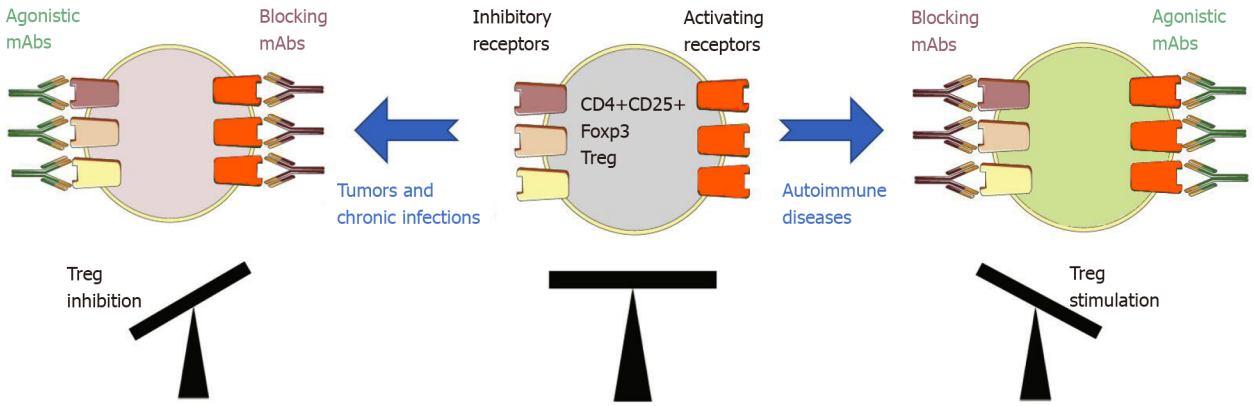


Figure 3 Distinct targeting of regulatory T cell activating and inhibitory receptor-targeted therapies in cancer and autoimmunity. Regulatory T cell (Treg) cells are equipped with a repertoire of activating and inhibitory receptors. For successful therapy of tumors and chronic infections, blockade of activating receptors and/or stimulation of inhibitory receptors shifts the balance toward inhibition of Tregs. In contrast, to achieve Treg activation in autoimmune diseases, blockade of inhibitory receptors and/or stimulation of activating receptors may be desirable. Treg: Regulatory T cell.

Hepatic toxicity associated with ICI is characterized by elevation of liver parameters values. The pattern of liver enzymes elevation is defined by the increase of alanine aminotransferase (ALT) or alkaline phosphatase (ALP) alone above a specific threshold or by the ratio of serum ALT to ALP levels [R value = [ALT/upper normal level (UNL)]/(ALP/UNL)] and can be categorized as hepatocellular (ALT ≥ 5-fold above UNL or R > 5), mixed (R > 2 to < 5), or cholestatic (ALP ≥ 2 fold above UNL or R < 2)[111].

The pattern of ICI-related liver toxicity is heterogenous since it may be cytolytic, cholestatic or mixed, although ICI-related cholestasis seems to be rarer[107].

In HCC patients receiving ICIs the incidence of liver toxicity ranges according to the type of drug and the dose received. It has been reported that liver toxicity is more frequent in HCC patients receiving anti-CTLA-4 therapies. In HCC patients receiving the anti-PD-1 antibody nivolumab (CHECKMATE 040 trial) and in those receiving another anti-PD-1 antibody pembrolizumab (KEYNOTE-224 trial), ALT elevation of any grade and of grade ≥ 3 was found in 15% and 6% (nivolumab) *vs* 9% and 4% (pembrolizumab), respectively[112,113].

Differently, therapy with the anti-CTLA-4 antibody tremelimumab was associated with an ALT elevation of any grade and of grade ≥ 3 in 19% and 9% of patients, respectively[114].

Interestingly, during HCC clinical studies, HCV and HBV positive patients exhibited a reduction in viral load during immunotherapy, more pronounced with anti-CTLA-4 agents, thus suggesting that the treatment induced immunological shift has favorable effect on antiviral response[112-116].

No definitive data has been reported about patients with autoimmune liver diseases treated with ICIs. However, in most of the HCC clinical trial, pre-existing autoimmune diseases was a contraindication for enrollment in light of previous data demonstrating that immunotherapies might trigger a flare-up of pre-existing autoimmune disease or the onset of additional immune-related disease[117-119].

No data are available concerning genetic and autoantibody profile of patients before starting ICI treatments, however autoantibodies such as antinuclear and anti-smooth muscle have been reported in patients after ICI-induced liver toxicity onset.

A management protocol for patients experiencing liver toxicity due to ICI treatment administered for non-liver tumors has been proposed and is based on corticosteroids or mycophenolate mofetil/tacrolimus (for patients not improving under corticosteroids), and ursodeoxycholic acid for those with a predominant cholestasis[120].

CONCLUSION

Increasing understanding of the immunologic mechanisms that characterize the TME of HCC has led to a better insight into the pathogenesis of HCC and its link to chronic inflammation and cirrhosis. As well as, the different prevalence in viral *vs* non-viral liver diseases, particularly autoimmune, confirms the key role of the liver immune microenvironment.

Although the precise pathogenetic mechanisms of irAEs remain largely undefined, several processes have been proposed to be involved in the development of irAEs such as genetic factors, gut microbiome, epitope spreading, and cross-presentation of neoantigens[121,122]. A thorough evaluation on the role of Tregs in the pathogenesis of irAEs is crucial. Since cancer and autoimmunity constitute two sides of the same coin, it is perhaps not surprising that when we manipulate the immune system to treat cancer through the use of checkpoint therapy, we inevitably unbalance the vital mechanisms that regulate self-tolerance, inducing a number of irAEs. This is, at least in part, related to the impairment of Treg homeostasis, which is crucial for maintaining immune tolerance[123].

Currently used ICIs may also target Tregs, since several checkpoint molecules including CTLA4 and PD-1 are highly expressed on their surface, therefore it is possible that the development of irAEs may be in part attributed to the Treg destabilization. Consistent with this line, it has been demonstrated that anti-CTLA4 disrupts the crosstalk between Foxp3 Tregs and antigen-presenting cells to promote autoimmunity[124]. In light of the effects of immunotherapy on the enhancement of the immune response, it should be investigated whether in cases of HCC occurring in patients with pre-existing autoimmune liver and non-liver diseases these therapies are safe and not potentially hepatotoxic, since presently this information is lacking due to the exclusion of patients with autoimmune diseases from clinical trials. Of interest, other approaches to target the immunosuppressive effect of Tregs are ongoing. Given the TGF-β mediated inhibitory role in HCC development and progression, studies are ongoing to assess how to target TGF-β. Galunisertib, an inhibitor of TGF-β, is currently in a phase II clinical trial for HCC patients[125,126].

Due to the potential effect of immunotherapies on Tregs and the possible effect of triggering autoimmune disorders, it is desirable that in future HCC trials the autoantibody profile, as well as genetic background (HLA) and change in the percentage of peripheral Treg during therapy be monitored to assess whether these

variables predict the risk of hepatotoxicity or extrahepatic autoimmune disorders development.

REFERENCES

1 **International Agency for Research on Cancer**. Global Cancer Observatory. [cited 10 Febraury 2021]. In: International Agency for Research on Cancer [Internet]. Available from: https://gco.iarc.fr/today/home

2 **El-Serag HB**. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012; **142**: 1264-1273. e1 [PMID: 22537432 DOI: 10.1053/j.gastro.2011.12.061]

3 **White DL**, Thrift AP, Kanwal F, Davila J, El-Serag HB. Incidence of Hepatocellular Carcinoma in All 50 United States, From 2000 Through 2012. *Gastroenterology* 2017; **152**: 812-820. e5 [PMID: 27889576 DOI: 10.1053/j.gastro.2016.11.020]

4 **Singal AG**, Lampertico P, Nahon P. Epidemiology and surveillance for hepatocellular carcinoma: New trends. *J Hepatol* 2020; **72**: 250-261 [PMID: 31954490 DOI: 10.1016/j.jhep.2019.08.025]

5 **Siegel AB**, Zhu AX. Metabolic syndrome and hepatocellular carcinoma: two growing epidemics with a potential link. *Cancer* 2009; **115**: 5651-5661 [PMID: 19834957 DOI: 10.1002/cncr.24687]

6 **Baffy G**, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *J Hepatol* 2012; **56**: 1384-1391 [PMID: 22326465 DOI: 10.1016/j.jhep.2011.10.027]

7 **Dyson J**, Jaques B, Chattopadyhay D, Lochan R, Graham J, Das D, Aslam T, Patanwala I, Gaggar S, Cole M, Sumpter K, Stewart S, Rose J, Hudson M, Manas D, Reeves HL. Hepatocellular cancer: the impact of obesity, type 2 diabetes and a multidisciplinary team. *J Hepatol* 2014; **60**: 110-117 [PMID: 23978719 DOI: 10.1016/j.jhep.2013.08.011]

8 **Singal AK**, Singh A, Jaganmohan S, Guturu P, Mummadi R, Kuo YF, Sood GK. Antiviral therapy reduces risk of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis. *Clin Gastroenterol Hepatol* 2010; **8**: 192-199 [PMID: 19879972 DOI: 10.1016/j.cgh.2009.10.026]

9 **Morgan TR**, Ghany MG, Kim HY, Snow KK, Shiffman ML, De Santo JL, Lee WM, Di Bisceglie AM, Bonkovsky HL, Dienstag JL, Morishima C, Lindsay KL, Lok AS; HALT-C Trial Group. Outcome of sustained virological responders with histologically advanced chronic hepatitis C. *Hepatology* 2010; **52**: 833-844 [PMID: 20564351 DOI: 10.1002/hep.23744]

10 **Santi V**, Buccione D, Di Micoli A, Fatti G, Frigerio M, Farinati F, Del Poggio P, Rapaccini G, Di Nolfo MA, Benvegnù L, Zoli M, Borzio F, Giannini EG, Caturelli E, Chiaramonte M, Bernardi M, Trevisani F. The changing scenario of hepatocellular carcinoma over the last two decades in Italy. *J Hepatol* 2012; **56**: 397-405 [PMID: 21756850 DOI: 10.1016/j.jhep.2011.05.026]

11 **Simmons B**, Saleem J, Heath K, Cooke GS, Hill A. Long-Term Treatment Outcomes of Patients Infected With Hepatitis C Virus: A Systematic Review and Meta-analysis of the Survival Benefit of Achieving a Sustained Virological Response. *Clin Infect Dis* 2015; **61**: 730-740 [PMID: 25987643 DOI: 10.1093/cid/civ396]

12 **Rigopoulou EI**, Dalekos GN. Current Trends and Characteristics of Hepatocellular Carcinoma in Patients with Autoimmune Liver Diseases. *Cancers (Basel)* 2021; **13** [PMID: 33804480 DOI: 10.3390/cancers13051023]

13 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

14 **Levrero M**, Zucman-Rossi J. Mechanisms of HBV-induced hepatocellular carcinoma. *J Hepatol* 2016; **64**: S84-S101 [PMID: 27084040 DOI: 10.1016/j.jhep.2016.02.021]

15 **Marrero JA**, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; **68**: 723-750 [PMID: 29624699 DOI: 10.1002/hep.29913]

16 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

17 **Ringelhan M**, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. *Nat Immunol* 2018; **19**: 222-232 [PMID: 29379119 DOI: 10.1038/s41590-018-0044-z]

18 **Jenne CN**, Kubes P. Immune surveillance by the liver. *Nat Immunol* 2013; **14**: 996-1006 [PMID: 24048121 DOI: 10.1038/ni.2691]

19 **Oo YH**, Sakaguchi S. Regulatory T-cell directed therapies in liver diseases. *J Hepatol* 2013; **59**: 1127-1134 [PMID: 23727305 DOI: 10.1016/j.jhep.2013.05.034]

20 **Granito A**, Muratori P, Ferri S, Pappas G, Quarneti C, Lenzi M, Bianchi FB, Muratori L. Diagnosis and therapy of autoimmune hepatitis. *Mini Rev Med Chem* 2009; **9**: 847-860 [PMID: 19519509 DOI: 10.2174/138955709788452676]

21 **Kole C**, Charalampakis N, Tsakatikas S, Vailas M, Moris D, Gkotsis E, Kykalos S, Karamouzis MV, Schizas D. Immunotherapy for Hepatocellular Carcinoma: A 2021 Update. *Cancers (Basel)* 2020; **12** [PMID: 33020428 DOI: 10.3390/cancers12102859]

22 **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and

risk factors. *Gastroenterology* 2004; **127**: S35-S50 [PMID: 15508101 DOI: 10.1053/j.gastro.2004.09.014]

23 **Forner A**, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018; **391**: 1301-1314 [PMID: 29307467 DOI: 10.1016/S0140-6736(18)30010-2]

24 **Tarao K**, Nozaki A, Ikeda T, Sato A, Komatsu H, Komatsu T, Taguri M, Tanaka K. Real impact of liver cirrhosis on the development of hepatocellular carcinoma in various liver diseases-meta-analytic assessment. *Cancer Med* 2019; **8**: 1054-1065 [PMID: 30791221 DOI: 10.1002/cam4.1998]

25 **Global Burden of Disease Liver Cancer Collaboration**, Akinyemiju T, Abera S, Ahmed M, Alam N, Alemayohu MA, Allen C, Al-Raddadi R, Alvis-Guzman N, Amoako Y, Artaman A, Ayele TA, Barac A, Bensenor I, Berhane A, Bhutta Z, Castillo-Rivas J, Chitheer A, Choi JY, Cowie B, Dandona L, Dandona R, Dey S, Dicker D, Phuc H, Ekwueme DU, Zaki MS, Fischer F, Fürst T, Hancock J, Hay SI, Hotez P, Jee SH, Kasaeian A, Khader Y, Khang YH, Kumar A, Kutz M, Larson H, Lopez A, Lunevicius R, Malekzadeh R, McAlinden C, Meier T, Mendoza W, Mokdad A, Moradi-Lakeh M, Nagel G, Nguyen Q, Nguyen G, Ogbo F, Patton G, Pereira DM, Pourmalek F, Qorbani M, Radfar A, Roshandel G, Salomon JA, Sanabria J, Sartorius B, Satpathy M, Sawhney M, Sepanlou S, Shackelford K, Shore H, Sun J, Mengistu DT, Topór-Mądry R, Tran B, Ukwaja KN, Vlassov V, Vollset SE, Vos T, Wakayo T, Weiderpass E, Werdecker A, Yonemoto N, Younis M, Yu C, Zaidi Z, Zhu L, Murray CJL, Naghavi M, Fitzmaurice C. The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level: Results From the Global Burden of Disease Study 2015. *JAMA Oncol* 2017; **3**: 1683-1691 [PMID: 28983565 DOI: 10.1001/jamaoncol.2017.3055]

26 **Tansel A**, Katz LH, El-Serag HB, Thrift AP, Parepally M, Shakhatreh MH, Kanwal F. Incidence and Determinants of Hepatocellular Carcinoma in Autoimmune Hepatitis: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2017; **15**: 1207-1217. e4 [PMID: 28215616 DOI: 10.1016/j.cgh.2017.02.006]

27 **Lleo A**, de Boer YS, Liberal R, Colombo M. The risk of liver cancer in autoimmune liver diseases. *Ther Adv Med Oncol* 2019; **11**: 1758835919861914 [PMID: 31320937 DOI: 10.1177/1758835919861914]

28 **Jones DE**, Metcalf JV, Collier JD, Bassendine MF, James OF. Hepatocellular carcinoma in primary biliary cirrhosis and its impact on outcomes. *Hepatology* 1997; **26**: 1138-1142 [PMID: 9362353 DOI: 10.1002/hep.510260508]

29 **Liang Y**, Yang Z, Zhong R. Primary biliary cirrhosis and cancer risk: a systematic review and meta-analysis. *Hepatology* 2012; **56**: 1409-1417 [PMID: 22504852 DOI: 10.1002/hep.25788]

30 **Tarao K**, Rino Y, Ohkawa S, Shimizu A, Tamai S, Miyakawa K, Aoki H, Imada T, Shindo K, Okamoto N, Totsuka S. Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. *Cancer* 1999; **86**: 589-595 [PMID: 10440686 DOI: 10.1002/(sici)1097-0142(19990815)86:4<589::aid-cncr7>3.0.co;2-k]

31 **Chen CF**, Lee WC, Yang HI, Chang HC, Jen CL, Iloeje UH, Su J, Hsiao CK, Wang LY, You SL, Lu SN, Chen CJ; Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer in HBV (REVEAL–HBV) Study Group. Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. *Gastroenterology* 2011; **141**: 1240-1248, 1248.e1-1248. e2 [PMID: 21703214 DOI: 10.1053/j.gastro.2011.06.036]

32 **Ishikawa T**, Ichida T, Yamagiwa S, Sugahara S, Uehara K, Okoshi S, Asakura H. High viral loads, serum alanine aminotransferase and gender are predictive factors for the development of hepatocellular carcinoma from viral compensated liver cirrhosis. *J Gastroenterol Hepatol* 2001; **16**: 1274-1281 [PMID: 11903747 DOI: 10.1046/j.1440-1746.2001.02616.x]

33 **Montano-Loza AJ**, Carpenter HA, Czaja AJ. Predictive factors for hepatocellular carcinoma in type 1 autoimmune hepatitis. *Am J Gastroenterol* 2008; **103**: 1944-1951 [PMID: 18564111 DOI: 10.1111/j.1572-0241.2008.01922.x]

34 **Shi JH**, Line PD. Effect of liver regeneration on malignant hepatic tumors. *World J Gastroenterol* 2014; **20**: 16167-16177 [PMID: 25473170 DOI: 10.3748/wjg.v20.i43.16167]

35 **Li H**, Zhang L. Liver regeneration microenvironment of hepatocellular carcinoma for prevention and therapy. *Oncotarget* 2017; **8**: 1805-1813 [PMID: 27655683 DOI: 10.18632/oncotarget.12101]

36 **Yu LX**, Ling Y, Wang HY. Role of nonresolving inflammation in hepatocellular carcinoma development and progression. *NPJ Precis Oncol* 2018; **2**: 6 [PMID: 29872724 DOI: 10.1038/s41698-018-0048-z]

37 **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867 [PMID: 12490959 DOI: 10.1038/nature01322]

38 **Ficht X**, Iannacone M. Immune surveillance of the liver by T cells. *Sci Immunol* 2020; **5**: eaba2351 [PMID: 32887842 DOI: 10.1126/sciimmunol.aba2351]

39 **Lian M**, Selmi C, Gershwin ME, Ma X. Myeloid Cells and Chronic Liver Disease: a Comprehensive Review. *Clin Rev Allergy Immunol* 2018; **54**: 307-317 [PMID: 29313221 DOI: 10.1007/s12016-017-8664-x]

40 **Pallett LJ**, Davies J, Colbeck EJ, Robertson F, Hansi N, Easom NJW, Burton AR, Stegmann KA, Schurich A, Swadling L, Gill US, Male V, Luong T, Gander A, Davidson BR, Kennedy PTF, Maini MK. IL-2^{high} tissue-resident T cells in the human liver: Sentinels for hepatotropic infection. *J Exp Med* 2017; **214**: 1567-1580 [PMID: 28526759 DOI: 10.1084/jem.20162115]

41 **Piconese S**, Cammarata I, Barnaba V. Viral hepatitis, inflammation, and cancer: A lesson for

autoimmunity. *J Autoimmun* 2018; **95**: 58-68 [PMID: 30509387 DOI: 10.1016/j.jaut.2018.10.021]
42 **Thornton AM**, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J Exp Med* 1998; **188**: 287-296 [PMID: 9670041 DOI: 10.1084/jem.188.2.287]
43 **Ng WF**, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD, Isaacs JD, Lechler RI. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 2001; **98**: 2736-2744 [PMID: 11675346 DOI: 10.1182/blood.v98.9.2736]
44 **Fontenot JD**, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; **4**: 330-336 [PMID: 12612578 DOI: 10.1038/ni904]
45 **Hori S**, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057-1061 [PMID: 12522256 DOI: 10.1126/science.1079490]
46 **Khattri R**, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 2003; **4**: 337-342
47 **McHugh RS**, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002; **16**: 311-323 [PMID: 11869690 DOI: 10.1016/s1074-7613(02)00280-7]
48 **Shimizu J**, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002; **3**: 135-142 [PMID: 11812990 DOI: 10.1038/ni759]
49 **Wing JB**, Sakaguchi S. Multiple treg suppressive modules and their adaptability. *Front Immunol* 2012; **3**: 178 [PMID: 22754556 DOI: 10.3389/fimmu.2012.00178]
50 **Oderup C**, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology* 2006; **118**: 240-249 [PMID: 16771859 DOI: 10.1111/j.1365-2567.2006.02362.x]
51 **Onishi Y**, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells *in vitro* and actively inhibit their maturation. *Proc Natl Acad Sci U S A* 2008; **105**: 10113-10118 [PMID: 18635688 DOI: 10.1073/pnas.0711106105]
52 **von Boehmer H**. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 2005; **6**: 338-344 [PMID: 15785759 DOI: 10.1038/ni1180]
53 **Izcue A**, Coombes JL, Powrie F. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* 2006; **212**: 256-271 [PMID: 16903919 DOI: 10.1111/j.0105-2896.2006.00423.x]
54 **Zou W**. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006; **6**: 295-307 [PMID: 16557261 DOI: 10.1038/nri1806]
55 **Chen X**, Du Y, Lin X, Qian Y, Zhou T, Huang Z. CD4+CD25+ regulatory T cells in tumor immunity. *Int Immunopharmacol* 2016; **34**: 244-249 [PMID: 26994448 DOI: 10.1016/j.intimp.2016.03.009]
56 **Zheng MH**, Gu DN, Braddock M, Leishman AJ, Jin C, Wen JS, Gong YW, Chen YP. CD4+ CD25+ regulatory T cells: a therapeutic target for liver diseases. *Expert Opin Ther Targets* 2008; **12**: 313-326 [PMID: 18269341 DOI: 10.1517/14728222.12.3.313]
57 **Xu D**, Fu J, Jin L, Zhang H, Zhou C, Zou Z, Zhao JM, Zhang B, Shi M, Ding X, Tang Z, Fu YX, Wang FS. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. *J Immunol* 2006; **177**: 739-747 [PMID: 16785573 DOI: 10.4049/jimmunol.177.1.739]
58 **Stoop JN**, van der Molen RG, Baan CC, van der Laan LJ, Kuipers EJ, Kusters JG, Janssen HL. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology* 2005; **41**: 771-778 [PMID: 15791617 DOI: 10.1002/hep.20649]
59 **Yang G**, Liu A, Xie Q, Guo TB, Wan B, Zhou B, Zhang JZ. Association of CD4+CD25+Foxp3+ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B. *Int Immunol* 2007; **19**: 133-140 [PMID: 17182968 DOI: 10.1093/intimm/dxl130]
60 **Peng G**, Li S, Wu W, Sun Z, Chen Y, Chen Z. Circulating CD4+ CD25+ regulatory T cells correlate with chronic hepatitis B infection. *Immunology* 2008; **123**: 57-65 [PMID: 17764450 DOI: 10.1111/j.1365-2567.2007.02691.x]
61 **Sugimoto K**, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated *ex vivo* in persistent HCV infection. *Hepatology* 2003; **38**: 1437-1448 [PMID: 14647055 DOI: 10.1016/j.hep.2003.09.026]
62 **Cabrera R**, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**: 1062-1071 [PMID: 15486925 DOI: 10.1002/hep.20454]
63 **Boettler T**, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von Weizsäcker F, Thimme R. T cells with a CD4+CD25+ regulatory phenotype suppress *in vitro* proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* 2005; **79**: 7860-7867 [PMID: 15919940 DOI: 10.1128/JVI.79.12.7860-7867.2005]
64 **Rushbrook SM**, Ward SM, Unitt E, Vowler SL, Lucas M, Klenerman P, Alexander GJ. Regulatory T cells suppress *in vitro* proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**: 7852-7859 [PMID: 15919939 DOI:

10.1128/JVI.79.12.7852-7859.2005]
65 **Granito A**, Maldini MC, Muratori P, Chiesa D, Bassi M et al Increased percentage of CD4+CD25high T cells in chronic hepatitis C patients correlates with higher viremia levels. J Hepatol 2005; 42: 158
66 **Ferri S**, Longhi MS, De Molo C, Lalanne C, Muratori P, Granito A, Hussain MJ, Ma Y, Lenzi M, Mieli-Vergani G, Bianchi FB, Vergani D, Muratori L. A multifaceted imbalance of T cells with regulatory function characterizes type 1 autoimmune hepatitis. *Hepatology* 2010; **52**: 999-1007 [PMID: 20683931 DOI: 10.1002/hep.23792]
67 **Longhi MS**, Meda F, Wang P, Samyn M, Mieli-Vergani G, Vergani D, Ma Y. Expansion and de novo generation of potentially therapeutic regulatory T cells in patients with autoimmune hepatitis. *Hepatology* 2008; **47**: 581-591 [PMID: 18220288 DOI: 10.1002/hep.22071]
68 **Bonilla CM**, McGrath NA, Fu J, Xie C. Immunotherapy of hepatocellular carcinoma with infection of hepatitis B or C virus. *Hepatoma Res* 2020; **6** [PMID: 33134550 DOI: 10.20517/2394-5079.2020.58]
69 **Chaoul N**, Mancarella S, Lupo L, Giannelli G, Dituri F. Impaired Anti-Tumor T cell Response in Hepatocellular Carcinoma. *Cancers (Basel)* 2020; **12** [PMID: 32182707 DOI: 10.3390/cancers12030627]
70 **Lapierre P**, Lamarre A. Regulatory T Cells in Autoimmune and Viral Chronic Hepatitis. *J Immunol Res* 2015; **2015**: 479703 [PMID: 26106627 DOI: 10.1155/2015/479703]
71 **Kobayashi N**, Hiraoka N, Yamagami W, Ojima H, Kanai Y, Kosuge T, Nakajima A, Hirohashi S. FOXP3+ regulatory T cells affect the development and progression of hepatocarcinogenesis. *Clin Cancer Res* 2007; **13**: 902-911 [PMID: 17289884 DOI: 10.1158/1078-0432.CCR-06-2363]
72 **Kurebayashi Y**, Kubota N, Sakamoto M. Immune microenvironment of hepatocellular carcinoma, intrahepatic cholangiocarcinoma and liver metastasis of colorectal adenocarcinoma: Relationship with histopathological and molecular classifications. *Hepatol Res* 2021; **51**: 5-18 [PMID: 32573056 DOI: 10.1111/hepr.13539]
73 **Bian J**, Lin J, Long J, Yang X, Lu X, Sang X, Zhao H. T lymphocytes in hepatocellular carcinoma immune microenvironment: insights into human immunology and immunotherapy. *Am J Cancer Res* 2020; **10**: 4585-4606 [PMID: 33415021]
74 **Wada Y**, Nakashima O, Kutami R, Yamamoto O, Kojiro M. Clinicopathological study on hepatocellular carcinoma with lymphocytic infiltration. *Hepatology* 1998; **27**: 407-414 [PMID: 9462638 DOI: 10.1002/hep.510270214]
75 **Ding W**, Xu X, Qian Y, Xue W, Wang Y, Du J, Jin L, Tan Y. Prognostic value of tumor-infiltrating lymphocytes in hepatocellular carcinoma: A meta-analysis. *Medicine (Baltimore)* 2018; **97**: e13301 [PMID: 30557978 DOI: 10.1097/MD.0000000000013301]
76 **Chiou SH**, Sheu BC, Chang WC, Huang SC, Hong-Nerng H. Current concepts of tumor-infiltrating lymphocytes in human malignancies. *J Reprod Immunol* 2005; **67**: 35-50 [PMID: 16111767 DOI: 10.1016/j.jri.2005.06.002]
77 **Gao Q**, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, Xu Y, Li YW, Tang ZY. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 2007; **25**: 2586-2593 [PMID: 17577038 DOI: 10.1200/JCO.2006.09.4565]
78 **Unitt E**, Rushbrook SM, Marshall A, Davies S, Gibbs P, Morris LS, Coleman N, Alexander GJ. Compromised lymphocytes infiltrate hepatocellular carcinoma: the role of T-regulatory cells. *Hepatology* 2005; **41**: 722-730 [PMID: 15791620 DOI: 10.1002/hep.20644]
79 **Ma J**, Zheng B, Goswami S, Meng L, Zhang D, Cao C, Li T, Zhu F, Ma L, Zhang Z, Zhang S, Duan M, Chen Q, Gao Q, Zhang X. $PD1^{Hi}$ $CD8^{+}$ T cells correlate with exhausted signature and poor clinical outcome in hepatocellular carcinoma. *J Immunother Cancer* 2019; **7**: 331 [PMID: 31783783 DOI: 10.1186/s40425-019-0814-7]
80 **Tauber C**, Schultheiss M, Luca R, Buettner N, Llewellyn-Lacey S, Emmerich F, Zehe S, Price DA, Neumann-Haefelin C, Schmitt-Graeff A, Hofmann M, Thimme R. Inefficient induction of circulating TAA-specific CD8+ T-cell responses in hepatocellular carcinoma. *Oncotarget* 2019; **10**: 5194-5206 [PMID: 31497249 DOI: 10.18632/oncotarget.27146]
81 **Bunt SK**, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. *Cancer Res* 2007; **67**: 10019-10026 [PMID: 17942936 DOI: 10.1158/0008-5472.CAN-07-2354]
82 **Vignali DA**, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008; **8**: 523-532 [PMID: 18566595 DOI: 10.1038/nri2343]
83 **Zhou SL**, Zhou ZJ, Hu ZQ, Huang XW, Wang Z, Chen EB, Fan J, Cao Y, Dai Z, Zhou J. Tumor-Associated Neutrophils Recruit Macrophages and T-Regulatory Cells to Promote Progression of Hepatocellular Carcinoma and Resistance to Sorafenib. *Gastroenterology* 2016; **150**: 1646-1658. e17 [PMID: 26924089 DOI: 10.1053/j.gastro.2016.02.040]
84 **Zhou J**, Ding T, Pan W, Zhu LY, Li L, Zheng L. Increased intratumoral regulatory T cells are related to intratumoral macrophages and poor prognosis in hepatocellular carcinoma patients. *Int J Cancer* 2009; **125**: 1640-1648 [PMID: 19569243 DOI: 10.1002/ijc.24556]
85 **Tovoli F**, De Lorenzo S, Trevisani F. Immunotherapy with Checkpoint Inhibitors for Hepatocellular Carcinoma: Where Are We Now? *Vaccines (Basel)* 2020; **8** [PMID: 33023131 DOI: 10.3390/vaccines8040578]
86 **Sakaguchi S**, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*

2008; **133**: 775-787 [PMID: 18510923 DOI: 10.1016/j.cell.2008.05.009]
87 **Tanaka A**, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res* 2017; **27**: 109-118 [PMID: 27995907 DOI: 10.1038/cr.2016.151]
88 **Pedroza-Pacheco I**, Madrigal A, Saudemont A. Interaction between natural killer cells and regulatory T cells: perspectives for immunotherapy. *Cell Mol Immunol* 2013; **10**: 222-229 [PMID: 23524654 DOI: 10.1038/cmi.2013.2]
89 **Ren L**, Yu Y, Wang L, Zhu Z, Lu R, Yao Z. Hypoxia-induced CCL28 promotes recruitment of regulatory T cells and tumor growth in liver cancer. *Oncotarget* 2016; **7**: 75763-75773 [PMID: 27716621 DOI: 10.18632/oncotarget.12409]
90 **Marra F**, Tacke F. Roles for chemokines in liver disease. *Gastroenterology* 2014; **147**: 577-594. e1 [PMID: 25066692 DOI: 10.1053/j.gastro.2014.06.043]
91 **Shen X**, Li N, Li H, Zhang T, Wang F, Li Q. Increased prevalence of regulatory T cells in the tumor microenvironment and its correlation with TNM stage of hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2010; **136**: 1745-1754 [PMID: 20221638 DOI: 10.1007/s00432-010-0833-8]
92 **Fu YP**, Yi Y, Cai XY, Sun J, Ni XC, He HW, Wang JX, Lu ZF, Huang JL, Cao Y, Zhou J, Fan J, Qiu SJ. Overexpression of interleukin-35 associates with hepatocellular carcinoma aggressiveness and recurrence after curative resection. *Br J Cancer* 2016; **114**: 767-776 [PMID: 27002937 DOI: 10.1038/bjc.2016.47]
93 **Chen J**, Gingold JA, Su X. Immunomodulatory TGF-β Signaling in Hepatocellular Carcinoma. *Trends Mol Med* 2019; **25**: 1010-1023 [PMID: 31353124 DOI: 10.1016/j.molmed.2019.06.007]
94 **Chen X**, Du Y, Hu Q, Huang Z. Tumor-derived CD4+CD25+regulatory T cells inhibit dendritic cells function by CTLA-4. *Pathol Res Pract* 2017; **213**: 245-249 [PMID: 28214198 DOI: 10.1016/j.prp.2016.12.008]
95 **Li C**, Jiang P, Wei S, Xu X, Wang J. Regulatory T cells in tumor microenvironment: new mechanisms, potential therapeutic strategies and future prospects. *Mol Cancer* 2020; **19**: 116 [PMID: 32680511 DOI: 10.1186/s12943-020-01234-1]
96 **Cabrera R**, Ararat M, Eksioglu EA, Cao M, Xu Y, Wasserfall C, Atkinson MA, Liu C, Nelson DR. Influence of serum and soluble CD25 (sCD25) on regulatory and effector T-cell function in hepatocellular carcinoma. *Scand J Immunol* 2010; **72**: 293-301 [PMID: 20883314 DOI: 10.1111/j.1365-3083.2010.02427.x]
97 **Li FJ**, Zhang Y, Jin GX, Yao L, Wu DQ. Expression of LAG-3 is coincident with the impaired effector function of HBV-specific CD8(+) T cell in HCC patients. *Immunol Lett* 2013; **150**: 116-122 [PMID: 23261718 DOI: 10.1016/j.imlet.2012.12.004]
98 **Cheng X**, Wu H, Jin ZJ, Ma D, Yuen S, Jing XQ, Shi MM, Shen BY, Peng CH, Zhao R, Qiu WH. Up-regulation of chemokine receptor CCR4 is associated with Human Hepatocellular Carcinoma malignant behavior. *Sci Rep* 2017; **7**: 12362 [PMID: 28959024 DOI: 10.1038/s41598-017-10267-4]
99 **Singh SK**, Mishra MK, Rivers BM, Gordetsky JB, Bae S, Singh R. Biological and Clinical Significance of the CCR5/CCL5 Axis in Hepatocellular Carcinoma. *Cancers (Basel)* 2020; **12** [PMID: 32260550 DOI: 10.3390/cancers12040883]
100 **Xue D**, Zheng Y, Wen J, Han J, Tuo H, Liu Y, Peng Y. Role of chemokines in hepatocellular carcinoma (Review). *Oncol Rep* 2021; **45**: 809-823 [PMID: 33650640 DOI: 10.3892/or.2020.7906]
101 **Caridade M**, Graca L, Ribeiro RM. Mechanisms Underlying CD4+ Treg Immune Regulation in the Adult: From Experiments to Models. *Front Immunol* 2013; **4**: 378 [PMID: 24302924 DOI: 10.3389/fimmu.2013.00378]
102 **Maj T**, Wang W, Crespo J, Zhang H, Wei S, Zhao L, Vatan L, Shao I, Szeliga W, Lyssiotis C, Liu JR, Kryczek I, Zou W. Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. *Nat Immunol* 2017; **18**: 1332-1341 [PMID: 29083399 DOI: 10.1038/ni.3868]
103 **Larmonier N**, Marron M, Zeng Y, Cantrell J, Romanoski A, Sepassi M, Thompson S, Chen X, Andreansky S, Katsanis E. Tumor-derived CD4(+)CD25(+) regulatory T cell suppression of dendritic cell function involves TGF-beta and IL-10. *Cancer Immunol Immunother* 2007; **56**: 48-59 [PMID: 16612596 DOI: 10.1007/s00262-006-0160-8]
104 **Bauer CA**, Kim EY, Marangoni F, Carrizosa E, Claudio NM, Mempel TR. Dynamic Treg interactions with intratumoral APCs promote local CTL dysfunction. *J Clin Invest* 2014; **124**: 2425-2440 [PMID: 24812664 DOI: 10.1172/JCI66375]
105 **Lai E**, Astara G, Ziranu P, Pretta A, Migliari M, Dubois M, Donisi C, Mariani S, Liscia N, Impera V, Persano M, Tolu S, Balconi F, Pinna G, Spanu D, Pireddu A, Saba G, Camera S, Musio F, Puzzoni M, Pusceddu V, Madeddu C, Casadei Gardini A, Scartozzi M. Introducing immunotherapy for advanced hepatocellular carcinoma patients: Too early or too fast? *Crit Rev Oncol Hematol* 2021; **157**: 103167 [PMID: 33271389 DOI: 10.1016/j.critrevonc.2020.103167]
106 **Wing K**, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 2008; **322**: 271-275 [PMID: 18845758 DOI: 10.1126/science.1160062]
107 **De Martin E**, Michot JM, Rosmorduc O, Guettier C, Samuel D. Liver toxicity as a limiting factor to the increasing use of immune checkpoint inhibitors. *JHEP Rep* 2020; **2**: 100170 [PMID: 33205034 DOI: 10.1016/j.jhepr.2020.100170]
108 **McGonagle D**, Bragazzi NL, Amital H, Watad A. Mechanistic classification of immune checkpoint inhibitor toxicity as a pointer to minimal treatment strategies to further improve survival. *Autoimmun Rev* 2020; **19**: 102456 [PMID: 31838166 DOI: 10.1016/j.autrev.2019.102456]

109 **Michot JM**, Bigenwald C, Champiat S, Collins M, Carbonnel F, Postel-Vinay S, Berdelou A, Varga A, Bahleda R, Hollebecque A, Massard C, Fuerea A, Ribrag V, Gazzah A, Armand JP, Amellal N, Angevin E, Noel N, Boutros C, Mateus C, Robert C, Soria JC, Marabelle A, Lambotte O. Immune-related adverse events with immune checkpoint blockade: a comprehensive review. *Eur J Cancer* 2016; **54**: 139-148 [PMID: 26765102 DOI: 10.1016/j.ejca.2015.11.016]

110 **Postow MA**, Sidlow R, Hellmann MD. Immune-Related Adverse Events Associated with Immune Checkpoint Blockade. *N Engl J Med* 2018; **378**: 158-168 [PMID: 29320654 DOI: 10.1056/NEJMra1703481]

111 **European Association for the Study of the Liver**. ; Clinical Practice Guideline Panel: Chair:; Panel members; EASL Governing Board representative:. EASL Clinical Practice Guidelines: Drug-induced liver injury. *J Hepatol* 2019; **70**: 1222-1261 [PMID: 30926241 DOI: 10.1016/j.jhep.2019.02.014]

112 **El-Khoueiry AB**, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]

113 **Zhu AX**, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, Verslype C, Zagonel V, Fartoux L, Vogel A, Sarker D, Verset G, Chan SL, Knox J, Daniele B, Webber AL, Ebbinghaus SW, Ma J, Siegel AB, Cheng AL, Kudo M; KEYNOTE-224 investigators. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. *Lancet Oncol* 2018; **19**: 940-952 [PMID: 29875066 DOI: 10.1016/S1470-2045(18)30351-6]

114 **Duffy AG**, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, Davis JL, Hughes MS, Heller T, ElGindi M, Uppala A, Korangy F, Kleiner DE, Figg WD, Venzon D, Steinberg SM, Venkatesan AM, Krishnasamy V, Abi-Jaoudeh N, Levy E, Wood BJ, Greten TF. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. *J Hepatol* 2017; **66**: 545-551 [PMID: 27816492 DOI: 10.1016/j.jhep.2016.10.029]

115 **Ravi S**, Spencer K, Ruisi M, Ibrahim N, Luke JJ, Thompson JA, Shirai K, Lawson D, Bartell H, Kudchadkar R, Gunter NT, Mehnert JM, Lipson EJ. Ipilimumab administration for advanced melanoma in patients with pre-existing Hepatitis B or C infection: a multicenter, retrospective case series. *J Immunother Cancer* 2014; **2**: 33 [PMID: 25317333 DOI: 10.1186/s40425-014-0033-1]

116 **Sangro B**, Gomez-Martin C, de la Mata M, Iñarrairaegui M, Garralda E, Barrera P, Riezu-Boj JI, Larrea E, Alfaro C, Sarobe P, Lasarte JJ, Pérez-Gracia JL, Melero I, Prieto J. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol* 2013; **59**: 81-88 [PMID: 23466307 DOI: 10.1016/j.jhep.2013.02.022]

117 **Danlos FX**, Voisin AL, Dyevre V, Michot JM, Routier E, Taillade L, Champiat S, Aspeslagh S, Haroche J, Albiges L, Massard C, Girard N, Dalle S, Besse B, Laghouati S, Soria JC, Mateus C, Robert C, Lanoy E, Marabelle A, Lambotte O. Safety and efficacy of anti-programmed death 1 antibodies in patients with cancer and pre-existing autoimmune or inflammatory disease. *Eur J Cancer* 2018; **91**: 21-29 [PMID: 29331748 DOI: 10.1016/j.ejca.2017.12.008]

118 **Abdel-Wahab N**, Shah M, Lopez-Olivo MA, Suarez-Almazor ME. Use of Immune Checkpoint Inhibitors in the Treatment of Patients With Cancer and Preexisting Autoimmune Disease: A Systematic Review. *Ann Intern Med* 2018; **168**: 121-130 [PMID: 29297009 DOI: 10.7326/M17-2073]

119 **Tison A**, Quéré G, Misery L, Funck-Brentano E, Danlos FX, Routier E, Robert C, Loriot Y, Lambotte O, Bonniaud B, Scalbert C, Maanaoui S, Lesimple T, Martinez S, Marcq M, Chouaid C, Dubos C, Brunet-Possenti F, Stavris C, Chiche L, Beneton N, Mansard S, Guisier F, Doubre H, Skowron F, Aubin F, Zehou O, Roge C, Lambert M, Pham-Ledard A, Beylot-Barry M, Veillon R, Kramkimel N, Giacchero D, De Quatrebarbes J, Michel C, Auliac JB, Gonzales G, Decroisette C, Le Garff G, Carpiuc I, Vallerand H, Nowak E, Cornec D, Kostine M; Groupe de Cancérologie Cutanée; Groupe Français de Pneumo-Cancérologie, and Club Rhumatismes et Inflammations. Safety and Efficacy of Immune Checkpoint Inhibitors in Patients With Cancer and Preexisting Autoimmune Disease: A Nationwide, Multicenter Cohort Study. *Arthritis Rheumatol* 2019; **71**: 2100-2111 [PMID: 31379105 DOI: 10.1002/art.41068]

120 **De Martin E**, Michot JM, Papouin B, Champiat S, Mateus C, Lambotte O, Roche B, Antonini TM, Coilly A, Laghouati S, Robert C, Marabelle A, Guettier C, Samuel D. Characterization of liver injury induced by cancer immunotherapy using immune checkpoint inhibitors. *J Hepatol* 2018; **68**: 1181-1190 [PMID: 29427729 DOI: 10.1016/j.jhep.2018.01.033]

121 **Day D**, Hansen AR. Immune-Related Adverse Events Associated with Immune Checkpoint Inhibitors. *BioDrugs* 2016; **30**: 571-584 [PMID: 27848165 DOI: 10.1007/s40259-016-0204-3]

122 **June CH**, Warshauer JT, Bluestone JA. Is autoimmunity the Achilles' heel of cancer immunotherapy? *Nat Med* 2017; **23**: 540-547 [PMID: 28475571 DOI: 10.1038/nm.4321]

123 **Sekai I**, Hagiwara S, Watanabe T, Kudo M. A case with hepatic immune-related adverse events caused by nivolumab exhibiting impaired accumulation of regulatory T cells. *Clin J Gastroenterol* 2021 [PMID: 33665689 DOI: 10.1007/s12328-020-01317-y]

124 **Alissafi T**, Banos A, Boon L, Sparwasser T, Ghigo A, Wing K, Vassilopoulos D, Boumpas D, Chavakis T, Cadwell K, Verginis P. Tregs restrain dendritic cell autophagy to ameliorate autoimmunity. *J Clin Invest* 2017; **127**: 2789-2804 [PMID: 28581446 DOI: 10.1172/JCI92079]

125 **Eli Lilly and Company**. A Randomized Phase 2 Study of LY2157299 Versus LY2157299 - Sorafenib Combination Versus Sorafenib in Patients With Advanced Hepatocellular Carcinoma. [cited 10 Febraury 2021]. In: ClinicalTrials.gov [Internet]. Indianapolis (IN): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT02178358?term=Galunisertib&cond=Hepatocellular+Carcinoma&draw=2&rank=3 ClinicalTrials.gov Identifier: NCT00417417

126 **Giannelli G**, Mikulits W, Dooley S, Fabregat I, Moustakas A, ten Dijke P, Portincasa P, Winter P, Janssen R, Leporatti S, Herrera B, Sanchez A. The rationale for targeting TGF-β in chronic liver diseases. *Eur J Clin Invest* 2016; **46**: 349-361 [PMID: 26823073 DOI: 10.1111/eci.12596]

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MINIREVIEWS

Role of bile acids in liver diseases mediated by the gut microbiome

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Abstract

The intensive crosstalk between the liver and the intestine performs many essential functions. This crosstalk is important for natural immune surveillance, adaptive immune response regulation and nutrient metabolism and elimination of toxic bacterial metabolites. The interaction between the gut microbiome and bile acids is bidirectional. The gut microbiome regulates the synthesis of bile acids and their biological signaling activity and circulation *via* enzymes. Similarly, bile acids also shape the composition of the gut microbiome by modulating the host's natural antibacterial defense and the intestinal immune system. The interaction between bile acids and the gut microbiome has been implicated in the pathophysiology of many intestinal and extra intestinal diseases, especially liver diseases. As essential mediators of the gut-liver crosstalk, bile acids regulate specific host metabolic pathways and modulate the inflammatory responses through farnesoid X-activated receptor and G protein-coupled bile acid receptor 1. Several clinical trials have demonstrated the signaling effects of bile acids in the context of liver diseases. We hypothesize the existence of a gut microbiome-bile acids-liver triangle and explore the potential therapeutic strategies for liver diseases targeting the triangle.

Key Words: Bile acids; Gut microbiome; Liver diseases; Farnesoid X-activated receptor; G protein-coupled bile acid receptor 1; Immune response

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Core Tip: This review explores the interaction between the gut microbiome, bile acids and liver disease. Bile acids are involved in liver immune and inflammatory responses *via* the membrane receptor G protein-coupled bile acid receptor 1 and the intracellular farnesoid-X receptor. The interaction between the gut microbiome and bile acids can control host immunological homeostasis and inhibit liver inflammation.

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INTRODUCTION

Approximately 70%-75% of the liver blood supply is derived from the intestine, which forms the basis of the gut-liver axis[1]. Liver plays an important role in the immune response of the human body; it serves as the central hub of the crosstalk between the host metabolism and the intestinal microenvironment. Therefore, the liver is exposed to a large number of bacterial components, metabolites and microbiome-derived signals. Recent studies have indicated that bile acids act as pleiotropic signaling molecules that mediate the gut-liver crosstalk[2,3]. The complex relationship between bile acids and the gut microbiome (mutual dependence and inhibition) plays an important role in the maintenance of mammalian homeostasis.

The gut microbiome participates in the conversion of bile acids and regulates the synthesis and reabsorption of bile acids in the liver (Figure 1). Studies have found decreased activity and gene expression levels of cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme in the classical synthesis pathway of bile acids, in CONV-R mice compared with those in germ-free mice[4,5]. Moreover, the volume of the bile acid pool in CONV-R mice is smaller, which may be related to the decreased expression of ileal sodium-dependent bile acid transporter, which leads to decreased reabsorption of bile acids in distal ileum and increased fecal excretion of bile acids[5]. The 7α-dehydroxylation reaction of the intestinal microbiome is an essential part of biological conversion[6]. The genus *Clostridium* exhibits 7α-dehydroxylation activity that can transform primary bile acids into secondary bile acids[7]. Most Gram-positive bacteria in the intestine have bile salt hydrolases activity, while only *Bacteroides* among the Gram-negative bacteria can hydrolyze conjugated bile acids into unconjugated bile acids. The biological functions of bile acids in the body vary depending upon their state[8]. Unconjugated bile acids can eliminate the pH differential across the cell membrane. The consequent disappearance of bio-energy driven by the proton pump can cause direct damage to the cell membrane. Eventually, bile acids inhibit the growth of certain bacteria and participate in the formation of the gut microbiome[9].

Bile acids, as essential signal molecules for bidirectional regulation between the liver and the intestinal tract, are mainly activated by the following two signaling pathways: binding of a signaling molecule to G-protein coupled bile acid receptor 1 (GPBAR1 or TGR5) and activation of the expression of the farnesoid X-activated receptor (FXR). The above two pathways control the balance of energy metabolism, regulate hepatic steatosis and inflammatory response and influence the composition of the gut microbiome by shaping intestinal immunity and some antimicrobial properties of endogenous peptides. Therefore, the use of bile acids as signaling molecules by the gut microbiome may play a role in the pathophysiology of liver diseases[10-13]. Better characterization of the specific sites of action of the gut microbiome and bile acids in different signaling pathways in liver diseases can lay the foundation for novel therapies targeting bile acids.

LIVER CANCER AND BILE ACIDS

In a seminal study by Ma *et al*[10], ABX (primaxin, neomycin and vancomycin) fed mice exhibited fewer and smaller primary or metastatic liver cancer lesions, and this result was not related to the age, strain or sex. These results suggested that regulation

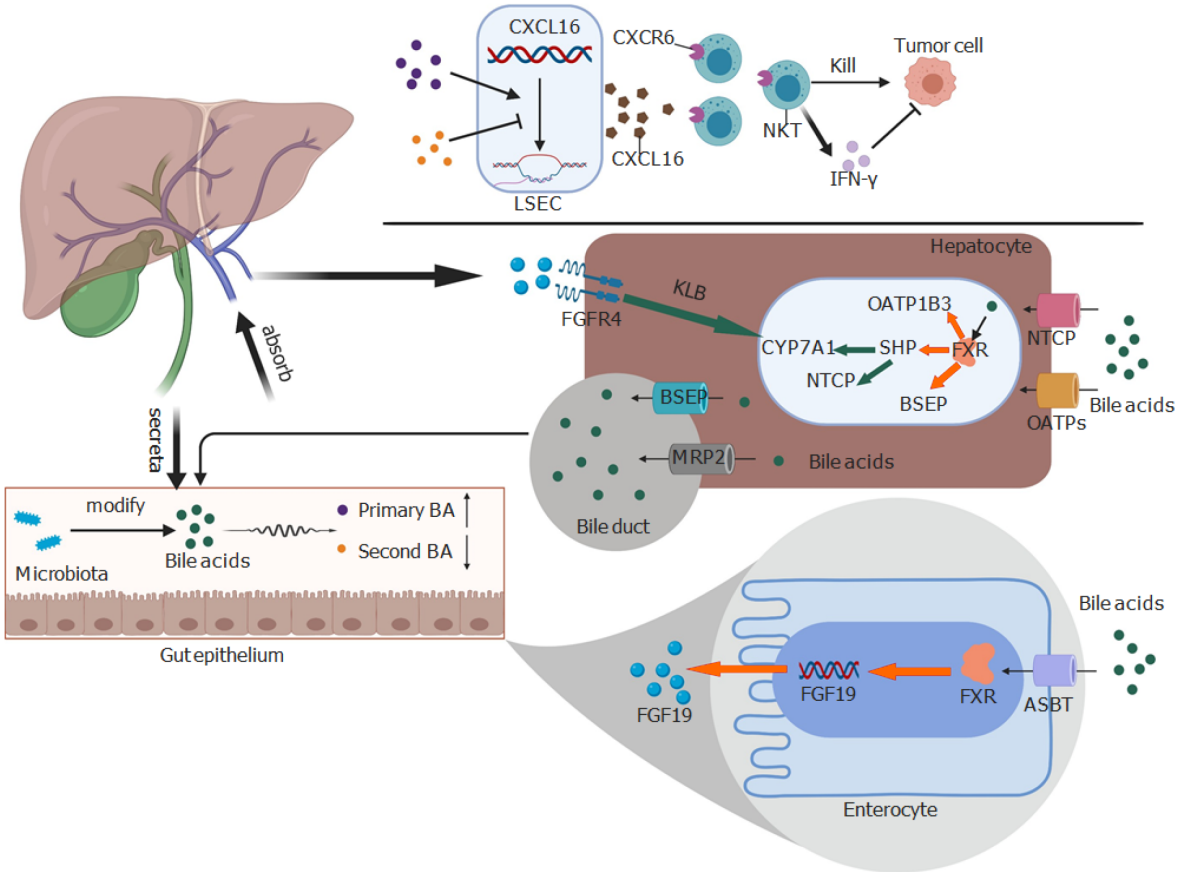


Figure 1 Bile acids play bidirectional action between the liver and the gut microbiome. Bile acids can upregulate CXCL16 on liver sinusoidal endothelial cells to induce the accumulation of CXCR6$^+$ natural killer T cells, which kill tumor cells directly or through interferon-.The farnesoid X receptor improves the expression of fibroblast growth factor 19 (FGF19) in the enterocyte after being activated by bile acids. FGF19 is the ligand of FGFR4 and can suppress the expression of cholesterol 7α-hydroxylase with the help of Klotho beta. The presence of Klotho beta can activate FGFR4. In other words, bile acids can activate the farnesoid X receptor to increase the expression of bile salt export protein, organic anion transporting polypeptides 1B3, and small heterodimer partner. However, small heterodimer partner can suppress the activation of cholesterol 7α-hydroxylase and Na$^+$-taurocholate cotransporting polypeptide. Red and green arrows indicate positive and negative effects, respectively. NKT: Natural killer T; BA: Bile acid; IFN-: Interferon-; BSEP: Bile salt export protein; FGF19: Fibroblast growth factor 19; FGFR4: Fibroblast growth factor receptor 4; FXR: Farnesoid X receptor; KLB: Klotho beta; NTCP: Na$^+$-taurocholate cotransporting polypeptide; SHP: Small heterodimer partner; ASBT: sodium-dependent bile acid transporter; CYP7A1: Cholesterol 7α-hydroxylase; MRP2: Multidrug resistance-associated protein 2; OATPs: Organic anion transporting polypeptides; LSEC: Liver sinusoidal endothelial cells; OATP1B3: Organic anion transporting polypeptides 1B3.

of the gut microbiome may usefully alter the growth kinetics of liver tumors.

Several studies have investigated the mechanism linking the gut microbiome and liver tumor immunity and surveillance. In a study, CD8$^+$ T cells and natural killer T (NKT) cells in the liver tissues of ABX-treated EL4-tumor-bearing mice were significantly increased compared with that in the control group. However, in the ABX-treated MYC mice, only NKT cells were increased, and similar results were observed in normal mice without tumors. Moreover, ABX-treated mice showed increased expression of *CXCL16* mRNA in the liver sinusoidal endothelial cells (LSEC). Further studies showed that CXCL16 is the only ligand of CXCR6, which can induce the accumulation of CXCR6$^+$ hepatic NKT cells in the liver[14]. NKT cells can directly kill CD1d-expressing tumors (B16, EL4 and A20) and can also suppress liver tumors by secreting interferon-.

To identify the potential link between bile acids and NKT cells, the bile acid profile of ABX-treated mice was determined. The results showed significantly increased primary bile acids compared with that in H_2O-treated mice. The findings were further confirmed after treatment of isolated LSECs with various bile acids or a combination of tauro-β-muricholic acid with -muricholic acid or tauro--muricholic acid; the results showed that primary bile acids could indeed upregulate the expression of *CXCL16* mRNA. Predictably, the secondary bile acids (lithocholic acid) reversed the ABX-induced suppression of the growth of intrahepatic tumors growth and increased liver surface metastasis.

The above findings suggest that, the level of CXCL16 on LSECs can be upregulated by the gut microbiome through the primary bile acids, which leads to the accumu-

lation of CXCR6$^+$ hepatic NKT cells in the liver, while the secondary bile acids have the opposite effect. Although, ABX kills most of the bacteria in the mice intestine, the regulatory impact of the remaining bacteria on NKT cells cannot be excluded. To address this issue, the above experiment was repeated with germ-free mice. The results showed greater accumulation of NKT cells and increased expression of *CXCL16* mRNA in the liver of germ-free mice. Some antibiotics such as cefoperazone and vancomycin were used against Gram-positive bacteria to increase primary bile acids and NKT cells in the liver, while depleting secondary bile acids. The 7-α dehydroxylation reaction is a key step in the transformation of primary bile acids into secondary bile acids[6]; *Clostridium XIV* among Gram-positive bacteria synthesizes 7-α dehydroxylase[7]. To explore the role of *Clostridium* in the accumulation of hepatic NKT cells, mice were first fed vancomycin to increase the NKT cells. One week later, the antibiotic treatment was replaced with *Clostridium scidens*, a type of *Clostridium* occurring in both humans and mice. On the second day after successful colonization of *Clostridium scidens*, hepatic NKT levels began to decline with a decrease in primary bile acids. The antibiotic effect was offset when antibiotic-treated mice were gavaged with bile acids metabolizing bacteria or were fed with secondary bile acids. Both the inhibition of intrahepatic tumor growth and accumulation of hepatic NKT cells were reversed in mice with an altered gut microbiome. *Clostridium* species is a key bacterium for regulating bile acid signal NKT cell accumulation.

LSECs isolated from human samples were treated with chenodeoxycholic acid (CDCA) and taurocholic acid. The results were similar to the mouse study in that primary bile acids were also found to upregulate the expression of *CXCL16* mRNA. Normal tissues excised from patients with cholangiocarcinoma and hepatocellular carcinoma showed a positive correlation between CDCA and CXCL16, while secondary bile acids were observed to have opposite results. The above findings indicate that gut microbiome-mediated bile acid metabolism regulates liver cancer *via* NKT cells, which is also applicable to the human body.

NONALCOHOLIC FATTY LIVER DISEASE AND BILE ACIDS

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathologic syndrome characterized by excessive fat deposition in hepatocytes and other tissues caused by factors other than alcohol and other definitive causes of liver damage. In the absence of effective interventions, NAFLD may progress to fibrosis, cirrhosis and hepatocellular carcinoma. Environmental, genetic and metabolic factors as well as altered gut microbiome have been implicated in the pathogenesis of NAFLD. Bile acids and their metabolites help maintain the homeostasis of glucose, cholesterol and triglycerides in the liver and regulate inflammation; this is considered a potential therapeutic target for NAFLD[15,16].

Recent studies have revealed that the role of bile acids as signaling molecules may affect the development of NAFLD at multiple levels[17]. These regulatory activities are mainly achieved by FXR and TGR5, and different bile acids have different effects on these two signaling pathways[18]. FXR can regulate lipid and glucose metabolism through different pathways, such as by inhibiting the expression of hepatic gluconeogenesis genes and increasing liver glycogen synthesis and insulin sensitivity[19,20]. It can also induce the expression and secretion of liver fibroblast growth factor 21(FGF21), which as a metabolic regulator can stimulate the uptake of glucose in adipose tissue[21]. In addition, FXR activation inhibits lipogenesis, promotes fatty acid oxidation and affects cholesterol transport[22]. Bile acids can also activate the G protein-coupled receptor. TGR5 is expressed in nonparenchymal hepatocytes, monocytes and a variety of macrophages that secrete various inflammatory mediators and play an important role in regulating inflammatory responses[23]. Bile acids can inhibit the lipopolysaccharide-induced secretion of interleukin (IL)-6, IL-1A and IL-1B; in addition, bile acids can inhibit the secretion of tumor necrosis factor by Kupffer cells through a TGR5-cAMP-dependent pathway[24]. TGR5 can also regulate glucose homeostasis by inducing the expression of glucagon-like peptide-1 and inhibiting the activation of Nod-like receptor protein 3 inflammasome. Activation of TGR5 results in increased energy expenditure and weight loss[25,26]. TGR5 or FXR agonists can reduce lipogenesis, improve cholesterolemia, induce energy consumption and reduce liver inflammation in NAFLD patients[27,28].

Clinical studies have implicated disorders of bile acid homeostasis and related signaling pathways in the occurrence of NAFLD[29]. The serum concentration of total bile acids in patients with nonalcoholic steatohepatitis was three times higher than that

in healthy individuals. Moreover, the composition of the bile acid pool was also different in these two groups[30]. Bile acids have a direct antibacterial effect; through FXR, they can induce the production of antibacterial peptides, such as angiogenin 1, which participates in the shaping of the gut microbiome[31]. During the development and progression of NAFLD, disruption of bile acid balance is also accompanied by disruption of the gut microbiome. Therefore, the liver-bile acid-gut microbiome triangle is a good entry point for the treatment of NAFLD.

In two human studies, obeticholic acid (OCA), an FXR agonist, was shown to decrease liver fibrosis markers and improve insulin resistance in patients with NAFLD [32]. Patients receiving OCA treatment showed a significant decrease in high-density lipoprotein and an increase in low-density lipoprotein levels. Compared with the placebo-treated group, OCA significantly improved the NAFLD Activity Score while significantly reducing liver fibrosis[33,34]. As a derivative of cholic acid, INT-777 is a selective agonist of TGR5 that increases energy expenditure and induces weight loss in high fat diet-fed mice[35]. McMahan *et al*[27] found that INT-767, a dual agonist of FXR/TGR5, can reduce the expression of proinflammatory factors, decrease hepatic steatosis and transform monocytes and macrophages to the anti-inflammatory M2 phenotype.

ALCOHOL-RELATED LIVER DISEASE AND BILE ACIDS

Alcohol-related liver disease (ALD) is caused by chronic consumption of alcohol. It is initially characterized by liver steatosis, which in turn can develop into alcoholic hepatitis, liver fibrosis and cirrhosis. Alcohol can cause damage to multiple target organs, especially the brain, intestines and liver. It worth noting that alcohol intake can alter the structure of the gut microbiome prior to the occurrence of overt liver diseases.

Continued drinking in patients with alcoholic cirrhosis can aggravate gut microbiome disorders, reduce the detection of gut commensal bacteria in feces and worsen the function of the duodenal and colonic mucosa. Chronic alcohol exposure is associated with gut microbiome dysbiosis in preclinical models and the human gut, which is associated with the pathogenesis of ALD. Previous studies have shown altered gut microbiome in patients with alcoholic cirrhosis, which was characterized by an increase in endotoxin-producing bacteria and a decrease in the gut commensal bacteria[36]. Changes in composition of the gut microbiome may alter brain function, and alcohol abuse can also affect the gut-brain axis; this may further aggravate alcohol dependence and induce affective disorders, ultimately, accelerating the development of hepatic encephalopathy. Compared to people who do not have the disease and do not drink alcohol, patients with alcoholic cirrhosis have higher levels of endotoxins and worse gut microbiome dysbiosis even after cessation of alcohol intake[36]. This indicates that alcohol-induced damage to the gut microbiome continues even after cessation of intake and that the damage also extends to the gut-brain axis, leading to cognitive impairment[37].

FXR negatively regulates CYP7A1, which is a rate-limiting enzyme for bile acid synthesis *via* FGF15. In long-term alcohol-fed mice, ethanol was shown to negate the negative regulation of bile acids by conjugated CDCA through the FXR pathway and increase the expression of CYP7A1 protein in liver cells; this ultimately led to an increased concentration of bile acids in the serum and liver. FXR can induce the expression of antimicrobial molecules in intestinal epithelial cells to prevent alcohol-induced damage to the enteric tight junctions and avoid loss of intestinal barrier integrity[38]. Intragastric administration of the FXR agonist fexaramine caused a decrease in serum alanine aminotransferase levels and hepatic IL-1B and tumor necrosis factor protein. This is because FXR can increase the small heterodimer partner protein to inhibit intestinal inflammation and protect the integrity of the intestinal barrier[11]. The above finding indicates that FXR agonists can negatively regulate the synthesis of bile acids and reduce the concentration of serum bile acids, which can alleviate alcohol-induced steatosis and liver inflammation. Administration of antibiotics to chronic drinkers was found to reduce alcohol-related liver disease. This is because antibiotics can kill gut commensal bacteria, reduce the concentration of bile acids and inhibit the hydrolysis of bile acids; this can only reduce the toxicity of deoxycholic acid to hepatocytes and stabilize the intestinal barrier function.

In summary, alcohol-related changes in the gut microbiome can ultimately alter the bile acid profile. Interventions targeting the bile acid-FXR-FGF15 signaling pathway to regulate the synthesis of CYP7A1 and lipid metabolism can reduce the occurrence of ALD in mice. As a signaling molecule, bile acids can modulate the complex

interactions between the gut microbiome and alcohol through the gut-liver-brain axis. Therefore, FGF19 and fexaramine are good candidates for the treatment of ALD.

CHOLESTATIC LIVER DISEASES AND BILE ACIDS

Chronic cholestasis can lead to liver damage by affecting the expression levels of nuclear receptors and bile acid transporters. Hydrophobic bile acids can induce hepatocyte injury. Bile salt can cause apoptosis at concentrations of 50-200 μmol/L, induce proinflammatory responses at concentration of 200 μmol/L and cause cell necrosis at concentrations of 200-2000 μmol/L[39-42]. The progress of autoimmune cholestatic liver diseases also affects the composition of the gut microbiome, aggravating the development of cholestasis in this interactive cycle[43].

Cholestatic liver diseases are often accompanied by gut microbiome dysbiosis and reduced bacterial diversity[44]. Kummen *et al*[45] found that the gut microbiome of patients with primary sclerosing cholangitis (PSC) was significantly different from that of patients with ulcerative colitis without liver disease as well as that of healthy individuals. The *Veillonella* genus was overexpressed only in the intestines of patients with PSC. It is worth noting that *Veillonella* showed a positive correlation with the pathogenesis of fibrosis, not only in PSC but also in other fibrotic diseases, such as idiopathic pulmonary fibrosis[46]. The changes in gut commensal bacteria are related to the pathogenesis of NAFLD and ALD and to primary biliary cholangitis (PBC) and PSC. This may be related to the abnormal development of immunity caused by an imbalance of the gut microbiome, resulting in imbalanced production of injurious *vs* cytoprotective metabolites[46].

In previous studies, bile acids have been considered a tissue-damaging factor that promotes inflammation owing to its chemical properties; its detergent effect can destroy the cell and mitochondrial membranes[47]. Overall, there are three important pathways of cytotoxicity induced by bile acids: (1) oxidative stress in the endoplasmic reticulum and mitochondria; (2) direct activation of death receptors Traill2 and Fas; and (3) lysis of the plasma membrane of liver cells. Thus, accumulation of hydrophobic bile acids is the leading cause of cholestatic liver diseases. However, a recent article suggested that two derivatives of lithocholic acid (LCA) (isoalloLCA and 3-oxolCA) can influence the adaptive immune response by regulating the differentiation of T helper (Th)17 and regulatory T (Treg) cells[48]. They are mutually restricted in function, and the change in their ratio has a decisive role in the pathogenesis and clinical prognosis of autoimmune and inflammatory diseases. Many studies have shown an imbalance between Th17 and Treg cells in patients with PBC[49-52]. These patients presented with a defective $CD8^+$ Treg cell subset and preferentially activated Th17 cells[53]. IsoalloLCA can promote the differentiation of Treg cells by increasing mitochondrial reactive oxygen species synthesis and the expression of H3K27ac in the Foxp3 promoter region under the induction of the TGF-β signal[48]. Another LCA derivative, 3-oxoLCA, inhibits Th17 cells differentiation, manifested by significantly reduced IL-17a, thereby inhibiting inflammation[48]. The results demonstrated that LCA derivatives (isoalloLCA and 3-oxoLCA) can regulate the balance between Th17 and Treg cells, which is of great significance for the treatment of cholestatic liver diseases. Moreover, isoalloLCA and 3-oxoLCA are expected to be used for the treatment of autoimmune or inflammatory diseases mediated by Th17/Treg cells imbalance in the future.

Bile acids play an important role in regulating both adaptive and innate immune systems through the gut-bile acids-liver triangle. Among them, ursodeoxycholic acid (UDCA) is currently the only drug that has been approved for the treatment of PBC. It can effectively reduce the retention of toxic bile acids in liver cells and alleviate liver damage[54,55]. However, UDCA has limited efficacy in cholestatic liver disease[56]; in addition, some patients are unable to tolerate the adverse effects of UDCA (such as gastrointestinal symptoms)[57].

Development of effective medical therapy for cholestatic liver disease is a key imperative. 24-norursodeoxycholic acid (norUDCA), a C23 homologue of UDCA with shortened lateral chains, has effective antifibrosis, anticholestatic and anti-inflammatory properties[58,59]. In a phase II clinical study, 12-wk treatment with norUDCA caused a significant dose-dependent reduction in serum alkaline phosphatase levels in PSC patients. A multicenter randomized controlled trial evaluated the efficacy and safety of norUDCA (500 mg/d, 1000 mg/d or 1500 mg/d) compared with placebo in PSC patients. NorUDCA was shown to have an excellent safety profile similar to placebo[60]. Another bile acid, OCA (an FXR agonist), has shown potential benefits for

PBC. It has approximately 100 times greater potency in activating FXR than CDCA [61]. OCA protects hepatocytes from the toxic effects of bile acids by activating the FXR receptor, reducing bile acids synthesis and improving choleresis. In addition to the effect of FXR on bile acid homeostasis, OCA monotherapy can improve the secretion of IgM and tumor necrosis factor-α and has direct immunomodulatory, antifibrosis and anti-inflammatory effects[62,63]. In clinical trials, OCA monotherapy caused a significantly greater decrease in alkaline phosphatase and bilirubin levels from baseline as compared to placebo. However, OCA treatment caused a dose-related increase in pruritus[34,57]. In conclusion, OCA may represent a new treatment option for PBC patients who cannot tolerate UDCA. Discovery of new bile acids and understanding the best way to use various bile acids may help develop new treatments for cholestatic liver disease.

LIVER FIBROSIS AND BILE ACIDS

Previous studies have shown that gut microbiome changes or dysbiosis in patients with chronic liver disease or cirrhosis are often accompanied by a significant reduction in total bile acids and secondary bile acid/primary bile acid ratio[8]. The dysbiosis is characterized by a decrease in bile acid 7α-dehydroxylating bacteria, a change in *Bacteroides*/*Firmicutes* ratio and an increase in pathogenic Gram-negative bacte-ria[64-66]. During the progression of cirrhosis, there is overgrowth of pathogenic bacteria in the small intestine, due to translocation of lipopolysaccharide, endotoxins and other metabolites and the resultant inflammation. In fact, studies have demonstrated a positive correlation of *Enterobacteriaceae* with endotoxemia inflammation and CDCA levels in feces. These metabolites derived from oxidative stress and the metabolism of ammonia and aromatic amino acids were positively related to *Porphyromonadaceae* and *Enterobacteriaceae* and closely related to the occurrence of hepatic encephalopathy[67].

Kakiyama *et al*[8] proposed that gut microbiome dysbiosis in cirrhotic patients is partly attributable to the decreased concentration of bile acids in the intestine. For example, the decrease in the number of bile acid 7α-dehydroxylating bacteria is caused by the reduction in the level of primary bile acids in the colon, which serves as an energy source[7,68]. Reduced levels of bile acids entering the small intestine can lead to overgrowth of proinflammatory and pathogenic bacteria and induce the release of inflammatory markers as well as an increase in liver inflammation[69]. Liver inflammation triggers a positive-feedback mechanism, which can further inhibit the synthesis of bile acids[70]. The size and composition of the bile acid pool can significantly regulate the gut microbiome structure and serve as an indicator of the severity of hepatic diseases. In summary, the balance of the liver-bile acid-gut microbiome axis is essential for human health and liver fibrosis.

DCA, the most effective antimicrobial in bile acids, is produced by bile acid 7α-dehydroxylating bacteria[71]. Studies have shown that increasing the DCA/cholic acid ratio in patients with cirrhosis can improve the toxic metabolites from the gut microbiome and increase the incidence of endotoxemia and hepatic encephalopathy, which may be related to the destruction of the intestinal mucosal barrier by DCA[8,72,73]. Compared with DCA, which exacerbates barrier dysfunction, LCA has a much less destructive effect on the gut microbiome due to its insolubility in water and easy excretion with feces[72,73]. TGR5 is a membrane receptor that can be activated by a variety of bile acids, among which LCA is its most potent natural agonist[18]. In the study by Guo *et al*[74], LCA inhibited the activation of Nod-like receptor protein 3 inflammasome *via* the TGR5-cAMP-protein kinase A axis, which significantly repressed the maturation of caspase-1 and the secretion of IL-B or IL-18. In addition, LCA was also shown to reduce the release of proinflammatory cytokines induced by lipopolysaccharide and the phagocytic activity of macrophages through TGR5, thus inhibiting liver inflammation[24,75-77]. Recent studies have shown that two different metabolites of LCA can also control host immune responses both in human and mice [78-80]. In clinical studies, LCA content in the stool of patients with advanced cirrhosis was significantly lower than that in patients with early cirrhosis[8]. Previous studies have focused on the cytotoxicity of bile acids. However, recent studies have revealed the anti-inflammatory and immunomodulatory effects of bile acids. Increasing evidence has shown that bile acids are a potential therapeutic target against inflammatory diseases. An appropriate increase in the concentration of bile acids in patients with liver cirrhosis may repress liver inflammation and improve liver fibrosis, which deserves further discussion.

Table 1 Bile acids regulate host immunological homeostasis and inflammatory response

Title	Ref.	Target	Result
Microbial bile acid metabolites modulate gut RORγ$^+$ regulatory T cell homeostasis	Song *et al*[83], 2020	BA nuclear receptors	Restoration of the intestinal BA pool can increase colonic RORγ$^+$ Treg cell counts and ameliorate host susceptibility to inflammatory colitis
Bile acid metabolites control Th17 and Treg cell differentiation	Hang *et al*[48], 2019	Derivatives of LCA, 3-oxoLCA and isoalloLCA	Administration of 3-oxoLCA and isoalloLCA to mice reduced Th17 cell differentiation and increased Treg cell differentiation, respectively, in the intestinal lamina propria
Bile acids control inflammation and metabolic disorder through inhibition of NLRP3 inflammasome	Guo *et al* [26], 2016	Membrane receptor TGR5	Bile acids inhibited activation of the NLRP3 inflammasome *via* the TGR5-cAMP-PKA axis.
Gut microbiome–mediated bile acid metabolism regulates liver cancer *via* NKT cells	Ma *et al* [10], 2018	Hepatic CXCR6$^+$ NKT cells	Microbiome uses bile acids as a messenger to accumulate NKT cells, which have an activated phenotype and inhibit liver tumor growth.
TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading	Pols *et al* [77], 2011	Membrane receptor TGR5	TGR5 activation in macrophages by 6a-ethyl-23(S)-methylcholic acid (6-EMCA, INT-777), a semisynthetic BA, inhibits proinflammatory cytokine production, an effect mediated by TGR5-induced cAMP signaling and subsequent NF-κB inhibition.

NKT: Natural killer T; BA: Bile acid; LCA: Lithocholic acid; TGR5: G-protein coupled bile acid receptor 1; Treg: Regulatory T; Th: T helper; PKA: Protein kinase A; RORγ: RAR-related orphan receptor gamma; NLRP3: NLR family pyrin domain containing 3.

CONCLUSION

Numerous studies in recent decades have shown that the function of bile acids is beyond that of "digestive surfactants." At the host level, there is a clear relationship between bile acid signaling and innate immunity in the liver and intestine. In other words, bile acids are the cornerstone of the immune axis between the liver and the gut microbiome. As a mediator in the gut-liver axis, bile acids can regulate the inflammatory response, host metabolism and innate immunity, which are effective therapeutic targets in the context of various hepatic diseases (Table 1). However, most contemporary research on bile acids is based on genetically modified mice models, while the immune system, bile acid metabolism and the gut microbiome of mice are vastly different from that of humans. Recent clinical trials of FXR agonists have yielded promising results. Preclinical data suggest that gut microbiome metabolism of bile acids is also a potential therapeutic target. Changes in the gut microbiome to regulate the composition of bile acids can improve liver health through the use of antibiotics, probiotics, prebiotics and fecal bacteria transplantation[81,82]. The complex interactions between bile acids and host-microbiome in the gut-liver axis are only beginning to be understood. Clinical trials and further in-depth studies can help characterize the different roles of bile acids in healthy individuals and patients with hepatic diseases, allowing their optimal utilization as potential therapeutic targets.

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REFERENCES

1 **Abdel-Misih SR**, Bloomston M. Liver anatomy. *Surg Clin North Am* 2010; **90**: 643-653 [PMID: 20637938 DOI: 10.1016/j.suc.2010.04.017]
2 **de Aguiar Vallim TQ**, Tarling EJ, Edwards PA. Pleiotropic roles of bile acids in metabolism. *Cell Metab* 2013; **17**: 657-669 [PMID: 23602448 DOI: 10.1016/j.cmet.2013.03.013]
3 **Wahlström A**, Sayin SI, Marschall HU, Bäckhed F. Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. *Cell Metab* 2016; **24**: 41-50 [PMID: 27320064 DOI: 10.1016/j.cmet.2016.05.005]
4 **Li-Hawkins J**, Gåfvels M, Olin M, Lund EG, Andersson U, Schuster G, Björkhem I, Russell DW, Eggertsen G. Cholic acid mediates negative feedback regulation of bile acid synthesis in mice. *J Clin Invest* 2002; **110**: 1191-1200 [PMID: 12393855 DOI: 10.1172/JCI16309]
5 **Sayin SI**, Wahlström A, Felin J, Jäntti S, Marschall HU, Bamberg K, Angelin B, Hyötyläinen T, Orešič M, Bäckhed F. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-

beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metab* 2013; **17**: 225-235 [PMID: 23395169 DOI: 10.1016/j.cmet.2013.01.003]
6 **Staley C**, Weingarden AR, Khoruts A, Sadowsky MJ. Interaction of gut microbiota with bile acid metabolism and its influence on disease states. *Appl Microbiol Biotechnol* 2017; **101**: 47-64 [PMID: 27888332 DOI: 10.1007/s00253-016-8006-6]
7 **Ridlon JM**, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* 2006; **47**: 241-259 [PMID: 16299351 DOI: 10.1194/jlr.R500013-JLR200]
8 **Kakiyama G**, Pandak WM, Gillevet PM, Hylemon PB, Heuman DM, Daita K, Takei H, Muto A, Nittono H, Ridlon JM, White MB, Noble NA, Monteith P, Fuchs M, Thacker LR, Sikaroodi M, Bajaj JS. Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. *J Hepatol* 2013; **58**: 949-955 [PMID: 23333527 DOI: 10.1016/j.jhep.2013.01.003]
9 **Ciocan D**, Voican CS, Wrzosek L, Hugot C, Rainteau D, Humbert L, Cassard AM, Perlemuter G. Bile acid homeostasis and intestinal dysbiosis in alcoholic hepatitis. *Aliment Pharmacol Ther* 2018; **48**: 961-974 [PMID: 30144108 DOI: 10.1111/apt.14949]
10 **Ma C**, Han M, Heinrich B, Fu Q, Zhang Q, Sandhu M, Agdashian D, Terabe M, Berzofsky JA, Fako V, Ritz T, Longerich T, Theriot CM, McCulloch JA, Roy S, Yuan W, Thovarai V, Sen SK, Ruchirawat M, Korangy F, Wang XW, Trinchieri G, Greten TF. Gut microbiome-mediated bile acid metabolism regulates liver cancer *via* NKT cells. *Science* 2018; **360** [PMID: 29798856 DOI: 10.1126/science.aan5931]
11 **Hartmann P**, Hochrath K, Horvath A, Chen P, Seebauer CT, Llorente C, Wang L, Alnouti Y, Fouts DE, Stärkel P, Loomba R, Coulter S, Liddle C, Yu RT, Ling L, Rossi SJ, DePaoli AM, Downes M, Evans RM, Brenner DA, Schnabl B. Modulation of the intestinal bile acid/farnesoid X receptor/fibroblast growth factor 15 axis improves alcoholic liver disease in mice. *Hepatology* 2018; **67**: 2150-2166 [PMID: 29159825 DOI: 10.1002/hep.29676]
12 **Wehr A**, Baeck C, Heymann F, Niemietz PM, Hammerich L, Martin C, Zimmermann HW, Pack O, Gassler N, Hittatiya K, Ludwig A, Luedde T, Trautwein C, Tacke F. Chemokine receptor CXCR6-dependent hepatic NK T Cell accumulation promotes inflammation and liver fibrosis. *J Immunol* 2013; **190**: 5226-5236 [PMID: 23596313 DOI: 10.4049/jimmunol.1202909]
13 **Jiao N**, Baker SS, Chapa-Rodriguez A, Liu W, Nugent CA, Tsompana M, Mastrandrea L, Buck MJ, Baker RD, Genco RJ, Zhu R, Zhu L. Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD. *Gut* 2018; **67**: 1881-1891 [PMID: 28774887 DOI: 10.1136/gutjnl-2017-314307]
14 **Balkwill F**. Cancer and the chemokine network. *Nat Rev Cancer* 2004; **4**: 540-550 [PMID: 15229479 DOI: 10.1038/nrc1388]
15 **Arab JP**, Karpen SJ, Dawson PA, Arrese M, Trauner M. Bile acids and nonalcoholic fatty liver disease: Molecular insights and therapeutic perspectives. *Hepatology* 2017; **65**: 350-362 [PMID: 27358174 DOI: 10.1002/hep.28709]
16 **Copple BL**, Li T. Pharmacology of bile acid receptors: Evolution of bile acids from simple detergents to complex signaling molecules. *Pharmacol Res* 2016; **104**: 9-21 [PMID: 26706784 DOI: 10.1016/j.phrs.2015.12.007]
17 **Chiang JY**. Bile acid metabolism and signaling. *Compr Physiol* 2013; **3**: 1191-1212 [PMID: 23897684 DOI: 10.1002/cphy.c120023]
18 **Schaap FG**, Trauner M, Jansen PL. Bile acid receptors as targets for drug development. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 55-67 [PMID: 23982684 DOI: 10.1038/nrgastro.2013.151]
19 **Zhang Y**, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, Willson TM, Edwards PA. Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. *Proc Natl Acad Sci U S A* 2006; **103**: 1006-1011 [PMID: 16410358 DOI: 10.1073/pnas.0506982103]
20 **De Fabiani E**, Mitro N, Gilardi F, Caruso D, Galli G, Crestani M. Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis *via* a novel mechanism of transcription regulation linked to the fasted-to-fed cycle. *J Biol Chem* 2003; **278**: 39124-39132 [PMID: 12865425 DOI: 10.1074/jbc.M305079200]
21 **Cyphert HA**, Ge X, Kohan AB, Salati LM, Zhang Y, Hillgartner FB. Activation of the farnesoid X receptor induces hepatic expression and secretion of fibroblast growth factor 21. *J Biol Chem* 2012; **287**: 25123-25138 [PMID: 22661717 DOI: 10.1074/jbc.M112.375907]
22 **Watanabe M**, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, Auwerx J. Bile acids lower triglyceride levels *via* a pathway involving FXR, SHP, and SREBP-1c. *J Clin Invest* 2004; **113**: 1408-1418 [PMID: 15146238 DOI: 10.1172/JCI21025]
23 **Gruenbacher G**, Gander H, Rahm A, Dobler G, Drasche A, Troppmair J, Nussbaumer W, Thurnher M. The Human G Protein-Coupled ATP Receptor $P2Y_{11}$ Is Associated With IL-10 Driven Macrophage Differentiation. *Front Immunol* 2019; **10**: 1870 [PMID: 31447857 DOI: 10.3389/fimmu.2019.01870]
24 **Keitel V**, Donner M, Winandy S, Kubitz R, Häussinger D. Expression and function of the bile acid receptor TGR5 in Kupffer cells. *Biochem Biophys Res Commun* 2008; **372**: 78-84 [PMID: 18468513 DOI: 10.1016/j.bbrc.2008.04.171]
25 **Ding L**, Sousa KM, Jin L, Dong B, Kim BW, Ramirez R, Xiao Z, Gu Y, Yang Q, Wang J, Yu D, Pigazzi A, Schones D, Yang L, Moore D, Wang Z, Huang W. Vertical sleeve gastrectomy activates GPBAR-1/TGR5 to sustain weight loss, improve fatty liver, and remit insulin resistance in mice. *Hepatology* 2016; **64**: 760-773 [PMID: 27312543 DOI: 10.1002/hep.28689]
26 **Guo C**, Xie S, Chi Z, Zhang J, Liu Y, Zhang L, Zheng M, Zhang X, Xia D, Ke Y, Lu L, Wang D.

Bile Acids Control Inflammation and Metabolic Disorder through Inhibition of NLRP3 Inflammasome. *Immunity* 2016; **45**: 802-816 [PMID: 27692610 DOI: 10.1016/j.immuni.2016.09.008]

27 **McMahan RH**, Wang XX, Cheng LL, Krisko T, Smith M, El Kasmi K, Pruzanski M, Adorini L, Golden-Mason L, Levi M, Rosen HR. Bile acid receptor activation modulates hepatic monocyte activity and improves nonalcoholic fatty liver disease. *J Biol Chem* 2013; **288**: 11761-11770 [PMID: 23460643 DOI: 10.1074/jbc.M112.446575]

28 **Jadhav K**, Xu Y, Li Y, Xu J, Zhu Y, Adorini L, Lee YK, Kasumov T, Yin L, Zhang Y. Reversal of metabolic disorders by pharmacological activation of bile acid receptors TGR5 and FXR. *Mol Metab* 2018; **9**: 131-140 [PMID: 29361497 DOI: 10.1016/j.molmet.2018.01.005]

29 **Chu H**, Duan Y, Yang L, Schnabl B. Small metabolites, possible big changes: a microbiota-centered view of non-alcoholic fatty liver disease. *Gut* 2019; **68**: 359-370 [PMID: 30171065 DOI: 10.1136/gutjnl-2018-316307]

30 **Gottlieb A**, Bechmann L, Canbay A. The Presence and Severity of Nonalcoholic Steatohepatitis Is Associated with Specific Changes in Circulating Bile Acids. *Ann Hepatol* 2018; **17**: 340-341 [PMID: 29735794 DOI: 10.5604/01.3001.0011.7378]

31 **Parséus A**, Sommer N, Sommer F, Caesar R, Molinaro A, Ståhlman M, Greiner TU, Perkins R, Bäckhed F. Microbiota-induced obesity requires farnesoid X receptor. *Gut* 2017; **66**: 429-437 [PMID: 26740296 DOI: 10.1136/gutjnl-2015-310283]

32 **Mudaliar S**, Henry RR, Sanyal AJ, Morrow L, Marschall HU, Kipnes M, Adorini L, Sciacca CI, Clopton P, Castelloe E, Dillon P, Pruzanski M, Shapiro D. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. *Gastroenterology* 2013; **145**: 574-82. e1 [PMID: 23727264 DOI: 10.1053/j.gastro.2013.05.042]

33 **Musso G**, Cassader M, Gambino R. Non-alcoholic steatohepatitis: emerging molecular targets and therapeutic strategies. *Nat Rev Drug Discov* 2016; **15**: 249-274 [PMID: 26794269 DOI: 10.1038/nrd.2015.3]

34 **Hirschfield GM**, Mason A, Luketic V, Lindor K, Gordon SC, Mayo M, Kowdley KV, Vincent C, Bodhenheimer HC Jr, Parés A, Trauner M, Marschall HU, Adorini L, Sciacca C, Beecher-Jones T, Castelloe E, Böhm O, Shapiro D. Efficacy of obeticholic acid in patients with primary biliary cirrhosis and inadequate response to ursodeoxycholic acid. *Gastroenterology* 2015; **148**: 751-61. e8 [PMID: 25500425 DOI: 10.1053/j.gastro.2014.12.005]

35 **Pellicciari R**, Gioiello A, Macchiarulo A, Thomas C, Rosatelli E, Natalini B, Sardella R, Pruzanski M, Roda A, Pastorini E, Schoonjans K, Auwerx J. Discovery of 6alpha-ethyl-23(S)-methylcholic acid (S-EMCA, INT-777) as a potent and selective agonist for the TGR5 receptor, a novel target for diabesity. *J Med Chem* 2009; **52**: 7958-7961 [PMID: 20014870 DOI: 10.1021/jm901390p]

36 **Bajaj JS**, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, Noble NA, Unser AB, Daita K, Fisher AR, Sikaroodi M, Gillevet PM. Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J Hepatol* 2014; **60**: 940-947 [PMID: 24374295 DOI: 10.1016/j.jhep.2013.12.019]

37 **Ahluwalia V**, Wade JB, Moeller FG, White MB, Unser AB, Gavis EA, Sterling RK, Stravitz RT, Sanyal AJ, Siddiqui MS, Puri P, Luketic V, Heuman DM, Fuchs M, Matherly S, Bajaj JS. The etiology of cirrhosis is a strong determinant of brain reserve: A multimodal magnetic resonance imaging study. *Liver Transpl* 2015; **21**: 1123-1132 [PMID: 25939692 DOI: 10.1002/lt.24163]

38 **Arroyo V**, Moreau R, Kamath PS, Jalan R, Ginès P, Nevens F, Fernández J, To U, García-Tsao G, Schnabl B. Acute-on-chronic liver failure in cirrhosis. *Nat Rev Dis Primers* 2016; **2**: 16041 [PMID: 27277335 DOI: 10.1038/nrdp.2016.41]

39 **Jansen PL**, Ghallab A, Vartak N, Reif R, Schaap FG, Hampe J, Hengstler JG. The ascending pathophysiology of cholestatic liver disease. *Hepatology* 2017; **65**: 722-738 [PMID: 27981592 DOI: 10.1002/hep.28965]

40 **Chen W**, Owsley E, Yang Y, Stroup D, Chiang JY. Nuclear receptor-mediated repression of human cholesterol 7alpha-hydroxylase gene transcription by bile acids. *J Lipid Res* 2001; **42**: 1402-1412 [PMID: 11518759]

41 **Allen K**, Jaeschke H, Copple BL. Bile acids induce inflammatory genes in hepatocytes: a novel mechanism of inflammation during obstructive cholestasis. *Am J Pathol* 2011; **178**: 175-186 [PMID: 21224055 DOI: 10.1016/j.ajpath.2010.11.026]

42 **Schoemaker MH**, Conde de la Rosa L, Buist-Homan M, Vrenken TE, Havinga R, Poelstra K, Haisma HJ, Jansen PL, Moshage H. Tauroursodeoxycholic acid protects rat hepatocytes from bile acid-induced apoptosis *via* activation of survival pathways. *Hepatology* 2004; **39**: 1563-1573 [PMID: 15185297 DOI: 10.1002/hep.20246]

43 **Li Y**, Tang R, Leung PSC, Gershwin ME, Ma X. Bile acids and intestinal microbiota in autoimmune cholestatic liver diseases. *Autoimmun Rev* 2017; **16**: 885-896 [PMID: 28698093 DOI: 10.1016/j.autrev.2017.07.002]

44 **Woodhouse CA**, Patel VC, Singanayagam A, Shawcross DL. Review article: the gut microbiome as a therapeutic target in the pathogenesis and treatment of chronic liver disease. *Aliment Pharmacol Ther* 2018; **47**: 192-202 [PMID: 29083037 DOI: 10.1111/apt.14397]

45 **Kummen M**, Holm K, Anmarkrud JA, Nygård S, Vesterhus M, Høivik ML, Trøseid M, Marschall HU, Schrumpf E, Moum B, Røsjø H, Aukrust P, Karlsen TH, Hov JR. The gut microbial profile in patients with primary sclerosing cholangitis is distinct from patients with ulcerative colitis without biliary disease and healthy controls. *Gut* 2017; **66**: 611-619 [PMID: 26887816 DOI: 10.1136/gutjnl-2015-310500]

46 **Santiago P**, Scheinberg AR, Levy C. Cholestatic liver diseases: new targets, new therapies. *Therap Adv Gastroenterol* 2018; **11**: 1756284818787400 [PMID: 30159035 DOI: 10.1177/1756284818787400]

47 **Trauner M**, Meier PJ, Boyer JL. Molecular pathogenesis of cholestasis. *N Engl J Med* 1998; **339**: 1217-1227 [PMID: 9780343 DOI: 10.1056/NEJM199810223391707]

48 **Hang S**, Paik D, Yao L, Kim E, Trinath J, Lu J, Ha S, Nelson BN, Kelly SP, Wu L, Zheng Y, Longman RS, Rastinejad F, Devlin AS, Krout MR, Fischbach MA, Littman DR, Huh JR. Bile acid metabolites control T_H17 and T_{reg} cell differentiation. *Nature* 2019; **576**: 143-148 [PMID: 31776512 DOI: 10.1038/s41586-019-1785-z]

49 **Rong G**, Zhou Y, Xiong Y, Zhou L, Geng H, Jiang T, Zhu Y, Lu H, Zhang S, Wang P, Zhang B, Zhong R. Imbalance between T helper type 17 and T regulatory cells in patients with primary biliary cirrhosis: the serum cytokine profile and peripheral cell population. *Clin Exp Immunol* 2009; **156**: 217-225 [PMID: 19302244 DOI: 10.1111/j.1365-2249.2009.03898.x]

50 **Acosta-Rodriguez EV**, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 2007; **8**: 639-646 [PMID: 17486092 DOI: 10.1038/ni1467]

51 **Yamazaki T**, Yang XO, Chung Y, Fukunaga A, Nurieva R, Pappu B, Martin-Orozco N, Kang HS, Ma L, Panopoulos AD, Craig S, Watowich SS, Jetten AM, Tian Q, Dong C. CCR6 regulates the migration of inflammatory and regulatory T cells. *J Immunol* 2008; **181**: 8391-8401 [PMID: 19050256 DOI: 10.4049/jimmunol.181.12.8391]

52 **Lim HW**, Lee J, Hillsamer P, Kim CH. Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. *J Immunol* 2008; **180**: 122-129 [PMID: 18097011 DOI: 10.4049/jimmunol.180.1.122]

53 **Fenoglio D**, Bernuzzi F, Battaglia F, Parodi A, Kalli F, Negrini S, De Palma R, Invernizzi P, Filaci G. Th17 and regulatory T lymphocytes in primary biliary cirrhosis and systemic sclerosis as models of autoimmune fibrotic diseases. *Autoimmun Rev* 2012; **12**: 300-304 [PMID: 22634708 DOI: 10.1016/j.autrev.2012.05.004]

54 **Lindor K**. Ursodeoxycholic acid for the treatment of primary biliary cirrhosis. *N Engl J Med* 2007; **357**: 1524-1529 [PMID: 17928600 DOI: 10.1056/NEJMct074694]

55 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: management of cholestatic liver diseases. *J Hepatol* 2009; **51**: 237-267 [PMID: 19501929 DOI: 10.1016/j.jhep.2009.04.009]

56 **Hirschfield GM**, Heathcote EJ, Gershwin ME. Pathogenesis of cholestatic liver disease and therapeutic approaches. *Gastroenterology* 2010; **139**: 1481-1496 [PMID: 20849855 DOI: 10.1053/j.gastro.2010.09.004]

57 **Kowdley KV**, Luketic V, Chapman R, Hirschfield GM, Poupon R, Schramm C, Vincent C, Rust C, Parés A, Mason A, Marschall HU, Shapiro D, Adorini L, Sciacca C, Beecher-Jones T, Böhm O, Pencek R, Jones D; Obeticholic Acid PBC Monotherapy Study Group. A randomized trial of obeticholic acid monotherapy in patients with primary biliary cholangitis. *Hepatology* 2018; **67**: 1890-1902 [PMID: 29023915 DOI: 10.1002/hep.29569]

58 **Moustafa T**, Fickert P, Magnes C, Guelly C, Thueringer A, Frank S, Kratky D, Sattler W, Reicher H, Sinner F, Gumhold J, Silbert D, Fauler G, Höfler G, Lass A, Zechner R, Trauner M. Alterations in lipid metabolism mediate inflammation, fibrosis, and proliferation in a mouse model of chronic cholestatic liver injury. *Gastroenterology* 2012; **142**: 140-151. e12 [PMID: 22001865 DOI: 10.1053/j.gastro.2011.09.051]

59 **Sombetzki M**, Fuchs CD, Fickert P, Österreicher CH, Mueller M, Claudel T, Loebermann M, Engelmann R, Langner C, Sahin E, Schwinge D, Guenther ND, Schramm C, Mueller-Hilke B, Reisinger EC, Trauner M. 24-nor-ursodeoxycholic acid ameliorates inflammatory response and liver fibrosis in a murine model of hepatic schistosomiasis. *J Hepatol* 2015; **62**: 871-878 [PMID: 25463533 DOI: 10.1016/j.jhep.2014.11.020]

60 **Fickert P**, Hirschfield GM, Denk G, Marschall HU, Altorjay I, Färkkilä M, Schramm C, Spengler U, Chapman R, Bergquist A, Schrumpf E, Nevens F, Trivedi P, Reiter FP, Tornai I, Halilbasic E, Greinwald R, Pröls M, Manns MP, Trauner M; European PSC norUDCA Study Group. norUrsodeoxycholic acid improves cholestasis in primary sclerosing cholangitis. *J Hepatol* 2017; **67**: 549-558 [PMID: 28529147 DOI: 10.1016/j.jhep.2017.05.009]

61 **Pellicciari R**, Fiorucci S, Camaioni E, Clerici C, Costantino G, Maloney PR, Morelli A, Parks DJ, Willson TM. 6alpha-ethyl-chenodeoxycholic acid (6-ECDCA), a potent and selective FXR agonist endowed with anticholestatic activity. *J Med Chem* 2002; **45**: 3569-3572 [PMID: 12166927 DOI: 10.1021/jm025529g]

62 **Neuschwander-Tetri BA**, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarathy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, Doo E; NASH Clinical Research Network. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* 2015; **385**: 956-965 [PMID: 25468160 DOI: 10.1016/S0140-6736(14)61933-4]

63 **Beuers U**, Trauner M, Jansen P, Poupon R. New paradigms in the treatment of hepatic cholestasis: from UDCA to FXR, PXR and beyond. *J Hepatol* 2015; **62**: S25-S37 [PMID: 25920087 DOI: 10.1016/j.jhep.2015.02.023]

64 **Bajaj JS**, Ridlon JM, Hylemon PB, Thacker LR, Heuman DM, Smith S, Sikaroodi M, Gillevet PM.

Linkage of gut microbiome with cognition in hepatic encephalopathy. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G168-G175 [PMID: 21940902 DOI: 10.1152/ajpgi.00190.2011]

65 **Chen Y**, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 2011; **54**: 562-572 [PMID: 21574172 DOI: 10.1002/hep.24423]

66 **Quigley EM**, Quera R. Small intestinal bacterial overgrowth: roles of antibiotics, prebiotics, and probiotics. *Gastroenterology* 2006; **130**: S78-S90 [PMID: 16473077 DOI: 10.1053/j.gastro.2005.11.046]

67 **Bajaj JS**, Heuman DM, Sanyal AJ, Hylemon PB, Sterling RK, Stravitz RT, Fuchs M, Ridlon JM, Daita K, Monteith P, Noble NA, White MB, Fisher A, Sikaroodi M, Rangwala H, Gillevet PM. Modulation of the metabiome by rifaximin in patients with cirrhosis and minimal hepatic encephalopathy. *PLoS One* 2013; **8**: e60042 [PMID: 23565181 DOI: 10.1371/journal.pone.0060042]

68 **Ridlon JM**, Hylemon PB. Identification and characterization of two bile acid coenzyme A transferases from Clostridium scindens, a bile acid 7α-dehydroxylating intestinal bacterium. *J Lipid Res* 2012; **53**: 66-76 [PMID: 22021638 DOI: 10.1194/jlr.M020313]

69 **Islam KB**, Fukiya S, Hagio M, Fujii N, Ishizuka S, Ooka T, Ogura Y, Hayashi T, Yokota A. Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 2011; **141**: 1773-1781 [PMID: 21839040 DOI: 10.1053/j.gastro.2011.07.046]

70 **Ridlon JM**, Alves JM, Hylemon PB, Bajaj JS. Cirrhosis, bile acids and gut microbiota: unraveling a complex relationship. *Gut Microbes* 2013; **4**: 382-387 [PMID: 23851335 DOI: 10.4161/gmic.25723]

71 **Begley M**, Gahan CG, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev* 2005; **29**: 625-651 [PMID: 16102595 DOI: 10.1016/j.femsre.2004.09.003]

72 **Stenman LK**, Holma R, Korpela R. High-fat-induced intestinal permeability dysfunction associated with altered fecal bile acids. *World J Gastroenterol* 2012; **18**: 923-929 [PMID: 22408351 DOI: 10.3748/wjg.v18.i9.923]

73 **Stenman LK**, Holma R, Eggert A, Korpela R. A novel mechanism for gut barrier dysfunction by dietary fat: epithelial disruption by hydrophobic bile acids. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G227-G234 [PMID: 23203158 DOI: 10.1152/ajpgi.00267.2012]

74 **Guo H**, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med* 2015; **21**: 677-687 [PMID: 26121197 DOI: 10.1038/nm.3893]

75 **Haselow K**, Bode JG, Wammers M, Ehlting C, Keitel V, Kleinebrecht L, Schupp AK, Häussinger D, Graf D. Bile acids PKA-dependently induce a switch of the IL-10/IL-12 ratio and reduce proinflammatory capability of human macrophages. *J Leukoc Biol* 2013; **94**: 1253-1264 [PMID: 23990628 DOI: 10.1189/jlb.0812396]

76 **Perino A**, Schoonjans K. TGR5 and Immunometabolism: Insights from Physiology and Pharmacology. *Trends Pharmacol Sci* 2015; **36**: 847-857 [PMID: 26541439 DOI: 10.1016/j.tips.2015.08.002]

77 **Pols TW**, Nomura M, Harach T, Lo Sasso G, Oosterveer MH, Thomas C, Rizzo G, Gioiello A, Adorini L, Pellicciari R, Auwerx J, Schoonjans K. TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. *Cell Metab* 2011; **14**: 747-757 [PMID: 22152303 DOI: 10.1016/j.cmet.2011.11.006]

78 **Kakiyama G**, Muto A, Takei H, Nittono H, Murai T, Kurosawa T, Hofmann AF, Pandak WM, Bajaj JS. A simple and accurate HPLC method for fecal bile acid profile in healthy and cirrhotic subjects: validation by GC-MS and LC-MS. *J Lipid Res* 2014; **55**: 978-990 [PMID: 24627129 DOI: 10.1194/jlr.D047506]

79 **Sakai K**, Makino T, Kawai Y, Mutai M. Intestinal microflora and bile acids. Effect of bile acids on the distribution of microflora and bile acid in the digestive tract of the rat. *Microbiol Immunol* 1980; **24**: 187-196 [PMID: 6447830 DOI: 10.1111/j.1348-0421.1980.tb00578.x]

80 **Robben J**, Caenepeel P, Van Eldere J, Eyssen H. Effects of intestinal microbial bile salt sulfatase activity on bile salt kinetics in gnotobiotic rats. *Gastroenterology* 1988; **94**: 494-502 [PMID: 3335321 DOI: 10.1016/0016-5085(88)90443-x]

81 **David LA**, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014; **505**: 559-563 [PMID: 24336217 DOI: 10.1038/nature12820]

82 **Dawson PA**, Hubbert M, Haywood J, Craddock AL, Zerangue N, Christian WV, Ballatori N. The heteromeric organic solute transporter alpha-beta, Ostalpha-Ostbeta, is an ileal basolateral bile acid transporter. *J Biol Chem* 2005; **280**: 6960-6968 [PMID: 15563450 DOI: 10.1074/jbc.M412752200]

83 **Song X**, Sun X, Oh SF, Wu M, Zhang Y, Zheng W, Geva-Zatorsky N, Jupp R, Mathis D, Benoist C, Kasper DL. Microbial bile acid metabolites modulate gut RORγ^{+} regulatory T cell homeostasis. *Nature* 2020; **577**: 410-415 [PMID: 31875848 DOI: 10.1038/s41586-019-1865-0]

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MINIREVIEWS

Liver injury in COVID-19: Detection, pathogenesis, and treatment

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Abstract

In the early December 2019, a novel coronavirus named severe acute respiratory syndrome coronavirus 2 was first reported in Wuhan, China, followed by an outbreak that spread around the world. Numerous studies have shown that liver injury is common in patients with coronavirus disease 2019 (COVID-19), and may aggravate the severity of the disease. However, the exact cause and specific mechanism of COVID-associated liver injury needs to be elucidated further. In this review, we present an analysis of the clinical features, potential mechanisms, and treatment strategies for liver injury associated with COVID-19. We hope that this review would benefit clinicians in devising better strategies for management of such patients.

Key Words: COVID-19; Liver injury; Clinical features; Potential mechanism; Treatment

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Core Tip: Coronavirus disease 2019 (COVID-19) has assumed pandemic proportions, and has resulted in several hundred thousand deaths globally. Although the lung is the main organ that is damaged in COVID-19, approximately 60% of the patients have been reported to develop various degrees of liver injury in several studies. Accumulating clinical data show that liver damage is related to the severity of COVID-19 and is a major cause of death from COVID-19, especially in the presence of hepatic failure. The exact cause of liver injury in patients with COVID-19 remains unclear and the specific underlying mechanism(s) need to be elucidated.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which first broke out in Wuhan, China in December 2019, has become a great threat to public health worldwide. As of August 4, 2020, more than 1000000 deaths from COVID-19 have been confirmed[1]. Although the lung is the main organ that is damaged in COVID-19, approximately 60% of the patients were reported to develop various degrees of liver injury in previous studies[2-5]. Accumulating clinical data show that liver damage is related to the severity of COVID-19 and is also a major cause of death from COVID-19, especially in the presence of hepatic failure[6,7]. Thus, early detection, effective treatment, and elucidation of the mechanisms underlying the pathogenesis of liver damage are urgently needed for COVID-19 patients. In this review, we summarize the characteristics of COVID-19-associated liver injury from multiple perspectives, including clinical features (manifestation, laboratory examinations, liver biopsy, *etc.*), underlying pathogenesis (direct viral cytotoxicity, uncontrolled cytokine storm, drug-induced toxicity, *etc.*), special population of patients (those with cirrhosis, hepatitis B, liver transplantation, *etc.*), and clinical management (drugs, oxygen therapy, artificial liver blood purification, *etc.*). Based on the latest data, we hope to provide a feasible reference for follow-up clinical management of COVID-19.

CLINICAL FEATURES OF HEPATIC INJURY IN COVID-19

In patients with COVID-19, the most commonly used indicators of liver function impairment are liver transaminase, bilirubin, and albumin levels. Abnormal levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin were reported in 11%-56.3%, 15.0%-86.8%, and 2.7%-30.6% of patients with COVID-19, respectively, whereas 2%-11% of such patients had pre-existing liver disease[3-8]. In a recent study involving 228 patients with COVID-19, who did not have chronic liver disease (CLD), abnormal liver function was observed in 129 (56.3%) patients, which included elevations in the levels of ALT [84 (36.8%)], AST [58 (25.4%)], total bilirubin [59 (25.9%)], and gamma-glutamyl transferase [67 (29.5%)][9]. In a study on 99 patients, Chen *et al*[10] reported elevated levels of ALT and AST in 28 (28%) and 35 (35%) patients, respectively, and hypoalbuminemia and hyperbilirubinemia in 97 (98%) and 18 (18%) patients, respectively. In addition, several studies revealed that liver damage is more prevalent in severe cases of COVID-19 than in mild cases. In a large sample multicenter study[11], abnormally elevated levels of ALT were observed in 28.1% of critically ill patients and in 19.8% of non-critically ill patients, and of AST in 18.2% of non-critically ill patients and 39.4% of critically ill patients. Huang *et al*[4] also indicated that patients admitted to the intensive care unit (ICU) not only had higher plasma levels of the inflammatory indices [interleukin (IL)-2, IL-7, IL-10, tumor

necrosis factor (TNF)-α, *etc.*], but also had abnormally high levels of AST [8 (62%) of 13 patients] compared with non-ICU patients [7 (25%) of 28 patients]. A recent descriptive study confirmed that the levels of ALT (35 *vs* 23, normal range 9-50 U/L, $P = 0.007$) and AST (52 *vs* 29, normal range 5-21 U/L, $P < 0.001$) were significantly higher in ICU patients[12]. In a Japanese cohort study concerning COVID-19, patients were classified into mild, moderate, and severe groups, respectively, based on gastrointestinal symptoms and severity of pneumonia; and the peak levels of AST (28 *vs* 48 *vs* 109, $P < 0.001$) and ALT (33 *vs* 47.5 *vs* 106, $P = 0.0114$) were significantly stratified according to these criteria[13].

Apart from the liver enzyme tests mentioned above, there are characteristic clinical manifestations of liver damage. In China, it has been reported that some patients recovering from severe COVID-19 exhibited darkening and pigmentation during the recovery process. Multiple organ damage, especially liver damage, is the main cause of darkening and hyperpigmentation[14]. Abnormal liver function may lead to pigmentation through the following three pathways: (1) Impaired liver function leads to hypofunction of the adrenal cortex. When the liver is unable to metabolize the melanin-stimulating hormone secreted by the anterior pituitary gland, the secretion of melanin increases[15]; (2) abnormal liver function hinders the inactivation of estrogen, leading to an increase in its level. The increase in estrogen levels in the body reduces the inhibition of tyrosinase by thiamine, thereby increasing the conversion of tyrosine to melanin[16]; and (3) liver damage increases the iron content in the blood. Iron delivered to the facial skin causes the darkening of the face.

Liver biopsy is also important in the etiological diagnosis of hepatic injury in COVID-19, particularly in cases where liver damage dominates the clinical manifestation, or where other alternative causes of damage need to be ruled out. Currently, most of the information on histological changes in the liver of patients with COVID-19 comes from autopsies. A case series by Bradley *et al*[17], based on ultrastructural findings and histopathology of samples from 14 fatal COVID-19 infections in the Washington State, showed centrilobular necrosis, consistent with hypoperfusion injury, in four patients; viral RNA was detected in the liver of these patients, as well. However, autopsies have several limitations. Death may occur long after the acute liver injury is noted; subsequently, histological changes may have been eliminated or obscured, and viral load would have diminished over time. Fiel *et al*[18] presented their findings from two liver biopsies performed on patients infected with SARS-CoV-2. Both patients had severe hepatic failure in the absence of obvious involvement of other organs. Detailed histological analysis, *in situ* hybridization, and electron microscopy revealed that apoptosis, abundant mitosis, mixed inflammatory infiltration in the portal area, severe bile duct injury, apparent viral particles, and viral RNA within hepatocytes are typical. These findings suggested hepatic involvement in infections with SARS-CoV-2. Another case report by Melquist *et al*[19] showed similar findings in a patient infected with SARS-CoV-2, manifesting as acute hepatitis without any respiratory symptoms, rapidly progressing to fulminant liver failure. Acute hepatitis (panacinar hepatitis, zone 3 necrosis, and focal hemophagocytosis) with viral-like changes was identified at the time of liver biopsy.

POTENTIAL MECHANISMS OF LIVER INJURY IN PATIENTS WITH COVID-19

The available evidence supports that hepatic injury in SARS-CoV-2 infection is a consequence of a multifactorial attack. The potential mechanisms of pathogenesis may be broad spectrum, ranging from direct cytotoxicity from viral infection to indirect involvement of the inflammatory cytokine storm, hypoxic changes caused by respiratory failure, endotheliitis, and drug-induced liver injury (DILI)[20] (Figure 1).

Direct effect of viral infection on the liver

Recently, it was determined by quantitative reverse transcription-polymerase chain reaction that SARS-CoV-2 RNA is widely present in other organs outside the respiratory tract, such as the liver[21], although the exact cell location of replication has not been determined because of the isolation of nucleic acids by whole tissue homogenization. Until recently, a typical hepatitis picture is yet to be observed, and hepatic tropism and direct cytopathic effects of SARS-CoV-2 should be considered as the underlying mechanism of COVID-19 associated liver injury[22]. A major determinant of viral tropism is the availability of viral receptors on the surface of host cells in specific tissues. Cellular entry of SARS-CoV-2 is mediated by the spike (S)

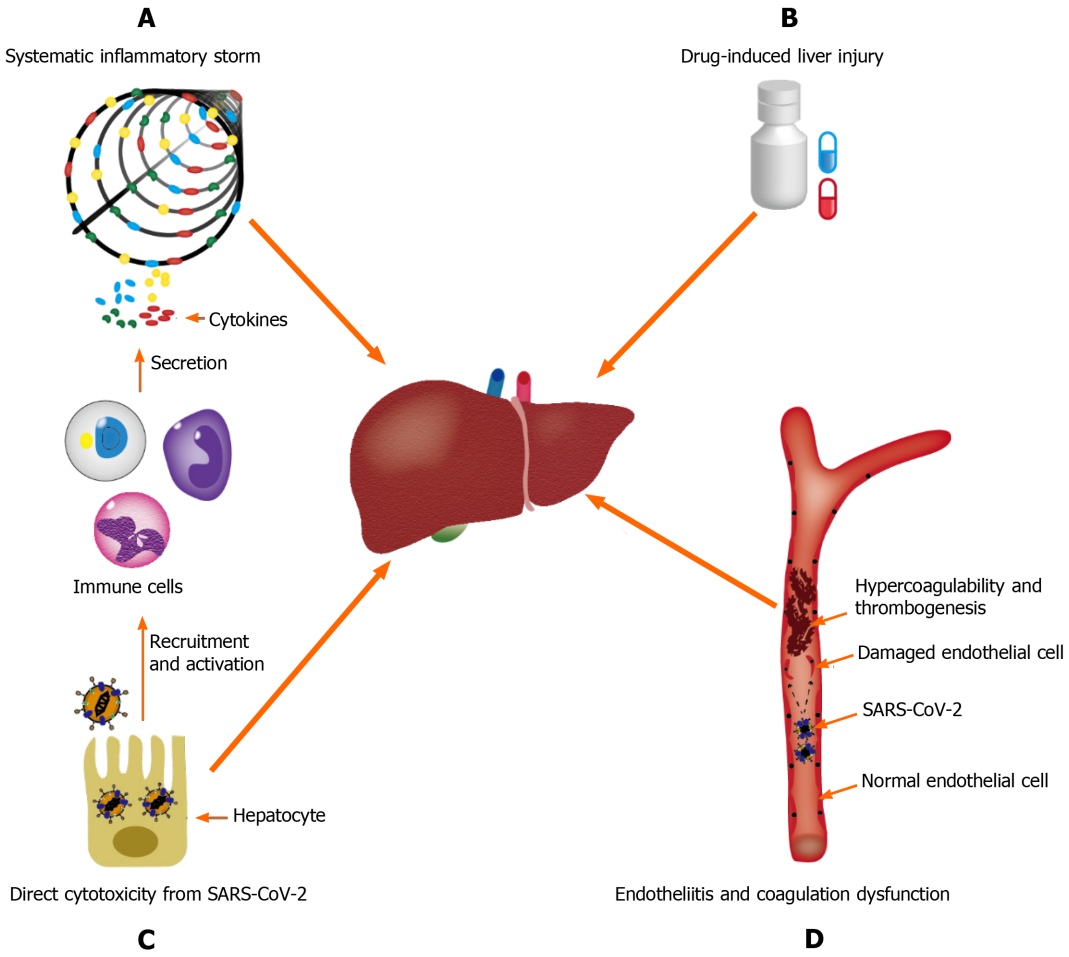


Figure 1 Underlying mechanisms of coronavirus disease-19-assocaited liver injury. A: Systematic inflammatory storm, which is generated by abnormal activation of the immune system; B: Drug-induced liver injury; C: Endotheliitis and coagulation dysfunction; D: Direct cytotoxicity from severe acute respiratory syndrome coronavirus 2. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

protein of the virus, which is cleaved by transmembrane serine protease 2/transmembrane serine protease 4 and specifically interacts with angiotensin converting enzyme 2 (ACE2) in the host[23] (Figure 2). According to Human Protein Atlas, ACE2 is highly expressed in the lung (type II alveolar cells), intestine, and gall bladder, but it seems to be almost absent in the liver. After in-depth research on ACE2 expression patterns, sinusoidal endothelial cells appear to be negative for ACE2, but this protein is expressed in the central hepatic vein and portal vein endothelial cells [24]. The expression level of ACE2 in the bile duct epithelium is comparable to that in alveolar epithelial cells, being almost 20-times higher than that in hepatocytes. Of note, Letko *et al*[25] revealed that compensatory differentiation and proliferation of liver parenchymal cells derived from bile duct cells leads to the upregulation of ACE2 expression in liver tissues, which might be the underlying mechanisms in COVID-19-associated liver injury.

Notably, typical coronavirus particles (characterized by S structures) were identified in the cytoplasm of hepatocytes in an ultrastructural examination by Wang *et al*[26]. In this study, typical lesions of viral infection, including conspicuous mitochondrial swelling, decreased glycogen granules, and endoplasmic reticulum dilatation, were also observed in the SARS-CoV-2-infected hepatocytes, which indicated that hepatic impairment might be directly caused by SARS-CoV-2. Interestingly, massive hepatic apoptosis and some binuclear hepatocytes were also identified in this study. In addition, autopsy results in patients with SARS, in the study by Guo *et al*[27], showed a large number of hepatocyte balloons, central lobular necrosis, and obvious apoptosis. Similar histological findings were also observed in a study of liver biopsy from patients with SARS by Chau *et al*[28], which suggested that SARS-CoV may induce apoptosis of hepatocytes, thereby leading to liver damage. Furthermore, Tan *et al*[29] demonstrated that overexpression of p7a, a protein specifically expressed in SARS-CoV-infected cells, could induce apoptosis in cell lines derived from different organs (including lung, kidney, and liver) *via* a caspase-dependent pathway. This further

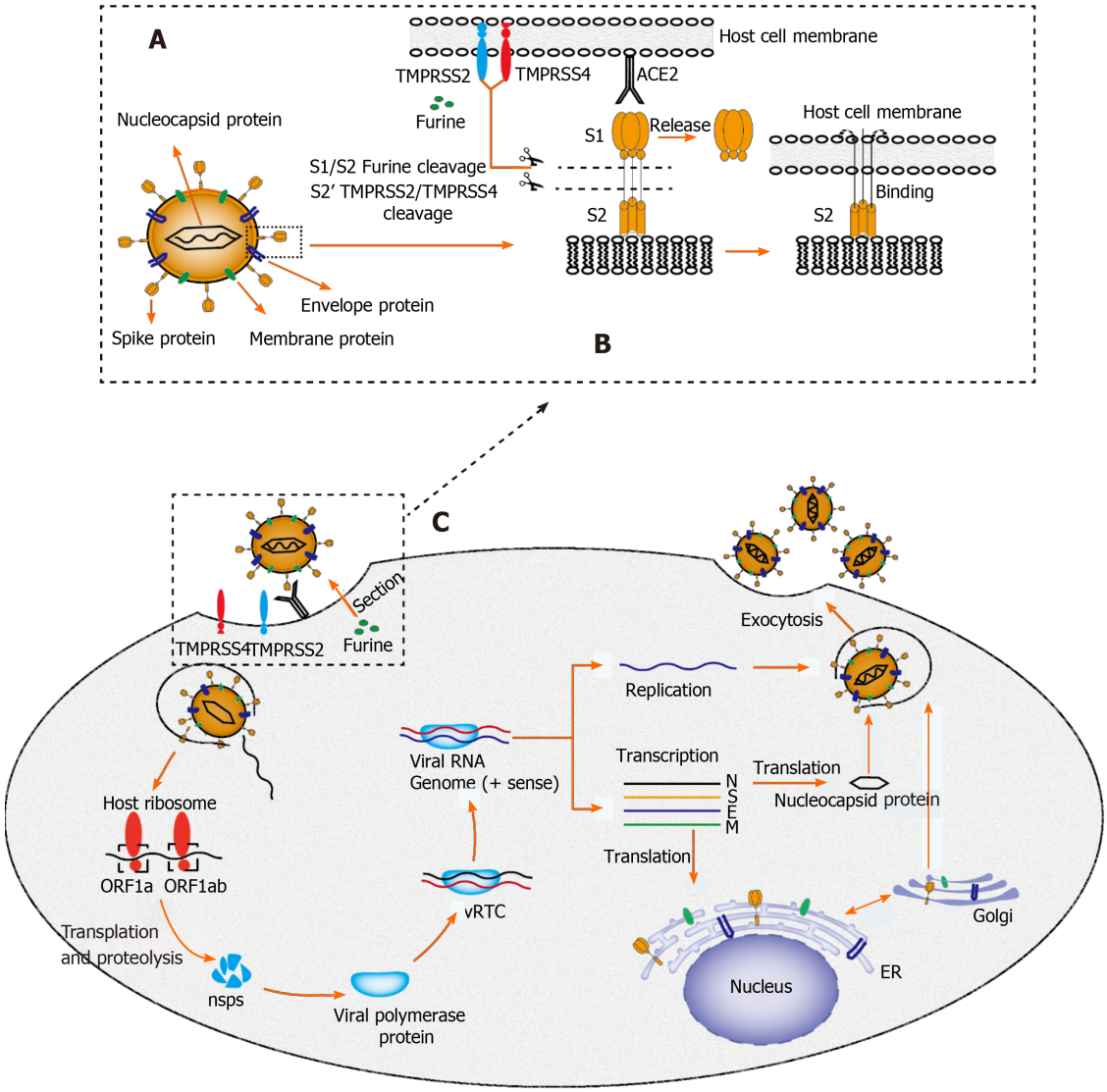


Figure 2 Proposed structure diagram of severe acute respiratory syndrome coronavirus 2 and its life cycle in host cells. A: Structural sketch of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); B: Recognition and entry of SARS-CoV-2 into host cell. Transmembrane spike (S) glycoprotein on the surface of SARS-CoV-2 forms a homotrimer to recognize the human host angiotensin converting enzyme 2 (ACE2) receptor. The S protein is specifically cleaved by two mucose-specific serine proteases [recombinant transmembrane protease serine 2 (TMPRSS2) and TMPRSS4] and furine. The subunit of S protein (S1) is released, and another subunit (S2) is exposed and mediates the viral entry into host cells; C: Life cycle of SARS-CoV-2 in host cells. First, the S protein of SARS-CoV-2 binds to ACE2 to form an S protein-ACE2 complex, which directly mediates the cellular entry of virus and the process is facilitated by TMPRSS2, TMPRSS4, and furine. Second, viral RNA is released into host cytoplasm. Open reading frame (ORF) 1a and ORF1ab are translated into large polyproteins by host ribosome, which are further proteolytically cleaved into 16 non-structural proteins (nsps). Viral polymerase protein is assembled by nsps and viral replication/transcription complex (vRTC) is subsequnently formed by polymerase protein and genomic RNA. Third, a negative sense viral RNA is synthesized and used as a template to replicate progeny (+) sense viral genome and transcribes to form various mRNAs. The nucleocapsid protein is translated in the cytoplasm, whereas the S protein, membrane (M) protein, and envelope (E) protein are translated in the endoplasmic reticulum and transported to the Golgi apparatus for further packaging. Finally, a completely new viral particle is assembled by viral RNA-nucleocapsid complex and S, M, and E proteins in endoplasmic reticulum–Golgi intermediate compartment and is released from host cell *via* exocytosis. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; M: Membrane; E: Envelope; S: Spike; ER: Endoplasmic reticulum; vRTC: Viral replication/transcription complex; ORF: Open reading frame; TMPRSS: Transmembrane protease serine; ACE2: Angiotensin converting enzyme 2.

confirmed the possibility that SARS-CoV can directly attack liver tissues and cause liver damage.

It is noteworthy that the expression level of ACE2 on hepatocytes is regulated by many factors. Several experimental studies, in both mice and humans, have confirmed increased expression of hepatic ACE2 under conditions of liver fibrosis/cirrhosis[30]. This may partially explain why pre-existing CLD increases the probability of liver damage in patients with COVID-19. Hypoxia, a typical feature in severe COVID-19 cases, has been proven to be a main regulator of ACE2 expression in hepatic cells[31]. This may explain why the dissemination of SARS-CoV-2 outside the lungs is mainly observed in patients with acute respiratory distress syndrome and other hypoxic

conditions. Notably, the affinity of S protein in SARS-CoV-2 for its receptor can be increased when it is proteolytically activated by trypsin, a protein commonly expressed in liver epithelial cells[32]. A clinical drug trial by Fantini *et al*[33] indicated that ganglioside (GM1) might be another target that influences the S protein–ACE2 interaction, using a combination of structural and molecular modeling approaches. In the near future, new molecular and therapeutic insights concerning the S protein–ACE2 interactor are expected to be uncovered with the advancement of research.

Inflammatory storm in COVID-19-associated hepatic injury

Inflammatory cytokine storm generated by the excessive immune response induced by coronavirus infection might also be one of the key factors in hepatic injury[34,35]. Higher plasma levels of inflammatory cytokines (IL-2, IL-7, IL-10, GSCF, IP10, MCP1, MIP1A, and TNF-α) and lower lymphocyte counts (both helper T cells and suppressor T cells) were commonly observed in patients with COVID-19, especially in the critically ill ones[4,36]. Qin *et al*[37] showed that COVID-19 is a systemic inflammatory viral response, first during the viral infection period and subsequently during the inflammatory period. This may explain why the conditions of illness in some patients with COVID-19 are not serious in the early stage, but if they do not receive timely medical treatment, the disease deteriorates rapidly in a short time and enters a state of multiple organ failure[38]. A cohort study of 192 patients revealed that an increase in IL-6 and IL-10 and a decrease in CD4+ T cells were independent risk factors related to severe liver damage[39]. In another study recently published in *World J Gastroenterol* [40], lymphopenia and C-reactive protein levels were found to be independently associated with hepatic injury (Figure 3).

Burra[14] confirmed that the incidence of liver damage in patients with COVID-19 having elevated ferritin levels was significantly higher (52.3% *vs* 20.0%) than that of patients with normal ferritin levels. This suggests that ferritin could be employed as an easy-to-use tool to ascertain liver injury. A possible reason for this is that ferritin, acting as an inflammatory cytokine like IL-6, participates in acute liver damage[41]. Inflammasome activation and apoptosis/pyrolysis in SARS-CoV-2 induced inflammatory cells may cause multiorgan dysfunction[42]. Interestingly, pathological changes, such as spleen atrophy and lymph node necrosis, were observed in severe cases of SARS infection, which indicated the presence of immune-mediated injury[43].

Endotheliitis in COVID-19-associated hepatic injury

COVID-19 is considered to be a thrombo-inflammatory disease that affects the lungs and, beyond that, endothelium, which is one of the largest organs in the human body. A variety of viruses, such as the HIV, dengue fever virus, and Ebola virus, have been previously reported to affect the coagulation system[44]. SARS-CoV-2 enters the endothelial cells by endocytosis *via* binding to the ACE2 receptor as well[45]. A recent study from Switzerland[42] showed the presence of viral inclusion structures within endothelial cells and diffuse endothelial inflammation. The vascular endothelium is indispensable in regulating the vascular tone and in maintaining vascular homeostasis, and intact endothelial cells provide potent anti-coagulant properties[46]. When the vascular endothelium is destroyed, either directly by viral infection or through immune-mediated inflammation, vasoconstriction and procoagulant behavior can occur rapidly. Spiezia *et al*[47] found that plasma levels of fibrinogen and D-dimer in severe cases of COVID-19 were significantly higher than those in healthy controls. In this study, markedly hypercoagulable thromboelastometry profiles, as reflected by shorter clot formation time and higher maximum clot firmness, were also observed in patients with COVID-19. In a recent study, multiple areas of microthrombi were revealed in a patient with COVID-19 by contrast-enhanced ultrasound of the lung[48], which confirmed the involvement of microvessels during the disease process. In addition, the frequency of acute pulmonary embolus in patients with COVID-19 was 30%[49] higher than that usually occurring in critically ill (1.3%)[50] or emergency (3% to 10%)[51] patients without COVID-19 (Figure 3).

SARS-CoV-2 infection causes inflammation of the vascular endothelium, which in turn leads to vascular dysfunction, especially in capillaries. Subsequently, microvascular dysfunction leads to a hypercoagulable state, tissue edema, and organ ischemia[44,52]. Liver ischemia reperfusion injury, a pathophysiological process commonly occurring after rapid recovery of blood circulation, may be the underlying mechanism of COVID-19-associated hepatic injury. Liver ischemia-reperfusion can activate neutrophils, Kupffer cells, and platelets, inducing a series of destructive cellular reactions, such as reactive oxygen species and calcium overload, which ultimately lead to an inflammatory response and cell damage. It has also been

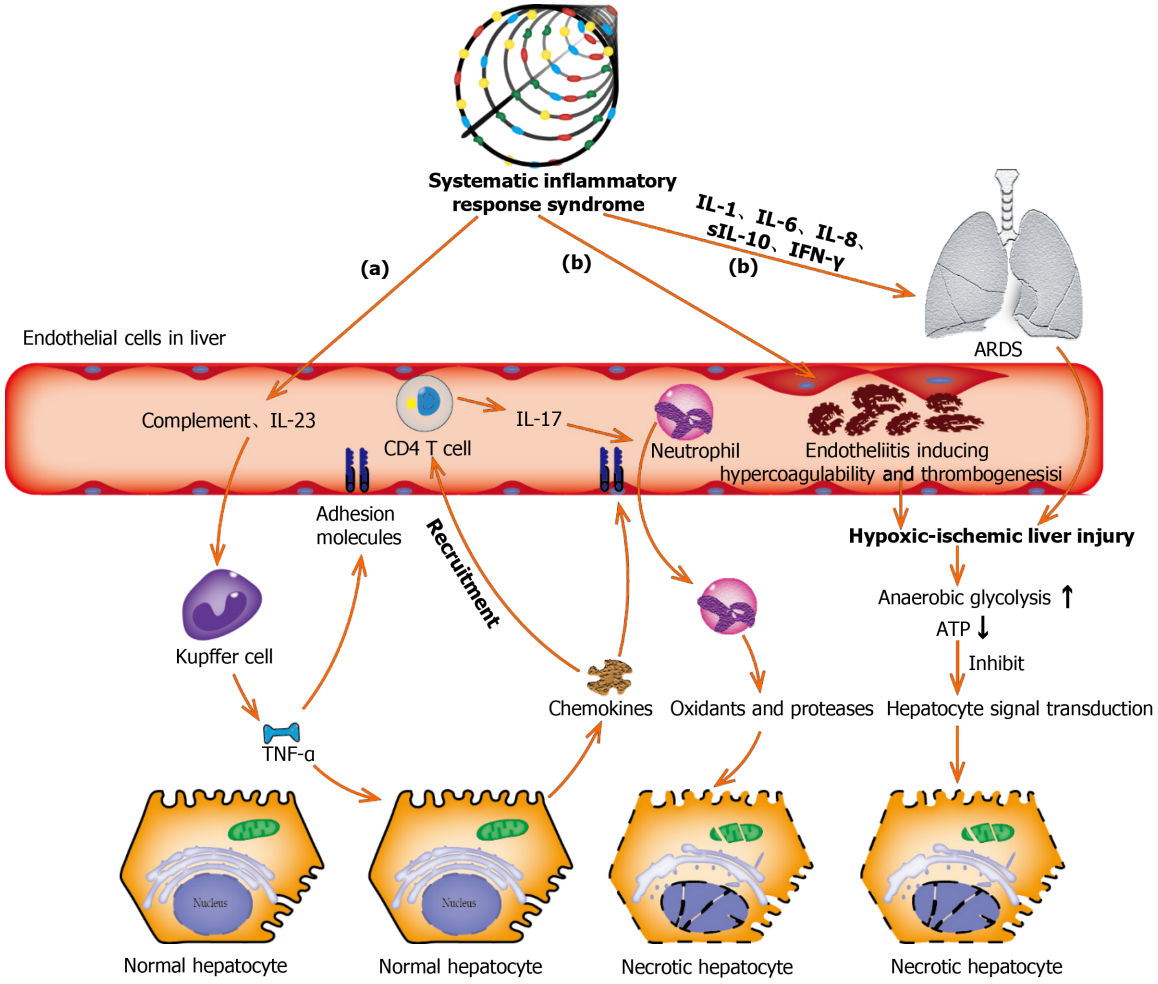


Figure 3 Underlying molecular mechanisms of coronavirus disease-19-associated liver injury caused by systematic inflammatory response syndrome and hypoxic ischemia. (a): Complement and interleukin-23 are released into the blood during the systemic inflammation, which subsequently activate Kupffer cells and induce their production of tumor necrosis factor α (TNF-α). As a pro-inflammatory cytokine, TNF-α aggravates the inflammation responses by up-regulating the expression of endothelial cell adhesion molecules and inducing hepatocytes to secrete chemokines. Under the induction of chemokines, CD4 T cells and neutrophils are rapidly recruited to the liver, in which CD4 T cells assist mucosal molecules to promote neutrophils into the liver parenchyma. Finally, neutrophils directly damage liver cells by releasing oxidants and proteases, leading to necrotic cell death; (b): Acute respiratory distress syndrome and endotheliitis are the two main causes leading to hypoxic-ischemic liver injury in the period of systematic inflammatory response syndrome. Increased anaerobic glycolysis leads to a decrease in ATP production, which ultimately leads to the death of hepatocytes by inhibiting hepatocyte signal transduction. ARDS: Acute respiratory distress syndrome; TNF-α: Tumor necrosis factor α; IL: Interleukin; IFN-γ: Interferon-γ.

reported that hepatic sinusoidal endothelial cell damage causes microcirculation disorders and further aggravates liver ischemia and hypoxia. Wang *et al*[9] observed different degrees of hypoxemia by blood gas analysis in more than 40% of patients with COVID-19. Patients receiving oxygen therapy have a faster recovery of liver function, and the average length of hospital stay is considerably shortened. In addition, lymphatic vessels are also reported to be involved in the pathological process of acute liver injury, prevent the occurrence of acute liver damage, and delay the progression of COVID-19[53]. Lymphatic vessels participate in the clearance of virus through absorption and transportation of inflammatory exudates, inflammatory cytokines, dead cell debris, and immune cells[54].

Drug-induced hepatic injury during treatment

DILI is defined as liver damage caused by the drug and/or its metabolites, or by hypersensitivity or reduced tolerance of the drug owing to special physique during the use of drugs[55]. Based on the available data, a variety of drugs widely used to treat COVID-19, such as antibiotics (macrolides and quinolones), antiviral drugs (ribavirin and lopinavir/ritonavir), and non-steroidal anti-inflammatory drugs, have been reported to cause liver damage[56]. A meta-analysis by Kulkarni *et al*[57] showed a pooled incidence of DILI of 25.4% in patients with confirmed SARS-CoV-2 infection. Interestingly, Fan *et al*[58] found that the proportion of patients with abnormal liver

function who received lopinavir/ritonavir after admission (57.8%) was significantly higher than that of patients with normal liver function (31.3%). Moreover, the average hospital stay of patients with abnormal liver function was significantly longer than that of patients with normal liver function (15.09 ± 4.79 d *vs* 12.76 ± 4.14 d, $P = 0.021$). Previous studies have indicated that patients with severe COVID-19 disease are prone to hepatic injury[4,5]. The reason for this might be that severe and critically ill patients require long-course and/or more metered administration of antiviral drugs, antibiotics, or other potentially hepatotoxic drugs during hospitalization. Similarly, Qin *et al*[37] reported that up to 70% of critically ill patients received systemic corticosteroid therapy. Furthermore, a case report from Italy suggested that tocilizumab, a drug used to reduce inflammation by blocking the IL-6 signal transduction pathway, played a beneficial role in the management of severe COVID-19 disease[59]. In the case of poor efficacy of azithromycin, hydroxychloroquine, and lopinavir, the administration of tocilizumab rapidly improved the clinical condition. However, mild to moderate elevations in transaminases have been observed in patients with COVID-19 treated with tocilizumab. A possible reason is that the use of immunosuppressive drugs, such as tocilizumab, tofacitinib, and dexamethasone, can reactivate hepatitis B virus (HBV) in patients with occult infection and induce liver damage[57]. However, randomized controlled clinical trials evaluating the safety of remdesvir and tocilizumab in the treatment of COVID-19 have not yet revealed any significant difference in the incidence of liver injury between the treatment and placebo groups [60,61].

The Novel Coronavirus Pneumonia Diagnosis and Treatment Scheme (Trial Version 8) issued by the National Health Commission of the People's Republic of China on August 19, 2020[62], provides a brief summary of the antiviral drugs under trial. Some drugs have been shown to have certain therapeutic effects in clinical observation studies, but no antiviral drugs have been determined to be effective in strict "randomized, double-blind, placebo-controlled studies." It is recommended that drugs with potential antiviral effects should be used early in the course of the disease and should be applied to patients with high-risk factors for severe illness and severe illness tendencies. It is not recommended to use lopinavir/ritonavir and ribavirin alone, as well as hydroxychloroquine, in combination with azithromycin. The trial of alpha-interferon, ribavirin (recommended to be used in combination with interferon or lopinavir/ritonavir), chloroquine phosphate, and arbidol can be continued, and their efficacy, adverse reactions, contraindications, and interactions with other drugs should be evaluated in further clinical applications. It is not recommended to use more than three antiviral drugs at the same time.

COVID-19-ASSOCIATED LIVER INJURY IN SPECIAL POPULATIONS

As of date, there are approximately 400 million patients with CLD in China, including those with chronic viral hepatitis, fatty liver, alcoholic liver disease, cirrhosis, or other liver diseases. Therefore, in patients with SARS-CoV-2 infection, the effect of pre-existing liver disease on the liver injury status cannot be ignored.

Previous studies have shown that patients having cirrhosis with relatively lower immunity are vulnerable to liver decompensation or acute chronic liver failure after influenza virus infection[63]. Notably, similar results were also reported in a large, multicenter, international cohort study; cirrhosis in patients with COVID-19 was closely associated with a poor model for end-stage liver disease score and decompensated events[64]. The authors indicated that mortality was strongly correlated with hepatic decompensation following SARS-CoV-2 infection; 63.2% of patients with new decompensated events died, whereas the proportion of death in those without new decompensation events was 26.2%. According to a study by Sarin *et al*[65], decompensating one-fifth of cirrhosis was observed, in which 57% of patients had progression of liver damage and the mortality rate was 43%. Notably, among patients with liver cirrhosis, the mortality rate of COVID-19 was significantly higher than that in patients hospitalized for bacterial infection[66]. However, in a contemporaneously enrolled study conducted in the United States[67], Bajaj *et al*[67] determined that the mortality rate in patients with cirrhosis + COVID-19 was similar to that in patients with cirrhosis alone (30% *vs* 20%, $P = 0.16$), but was higher than that in patients with COVID-19 alone (30% *vs* 13%, $P = 0.03$) after matching for age/sex. In this study, the Charlson Comorbidity Index, a prognostic comorbidity score, was identified as the only independent variable predictive of mortality in the entire matched cohort [odds ratio 1.23, 95% confidence interval (CI): 1.11-1.37; $P < 0.001$].

Thus, whether the mortality rate in patients with cirrhosis infected with SARS-CoV-2 is higher than in those infected with other viruses or bacteria is yet to be determined.

Based on previous studies[68], patients with chronic hepatitis B co-infected with severe acute respiratory syndrome (SARS) virus are more likely to develop severe hepatitis. A possible reason is that the SARS virus triggers HBV reactivation and massive replication, and chronic hepatitis B patients co-infected with SARS virus may require a longer time to fully clear the SARS virus from their bodies. Very recently, an observation from China[69] revealed that two patients with HBV infection had a slower clearance of SARS-CoV-2 (mean difference 10.6 d; 95%CI: 6.2-15.1 d). The mechanism may involve the dysfunction of T cells in HBV-infected patients, which causes the body's immune response to other viruses to weaken, but whether there is an exact connection between the two remains to be elucidated. However, Chen *et al*[70] reviewed the clinical characteristics of patients with SARS-CoV-2/HBV coinfection and found no significant difference in the hepatic function index between 20 patients with HBV infection (6.1%) and 306 patients without HBV infection (93.9%). Moreover, there is no evidence that SARS-CoV-2/HBV coinfection would reduce the discharge rate and lengthen the hospital stay. A similar phenomenon was observed in another study[71] showing that patients with COVID-19 co-infected with hepatitis B were not significantly associated with worse outcomes compared to those without hepatitis B.

Patients with non-alcoholic fatty liver disease (NAFLD) were previously reported to be more prone to liver damage when infected with SARS-CoV-2; many of these were cases of mild to moderate liver damage, and severe disease was rare[72,73]. Ji *et al*[73] showed that patients with pre-existing NAFLD infected with SARS-CoV-2 had a higher possibility of abnormal hepatic function during hospitalization, higher risk of disease progression, and longer duration of virus shedding compared to those without NAFLD. In this study, the pattern of liver injury was mainly hepatocellular rather than cholestatic, which was contrary to the existing finding that SARS-CoV-2 has a high affinity for the ACE2 receptor highly expressed in biliary tract cells. Metabolic diseases, such as obesity, hypertension, diabetes, and cardiovascular disease, are common in patients with NAFLD. Studies have shown that coexisting metabolic risk factors in patients with NAFLD are independent risk factors for severe COVID-19 disease, and the risk of severity increases with the number of metabolic risk factors present[74]. To further verify whether NAFLD itself affects liver function in patients with COVID-19, Hashemi *et al*[75] adjusted for potential confounding factors (age, sex, hypertension, diabetes, obesity, hyperlipidemia, heart diseases, and pulmonary disorders), and determined that NAFLD was still independently associated with ICU admission (49.3% *vs* 35.0%, $P = 0.028$) and mechanical ventilation (47.8% *vs* 30.3%, $P = 0.0055$), but was not associated with mortality. Patients with NAFLD who were infected with SARS-CoV-2 had a higher prevalence of elevated transaminases on admission. Coincidently, a very recent study[76] revealed a similar result that NAFLD is an independent predictor of liver injury in COVID-19, but not a predictor of death and disease severity (presentation or progression). However, the debate concerning whether NAFLD increases the risk of death in patients with COVID-19 continues.

Liver transplant recipients are also a special population affected by the global spread of COVID-19. Long-term immunosuppressive treatment may increase the risk of contracting respiratory viruses, especially in patients with preoperative organ decompensation and chronic disease[77]. A prospective cohort study of 111 cases[75] showed that liver transplant patients had an increased risk of contracting SARS-CoV-2 owing to chronic immunosuppression, but the mortality rate was lower than that in the matched general population. Mycophenolate, a baseline immunosuppressive drug, was identified as an independent predictor of severe COVID-19, but no such deterioration was observed with calcineurin inhibitors or everolimus. In a series of cases in Brazil[78], a negative effect of COVID-19 on liver transplantation was reported, especially in elderly patients with comorbidities. One case was a 69-year-old patient with severe cardiovascular disease who showed a rapid deterioration after being diagnosed with COVID-19, and another case was a patient with NAFLD complicated with kidney failure, who eventually died of a secondary bacterial infection. However, different opinions have been forwarded in other studies. A large international observational study conducted by Webb *et al*[79] indicated that liver transplantation did not significantly increase the proportion of ICU admission and the risk of death. Similarly, D'Antiga *et al*[80] also showed that patients with COVID-19 receiving liver transplantation were not at an increased risk of severe pulmonary disease, despite their immunosuppressed status. Moreover, three COVID-19-related deaths observed at an Italian transplant center were of patients undergoing long-term treatment with a minimal immunosuppressive regimen, rather than of fully immunosuppressed patients who recently received transplants[81].

TREATMENT STRATEGIES FOR LIVER INJURY IN PATIENTS WITH COVID-19

Although liver damage is a common complication of COVID-19, most cases of COVID-19 show mildly abnormal liver function, which is usually temporary and can return to normal without any special treatment[7]. According to the Chinese Pharmaceutical Association, the "Four-Anti and Two-Balance" strategy is recommended, which includes antivirus, anti-shock, anti-hyoxemia, anti-secondary infection therapy, and maintenance of water, electrolyte, acid–base, and microecological balance. Patients with COVID-19 exhibiting obvious liver damage could be treated with hepatoprotective, anti-jaundice, or anti-inflammatory drugs, such as polyene phosphatidylcholine, glycyrrhizic acid, ursodeoxycholic acid, and adenosylmethionine. For liver injury in critically ill patients infected with SARS-CoV-2, one or two kinds of drugs can be chosen to avoid drug abuse and aggravation of liver burden, and reduce drug interactions. A recent study by Hoever *et al*[82] revealed that glycyrrhizic acid derivatives, which are preferred anti-hepatitis drugs, may also have antiviral activity against SARS-CoV-2. Glycyrrhizic acid has a strong affinity for liver steroid metabolism enzymes, and hinders the inactivation of cortisol and aldosterone; it also shows obvious corticosteroid-like effects, such as anti-inflammatory, anti-allergic, and protective film structures, without obvious cortical hormone-like side effects.

Hu *et al*[34] retrospectively analyzed the clinical characteristics, susceptible population, and treatment strategies for patients with new coronavirus infection and showed that the mainstay to manage COVID-19-associated liver injury was to suppress inflammatory response, correct hypoxemia, and provide symptomatic support. Conservative oxygen therapy is preferred, and ventilator-associated pneumonia should be strictly supervised in patients receiving mechanical ventilation [83]. Xu *et al*[83] showed that an artificial liver blood purification system could improve the treatment effect in critically ill patients by rapidly removing inflammatory mediators, blocking cytokine storms, and favoring the water–electrolyte balance. Similar findings were reported by Liu *et al*[84] who showed a significant declining trend in the levels of cytokines and inflammatory factors (IL-6 and C-reactive protein) in patients with COVID-19 after a course of artificial liver blood purification. For patients with COVID-19 who are suspected of having liver damage caused by drugs, consideration should be given to stop or reduce the drug dose. Prevention is better than management. Monitoring the liver function and avoiding liver damage play key roles in the treatment of COVID-19-associated liver injury.

CONCLUSION

In this review, we summarize the latest advances in research on the clinical features, potential mechanisms, exacerbation of underlying hepatic dysfunction in patients with CLD, and treatment strategies for patients with COVID-19 as of January 2021. Patients with COVID-19 showing liver injury may experience darkening of the skin and hyperpigmentation. To ascertain the presence of liver injury, liver enzymes are the most commonly used markers. Liver biopsy is strongly recommended for those with unexplained acute liver failure. Apparent viral particles have been observed in hepatocytes. However, to date, studies involving liver biopsy in COVID-19 are still limited to case reports. The potential mechanisms of COVID-19-associated liver injury may include the direct effects of viral infection, inflammatory storm, hypoxemia, endotheliitis, and drugs. The S protein-ACE2 interactor may be the main tunnel for the entry of virus, the activity of which can be regulated by multiple factors, such as hypoxia, fibrosis/cirrhosis, and GM1. Among patients with CLD, NAFLD was indicated as an independent factor associated with ICU admission and mechanical ventilation after adjusting for comorbidities, such as hypertension, diabetes, and obesity. Patients with cirrhosis, co-infected with SRAS-CoV-2, experience high mortality, whereas whether hepatitis B and liver transplantation increase the severity of COVID-19 disease remains an open question. The "Four-Anti and Two-Balance" strategy, in which it is necessary to avoid drug abuse that aggravates liver burden and prevention is preferred to management, is recommended to manage COVID-19-associated liver injury.

REFERENCES

1 **World Health Organization**. Coronavirus disease (COVID-19) pandemic, 2020. [cited 10 January 2021]. Available from: https://www.who.int/health-topics/coronavirus#tab=tab_1

2 **Wu J**, Song S, Cao HC, Li LJ. Liver diseases in COVID-19: Etiology, treatment and prognosis. *World J Gastroenterol* 2020; **26**: 2286-2293 [PMID: 32476793 DOI: 10.3748/wjg.v26.i19.2286]

3 **Holshue ML**, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, Spitters C, Ericson K, Wilkerson S, Tural A, Diaz G, Cohn A, Fox L, Patel A, Gerber SI, Kim L, Tong S, Lu X, Lindstrom S, Pallansch MA, Weldon WC, Biggs HM, Uyeki TM, Pillai SK; Washington State 2019-nCoV Case Investigation Team. First Case of 2019 Novel Coronavirus in the United States. *N Engl J Med* 2020; **382**: 929-936 [PMID: 32004427 DOI: 10.1056/NEJMoa2001191]

4 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

5 **Shi H**, Han X, Jiang N, Cao Y, Alwalid O, Gu J, Fan Y, Zheng C. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. *Lancet Infect Dis* 2020; **20**: 425-434 [PMID: 32105637 DOI: 10.1016/S1473-3099(20)30086-4]

6 **Xu XW**, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, Li SB, Wang HY, Zhang S, Gao HN, Sheng JF, Cai HL, Qiu YQ, Li LJ. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-Cov-2) outside of Wuhan, China: retrospective case series. *BMJ* 2020; **368**: m606 [PMID: 32075786 DOI: 10.1136/bmj.m606]

7 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]

8 **Xu L**, Liu J, Lu M, Yang D, Zheng X. Liver injury during highly pathogenic human coronavirus infections. *Liver Int* 2020; **40**: 998-1004 [PMID: 32170806 DOI: 10.1111/liv.14435]

9 **Wang J**, Zhu L, Xue L, Liu L, Yan X, Huang S, Zhang B, Xu T, Li C, Ji F, Ming F, Zhao Y, Cheng J, Shao H, Chen K, Zhao XA, Sang D, Zhao H, Guan X, Chen X, Chen Y, Liu J, Huang R, Zhu C, Wu C. Risk factors of liver injury in patients with coronavirus disease 2019 in Jiangsu, China: A retrospective, multi-center study. *J Med Virol* 2020 [PMID: 33174624 DOI: 10.1002/jmv.26663]

10 **Chen N**, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020; **395**: 507-513 [PMID: 32007143 DOI: 10.1016/S0140-6736(20)30211-7]

11 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

12 **Wang D**, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 2020; **323**: 1061-1069 [PMID: 32031570 DOI: 10.1001/jama.2020.1585]

13 **Kaneko S**, Kurosaki M, Nagata K, Taki R, Ueda K, Hanada S, Takayama K, Suzaki S, Harada N, Sugiyama T, Nagasawa M, Izumi N. Liver injury with COVID-19 based on gastrointestinal symptoms and pneumonia severity. *PLoS One* 2020; **15**: e0241663 [PMID: 33147270 DOI: 10.1371/journal.pone.0241663]

14 **Burra P**. Liver abnormalities and endocrine diseases. *Best Pract Res Clin Gastroenterol* 2013; **27**: 553-563 [PMID: 24090942 DOI: 10.1016/j.bpg.2013.06.014]

15 **Videira IF**, Moura DF, Magina S. Mechanisms regulating melanogenesis. *An Bras Dermatol* 2013; **88**: 76-83 [PMID: 23539007 DOI: 10.1590/s0365-05962013000100009]

16 **Jee SH**, Lee SY, Chiu HC, Chang CC, Chen TJ. Effects of estrogen and estrogen receptor in normal human melanocytes. *Biochem Biophys Res Commun* 1994; **199**: 1407-1412 [PMID: 8147884 DOI: 10.1006/bbrc.1994.1387]

17 **Bradley BT**, Maioli H, Johnston R, Chaudhry I, Fink SL, Xu H, Najafian B, Deutsch G, Lacy JM, Williams T, Yarid N, Marshall DA. Histopathology and ultrastructural findings of fatal COVID-19 infections in Washington State: a case series. *Lancet* 2020; **396**: 320-332 [PMID: 32682491 DOI: 10.1016/S0140-6736(20)31305-2]

18 **Fiel MI**, El Jamal SM, Paniz-Mondolfi A, Gordon RE, Reidy J, Bandovic J, Advani R, Kilaru S, Pourmand K, Ward S, Thung SN, Schiano T. Findings of Hepatic Severe Acute Respiratory Syndrome Coronavirus-2 Infection. *Cell Mol Gastroenterol Hepatol* 2021; **11**: 763-770 [PMID: 32992052 DOI: 10.1016/j.jcmgh.2020.09.015]

19 **Melquist S**, Estepp K, Aleksandrovich Y, Lee A, Beiseker A, Hamedani FS, Bassett J. COVID-19 presenting as fulminant hepatic failure: A case report. *Medicine (Baltimore)* 2020; **99**: e22818 [PMID: 33120805 DOI: 10.1097/MD.0000000000022818]

20 **Cha MH**, Regueiro M, Sandhu DS. Gastrointestinal and hepatic manifestations of COVID-19: A comprehensive review. *World J Gastroenterol* 2020; **26**: 2323-2332 [PMID: 32476796 DOI: 10.3748/wjg.v26.i19.2323]

21 **Puelles VG**, Lütgehetmann M, Lindenmeyer MT, Sperhake JP, Wong MN, Allweiss L, Chilla S, Heinemann A, Wanner N, Liu S, Braun F, Lu S, Pfefferle S, Schröder AS, Edler C, Gross O, Glatzel M, Wichmann D, Wiech T, Kluge S, Pueschel K, Aepfelbacher M, Huber TB. Multiorgan and Renal Tropism of SARS-CoV-2. *N Engl J Med* 2020; **383**: 590-592 [PMID: 32402155 DOI: 10.1056/NEJMc2011400]

22 **Sonzogni A**, Previtali G, Seghezzi M, Grazia Alessio M, Gianatti A, Licini L, Morotti D, Zerbi P, Carsana L, Rossi R, Lauri E, Pellegrinelli A, Nebuloni M. Liver histopathology in severe COVID 19 respiratory failure is suggestive of vascular alterations. *Liver Int* 2020; **40**: 2110-2116 [PMID: 32654359 DOI: 10.1111/liv.14601]

23 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280. e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]

24 **Pirola CJ**, Sookoian S. SARS-CoV-2 virus and liver expression of host receptors: Putative mechanisms of liver involvement in COVID-19. *Liver Int* 2020; **40**: 2038-2040 [PMID: 32352224 DOI: 10.1111/liv.14500]

25 **Letko M**, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol* 2020; **5**: 562-569 [PMID: 32094589 DOI: 10.1038/s41564-020-0688-y]

26 **Wang Y**, Liu S, Liu H, Li W, Lin F, Jiang L, Li X, Xu P, Zhang L, Zhao L, Cao Y, Kang J, Yang J, Li L, Liu X, Li Y, Nie R, Mu J, Lu F, Zhao S, Lu J, Zhao J. SARS-CoV-2 infection of the liver directly contributes to hepatic impairment in patients with COVID-19. *J Hepatol* 2020; **73**: 807-816 [PMID: 32437830 DOI: 10.1016/j.jhep.2020.05.002]

27 **Guo Y**, Korteweg C, McNutt MA, Gu J. Pathogenetic mechanisms of severe acute respiratory syndrome. *Virus Res* 2008; **133**: 4-12 [PMID: 17825937 DOI: 10.1016/j.virusres.2007.01.022]

28 **Chau TN**, Lee KC, Yao H, Tsang TY, Chow TC, Yeung YC, Choi KW, Tso YK, Lau T, Lai ST, Lai CL. SARS-associated viral hepatitis caused by a novel coronavirus: report of three cases. *Hepatology* 2004; **39**: 302-310 [PMID: 14767982 DOI: 10.1002/hep.20111]

29 **Tan YJ**, Fielding BC, Goh PY, Shen S, Tan TH, Lim SG, Hong W. Overexpression of 7a, a protein specifically encoded by the severe acute respiratory syndrome coronavirus, induces apoptosis *via* a caspase-dependent pathway. *J Virol* 2004; **78**: 14043-14047 [PMID: 15564512 DOI: 10.1128/JVI.78.24.14043-14047.2004]

30 **Paizis G**, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, Shaw T, Warner FJ, Zuilli A, Burrell LM, Angus PW. Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. *Gut* 2005; **54**: 1790-1796 [PMID: 16166274 DOI: 10.1136/gut.2004.062398]

31 **Huang Q**, Xie Q, Shi CC, Xiang XG, Lin LY, Gong BD, Zhao GD, Wang H, Jia NN. Expression of angiotensin-converting enzyme 2 in CCL4-induced rat liver fibrosis. *Int J Mol Med* 2009; **23**: 717-723 [PMID: 19424597 DOI: 10.3892/ijmm_00000185]

32 **Mohammed FF**, Khokha R. Thinking outside the cell: proteases regulate hepatocyte division. *Trends Cell Biol* 2005; **15**: 555-563 [PMID: 16150595 DOI: 10.1016/j.tcb.2005.08.009]

33 **Fantini J**, Di Scala C, Chahinian H, Yahi N. Structural and molecular modelling studies reveal a new mechanism of action of chloroquine and hydroxychloroquine against SARS-CoV-2 infection. *Int J Antimicrob Agents* 2020; **55**: 105960 [PMID: 32251731 DOI: 10.1016/j.ijantimicag.2020.105960]

34 **Hu LL**, Wang WJ, Zhu QJ, Yang L. [Novel coronavirus pneumonia-related liver injury: etiological analysis and treatment strategy]. *Zhonghua Gan Zang Bing Za Zhi* 2020; **28**: 97-99 [PMID: 32075364 DOI: 10.3760/cma.j.issn.1007-3418.2020.02.001]

35 **Cao X**. COVID-19: immunopathology and its implications for therapy. *Nat Rev Immunol* 2020; **20**: 269-270 [PMID: 32273594 DOI: 10.1038/s41577-020-0308-3]

36 **Maggi E**, Canonica GW, Moretta L. COVID-19: Unanswered questions on immune response and pathogenesis. *J Allergy Clin Immunol* 2020; **146**: 18-22 [PMID: 32389590 DOI: 10.1016/j.jaci.2020.05.001]

37 **Qin C**, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, Xie C, Ma K, Shang K, Wang W, Tian DS. Dysregulation of Immune Response in Patients With Coronavirus 2019 (COVID-19) in Wuhan, China. *Clin Infect Dis* 2020; **71**: 762-768 [PMID: 32161940 DOI: 10.1093/cid/ciaa248]

38 **Xu Z**, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med* 2020; **8**: 420-422 [PMID: 32085846 DOI: 10.1016/S2213-2600(20)30076-X]

39 **Zhan K**, Liao S, Li J, Bai Y, Lv L, Yu K, Qiu L, Li C, Yuan G, Zhang A, Mei Z. Risk factors in patients with COVID-19 developing severe liver injury during hospitalisation. *Gut* 2021; **70**: 628-629 [PMID: 32571973 DOI: 10.1136/gutjnl-2020-321913]

40 **Zhang H**, Liao YS, Gong J, Liu J, Zhang H. Clinical characteristics and risk factors for liver injury in COVID-19 patients in Wuhan. *World J Gastroenterol* 2020; **26**: 4694-4702 [PMID: 32884226 DOI: 10.3748/wjg.v26.i31.4694]

41 **Phipps MM**, Barraza LH, LaSota ED, Sobieszczyk ME, Pereira MR, Zheng EX, Fox AN, Zucker J, Verna EC. Acute Liver Injury in COVID-19: Prevalence and Association with Clinical Outcomes in a Large U.S. Cohort. *Hepatology* 2020; **72**: 807-817 [PMID: 32473607 DOI: 10.1002/hep.31404]

42 **Varga Z**, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, Mehra MR,

Schuepbach RA, Ruschitzka F, Moch H. Endothelial cell infection and endotheliitis in COVID-19. *Lancet* 2020; **395**: 1417-1418 [PMID: 32325026 DOI: 10.1016/S0140-6736(20)30937-5]

43 **Zhao JM**, Zhou GD, Sun YL, Wang SS, Yang JF, Meng EH, Pan D, Li WS, Zhou XS, Wang YD, Lu JY, Li N, Wang DW, Zhou BC, Zhang TH. [Clinical pathology and pathogenesis of severe acute respiratory syndrome]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 2003; **17**: 217-221 [PMID: 15340561]

44 **Antoniak S**, Mackman N. Multiple roles of the coagulation protease cascade during virus infection. *Blood* 2014; **123**: 2605-2613 [PMID: 24632711 DOI: 10.1182/blood-2013-09-526277]

45 **Wang Q**, Zhang Y, Wu L, Niu S, Song C, Zhang Z, Lu G, Qiao C, Hu Y, Yuen KY, Wang Q, Zhou H, Yan J, Qi J. Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. *Cell* 2020; **181**: 894-904. e9 [PMID: 32275855 DOI: 10.1016/j.cell.2020.03.045]

46 **Krüger-Genge A**, Blocki A, Franke RP, Jung F. Vascular Endothelial Cell Biology: An Update. *Int J Mol Sci* 2019; **20** [PMID: 31500313 DOI: 10.3390/ijms20184411]

47 **Spiezia L**, Boscolo A, Poletto F, Cerruti L, Tiberio I, Campello E, Navalesi P, Simioni P. COVID-19-Related Severe Hypercoagulability in Patients Admitted to Intensive Care Unit for Acute Respiratory Failure. *Thromb Haemost* 2020; **120**: 998-1000 [PMID: 32316063 DOI: 10.1055/s-0040-1710018]

48 **Tee A**, Wong A, Yusuf GT, Rao D, Sidhu PS. Contrast-enhanced ultrasound (CEUS) of the lung reveals multiple areas of microthrombi in a COVID-19 patient. *Intensive Care Med* 2020; **46**: 1660-1662 [PMID: 32424480 DOI: 10.1007/s00134-020-06085-4]

49 **Léonard-Lorant I**, Delabranche X, Séverac F, Helms J, Pauzet C, Collange O, Schneider F, Labani A, Bilbault P, Molière S, Leyendecker P, Roy C, Ohana M. Acute Pulmonary Embolism in Patients with COVID-19 at CT Angiography and Relationship to d-Dimer Levels. *Radiology* 2020; **296**: E189-E191 [PMID: 32324102 DOI: 10.1148/radiol.2020201561]

50 **Lim W**, Meade M, Lauzier F, Zarychanski R, Mehta S, Lamontagne F, Dodek P, McIntyre L, Hall R, Heels-Ansdell D, Fowler R, Pai M, Guyatt G, Crowther MA, Warkentin TE, Devereaux PJ, Walter SD, Muscedere J, Herridge M, Turgeon AF, Geerts W, Finfer S, Jacka M, Berwanger O, Ostermann M, Qushmaq I, Friedrich JO, Cook DJ; PROphylaxis for ThromboEmbolism in Critical Care Trial Investigators. Failure of anticoagulant thromboprophylaxis: risk factors in medical-surgical critically ill patients*. *Crit Care Med* 2015; **43**: 401-410 [PMID: 25474533 DOI: 10.1097/CCM.0000000000000713]

51 **Corrigan D**, Prucnal C, Kabrhel C. Pulmonary embolism: the diagnosis, risk-stratification, treatment and disposition of emergency department patients. *Clin Exp Emerg Med* 2016; **3**: 117-125 [PMID: 27752629 DOI: 10.15441/ceem.16.146]

52 **Antoniak S**. The coagulation system in host defense. *Res Pract Thromb Haemost* 2018; **2**: 549-557 [PMID: 30046760 DOI: 10.1002/rth2.12109]

53 **Witte MH**, Daley SK. SARS-CoV-2/COVID-19, Lymphatic vessels, lymph, and lymphology. *Lymphology* 2020; **53**: 97-98 [PMID: 33350283]

54 **Sprent J**, Tough DF. T cell death and memory. *Science* 2001; **293**: 245-248 [PMID: 11452113 DOI: 10.1126/science.1062416]

55 **Feng G**, Zheng KI, Yan QQ, Rios RS, Targher G, Byrne CD, Poucke SV, Liu WY, Zheng MH. COVID-19 and Liver Dysfunction: Current Insights and Emergent Therapeutic Strategies. *J Clin Transl Hepatol* 2020; **8**: 18-24 [PMID: 32274342 DOI: 10.14218/JCTH.2020.00018]

56 **Yang Z**, Xu M, Yi JQ, Jia WD. Clinical characteristics and mechanism of liver damage in patients with severe acute respiratory syndrome. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 60-63 [PMID: 15730921]

57 **Kulkarni AV**, Kumar P, Tevethia HV, Premkumar M, Arab JP, Candia R, Talukdar R, Sharma M, Qi X, Rao PN, Reddy DN. Systematic review with meta-analysis: liver manifestations and outcomes in COVID-19. *Aliment Pharmacol Ther* 2020; **52**: 584-599 [PMID: 32638436 DOI: 10.1111/apt.15916]

58 **Fan Z**, Chen L, Li J, Cheng X, Yang J, Tian C, Zhang Y, Huang S, Liu Z, Cheng J. Clinical Features of COVID-19-Related Liver Functional Abnormality. *Clin Gastroenterol Hepatol* 2020; **18**: 1561-1566 [PMID: 32283325 DOI: 10.1016/j.cgh.2020.04.002]

59 **Serviddio G**, Villani R, Stallone G, Scioscia G, Foschino-Barbaro MP, Lacedonia D. Tocilizumab and liver injury in patients with COVID-19. *Therap Adv Gastroenterol* 2020; **13**: 1756284820959183 [PMID: 33101458 DOI: 10.1177/1756284820959183]

60 **Beigel JH**, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, Hohmann E, Chu HY, Luetkemeyer A, Kline S, Lopez de Castilla D, Finberg RW, Dierberg K, Tapson V, Hsieh L, Patterson TF, Paredes R, Sweeney DA, Short WR, Touloumi G, Lye DC, Ohmagari N, Oh MD, Ruiz-Palacios GM, Benfield T, Fätkenheuer G, Kortepeter MG, Atmar RL, Creech CB, Lundgren J, Babiker AG, Pett S, Neaton JD, Burgess TH, Bonnett T, Green M, Makowski M, Osinusi A, Nayak S, Lane HC; ACTT-1 Study Group Members. Remdesivir for the Treatment of Covid-19 - Final Report. *N Engl J Med* 2020; **383**: 1813-1826 [PMID: 32445440 DOI: 10.1056/NEJMoa2007764]

61 **Stone JH**, Frigault MJ, Serling-Boyd NJ, Fernandes AD, Harvey L, Foulkes AS, Horick NK, Healy BC, Shah R, Bensaci AM, Woolley AE, Nikiforow S, Lin N, Sagar M, Schrager H, Huckins DS, Axelrod M, Pincus MD, Fleisher J, Sacks CA, Dougan M, North CM, Halvorsen YD, Thurber TK, Dagher Z, Scherer A, Wallwork RS, Kim AY, Schoenfeld S, Sen P, Neilan TG, Perugino CA, Unizony SH, Collier DS, Matza MA, Yinh JM, Bowman KA, Meyerowitz E, Zafar A, Drobni ZD, Bolster MB, Kohler M, D'Silva KM, Dau J, Lockwood MM, Cubbison C, Weber BN, Mansour MK; BACC Bay Tocilizumab Trial Investigators. Efficacy of Tocilizumab in Patients Hospitalized with Covid-19. *N Engl J Med* 2020; **383**: 2333-2344 [PMID: 33085857 DOI: 10.1056/NEJMoa2028836]

62 **Medical Administration Board**. China National Health Commission (2020) Diagnosis and treatment of 2019-nCoV pneumonia in China (version 8) (in Chinese). [cited 10 January 2021]. Available from: http://www.nhc.gov.cn/yzygj/s7653p/202008/0a7bdf12bd4b46e5bd28ca7f9a7f5e5a.shtml. Accessed 19 August 2020

63 **Schütte A**, Ciesek S, Wedemeyer H, Lange CM. Influenza virus infection as precipitating event of acute-on-chronic liver failure. *J Hepatol* 2019; **70**: 797-799 [PMID: 30635243 DOI: 10.1016/j.jhep.2018.11.015]

64 **Moon AM**, Webb GJ, Aloman C, Armstrong MJ, Cargill T, Dhanasekaran R, Genescà J, Gill US, James TW, Jones PD, Marshall A, Mells G, Perumalswami PV, Qi X, Su F, Ufere NN, Barnes E, Barritt AS, Marjot T. High mortality rates for SARS-CoV-2 infection in patients with pre-existing chronic liver disease and cirrhosis: Preliminary results from an international registry. *J Hepatol* 2020; **73**: 705-708 [PMID: 32446714 DOI: 10.1016/j.jhep.2020.05.013]

65 **Sarin SK**, Choudhury A, Lau GK, Zheng MH, Ji D, Abd-Elsalam S, Hwang J, Qi X, Cua IH, Suh JI, Park JG, Putcharoen O, Kaewdech A, Piratvisuth T, Treeprasertsuk S, Park S, Wejnaruemarn S, Payawal DA, Baatarkhuu O, Ahn SH, Yeo CD, Alonzo UR, Chinbayar T, Loho IM, Yokosuka O, Jafri W, Tan S, Soo LI, Tanwandee T, Gani R, Anand L, Esmail ES, Khalaf M, Alam S, Lin CY, Chuang WL, Soin AS, Garg HK, Kalista K, Batsukh B, Purnomo HD, Dara VP, Rathi P, Al Mahtab M, Shukla A, Sharma MK, Omata M; APASL COVID Task Force, APASL COVID Liver Injury Spectrum Study (APCOLIS Study-NCT 04345640). Pre-existing liver disease is associated with poor outcome in patients with SARS CoV2 infection; The APCOLIS Study (APASL COVID-19 Liver Injury Spectrum Study). *Hepatol Int* 2020; **14**: 690-700 [PMID: 32623632 DOI: 10.1007/s12072-020-10072-8]

66 **Iavarone M**, D'Ambrosio R, Soria A, Triolo M, Pugliese N, Del Poggio P, Perricone G, Massironi S, Spinetti A, Buscarini E, Viganò M, Carriero C, Fagiuoli S, Aghemo A, Belli LS, Lucà M, Pedaci M, Rimondi A, Rumi MG, Invernizzi P, Bonfanti P, Lampertico P. High rates of 30-day mortality in patients with cirrhosis and COVID-19. *J Hepatol* 2020; **73**: 1063-1071 [PMID: 32526252 DOI: 10.1016/j.jhep.2020.06.001]

67 **Bajaj JS**, Garcia-Tsao G, Biggins SW, Kamath PS, Wong F, McGeorge S, Shaw J, Pearson M, Chew M, Fagan A, de la Rosa Rodriguez R, Worthington J, Olofson A, Weir V, Trisolini C, Dwyer S, Reddy KR. Comparison of mortality risk in patients with cirrhosis and COVID-19 compared with patients with cirrhosis alone and COVID-19 alone: multicentre matched cohort. *Gut* 2021; **70**: 531-536 [PMID: 32660964 DOI: 10.1136/gutjnl-2020-322118]

68 **Peiris JS**, Chu CM, Cheng VC, Chan KS, Hung IF, Poon LL, Law KI, Tang BS, Hon TY, Chan CS, Chan KH, Ng JS, Zheng BJ, Ng WL, Lai RW, Guan Y, Yuen KY; HKU/UCH SARS Study Group. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* 2003; **361**: 1767-1772 [PMID: 12781535 DOI: 10.1016/s0140-6736(03)13412-5]

69 **Zha L**, Li S, Pan L, Tefsen B, Li Y, French N, Chen L, Yang G, Villanueva EV. Corticosteroid treatment of patients with coronavirus disease 2019 (COVID-19). *Med J Aust* 2020; **212**: 416-420 [PMID: 32266987 DOI: 10.5694/mja2.50577]

70 **Chen L**, Huang S, Yang J, Cheng X, Shang Z, Lu H, Cheng J. Clinical characteristics in patients with SARS-CoV-2/HBV co-infection. *J Viral Hepat* 2020; **27**: 1504-1507 [PMID: 32668494 DOI: 10.1111/jvh.13362]

71 **Zou X**, Fang M, Li S, Wu L, Gao B, Gao H, Ran X, Bian Y, Li R, ShanshanYu, Ling J, Li D, Tian D, Huang J. Characteristics of Liver Function in Patients With SARS-CoV-2 and Chronic HBV Coinfection. *Clin Gastroenterol Hepatol* 2021; **19**: 597-603 [PMID: 32553907 DOI: 10.1016/j.cgh.2020.06.017]

72 **Huang R**, Zhu L, Wang J, Xue L, Liu L, Yan X, Huang S, Li Y, Zhang B, Xu T, Li C, Ji F, Ming F, Zhao Y, Cheng J, Wang Y, Zhao H, Hong S, Chen K, Zhao XA, Zou L, Sang D, Shao H, Guan X, Chen X, Chen Y, Wei J, Zhu C, Wu C. Clinical features of COVID-19 patients with non-alcoholic fatty liver disease. *Hepatol Commun* 2020 [PMID: 32838108 DOI: 10.1002/hep4.1592]

73 **Ji D**, Qin E, Xu J, Zhang D, Cheng G, Wang Y, Lau G. Non-alcoholic fatty liver diseases in patients with COVID-19: A retrospective study. *J Hepatol* 2020; **73**: 451-453 [PMID: 32278005 DOI: 10.1016/j.jhep.2020.03.044]

74 **Zhou YJ**, Zheng KI, Wang XB, Sun QF, Pan KH, Wang TY, Ma HL, Chen YP, George J, Zheng MH. Metabolic-associated fatty liver disease is associated with severity of COVID-19. *Liver Int* 2020; **40**: 2160-2163 [PMID: 32573883 DOI: 10.1111/liv.14575]

75 **Hashemi N**, Viveiros K, Redd WD, Zhou JC, McCarty TR, Bazarbashi AN, Hathorn KE, Wong D, Njie C, Shen L, Chan WW. Impact of chronic liver disease on outcomes of hospitalized patients with COVID-19: A multicentre United States experience. *Liver Int* 2020; **40**: 2515-2521 [PMID: 32585065 DOI: 10.1111/liv.14583]

76 **Mushtaq K**, Khan MU, Iqbal F, Alsoub DH, Chaudhry HS, Ata F, Iqbal P, Elfert K, Balaraju G, Almaslamani M, Al-Ejji K, AlKaabi S, Kamel YM. NAFLD is a predictor of liver injury in COVID-19 hospitalized patients but not of mortality, disease severity on the presentation or progression - The debate continues. *J Hepatol* 2021; **74**: 482-484 [PMID: 33223215 DOI: 10.1016/j.jhep.2020.09.006]

77 **Sessa A**, Mazzola A, Lim C, Atif M, Pappatella J, Pourcher V, Scatton O, Conti F. COVID-19 in a liver transplant recipient: Could iatrogenic immunosuppression have prevented severe pneumonia? *World J Gastroenterol* 2020; **26**: 7076-7084 [PMID: 33311951 DOI: 10.3748/wjg.v26.i44.7076]

78 **Colmenero J**, Rodríguez-Perálvarez M, Salcedo M, Arias-Milla A, Muñoz-Serrano A, Graus J, Nuño J, Gastaca M, Bustamante-Schneider J, Cachero A, Lladó L, Caballero A, Fernández-Yunquera A, Loinaz C, Fernández I, Fondevila C, Navasa M, Iñarrairaegui M, Castells L, Pascual S, Ramírez P, Vinaixa C, González-Dieguez ML, González-Grande R, Hierro L, Nogueras F, Otero A, Álamo JM, Blanco-Fernández G, Fábrega E, García-Pajares F, Montero JL, Tomé S, De la Rosa G, Pons JA. Epidemiological pattern, incidence, and outcomes of COVID-19 in liver transplant patients. *J Hepatol* 2021; **74**: 148-155 [PMID: 32750442 DOI: 10.1016/j.jhep.2020.07.040]

79 **Webb GJ**, Marjot T, Cook JA, Aloman C, Armstrong MJ, Brenner EJ, Catana MA, Cargill T, Dhanasekaran R, García-Juárez I, Hagström H, Kennedy JM, Marshall A, Masson S, Mercer CJ, Perumalswami PV, Ruiz I, Thaker S, Ufere NN, Barnes E, Barritt AS 4th, Moon AM. Outcomes following SARS-CoV-2 infection in liver transplant recipients: an international registry study. *Lancet Gastroenterol Hepatol* 2020; **5**: 1008-1016 [PMID: 32866433 DOI: 10.1016/S2468-1253(20)30271-5]

80 **D'Antiga L**. Coronaviruses and Immunosuppressed Patients: The Facts During the Third Epidemic. *Liver Transpl* 2020; **26**: 832-834 [PMID: 32196933 DOI: 10.1002/lt.25756]

81 **Bhoori S**, Rossi RE, Citterio D, Mazzaferro V. COVID-19 in long-term liver transplant patients: preliminary experience from an Italian transplant centre in Lombardy. *Lancet Gastroenterol Hepatol* 2020; **5**: 532-533 [PMID: 32278366 DOI: 10.1016/S2468-1253(20)30116-3]

82 **Hoever G**, Baltina L, Michaelis M, Kondratenko R, Tolstikov GA, Doerr HW, Cinatl J Jr. Antiviral activity of glycyrrhizic acid derivatives against SARS-coronavirus. *J Med Chem* 2005; **48**: 1256-1259 [PMID: 15715493 DOI: 10.1021/jm0493008]

83 **Xu K**, Cai H, Shen Y, Ni Q, Chen Y, Hu S, Li J, Wang H, Yu L, Huang H, Qiu Y, Wei G, Fang Q, Zhou J, Sheng J, Liang T, Li L. [Management of COVID-19: the Zhejiang experience]. *Zhejiang Da Xue Xue Bao Yi Xue Ban* 2020; **49**: 147-157 [PMID: 32391658 DOI: 10.3785/j.issn.1008-9292.2020.02.02]

84 **Liu J**, Dong YQ, Yin J, He G, Wu X, Li J, Qiu Y, He X. Critically ill patients with COVID-19 with ECMO and artificial liver plasma exchange: A retrospective study. *Medicine (Baltimore)* 2020; **99**: e21012 [PMID: 32590819 DOI: 10.1097/MD.0000000000021012]

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MINIREVIEWS

Role of imaging in evaluating the response after neoadjuvant treatment for pancreatic ductal adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy. Despite the development of multimodality treatments, including surgical resection, radiotherapy, and chemotherapy, the long-term prognosis of patients with PDAC remains poor. Recently, the introduction of neoadjuvant treatment (NAT) has made more patients amenable to surgery, increasing the possibility of R0 resection, treatment of occult micro-metastasis, and prolongation of overall survival. Imaging plays a vital role in tumor response evaluation after NAT. However, conventional imaging modalities such as multidetector computed tomography have limited roles in the assessment of tumor resectability after NAT for PDAC because of the similar appearance of tissue fibrosis and tumor infiltration. Perfusion computed tomography, using blood perfusion as a biomarker, provides added value in predicting the histopathologic response of PDAC to NAT by reflecting the changes in tumor matrix and fibrosis content. Other imaging technologies, including diffusion-weighted imaging of magnetic resonance imaging and positron emission tomography, can reveal the tumor response by monitoring the structural changes in tumor cells and functional metabolic changes in tumors after NAT. In addition, with the renewed interest in data acquisition and analysis, texture analysis and radiomics have shown potential for the early evaluation of the response to NAT, thus improving patient stratification to achieve accurate and intensive treatment. In this review, we briefly introduce the application and value of NAT in resectable and unresectable PDAC. We also summarize the role of imaging in evaluating the response to NAT for PDAC, as well as the advantages, limitations, and future development directions of current imaging techniques.

Key Words: Pancreatic ductal adenocarcinoma; Neoadjuvant treatment; Imaging; Resectability; Tumor response; Prognosis

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Core Tip: The timely and accurate evaluation of tumor response in patients with pancreatic ductal adenocarcinoma (PDAC) after neoadjuvant therapy (NAT) is of great significance to increase the probability of tumor R0 resection and prolong survival. Ultrasound and conventional computed tomography imaging features show limited roles in the evaluation of NAT response for PDAC. Novel imaging biomarkers extracted from functional imaging technologies show promise in providing further important information for the assessment of tumor resectability and survival prediction. We reviewed the application and value of NAT in PDAC, as well as the advantages, limitations, and future development directions of current imaging techniques in tumor response assessment of PDAC after NAT in this article.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) has become a major public health issue globally[1,2]. Due to the late onset of symptoms, the high invasive potential of the disease, and the lack of accurate diagnostic markers to detect micro-metastasis, the prognosis of PDAC has not significantly improved over the past several decades[3].

Neoadjuvant treatment (NAT) is an emerging therapy for PDAC. It provides the theoretical advantages of downstaging borderline resectable or locally advanced pancreatic tumors, allowing more patients to benefit from surgery, increasing the possibility of R0 resection, treating occult micro-metastasis, and prolonging overall survival (OS)[4,5].

With the advancements in imaging technology and the renewed interest in data acquisition and analysis, there are increasingly more studies reporting the accuracy of imaging for evaluating the response to NAT for PDAC. This review focuses on the role of imaging in the response assessment to NAT for PDAC, as well as the advantages, limitations, and future directions of current imaging techniques.

CLASSIFICATION AND TREATMENT STRATEGY OF PDAC

Various guidelines recommend the use of multiphase contrast-enhanced computed tomography (CT) imaging for staging PDAC and determining tumor resectability[6]. According to the National Comprehensive Cancer Network guidelines, PDAC can be divided into four categories based on the preliminary assessment of tumor location and metastasis as follows[7]: (1) Resectable. The possibility of R0 resection is very high. The tumor has no contact with the adjacent arteries [superior mesenteric artery, hepatic artery, or celiac artery (CA)] and veins [superior mesenteric vein (SMV), portal vein (PV), or their confluence (SMV/PV)], or the degree of contact is less than 180° the circumference of the vessel wall; (2) Borderline resectable. The possibility of incomplete resection of R1 or R2 is high. The degree of contact between the tumor and arteries (superior mesenteric artery or CA) is less than 180°, the tumor invades a short segment of hepatic artery that can be resected or reconstructed, but does not involve the CA, or tumor contact with the adjoining vein (SMV-PV) exceeds the circumference of the vessel wall by 180°; (3) Locally advanced. The tumor cannot be resected due to invasion of the adjacent structures; and (4) Metastasis. The tumor has distant metastasis. As seen from this classification, the tumor–vessel contact is critical to determining whether the tumor is resectable. At the same time, the realization of R0 resection (marginal negative resection) is key to prolonging the life of patients with PDAC. Surgical resection is recommended for resectable PDAC, and the 5-year survival rate of R0 resection is 18%-24%[8]. However, the 5-year survival rate of borderline and locally advanced PDAC is poor, about 8%-11%[9,10]. In addition, almost 50% of patients relapse within a short period of time after tumor resection[11].

Other therapies such as chemotherapy and radiotherapy are usually used in unresectable cases to reduce the disease burden and prolong the survival time. However, even then, the prognosis remains poor with a median survival time of 6.8-11.1 mo in patients with unresectable tumor[12,13]. Hence, to achieve better survival, downstaging of the disease by NAT followed by surgical resection if possible has been advocated for unresectable PDAC.

NAT FOR RESECTABLE PDAC

In the past, the standard treatment for resectable PDAC was surgical resection followed by adjuvant chemotherapy. In 2008, a large retrospective study included surgically resectable PDAC and studied the impact of NAT on the prognosis of resectable PDAC[14]. The results showed that NAT followed by surgery had a survival advantage over upfront surgery with or without adjuvant treatment, which suggested the possible role of NAT in treating resectable PDAC. A recent meta-analysis[15] showed that the median OS of patients receiving NAT was longer than that with surgery (18.8 mo *vs* 14.8 mo). Although the overall resection rate of NAT was lower than that of previous surgery (66% *vs* 81.3%), the R0 rate was higher (86.8% *vs* 66.9%). In addition, other studies have reported that the tumor resection rate after NAT is between 50% and 90%, with a median survival time of 23.5 mo[16-19].

NAT FOR BORDERLINE AND UNRESECTABLE PDAC

Accumulating evidence indicates that patients with borderline PDAC can benefit from NAT, as it increases the chance of R0 resection, improves survival, and identifies cases of PDAC with rapid progression and poor response to treatment[20-22]. An international consensus has proposed that patients with borderline PDAC undergo surgical resection after NAT when there are no anatomical contraindications or metastatic disease[23]. A multicenter trial showed that neoadjuvant S-1 therapy in combination with radiotherapy followed by surgery achieved an R0 resection rate of 63%[24]. Inoue *et al*[25] reported that NAT with gemcitabine and nab-paclitaxel improved downstaging of the tumor and allowed patient selection. Anger *et al*[26] investigated the role of NAT in different types of borderline PDAC according to international consensus criteria. Their results showed that PDAC patients with biological (borderline resectable-B) have a relatively poor prognosis and should be considered for multimodal NAT.

In unresectable PDAC, palliative systemic chemotherapy is commonly used. In these patients, the main purpose is to extend the survival time under acceptable general conditions. FOLFIRINOX and gemcitabine in combination with other drugs are the preferred regimens for patients with good performance status, whereas capecitabine, gemcitabine, and 5-fluorouracil monotherapy are usually given to patients in poor general condition[12,27-29].

In summary, NAT for resectable PDAC performed before primary surgery increases the R0 resection rate and prolongs postoperative survival. In borderline and unresectable PDAC, NAT increases the chance of R0 resection, providing strong evidence for individualized patient treatment, eventually resulting in extending survival (Figure 1). However, there are some limitations in the current research. First, there is a lack of consensus on the best regimen for NAT. Second, some studies have not strictly separated resectable PDAC from unresectable PDAC during NAT. Finally, there is no clear consensus on the duration of NAT.

RESPONSE ASSESSMENT OF PDAC AFTER NAT

Conventional ultrasound

Conventional ultrasound (US) is an economical and radiation-free investigation. It helps to visualize pancreatic masses that are iso-attenuating on non-contrast CT images. However, its role in assessment of tumor response is very limited. US can be, however, a useful tool for detecting abdominal complications and drug toxicity during NAT in metastatic pancreatic cancer. The most common adverse effects of NAT regimens for metastatic pancreatic cancer are neutropenic colitis and venous thrombosis, which can be readily detected by abdominal US[30].

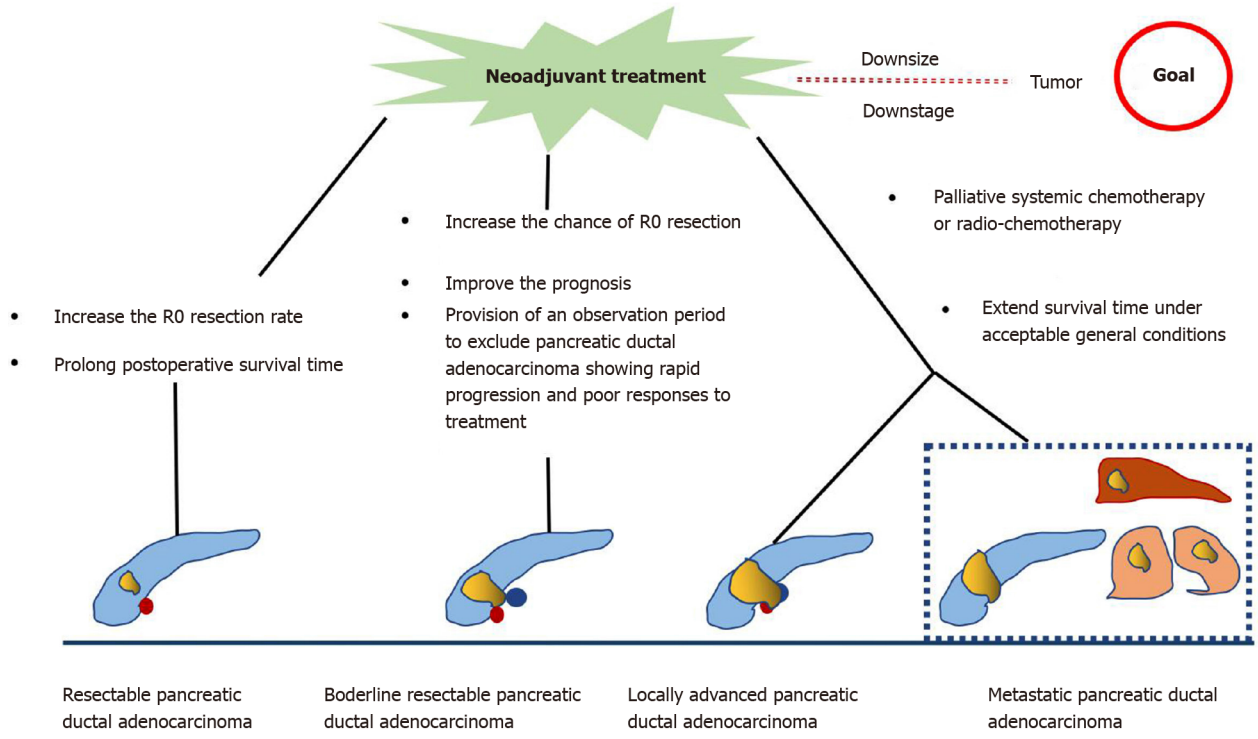


Figure 1 Role of neoadjuvant treatment for different types of pancreatic ductal adenocarcinoma.

Endoscopic ultrasound

Endoscopic US (EUS) has developed from a strictly imaging modality to one that allows for tissue diagnosis through fine needle aspiration. It has been proved to be a valuable means for early detection and staging of PDAC, especially for lesions ≤ 3 cm, which is superior to multi-detector CT[31,32]. Recently, the role of EUS in the delivery of NAT in PDAC and its response assessment is rapidly emerging. Das *et al*[33] conducted a large sample study to investigate the value of EUS in preoperative tumor response prediction of PDAC after NAT. The results showed that the change in the tumor size after NAT on EUS was a sensitive marker for tumor response evaluation, and tumor size reduction ≥ 47% was an independent prognostic factor for OS in these patients. A systematic review from Barreto *et al*[34] compared the accuracy of imaging modalities to predict resectability and R0 resection for borderline or locally advanced PDAC after NAT. They showed that effective imaging evaluation allowed prediction of tumor resectability. Moreover, decrease in tumor stiffness of PDAC on EUS elastography may be used as a potential marker for NAT response and tumor resectability assessment. In addition, Figueiredo *et al*[35] reported the role of EUS-guided technology in the implementation of NAT for PDAC. The authors indicated that EUS-guided placement of fiducial markers for stereotactic body radiation therapy in PDAC helped to ensure the feasibility and security of subsequent NAT. The above studies illustrate the importance of EUS in the process of NAT for PDAC.

Multi-detector CT

Multidetector CT (MDCT) is the most frequently used imaging method to evaluate the response of PDAC after NAT. Compared with other imaging techniques, its advantages include higher spatial resolution and multiplanar reconstruction ability [36]. However, recent studies have shown that the diagnostic performance of MDCT in evaluating tumor resectability and re-staging of borderline tumors is not very satisfactory. In a study of 129 patients with borderline PDAC, the authors found that the commonly used response evaluation criteria in solid tumors (RECIST) criteria were not suitable for evaluating tumor response after NAT, because there were few morphological changes in the imaging after treatment[37]. A systematic review reported that only a small number of patients showed tumor shrinkage after NAT (Figure 2), and most patients (53%-80%) had stable disease[34]. Similar results were reported by a recent study[38] that showed that the assessment of resectability by MDCT after NAT is relatively insensitive and non-specific to predict R0 resection, because MDCT cannot accurately distinguish between residual tumor and tissue

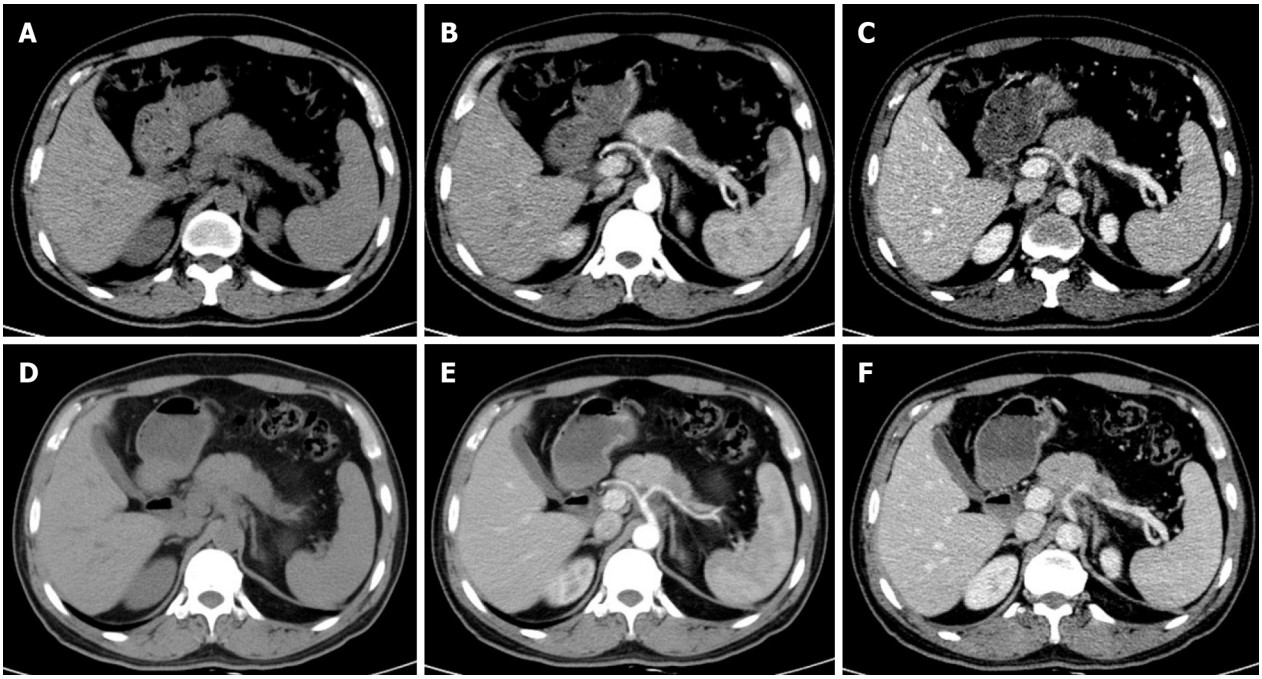


Figure 2 Response assessment with contrast-enhanced computed tomography after neoadjuvant treatment. A-C: A 55-year-old man with a 2.4 cm tumor in the pancreas with non-uniform low density (A), showing no obvious enhancement relative to the surrounding pancreatic parenchyma in the arterial phase (B), without hyperenhancement or distinct 'wash-out' appearance in the portal venous phase (C); D-F: After 20 d of neoadjuvant treatment (FOLFOX), tumor size was reduced to 2.1 cm, with low enhancement relative to the surrounding pancreatic parenchyma on contrast-enhanced computed tomography images.

scarring after tumor regression[39]. Moreover, local inflammatory pancreatitis also cannot be distinguished from tumor infiltration and the area of tumor infiltration is replaced by fibrotic tissue, which does not lead to apparent changes in tumor size (Figure 3). All of these factors lead to under-evaluation of tumor resectability[40-42].

Recently, some studies have started to explore whether imaging features other than tumor size and enhancement on MDCT images can be used to assess tumor response in PDAC. A study by Cassinotto *et al*[43] showed that the partial regression of tumor–vessel contact after NAT indicates suitability for surgical exploration, regardless of the reduction in tumor size or residual vascular involvement. Another study by Amer *et al*[44] suggested that changes at the PDAC/parenchyma interface may be used as an early predictor of response to NAT. A recent study from Wei *et al*[45] showed that the largest tumor diameter and radiological tumor volume on post-therapy MDCT were associated with the pathologic tumor staging and tumor response to NAT.

Although MDCT has a high resolution in displaying morphological characteristics of the tumor and the surrounding vascular structures, it has low specificity due to the lack of obvious tumor reduction after NAT in PDAC, as well as the presence of fibrous tissue and local pancreatitis. Hence, MDCT has low specificity and sensitivity in restaging of PDAC after NAT. However, further quantification and evaluation of imaging indicators on MDCT images can significantly improve the assessment of tumor response and prognostic value of patients with PDAC after NAT.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) provides better visualization of the soft tissues and pancreatic and biliary ductal abnormalities. In PDAC, the high cellularity and potential fibrosis of the tumor hinders the free movement of water molecules. This can be quantified by diffusion-weighted imaging (DWI) on MRI, which results in a low mean apparent diffusion coefficient (ADC) on the ADC map. Several studies have investigated the utility of DWI for assessment of the NAT response in patients with PDAC[46-50]. A previous study conducted by Cuneo *et al*[46] reported that there was an obvious correlation between the mean ADC values before treatment and the amount of destruction of tumor cells after neoadjuvant chemoradiation. The mean pre-treatment ADC (161×10^{-5} mm^2/s) of patients with a good response was significantly higher than that of non-responders (125×10^{-5} mm^2/s), which may provide evidence for candidate selection of intensified therapy. Dalah *et al*[47] investigated the relationship between ADC values after neoadjuvant chemoradiation and the

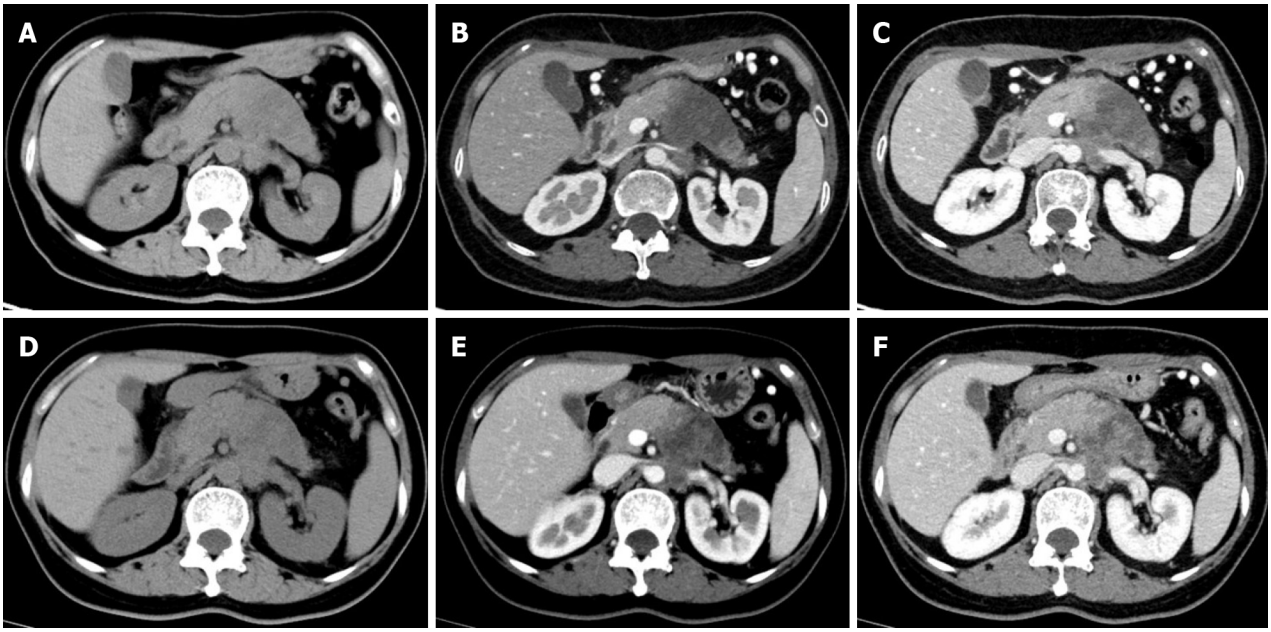


Figure 3 Response assessment with contrast-enhanced computed tomography after neoadjuvant therapy. A-C: A 58-year-old woman with a 5.6 cm × 4.2 cm tumor in the pancreatic body and tail with non-uniform low-density (A), showing slightly enhancement relative to the surrounding parenchyma in the arterial phase (B), with invasion of the left renal vein as seen in the portal venous phase (C); D-F: After 2.5 mo of neoadjuvant treatment (nab-paclitaxel combined with gemcitabine), the tumor size was reduced to 4.7 cm × 4.2 cm (D), small patchy enhancement was seen in the original lesion (E), and the degree of invasion of the left renal vein was reduced (F).

pathological treatment response in PDAC and showed that the post-mean ADC values were moderately correlated with the pathological tumor responses. The study also noted that, compared to tumors with poor pathological response, tumors with a good pathological response had a higher ratio of fibrosis to tumor cells. However, different results were reported by a recent study from Zimmermann *et al*[48]. The authors found that mean ADC cannot be used as a reliable marker to identify PDAC patients with a good response to NAT.

A prospective study by Okada *et al*[49] of 28 patients with borderline PDAC found that the post-treatment whole-tumor ADC value predicted R0 resection with 75% accuracy and histological response with 89% accuracy. Based on these findings, the authors suggested that whole-tumor ADC value can be used as the new marker for treatment response evaluation in PDAC. Bali *et al*[50] compared the effectiveness of DWI and RECIST criteria in evaluating tumor response to systemic chemotherapy in unresectable PDAC. This study selected three imaging biomarkers, ROI-ADC, DW volume, and diffusion parameters derived from histograms, and showed that these markers more accurately classified between responders and non-responders compared to RECIST 1.1 criteria. So, the ADC value of DWI shows better performance than morphological features in evaluating the tumor response to NAT in PDAC. However, at present, there are few related studies with inconsistent conclusions, and there are no unified standard criteria for the selection of DWI parameters. In addition, DWI has the disadvantages of large motion artifacts, long time-consuming procedures, and high cost, limiting its clinical application.

Positron emission tomography

^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG-PET) is a diagnostic test that reflects the genetic, molecular, metabolic, and functional status of the lesions. The maximum standardized uptake value (SUVmax) obtained by PET imaging reflects the glucose metabolism of the tumors. Choi *et al*[51] explored the relationship between the early treatment response after neoadjuvant chemo-radiotherapy using FDG-PET and surgical outcome in locally advanced PDAC. The results suggested that FDG-PET is helpful to monitor the clinical efficacy of NAT in the treatment of locally advanced PDAC. In patients with a good tumor response (≥ 50% decrease in SUV after cycle 1), surgical resection was accomplished. Lee *et al*[52] investigated the role of pre-operative ^{18}F-FDG PET/CT in predicting survival of patients with PDAC. The results showed that metabolic tumor volume and total lesion glycolysis were independent prognostic factors for predicting recurrence-free survival and OS of patients, and PET/CT

provides useful prognostic information for patients after resection of PDAC with curative intent irrespective of NAT.

Recently, some studies have further studied the value of SUVmax in PDAC to assess the tumor response after NAT[48,53,54]. The results showed that the post-NAT SUV_{max} can be an effective indicator to predict the prognosis and treatment response to NAT for PDAC. Moreover, the post-treatment metabolic parameters of PET/MRI also reportedly correlate with the pathological response. However, the sample size of the above studies was relatively small (< 50 cases in 2/3 articles), and the results were not convincing. Barnes *et al*[55] enrolled 201 consecutive patients with localized PDAC treated with NAT. The results showed that the pre-treatment SUVmax cut-off value of 7.5 on PET/CT could accurately predict the OS. The results from Yokose *et al*[56] showed that PET response criteria in solid tumor was more accurate in determining the NAT effects for PDAC than RECIST criteria (72.7% *vs* 36.4%), which puts forward a new direction for future research in this field.

The application of PET imaging indicates that the response assessment to NAT in PDAC has changed from morphological to metabolic evaluation, especially with the proposal of PET/MRI, which plays a significant role in the prediction of treatment outcome and clinical decision-making in these patients. However, due to the lack of literature, the results need to be further investigated. In addition, the PET response criteria in solid tumor criteria need to be compared with traditional evaluation criteria in future studies.

Perfusion CT

PDAC is a matrix-rich tumor, characterized by the activation of pancreatic stellate cells, which deposit a large amount of extracellular matrix[57]. The accumulation of extracellular matrix, including collagen, fibronectin, proteoglycan, and hyaluronic acid, can induce the formation of rigid extracellular matrix to compress blood vessels, leading to perfusion damage and ultimately hindering the transmission of anti-cancer drugs to the tumor cells[58] (Figure 3). Based on the above theoretical assumptions, Hamdy *et al*[59] investigated the value of perfusion CT in predicting the response of PDAC to neoadjuvant chemotherapy and radiation therapy. The results showed that participants who responded to NAT had higher baseline blood flow than those who did not respond (median, 44 mL/100 g/min *vs* 28 mL/100 g/min, respectively). In responders, the perfusion parameters increased after treatment, whereas there were no significant changes in perfusion parameters of non-responders. The authors suggested that pre-treatment perfusion CT can be helpful to predict the histopathologic response of PDAC to NAT. However, the exact role of perfusion imaging in PDAC needs to be further evaluated in a larger number of patients.

Texture analysis and radiomics

In recent years, many studies have emphasized the role of radiomics in various aspects of pancreatic tumors, such as tumor characterization, assessment of resectability, risk of recurrence, and prediction of survival. A previous study from Chen *et al*[42] showed changes in the CT radiomic features, such as the histograms of mean CT number, skewness, and kurtosis during the chemoradiation in patients with PDAC. The authors suggested that these changes may potentially be used for the early evaluation of the treatment response and patient stratification to achieve accurate and intensive treatment. Chakraborty *et al*[60] conducted a preliminary study to investigate the value of CT texture analysis in quantifying tumor heterogeneity and predicting 2-year survival in patients with PDAC. The results revealed that CT texture features can predict the heterogeneity in pancreatic tumors. Using fuzzy minimum-redundancy maximum-relevance feature selection and a naïve Bayes classifier, the area under the curve scaled up to 90%. At the same time, the accuracy of CT texture analysis in predicting the 2-year survival rate can reach 82.86%. Thus, it can be used to formulate the optimal treatment plan for PDAC patients. Ciaravino *et al*[61] investigated the added value of CT texture analysis in estimating tissue changes in PDAC downsized and resected after chemotherapy. The results suggested that the change of kurtosis before and after treatment showed a statistically significant difference, suggesting that CT texture analysis can assess tumor heterogeneity, tissue changes, and tumor downstaging in PDAC cases with no significant alteration in tumor size after NAT.

Recently, some studies have explored the utility of CT texture analysis in predicting resectability and prognosis in patients with PDAC[62] and the relationship between texture features and the tumor pathological response[63]. The results showed that the CT texture feature was more accurate in defining the tumor as resectable than unresectable. Moreover, higher subtracted entropy and lower subtracted gray-level co-occurrence matrix entropy indicated longer OS[62]. In the study by Borhani *et al*[63],

texture parameters, such as pre-treatment mean positive pixel, pre-treatment kurtosis, changes in kurtosis, and pre-treatment tumor SD, were found to be statistically different between patients with poor histologic response and those with favorable histologic response. The authors concluded that pre-treatment textural features of baseline CT imaging and longitudinal changes in tumor heterogeneity can be used as biomarkers for predicting histologic response to neoadjuvant chemotherapy and disease-free survival. In addition, Nasief *et al*[64] reported the value of radiomics combined with carbohydrate antigen 19-9 (CA19-9) in the evaluation of NAT for PDAC. The results showed that reduction of CA19-9 levels and delta radiomics features were predictors of survival in these patients. The delta radiomics features-CA19-9 combination has the potential to increase the possibility for response-based treatment adaptation. There is no denying that radiomics or texture analysis has wide prospects in the management of PDAC after NAT. The current limitations of radiomics include the time-consuming segmentation and non-robustness conclusion. Further large-scale studies are required to determine its real potential.

MERITS AND SHORTCOMINGS OF CURRENT IMAGING IN NAT RESPONSE FOR PDAC

In all stages of NAT for PDAC, imaging plays an essential role in the diagnosis, assessment of resectability, tumor re-staging, and response evaluation after NAT (Table 1). The change in tumor size as assessed by EUS after NAT can provide valuable information on tumor response and survival prediction. Moreover, change in tumor stiffness of PDAC on EUS elastography may be used as a potential marker for NAT response and tumor resectability assessment. MDCT, which relies on its high-density resolution and the speed of data acquisition, is often used to evaluate the resectability and tumor response of PDAC after NAT. However, because of the fibrotic and infiltrative nature of PDAC, changes in the size of the tumor after NAT are not apparent, and it is difficult to distinguish accurately between residual tumor and scarring from tumor regression. Hence, the value of conventional CT imaging features in evaluating NAT for PDAC is limited. ADC value quantified by DWI on MRI can reflect the cellularity and potential fibrotic changes of PDAC after NAT. Moreover, pre-treatment ADC can be used as an imaging biomarker to distinguish responders from non-responders after NAT. However, due to the shortcomings of large motion artifacts, the time-consuming process and high cost, as well as the inconsistency of existing results, the application of DWI in NAT for PDAC needs further confirmation. PET has great prospects for the prediction and evaluation of NAT for PDAC, which represents the transformation of imaging markers from morphological to metabolic. However, due to the lack of substantial evidence and the costly nature of the PET technique, the role of PET in PDAC is limited and needs further investigation. Perfusion CT, as a new imaging method to assess the response to NAT in PDAC, has proved to be beneficial. It can reveal the changes of extracellular matrix by monitoring the changes in the tumor blood perfusion, so as to predict and evaluate the efficacy of NAT in PDAC. However, more research is needed to confirm the findings of the preliminary studies. Radiomics and texture analysis have gradually become research hotspots in the field of NAT for PDAC, providing additional values for evaluating tumor heterogeneity. However, the time-consuming segmentation and non-robustness of current data cannot be denied.

CONCLUSION

The role of conventional CT imaging features in the evaluation of NAT response for PDAC is limited. Other imaging techniques, including EUS, DWI, PET, and perfusion CT, have enormous potential to become powerful tools for the assessment of tumor resectability and survival prediction of PDAC after NAT. In addition, the derivate techniques based on artificial intelligence, such as texture analysis and radiomics, have gradually begun to show their prominence in the field of NAT for PDAC. Although current research is limited and the conclusions are inconsistent, additional research conducted in this field will address the shortcomings of the existing evaluation system for PDAC and promote the implementation of precision medicine.

Table 1 Role of current imaging in the response assessment after neoadjuvant treatment for pancreatic ductal adenocarcinoma

Imaging technologies	Imaging parameters	Pathological basis	Roles and advantages	Limitations	Improvement
US	Conventional US imaging features	Iso-/low-attenuating pancreatic masses	Non-radiation and high economy; a tool for PDAC early detection and staging; detection of systemic therapies associated adverse events in metastatic PDAC	Play the limited role for tumor response of PDAC after NAT	Further exploration
EUS	Tumor size	Change in tumor size after NAT	Provides additional information for response assessment and survival prediction	Invasive procedure, preliminary research, lack of credible results	Further large-scale research is needed in the future
	Tumor stiffness (EUS elastography)	Decrease of change in tumor stiffness after NAT	Potential marker for NAT response and tumor resectability assessment	Poor objectivity and low reproducibility; the pressure of the probe on the lesion area is not easily controlled	Technology Optimization; further investigation is needed
CT	MDCT conventional imaging features Tumor size; Enhancement; Tumor-vessel contact	Size or enhancement reduction; changes in tumor-vessel contact	High-density resolution; speed of data acquisition; multiplanar reconstruction ability	Low specificity and sensitivity (53%-80% of patients are in a stable state on MDCT after NAT)	Further quantification, exploration and evaluation of imaging indicators on MDCT conventional imaging features
	Perfusion CT; Blood flow; Blood volume Permeability–surface area product	Changes in extracellular matrix causing changes in the tumor blood flow	Pre-treatment perfusion CT can be helpful to predict the histopathologic response of PDAC to NAT	Preliminary research, lack of credible results	Further studies are needed to evaluate the value of perfusion imaging in a large number of patients
	Texture analysis; Texture features	CT texture features after NAT can reveal the heterogeneity in PDAC	Providing additional value for judging tumor heterogeneity; judging tissue changes and tumor downstaging in those cases with no significant alteration in tumor size after NAT	Time consuming segmentation and non-robustness conclusion	Further large-scale research is needed in the future
	Radiomics; Radiomics features and delta radiomics features combined with common clinical index (*e.g.*, CA-199)	Radiomics features after NAT can reveal the heterogeneity change of PDAC	Increasing the possibility for response-based treatment adaptation; has a broad prospect in the management of PDAC after NAT	The lack of enough research; time consuming segmentation and non-robustness conclusion	
MRI	ADC value of DWI	Cellularity and potential fibrotic changes of PDAC after NAT	Improve the prediction possibility of R0 resection rate (75% accuracy) in resectable PDAC; pre-treatment ADC can be used to distinguish responding patients from non-responding patients after NAT	Few related studies and inconsistent conclusions; no unified standard for the selection of DWI scanning technology and parameters; large motion artifacts, time-consuming and high cost	Large-scale studies to validate the role of DWI in PDAC to NAT; establish a unified scanning standard; reduce motion artifacts and scan time; reduce costs by optimizing sequences
PET imaging	SUV_{max}	Changes in glucose metabolism of tumors before and after NAT	Aid in monitoring the clinical outcome of patients with locally advanced PDAC treated with NAT; distinguish responding patients from non-responding patients after NAT; plays a significant role in the clinical decision-making of patients	Lack of relevant research and high cost of inspection	Application of PERCIST criteria and comparison with the accuracy of traditional evaluation criteria will be the future research direction

ADC: Apparent diffusion coefficient; CT: Computed tomography; DWI: Diffusion-weighted imaging; EUS: Endoscopic ultrasound; MRI: Magnetic resonance imaging; NAT: Neoadjuvant treatment; PDAC: Pancreatic ductal adenocarcinoma; PERCIST: Positron emission tomography response criteria in solid tumor; PET: Positron emission tomography; SUV: Standardized uptake value; US: Ultrasound.

REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Mizrahi JD**, Surana R, Valle JW, Shroff RT. Pancreatic cancer. *Lancet* 2020; **395**: 2008-2020 [PMID: 32593337 DOI: 10.1016/S0140-6736(20)30974-0]

3 **Deplanque G**, Demartines N. Pancreatic cancer: are more chemotherapy and surgery needed? *Lancet* 2017; **389**: 985-986 [PMID: 28129986 DOI: 10.1016/S0140-6736(17)30126-5]

4 **Evans DB**, Varadhachary GR, Crane CH, Sun CC, Lee JE, Pisters PW, Vauthey JN, Wang H, Cleary KR, Staerkel GA, Charnsangavej C, Lano EA, Ho L, Lenzi R, Abbruzzese JL, Wolff RA. Preoperative gemcitabine-based chemoradiation for patients with resectable adenocarcinoma of the pancreatic head. *J Clin Oncol* 2008; **26**: 3496-3502 [PMID: 18640930 DOI: 10.1200/JCO.2007.15.8634]

5 **Roland CL**, Yang AD, Katz MH, Chatterjee D, Wang H, Lin H, Vauthey JN, Pisters PW, Varadhachary GR, Wolff RA, Crane CH, Lee JE, Fleming JB. Neoadjuvant therapy is associated with a reduced lymph node ratio in patients with potentially resectable pancreatic cancer. *Ann Surg Oncol* 2015; **22**: 1168-1175 [PMID: 25352267 DOI: 10.1245/s10434-014-4192-6]

6 **Abrams RA**, Lowy AM, O'Reilly EM, Wolff RA, Picozzi VJ, Pisters PW. Combined modality treatment of resectable and borderline resectable pancreas cancer: expert consensus statement. *Ann Surg Oncol* 2009; **16**: 1751-1756 [PMID: 19390900 DOI: 10.1245/s10434-009-0413-9]

7 **Tempero MA**, Malafa MP, Al-Hawary M, Asbun H, Bain A, Behrman SW, Benson AB 3rd, Binder E, Cardin DB, Cha C, Chiorean EG, Chung V, Czito B, Dillhoff M, Dotan E, Ferrone CR, Hardacre J, Hawkins WG, Herman J, Ko AH, Komanduri S, Koong A, LoConte N, Lowy AM, Moravek C, Nakakura EK, O'Reilly EM, Obando J, Reddy S, Scaife C, Thayer S, Weekes CD, Wolff RA, Wolpin BM, Burns J, Darlow S. Pancreatic Adenocarcinoma, Version 2.2017, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw* 2017; **15**: 1028-1061 [PMID: 28784865 DOI: 10.6004/jnccn.2017.0131]

8 **Gilbert JW**, Wolpin B, Clancy T, Wang J, Mamon H, Shinagare AB, Jagannathan J, Rosenthal M. Borderline resectable pancreatic cancer: conceptual evolution and current approach to image-based classification. *Ann Oncol* 2017; **28**: 2067-2076 [PMID: 28407088 DOI: 10.1093/annonc/mdx180]

9 **Konstantinidis IT**, Warshaw AL, Allen JN, Blaszkowsky LS, Castillo CF, Deshpande V, Hong TS, Kwak EL, Lauwers GY, Ryan DP, Wargo JA, Lillemoe KD, Ferrone CR. Pancreatic ductal adenocarcinoma: is there a survival difference for R1 resections versus locally advanced unresectable tumors? *Ann Surg* 2013; **257**: 731-736 [PMID: 22968073 DOI: 10.1097/SLA.0b013e318263da2f]

10 **Bilimoria KY**, Talamonti MS, Sener SF, Bilimoria MM, Stewart AK, Winchester DP, Ko CY, Bentrem DJ. Effect of hospital volume on margin status after pancreaticoduodenectomy for cancer. *J Am Coll Surg* 2008; **207**: 510-519 [PMID: 18926452 DOI: 10.1016/j.jamcollsurg.2008.04.033]

11 **Ferrone CR**, Pieretti-Vanmarcke R, Bloom JP, Zheng H, Szymonifka J, Wargo JA, Thayer SP, Lauwers GY, Deshpande V, Mino-Kenudson M, Fernández-del Castillo C, Lillemoe KD, Warshaw AL. Pancreatic ductal adenocarcinoma: long-term survival does not equal cure. *Surgery* 2012; **152**: S43-S49 [PMID: 22763261 DOI: 10.1016/j.surg.2012.05.020]

12 **Conroy T**, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, Choné L, Francois E, Artru P, Biagi JJ, Lecomte T, Assenat E, Faroux R, Ychou M, Volet J, Sauvanet A, Breysacher G, Di Fiore F, Cripps C, Kavan P, Texereau P, Bouhier-Leporrier K, Khemissa-Akouz F, Legoux JL, Juzyna B, Gourgou S, O'Callaghan CJ, Jouffroy-Zeller C, Rat P, Malka D, Castan F, Bachet JB; Canadian Cancer Trials Group and the Unicancer-GI–PRODIGE Group. FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer. *N Engl J Med* 2018; **379**: 2395-2406 [PMID: 30575490 DOI: 10.1056/NEJMoa1809775]

13 **Stein SM**, James ES, Deng Y, Cong X, Kortmansky JS, Li J, Staugaard C, Indukala D, Boustani AM, Patel V, Cha CH, Salem RR, Chang B, Hochster HS, Lacy J. Final analysis of a phase II study of modified FOLFIRINOX in locally advanced and metastatic pancreatic cancer. *Br J Cancer* 2016; **114**: 737-743 [PMID: 27022826 DOI: 10.1038/bjc.2016.45]

14 **Stessin AM**, Meyer JE, Sherr DL. Neoadjuvant radiation is associated with improved survival in patients with resectable pancreatic cancer: an analysis of data from the surveillance, epidemiology, and end results (SEER) registry. *Int J Radiat Oncol Biol Phys* 2008; **72**: 1128-1133 [PMID: 18538501 DOI: 10.1016/j.ijrobp.2008.02.065]

15 **Versteijne E**, Vogel JA, Besselink MG, Busch ORC, Wilmink JW, Daams JG, van Eijck CHJ, Groot Koerkamp B, Rasch CRN, van Tienhoven G; Dutch Pancreatic Cancer Group. Meta-analysis comparing upfront surgery with neoadjuvant treatment in patients with resectable or borderline resectable pancreatic cancer. *Br J Surg* 2018; **105**: 946-958 [PMID: 29708592 DOI: 10.1002/bjs.10870]

16 **Kim SS**, Nakakura EK, Wang ZJ, Kim GE, Corvera CU, Harris HW, Kirkwood KS, Hirose R, Tempero MA, Ko AH. Preoperative FOLFIRINOX for borderline resectable pancreatic cancer: Is radiation necessary in the modern era of chemotherapy? *J Surg Oncol* 2016; **114**: 587-596 [PMID: 27444658 DOI: 10.1002/jso.24375]

17 **Motoi F**, Kosuge T, Ueno H, Yamaue H, Satoi S, Sho M, Honda G, Matsumoto I, Wada K, Furuse J, Matsuyama Y, Unno M; Study Group of Preoperative Therapy for Pancreatic Cancer (Prep) and Japanese Study Group of Adjuvant Therapy for Pancreatic cancer (JSAP). Randomized phase II/III trial of neoadjuvant chemotherapy with gemcitabine and S-1 versus upfront surgery for resectable pancreatic cancer (Prep-02/JSAP05). *Jpn J Clin Oncol* 2019; **49**: 190-194 [PMID: 30608598 DOI: 10.1093/jjco/hyy190]

18 **Katz MH**, Shi Q, Ahmad SA, Herman JM, Marsh Rde W, Collisson E, Schwartz L, Frankel W, Martin R, Conway W, Truty M, Kindler H, Lowy AM, Bekaii-Saab T, Philip P, Talamonti M, Cardin

D, LoConte N, Shen P, Hoffman JP, Venook AP. Preoperative Modified FOLFIRINOX Treatment Followed by Capecitabine-Based Chemoradiation for Borderline Resectable Pancreatic Cancer: Alliance for Clinical Trials in Oncology Trial A021101. *JAMA Surg* 2016; **151**: e161137 [PMID: 27275632 DOI: 10.1001/jamasurg.2016.1137]

19 **Versteijne E**, Suker M, Groen JV, Besselink MG, Bonsing BA, Bosscha K, Busch OR, de Hingh IHJT, De Jong KP, Molenaar IQ, van Santvoort HC, Verkooijen HM, Van Eijck CH, van Tienhoven G; Dutch Pancreatic Cancer Group. External Validity of the Multicenter Randomized PREOPANC Trial on Neoadjuvant Chemoradiotherapy in Pancreatic Cancer: Outcome of Eligible But Non-Randomized Patients. *Ann Surg* 2020 [PMID: 33273349 DOI: 10.1097/SLA.0000000000004364]

20 **di Sebastiano P**, Grottola T, di Mola FF. Borderline resectable pancreatic cancer and the role of neoadjuvant chemoradiotherapy. *Updates Surg* 2016; **68**: 235-239 [PMID: 27629483 DOI: 10.1007/s13304-016-0392-x]

21 **Barnes CA**, Chavez MI, Tsai S, Aldakkak M, George B, Ritch PS, Dua K, Clarke CN, Tolat P, Hagen C, Hall WA, Erickson BA, Evans DB, Christians KK. Survival of patients with borderline resectable pancreatic cancer who received neoadjuvant therapy and surgery. *Surgery* 2019; **166**: 277-285 [PMID: 31272811 DOI: 10.1016/j.surg.2019.05.010]

22 **Rose JB**, Rocha FG, Alseidi A, Biehl T, Moonka R, Ryan JA, Lin B, Picozzi V, Helton S. Extended neoadjuvant chemotherapy for borderline resectable pancreatic cancer demonstrates promising postoperative outcomes and survival. *Ann Surg Oncol* 2014; **21**: 1530-1537 [PMID: 24473642 DOI: 10.1245/s10434-014-3486-z]

23 **Kulkarni NM**, Mannelli L, Zins M, Bhosale PR, Arif-Tiwari H, Brook OR, Hecht EM, Kastrinos F, Wang ZJ, Soloff EV, Tolat PP, Sangster G, Fleming J, Tamm EP, Kambadakone AR. White paper on pancreatic ductal adenocarcinoma from society of abdominal radiology's disease-focused panel for pancreatic ductal adenocarcinoma: Part II, update on imaging techniques and screening of pancreatic cancer in high-risk individuals. *Abdom Radiol (NY)* 2020; **45**: 729-742 [PMID: 31768594 DOI: 10.1007/s00261-019-02290-y]

24 **Takahashi S**, Ohno I, Ikeda M, Konishi M, Kobayashi T, Akimoto T, Kojima M, Morinaga S, Toyama H, Shimizu Y, Miyamoto A, Tomikawa M, Takakura N, Takayama W, Hirano S, Otsubo T, Nagino M, Kimura W, Sugimachi K, Uesaka K. Neoadjuvant S-1 With Concurrent Radiotherapy Followed by Surgery for Borderline Resectable Pancreatic Cancer: A Phase II Open-Label Multicenter Prospective Trial (JASPAC05). *Ann Surg* 2020 [PMID: 33065644 DOI: 10.1097/SLA.0000000000004535]

25 **Inoue Y**, Saiura A, Oba A, Ono Y, Mise Y, Ito H, Sasaki T, Ozaka M, Sasahira N, Takahashi Y. Neoadjuvant gemcitabine and nab-paclitaxel for borderline resectable pancreatic cancers: Intention-to-treat analysis compared with upfront surgery. *J Hepatobiliary Pancreat Sci* 2021; **28**: 143-155 [PMID: 33058515 DOI: 10.1002/jhbp.844]

26 **Anger F**, Döring A, van Dam J, Lock JF, Klein I, Bittrich M, Germer CT, Wiegering A, Kunzmann V, van Eijck C, Löb S. Impact of Borderline Resectability in Pancreatic Head Cancer on Patient Survival: Biology Matters According to the New International Consensus Criteria. *Ann Surg Oncol* 2021; **28**: 2325-2336 [PMID: 32920720 DOI: 10.1245/s10434-020-09100-6]

27 **Conroy T**, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817-1825 [PMID: 21561347 DOI: 10.1056/NEJMoa1011923]

28 **Neoptolemos JP**, Palmer DH, Ghaneh P, Psarelli EE, Valle JW, Halloran CM, Faluyi O, O'Reilly DA, Cunningham D, Wadsley J, Darby S, Meyer T, Gillmore R, Anthoney A, Lind P, Glimelius B, Falk S, Izbicki JR, Middleton GW, Cummins S, Ross PJ, Wasan H, McDonald A, Crosby T, Ma YT, Patel K, Sherriff D, Soomal R, Borg D, Sothi S, Hammel P, Hackert T, Jackson R, Büchler MW; European Study Group for Pancreatic Cancer. Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. *Lancet* 2017; **389**: 1011-1024 [PMID: 28129987 DOI: 10.1016/S0140-6736(16)32409-6]

29 **Mornex F**, Girard N, Delpero JR, Partensky C. Radiochemotherapy in the management of pancreatic cancer--part I: neoadjuvant treatment. *Semin Radiat Oncol* 2005; **15**: 226-234 [PMID: 16183476 DOI: 10.1016/j.semradonc.2005.04.008]

30 **Smith DA**, Somarouthu B, Ramaiya NH. An imaging-based review of systemic therapies and associated toxicities in metastatic pancreatic cancer as per the 2018 ASCO guidelines: what every radiologist should know. *Abdom Radiol (NY)* 2019; **44**: 2182-2195 [PMID: 30820626 DOI: 10.1007/s00261-019-01954-z]

31 **Vincent A**, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. *Lancet* 2011; **378**: 607-620 [PMID: 21620466 DOI: 10.1016/S0140-6736(10)62307-0]

32 **Dewitt J**, Devereaux BM, Lehman GA, Sherman S, Imperiale TF. Comparison of endoscopic ultrasound and computed tomography for the preoperative evaluation of pancreatic cancer: a systematic review. *Clin Gastroenterol Hepatol* 2006; **4**: 717-25; quiz 664 [PMID: 16675307 DOI: 10.1016/j.cgh.2006.02.020]

33 **Das R**, McGrath K, Seiser N, Smith K, Uttam S, Brand RE, Fasanella KE, Khalid A, Chennat JS, Sarkaria S, Singh H, Slivka A, Zeh HJ, Zureikat AH, Hogg ME, Lee KK, Paniccia A, Ongchin MC, Pingpank JF, Boone BA, Dasyam AK, Bahary N, Gorantla VC, Rhee JC, Thomas R, Ellsworth S,

Landau MS, Ohori NP, Henn P, Shyu S, Theisen BK, Singhi AD. Tumor Size Differences Between Preoperative Endoscopic Ultrasound and Postoperative Pathology for Neoadjuvant-Treated Pancreatic Ductal Adenocarcinoma Predict Patient Outcome. *Clin Gastroenterol Hepatol* 2020 [PMID: 33278573 DOI: 10.1016/j.cgh.2020.11.041]

34 **Barreto SG**, Loveday B, Windsor JA, Pandanaboyana S. Detecting tumour response and predicting resectability after neoadjuvant therapy for borderline resectable and locally advanced pancreatic cancer. *ANZ J Surg* 2019; **89**: 481-487 [PMID: 30117669 DOI: 10.1111/ans.14764]

35 **Figueiredo M**, Bouchart C, Moretti L, Mans L, Engelholm JL, Bali MA, Van Laethem JL, Eisendrath P. EUS-guided placement of fiducial markers for stereotactic body radiation therapy in pancreatic cancer: feasibility, security and a new quality score. *Endosc Int Open* 2021; **9**: E253-E257 [PMID: 33553589 DOI: 10.1055/a-1324-2892]

36 **Soriano A**, Castells A, Ayuso C, Ayuso JR, de Caralt MT, Ginès MA, Real MI, Gilabert R, Quintó L, Trilla A, Feu F, Montanyà X, Fernández-Cruz L, Navarro S. Preoperative staging and tumor resectability assessment of pancreatic cancer: prospective study comparing endoscopic ultrasonography, helical computed tomography, magnetic resonance imaging, and angiography. *Am J Gastroenterol* 2004; **99**: 492-501 [PMID: 15056091 DOI: 10.1111/j.1572-0241.2004.04087.x]

37 **Katz MH**, Fleming JB, Bhosale P, Varadhachary G, Lee JE, Wolff R, Wang H, Abbruzzese J, Pisters PW, Vauthey JN, Charnsangavej C, Tamm E, Crane CH, Balachandran A. Response of borderline resectable pancreatic cancer to neoadjuvant therapy is not reflected by radiographic indicators. *Cancer* 2012; **118**: 5749-5756 [PMID: 22605518 DOI: 10.1002/cncr.27636]

38 **Jang JK**, Byun JH, Kang JH, Son JH, Kim JH, Lee SS, Kim HJ, Yoo C, Kim KP, Hong SM, Seo DW, Kim SC, Lee MG. CT-determined resectability of borderline resectable and unresectable pancreatic adenocarcinoma following FOLFIRINOX therapy. *Eur Radiol* 2021; **31**: 813-823 [PMID: 32845389 DOI: 10.1007/s00330-020-07188-8]

39 **Windsor JA**, Barreto SG. The concept of 'borderline resectable' pancreatic cancer: limited foundations and limited future? *J Gastrointest Oncol* 2017; **8**: 189-193 [PMID: 28280624 DOI: 10.21037/jgo.2016.12.06]

40 **Balthazar EJ**. Pancreatitis associated with pancreatic carcinoma. Preoperative diagnosis: role of CT imaging in detection and evaluation. *Pancreatology* 2005; **5**: 330-344 [PMID: 16015017 DOI: 10.1159/000086868]

41 **Ferrone CR**, Marchegiani G, Hong TS, Ryan DP, Deshpande V, McDonnell EI, Sabbatino F, Santos DD, Allen JN, Blaszkowsky LS, Clark JW, Faris JE, Goyal L, Kwak EL, Murphy JE, Ting DT, Wo JY, Zhu AX, Warshaw AL, Lillemoe KD, Fernández-del Castillo C. Radiological and surgical implications of neoadjuvant treatment with FOLFIRINOX for locally advanced and borderline resectable pancreatic cancer. *Ann Surg* 2015; **261**: 12-17 [PMID: 25599322 DOI: 10.1097/SLA.0000000000000867]

42 **Chen X**, Oshima K, Schott D, Wu H, Hall W, Song Y, Tao Y, Li D, Zheng C, Knechtges P, Erickson B, Li XA. Assessment of treatment response during chemoradiation therapy for pancreatic cancer based on quantitative radiomic analysis of daily CTs: An exploratory study. *PLoS One* 2017; **12**: e0178961 [PMID: 28575105 DOI: 10.1371/journal.pone.0178961]

43 **Cassinotto C**, Mouries A, Lafourcade JP, Terrebonne E, Belleannée G, Blanc JF, Lapuyade B, Vendrely V, Laurent C, Chiche L, Wagner T, Sa-Cunha A, Gaye D, Trillaud H, Laurent F, Montaudon M. Locally advanced pancreatic adenocarcinoma: reassessment of response with CT after neoadjuvant chemotherapy and radiation therapy. *Radiology* 2014; **273**: 108-116 [PMID: 24960211 DOI: 10.1148/radiol.14132914]

44 **Amer AM**, Zaid M, Chaudhury B, Elganainy D, Lee Y, Wilke CT, Cloyd J, Wang H, Maitra A, Wolff RA, Varadhachary G, Overman MJ, Lee JE, Fleming JB, Tzeng CW, Katz MH, Holliday EB, Krishnan S, Minsky BD, Herman JM, Taniguchi CM, Das P, Crane CH, Le O, Bhosale P, Tamm EP, Koay EJ. Imaging-based biomarkers: Changes in the tumor interface of pancreatic ductal adenocarcinoma on computed tomography scans indicate response to cytotoxic therapy. *Cancer* 2018; **124**: 1701-1709 [PMID: 29370450 DOI: 10.1002/cncr.31251]

45 **Wei D**, Zaid MM, Katz MH, Prakash LR, Kim M, Tzeng CD, Lee JE, Agrawal A, Rashid A, Wang H, Varadhachary G, Wolff RA, Tamm EP, Bhosale PR, Maitra A, Koay EJ. Clinicopathological correlation of radiologic measurement of post-therapy tumor size and tumor volume for pancreatic ductal adenocarcinoma. *Pancreatology* 2021; **21**: 200-207 [PMID: 33221151 DOI: 10.1016/j.pan.2020.11.003]

46 **Cuneo KC**, Chenevert TL, Ben-Josef E, Feng MU, Greenson JK, Hussain HK, Simeone DM, Schipper MJ, Anderson MA, Zalupski MM, Al-Hawary M, Galban CJ, Rehemtulla A, Feng FY, Lawrence TS, Ross BD. A pilot study of diffusion-weighted MRI in patients undergoing neoadjuvant chemoradiation for pancreatic cancer. *Transl Oncol* 2014; **7**: 644-649 [PMID: 25389460 DOI: 10.1016/j.tranon.2014.07.005]

47 **Dalah E**, Erickson B, Oshima K, Schott D, Hall WA, Paulson E, Tai A, Knechtges P, Li XA. Correlation of ADC With Pathological Treatment Response for Radiation Therapy of Pancreatic Cancer. *Transl Oncol* 2018; **11**: 391-398 [PMID: 29455085 DOI: 10.1016/j.tranon.2018.01.018]

48 **Zimmermann C**, Distler M, Jentsch C, Blum S, Folprecht G, Zöphel K, Polster H, Troost EGC, Abolmaali N, Weitz J, Baumann M, Saeger HD, Grützmann R. Evaluation of response using FDG-PET/CT and diffusion weighted MRI after radiochemotherapy of pancreatic cancer: a non-randomized, monocentric phase II clinical trial-PaCa-DD-041 (Eudra-CT 2009-011968-11). *Strahlenther Onkol* 2021; **197**: 19-26 [PMID: 32638040 DOI: 10.1007/s00066-020-01654-4]

49 **Okada KI**, Kawai M, Hirono S, Kojima F, Tanioka K, Terada M, Miyazawa M, Kitahata Y, Iwahashi Y, Ueno M, Hayami S, Murata SI, Shimokawa T, Yamaue H. Diffusion-weighted MRI predicts the histologic response for neoadjuvant therapy in patients with pancreatic cancer: a prospective study (DIFFERENT trial). *Langenbecks Arch Surg* 2020; **405**: 23-33 [PMID: 31993737 DOI: 10.1007/s00423-020-01857-4]

50 **Bali MA**, Pullini S, Metens T, Absil J, Chao SL, Marechal R, Matos C, Peerboccus BM, Van Laethem JL. Assessment of response to chemotherapy in pancreatic ductal adenocarcinoma: Comparison between diffusion-weighted MR quantitative parameters and RECIST. *Eur J Radiol* 2018; **104**: 49-57 [PMID: 29857866 DOI: 10.1016/j.ejrad.2018.04.024]

51 **Choi M**, Heilbrun LK, Venkatramanamoorthy R, Lawhorn-Crews JM, Zalupski MM, Shields AF. Using 18F-fluorodeoxyglucose positron emission tomography to monitor clinical outcomes in patients treated with neoadjuvant chemo-radiotherapy for locally advanced pancreatic cancer. *Am J Clin Oncol* 2010; **33**: 257-261 [PMID: 19806035 DOI: 10.1097/COC.0b013e3181a76a0b]

52 **Lee JW**, Kang CM, Choi HJ, Lee WJ, Song SY, Lee JH, Lee JD. Prognostic Value of Metabolic Tumor Volume and Total Lesion Glycolysis on Preoperative ^{18}F-FDG PET/CT in Patients with Pancreatic Cancer. *J Nucl Med* 2014; **55**: 898-904 [PMID: 24711649 DOI: 10.2967/jnumed.113.131847]

53 **Panda A**, Garg I, Truty MJ, Kline TL, Johnson MP, Ehman EC, Suman G, Anaam DA, Kemp BJ, Johnson GB, Halfdanarson TR, Venkatesh SK, Fidler JL, Goenka AH. Borderline Resectable and Locally Advanced Pancreas Cancer: FDG PET/MRI and CT Tumor Metrics for Assessment of Neoadjuvant Therapy Pathologic Response and Prediction of Survival. *AJR Am J Roentgenol* 2020 [PMID: 33084382 DOI: 10.2214/AJR.20.24567]

54 **Itchins M**, Chua TC, Arena J, Jamieson NB, Nahm CB, O'Connell RL, Bailey EA, Schembri GP, Gill AJ, Kneebone A, Hruby G, Mittal A, Pavlakis N, Clarke SJ, Samra JS. Evaluation of Fluorodeoxyglucose Positron Emission Tomography Scanning in the Neoadjuvant Therapy Paradigm in Pancreatic Ductal Adenocarcinoma. *Pancreas* 2020; **49**: 224-229 [PMID: 32011525 DOI: 10.1097/MPA.0000000000001472]

55 **Barnes CA**, Aldakkak M, Clarke CN, Christians KK, Bucklan D, Holt M, Tolat P, Ritch PS, George B, Hall WA, Erickson BA, Evans DB, Tsai S. Value of Pretreatment ^{18}F-fluorodeoxyglucose Positron Emission Tomography in Patients With Localized Pancreatic Cancer Treated With Neoadjuvant Therapy. *Front Oncol* 2020; **10**: 500 [PMID: 32363161 DOI: 10.3389/fonc.2020.00500]

56 **Yokose T**, Kitago M, Matsusaka Y, Masugi Y, Shinoda M, Yagi H, Abe Y, Oshima G, Hori S, Endo Y, Toyama K, Iwabuchi Y, Takemura R, Ishii R, Nakahara T, Okuda S, Jinzaki M, Kitagawa Y. Usefulness of ^{18} F-fluorodeoxyglucose positron emission tomography/computed tomography for predicting the prognosis and treatment response of neoadjuvant therapy for pancreatic ductal adenocarcinoma. *Cancer Med* 2020; **9**: 4059-4068 [PMID: 32281301 DOI: 10.1002/cam4.3044]

57 **Dunér S**, Lopatko Lindman J, Ansari D, Gundewar C, Andersson R. Pancreatic cancer: the role of pancreatic stellate cells in tumor progression. *Pancreatology* 2010; **10**: 673-681 [PMID: 21242706 DOI: 10.1159/000320711]

58 **Neesse A**, Michl P, Frese KK, Feig C, Cook N, Jacobetz MA, Lolkema MP, Buchholz M, Olive KP, Gress TM, Tuveson DA. Stromal biology and therapy in pancreatic cancer. *Gut* 2011; **60**: 861-868 [PMID: 20966025 DOI: 10.1136/gut.2010.226092]

59 **Hamdy A**, Ichikawa Y, Toyomasu Y, Nagata M, Nagasawa N, Nomoto Y, Sami H, Sakuma H. Perfusion CT to Assess Response to Neoadjuvant Chemotherapy and Radiation Therapy in Pancreatic Ductal Adenocarcinoma: Initial Experience. *Radiology* 2019; **292**: 628-635 [PMID: 31287389 DOI: 10.1148/radiol.2019182561]

60 **Chakraborty J**, Langdon-Embry L, Cunanan KM, Escalon JG, Allen PJ, Lowery MA, O'Reilly EM, Gönen M, Do RG, Simpson AL. Preliminary study of tumor heterogeneity in imaging predicts two year survival in pancreatic cancer patients. *PLoS One* 2017; **12**: e0188022 [PMID: 29216209 DOI: 10.1371/journal.pone.0188022]

61 **Ciaravino V**, Cardobi N, DE Robertis R, Capelli P, Melisi D, Simionato F, Marchegiani G, Salvia R, D'Onofrio M. CT Texture Analysis of Ductal Adenocarcinoma Downstaged After Chemotherapy. *Anticancer Res* 2018; **38**: 4889-4895 [PMID: 30061265 DOI: 10.21873/anticanres.12803]

62 **Kim BR**, Kim JH, Ahn SJ, Joo I, Choi SY, Park SJ, Han JK. CT prediction of resectability and prognosis in patients with pancreatic ductal adenocarcinoma after neoadjuvant treatment using image findings and texture analysis. *Eur Radiol* 2019; **29**: 362-372 [PMID: 29931561 DOI: 10.1007/s00330-018-5574-0]

63 **Borhani AA**, Dewan R, Furlan A, Seiser N, Zureikat AH, Singhi AD, Boone B, Bahary N, Hogg ME, Lotze M, Zeh HJ III, Tublin ME. Assessment of Response to Neoadjuvant Therapy Using CT Texture Analysis in Patients With Resectable and Borderline Resectable Pancreatic Ductal Adenocarcinoma. *AJR Am J Roentgenol* 2020; **214**: 362-369 [PMID: 31799875 DOI: 10.2214/AJR.19.21152]

64 **Nasief H**, Hall W, Zheng C, Tsai S, Wang L, Erickson B, Li XA. Improving Treatment Response Prediction for Chemoradiation Therapy of Pancreatic Cancer Using a Combination of Delta-Radiomics and the Clinical Biomarker CA19-9. *Front Oncol* 2019; **9**: 1464 [PMID: 31970088 DOI: 10.3389/fonc.2019.01464]

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MINIREVIEWS

Current approach to treatment of minimal hepatic encephalopathy in patients with liver cirrhosis

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Abstract

Minimal hepatic encephalopathy (MHE) corresponds to the earliest stage of hepatic encephalopathy (HE). MHE does not present clinically detectable neurological-psychiatric abnormalities but is characterized by imperceptible neurocognitive alterations detected during routine clinical examination *via* neuropsychological or psychometrical tests. MHE may affect daily activities and reduce job performance and quality of life. MHE can increase the risk of accidents and may develop into overt encephalopathy, worsening the prognosis of patients with liver cirrhosis. Despite a lack of consensus on the therapeutic indication, interest in finding novel strategies for prevention or reversion has led to numerous clinical trials; their results are the main objective of this review. Many studies address the treatment of MHE, which is mainly based on the strategies and previous management of overt HE. Current alternatives for the management of MHE include measures to maintain nutritional status while avoiding sarcopenia, and manipulation of intestinal microbiota with non-absorbable disaccharides such as lactulose, antibiotics such as rifaximin, and administration of different probiotics. This review analyzes the results of clinical studies that evaluated the effects of different treatments for MHE.

Key Words: Minimal hepatic encephalopathy; Sarcopenia; Probiotics; Non-absorbable disaccharides, Rifaximin; L-ornithine-L-aspartate

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Core Tip: Minimal hepatic encephalopathy (MHE) is a frequent complication in patients with liver cirrhosis. MHE affects the quality of life and increases the risk of falls and car accidents, worsening patients' prognoses; however, there is no standard of care. Current alternatives for the management of MHE include measures to maintain the nutritional status while avoiding sarcopenia, manipulation of intestinal microbiota with non-absorbable disaccharides such as lactulose, antibiotics such as rifaximin, and administration of different probiotics. This review analyzes the results of clinical studies that evaluated the effects of different treatments for MHE.

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INTRODUCTION

Minimal hepatic encephalopathy (MHE) corresponds to the earliest stage of hepatic encephalopathy (HE) (Table 1) and is characterized by imperceptible neurocognitive alterations detected during routine clinical examination through neuropsychological or psychometrical tests[1-3]. The prevalence of MHE in patients with liver cirrhosis varies from 23.7% to 56.6%, depending on the diagnostic criteria and studied population[4-8]. MHE predisposes patients to the development of overt HE (OHE)[9-11]; it has been reported that 50% of patients with MHE develop OHE within 3 years [12]. MHE may also affect daily activities, negatively reduce job performance and quality of life (QOL), and increase the risk of causing car accidents[6,13,14].

ASSESSMENT OF MHE

Despite the existence of several grading instruments that allow for the distinction of patients with MHE among those without neurocognitive alterations, there are no specific tests that discriminate the different grades of covert HE (CHE). Table 2 shows some of the tools used for the diagnosis of MHE. Although formal neuropsychological assessment is the best method to identify neurocognitive deficits, there is no standard battery for MHE recognition, so neuropsychologists must interpret and assess these tests. Therefore, other neuropsychological, computerized, and short neuropsychological batteries have been suggested for its identification[15-17].

Even when surveillance of MHE is possible in every patient with liver cirrhosis, a screening that enables us to distinguish patients at risk of suffering from or causing car accidents among active drivers or distinguish patients manifesting cognitive deterioration or noticeable low job performance has been suggested[15].

ASSOCIATED CLINICAL CONDITIONS

Certain conditions that have been addressed as contributors to the development of MHE and are therefore considered potential therapeutic targets. They are as follows.

Hyperammonaemia

The increase in arterial ammonia has been found in patients manifesting some signs of cerebral dysfunction such as mild lethargy, brisk deep tendon reflexes, or increased resistance to passive arm movement[18], and in patients with the specific diagnosis of MHE[19-23] including those with concomitant MHE and sarcopenia[7,23]. An increase in the production of ammonia may be related to dysbiosis, small intestinal bacterial overgrowth (SIBO), or *Helicobacter pylori* infection as a result of increased activity of bacterial urease, intestinal urea hydrolysis, and absorption of nitrogenous products[20,24]. This, together with decreased intestinal motility and altered integrity of the intestinal barrier, facilitate the increase in bacterial translocation and endotoxin release

Table 1 Classification of the different stages of hepatic encephalopathy

Diagnostic criteria	Classification stages					
ISHEN	Unimpaired	Covert hepatic encephalopathy		Overt hepatic encephalopathy		
WHC		MHE	Grade I	Grade II	Grade III	Grade IV
	No encephalopathy, no history of OHE	Imperceptible cognitive alterations during routine clinical examination	Trivial lack of awareness; Euphoria or anxiety; Shortened attention span; Impairment of addition or subtraction; Altered sleep rhythm	Lethargy or apathy; Disorientation for time; Obvious personality change; Inappropriate behavior; Dyspraxia asterixis	Somnolence to semistupor; Responsive to stimuli; Confused; Gross disorientation; Bizarre behavior	Coma

ISHEN: International Society for Hepatic Encephalopathy and Nitrogen Metabolism; MHE: Minimal hepatic encephalopathy; OHE: Overt hepatic encephalopathy; WHC: West Haven Criteria.

Table 2 Diagnostic tools for the diagnosis of minimal hepatic encephalopathy

Test type	Test approach to differentiate MHE form unimpaired subjects
Formal neuropsychological; Assessment	No standard battery for MHE has been designed, but could include test of attention, executive function, psychomotor ability, and speed information processing to evaluate cognition, and mental activity.
Neuropsychological	EEG: Detect changes in cortical cerebral activity; Evoked potentials: Measurement of firing patterns of single cells or cell clusters.
Computerised	SCAN test: Measures speed and accuracy to perform a digit recognition memory task of increasing complexity; CFF: Degree of vigilance; CRT: Relies on the repeated registration of the motor reaction time to auditory stimuli. Measures the stability of the reaction time; Stroop test: Evaluates psychomotor speed and cognitive flexibility; ICT: Test of response inhibition and working memory.
Imaging	MRI: Through mean kurtosis values, evaluates six regions of interest, and amplitude of low frequency fluctuation values, which correlate with PHES values.
Short neuropsychological batteries	PHES: Evaluates cognitive/psychomotor processing speed and visuomotor coordination (NCT-A, NCT-B, SDT, LTT, DST); ANT: Cognitive function related to prefrontal anterior/cortex cortical areas.

ANT: Animal naming test; CFF: Critical flicker frequency; CRT: Continuous reaction time; DST: Digit symbol test; EEG: Electroencephalogram; ICT: Inhibitory control test; LTT: Line tracing test; MHE: Minimal hepatic encephalopathy; MRI: Magnetic resonance imaging; NCT-A: Number connection test A; NCT-B: Number connection test B; PHES: Psychometric hepatic encephalopathy score; SDT: Serial dotting test.

to the circulation[25,26].

Sarcopenia

A low skeletal muscle index (SMI) is found in 58% of patients with liver cirrhosis; however, its prevalence increases to 84% in patients with MHE compared to patients without MHE, of which 17-30% are sarcopenic[7,21,23]. In all, 41% to 49% of patients with MHE show muscle depletion according to a mid-arm muscle circumference (MAMC) and triceps skinfold thickness (TSF) below the 5th percentile and a decreased muscle function[7,21]. Protein malnutrition is associated with MHE; 49% of these patients are undernourished compared to 28-30% of patients without cognitive alterations[7].

SIBO

The concurrence of SIBO is greater in patients with MHE (38.6-65.4%) than in those without neurocognitive deterioration (8.8%-16.13%)[22,25-27].

Dysbiosis

An association of specific bacterial species and HE has been suggested[28,29]. An increase in *Enterobacteriaceae* (*Escherichia coli)* and *Staphylococcus spp.* has been related to MHE, and nearly 22% of patients with cirrhosis and MHE have *H. pylori* infection[20,29].

Inflammatory factors

Interleukin 6 (IL-6), IL-17a, and IL-18 cytokine concentrations are increased in cirrhotic patients with alterations in the psychometric tests but with no clinical manifestations of HE[30-32].

Hyponatremia

In all, 31% of patients with MHE have hyponatremia compared to 16% of patients without neurocognitive alterations[7].

CLINICAL TRIALS FOR TREATMENT OF MHE

There has been a striking increase of studies addressing the treatment of MHE, which is mainly based on the strategies and previous management of OHE. Tables 3-5 evaluate strategies aimed toward achieving greater detoxification and lower production of ammonia.

STRATEGIES TO INCREASE THE DETOXIFICATION OF AMMONIA

Energy-protein intake

The rationale for increasing the caloric and protein intake for the treatment of MHE (30-45 kcal/kg/d and 1.2-1.5 g protein/kg/d, respectively) considers the association of the neurocognitive alterations and sarcopenia in patients with MHE, together with the enhancement of ammonia detoxification by preserving muscle mass and avoiding protein malnutrition[7,33-36]. Only in one of these studies was the protein intake set to provide 1.0-1.5 g/kg/d for the treatment of MHE; this protein was solely of vegetable origin[33].

In patients without previous HE, the reversion of MHE upon normoprotein diet implementation (1.0-1.5 g/kg of ideal body weight/d) was attested to in 57.8% of the patients after 4 wk of dietary treatment. The response rate further increased to 68.4% by week 8; however, neither dietary compliance nor other factors possibly related to the reversion such as amino acid supplementation or use of lactulose and/or antibiotics were assessed. Moreover, results were not compared against a control group[37].

Regarding the effect of ammonia concentration, a dietary intake of at least 1 g vegetable protein/kg/d and 30 kcal/kg/d reported an average change of -6.53 μmol ammonia/L *vs* the basal concentrations after 6 mo of treatment[33]. An average decrease of 20.8 μmol ammonia/L with respect to the basal concentration was achieved with the administration of branched-chain amino acids (0.25 g/kg/d) and a standardized diet (35 kcal/kg/d and 1 g of protein/kg/d); however, patients were also receiving lactulose at the time of the study[34]. These dietary modifications have also improved some of the sarcopenia indicators, such as TSF and handgrip values in patients with MHE receiving dietary counseling; this study does not report what these values were before the study was started[33].

In some studies, a starting diet is standardized according to the minimum energy and protein requirements before the implementation of a specific treatment; however, dietary compliance was not measured in any of the aforementioned studies (Tables 3-5).

L-ornithine-L-aspartate

This compound provides substrates to metabolic pathways involved in the detoxification of ammonia by stimulating both the urea synthesis and glutamine synthesis [38]. The administration of L-ornithine-L-aspartate (LOLA) improved MHE in 35% of the patients, 47% with lactulose, 35% with VSL#3 probiotic, and 10% with a placebo during a 3-mo treatment (Table 3)[39]. The administration of LOLA also showed a 11.42% improvement in the mean critical flicker frequency threshold (CFF) after 8 wk of treatment[40]. Ammonia decreased an average of 8.94% compared to the initial concentrations in patients who were treated with an infusion of LOLA for just 7 d; however, nausea was a side effect in 15% of the patients[41].

Table 3 Published studies using several options for minimal hepatic encephalopathy treatment: Diet, branched-chain amino acids or L-ornithine-L-aspartate supplementation, and probiotics/symbiotics

Ref.	Study type	Follow-up (wk)	MHE diagnosis	Active treatment (s)	Objectives	Patients (*n*)	Main results/impact measures
No history of OHE[1]							
Kato *et al*[37], 2012	Quasi-experimental	8	NCT-A, NCT-B, BDT, DST	30-35 Kcal + 1.0-1.5 g/kg of protein/d[3,4]	Reversal of MHE	19	11/19 (57.8%) recovered at 4 wk, and 13/19 (68.4%) at 8 wk[8]
Maharshi *et al* [33], 2016	Randomized[2]	24	NCT-A/FCT-A, NCT-B/FCT-B, DST, LTT, SDT	Nutritional education/no nutritional therapy[3,5]	Reversal of MHE	60/60	27/38 (71.1%) *vs* 8/35 (22.8%); 27/60 (45%) *vs* 8/60 (13.3%), when considering PPS and ITT analysis.
Malaguarnera *et al*[64], 2008	Randomized, double-blind	13	NCT-A, NCT-B, BDT, SDT, TMT, AVL, EGG	Acetyl-L-carnitine /placebo[5]	Cognitive scores[7]	60/55	Changes of mean values in at least 20.71% to 32.79% respect to basal values[8]
Bajaj *et al*[34], 2014	Randomized, double-blind	8	NCT-A, NCT-B, DST/BDT	LGG/placebo	Psychometric scores[7]	18/19	Improvement from 1.02% to 15.89% from baseline values
Bajaj *et al*[44], 2008	Randomized[2]	8	NCT-A, BDT, DST	Probiotic yogurt/no treatment[4,5]	Reversal of MHE	17/8	ITT analysis: Reversal in 12/17 (70.58%) *vs* 0/7 (0%)
Mittal *et al*[39], 2011	Randomized[2]	12	NCT-A/FCT-A, NCT-B/FCT-B, BDT, PC	Lactulose/VSL#3, LOLA/no treatment	Reversal of MHE	40/40/40/40	ITT analysis: Reversal of 4 (10%) in no treatment group, 19 (47.5%), 14 (35%) and 14 (35%)
Possible history of OHE[1]							
Egberts *et al*[35], 1985	Crossover, double blind	6	EGG, DST, MVT-B	BCAAs/placebo	Psychometry and EGG	11/11	Improvement in psychometric test from 0 to 13.63% respect to basal values in DST[8]
Ndraha *et al* [36], 2011	Double blind, randomized	2	CFF	BCAAs + LOLA/BCAAs[4]	CFF	17/17	Improvement in CFF 7.0% and 1.96% values (Hz), respect to baseline
Kircheis *et al* [41], 1997	Randomized, double-blind	1	NCT-A	LOLA infusion vs Placebo[5]	Psychometry[7]	26/27	Improvement in mean time to respond NCT-A from baseline (29% *vs* 9.73%)
Liu *et al*[29], 2004	Randomized[2]	4	NCT, BAEP	Symbiotics + fermentable fibers/fermentable fibers/placebo[4,5]	Reversal of MHE[7]	20/20/15	Reversal of 50% in symbiotic group, 50% in fermentable fibers group and 13% in placebo. Not statistically significant until compression of treatment groups *vs* placebo ($P = 0.03$)[9]
Dhiman *et al* [65], 2014	Double blind, randomized	24	NCT-A/FCT-A, NCT-B, SDT, DST, LTT	VSL#3/placebo	Psychometric scores[7]	16/13	Mean psychometric scores before and after probiotics -9.9 (-13.3- to -6.5) *vs* -5.7 (-8.4 to 2.9) $P = 0.014$. Proportion of patients with scores < -5 did not change in either group[10]
Malaguarnera *et al*[45], 2007	Randomized, double-blind	17	TMT-A, TMT-B, BDT, MMSE	*Bifidobacterium longum* + FOS/placebo[5]	Psychometry	30/30	No statistical or clinical change was found respect to basal values at, 30, 60, 90 and 120 d
Ziada *et al*[48], 2013	Randomized[2]	4	NCT-A, DST, SDT	Lactulose/L, acidophilus/control[5,6]	Psychometry	24/26/25	Normalization of test occurred in 13/24 (54.2%), 14/26 (53.8%), and 3/25 (12%)

[1]Studies in which the clinical history to the OHE events is specified or no is specified within the inclusion criteria.
[2]Unblinded study.
[3]The effect of other treatments for MHE was not studied.
[4]Treatment compliance was not assessed in one or different group of treatments.
[5]Diet was not standardized together with the suggested treatment.
[6]The treatment that the control group received was not specified.
[7]Secondary objectives within the study.
[8]The analysis was performed within the same group (before–after treatment) despite the study design.
[9]Addition of the effect of two maneuvers.
[10]MHE reversion was not measured. AVL: Auditory verbal learning test; BAEP: Brainstem auditory evoked potential; BCAAs: Branched-chain amino acids; BDT: Block design test; CFF: Critical flicker frequency; DST: Digit symbol test; EEG: Electroencephalogram; FCT-A: Figure connection test A; FCT-B: Figure connection test B; FOS: Fructooligosaccharides; ITT: Intention to treat; LOLA: L-ornithine-L-aspartate; LTT: Line tracing test; MHE: Minimal hepatic encephalopathy; MMSE: Mini mental state examination; MVT-B: Multiple choice vocabulary test B; NCT-A: Number connection test A; NCT-B: Number connection test B; PC: Picture completion; PPS: Per Protocol Analysis Set; SDT: Serial dotting test; TMT: Trail making test; TMT-A: Trail making test A; TMT-B: Trail making test B.

Table 4 Published studies using non-absorbable disaccharides for minimal hepatic encephalopathy treatment

Ref.	Study type	Follow-up (wk)	MHE diagnosis	Active treatment (s)	Objectives	Patients (*n*)	Main results/impact measures
No history of OHE[1]							
Prasad *et al* [47], 2007	Randomized[2]	12	NCT-A/FCT-A, NCT-B/FCT-B, PCT, BDT	Lactulose *vs* no treatment	Psychometry	31/30	ITT analysis: Improvement in 20/31 (64.5%) *vs* 2/30 (6.7%); NNT:2
Horsmans *et al*[55], 1997	Randomized, double-blind	2	NCT, RTT.	Lactulose *vs* lactose as placebo[4,5]	Psychometry	7/7	Improvement in time on Psychometric test on lactulose group respect to basal values.[7] Rate of improvement NCT: 5/7 (71.42%) *vs* 1/7 (14.28%); NNT:2
Sharma *et al* [50], 2008	Randomized[2]	4	NCT-A/FCT-A, NCT-B/FCT-B, P300ERP	Lactulose, probiotics, and lactulose + probiotics[5]	Psychometry, P300ERP[6]	35/35/35	Normalization in 17/31 (54.8%), 16/31 (51.6%), and 17/30 (56.6%) of MHE patients
Morgan *et al* [60], 1989	Cross-over, randomized	8[3]	EEG, NCT, DST, DCT	Lactulose *vs* lactitol[4]	Psychometry	14/14	No differences between treatments in median change in psychometric time or scores
Possible history of OHE[1]							
Dhiman *et al* [51], 2000	Randomized[2]	12	NCT-A/FCT-A, NCT-B /FCT-B, PCT, BDT.	Lactulose *vs* no lactulose[4]	MHE improvement	14/12	Improvement in 8/10 (80.0%) *vs* 0/8 (0.0%), $P < 0.001$[8]
Wang *et al* [49], 2019	Randomized[2]	8	NCT-A, DST	Lactulose *vs* no lactulose	MHE reversal	67/31	ITT analysis: 43/67 (64.2%) *vs* 7/31 (22.6%); NNT: 3PPS: 41/59 (69.5%) *vs* 6/28 (21.4%); NNT: 2

[1]Studies in which the inclusion criteria comprise patients with clinical history to the overt hepatic encephalopathy events.
[2]Unblinded study.
[3]Washout period of 4-6 wk between treatments.
[4]Compliance to treatment was not measured in one or the different treatment groups.
[5]Diet was not standardized according to the suggested treatment.
[6]Secondary objectives within the study.
[7]Analysis was performed within the same group (before–after treatment) despite the experimental design.
[8]Minimal hepatic encephalopathy reversion not measured. BDT: Block design test; DCT: Digit copying test; DST: Digit symbol test; EEG: Electroencephalogram; FCT-A: Figure connection test A; FCT-B: Figure connection test B; ITT: Intention to treat; MHE: Minimal hepatic encephalopathy; NCT: Number connection test; NCT-A: Number connection test A; NCT-B: Number connection test B; NNT: Number needed to treat; OHE: Overt hepatic encephalopathy; PCT: Picture completion test; PPS: Per protocol analysis set; RTT: Race track test.

STRATEGIES TO REDUCE THE PRODUCTION OF AMMONIA

Fiber supplementation

Dietary fiber as a prebiotic is a non-digestible food component that has a positive effect

Table 5 Published studies using antibiotics for minimal hepatic encephalopathy treatment

Ref.	Study type	Follow-up (wk)	MHE diagnosis	Active treatment (s)	Objectives	Patients (*n*)	Main results/impact measures
No history of OHE[1]							
Ahluwalia *et al*[66], 2014	Quasi-experimental	8	NCT-A, NCT-B, DST, BDS, LTT, SDT, ICT	Rifaximin[4]	fMRI, ICT, MRS[5]	20	Changes in ICT, improvement of 12% respect to baseline, indicating a better cognition
Bajaj *et al* [61], 2013	Quasi-experimental	8	NCT-A, NCT-B, DST, BDT, LTT	Rifaximin[4]	Psychometry[5]	20	Improvement in NCT-A time (11.8%), NCT-B time (11.8%), DST raw score (9.1%), BDT raw score (0.0%), LTT time (20.7%), LTT errors (39.8%), SDT time (12.3%) from basal values
Bajaj *et al* [67], 2011	Randomized, single-blinded	8	NCT-A, DST, BDT, ICT	Rifaximin/placebo[4]	Driving performance, psychometry scores	21/21	Decrease of 46.6% of total errors respect to baseline in rifaximin group ($P < 0.001$)[6]. Improvement in NCT-A 91% *vs* 61% (NNT: 4); NCT-B: 81% *vs* 33% (NNT: 2); and ICT lures: 76% *vs* 43% (NNT: 3)[7]
Sidhu *et al* [59], 2015	Randomized, non-inferiority trial	12	NCT-A, FCT-A, DST, PCT, and BDT	Rifaximin/lactulose	Reversal of MHE	57/55	ITT analysis shows a reversal at 2 wk: lactulose 40.0% *vs* rifaximin 52.63% (NNT: 8). ITT analysis at 3 mo shows reversal in 69.1% and 73.7% of lactulose and rifaximin, (NNT: 22)
Goyal *et al* [62], 2017	Prospective cohort	24	NCT-A, FCT-A, DST, PCT, BDT	Previous intake of Rifaximin compared to lactulose[3,4]	Maintenance of remission for MHE	42/38	Still free of MHE: Rifaximin 42.8% *vs* lactulose 50.0% (NNT: 14)
Possible history of OHE[1]							
Agrawal *et al*[24], 2011	Quasi-experimental	1	NCT, FTC, LTT.	Clarithromycin, lansoprazole, tinidazole[3,4]	Psychometric scores[5]	35	Improvement in 12.7%, 13.3%, and 18.7% respect to basal mean time in NCT, FCT and LTT, respectively
Zhang *et al* [27], 2015	Quasi-experimental	5	NCT-A, NCT-B, DST	Rifaximin 1 wk[3,4]	Reversal of MHE	26	After a week, reversal present in 11/26 (42.3%)
Sidhu *et al* [56], 2011	Double-blind, randomized	8	NCT-A/FCT-A, DST, PCT, BDT	Rifaximin/placebo	Reversal of MHE	49/45	Reversal at 2 wk: 57% *vs* 18% (NNT: 3)At 8 wk: Reversal of 75.5% *vs* 20% (NNT: 2)
Sharma *et al*[40], 2014	Randomized[2]	8	NCT-A/FCT-A, DST and/ or CFF	LOLA/rifaximin/Probiotics/Placebo[4]	Reversal of MHE[5]	31/31/32/30	ITT analysis: Improvement in CFF values (Hz) from baseline in 11.42%, 6.5%, 8.68%, and 2.28%

[1]Studies in which the inclusion criteria comprise patient with clinical history to the overt hepatic encephalopathy events.
[2]Unblinded study.
[3]Compliance to treatment was not measured in one or the different treatment groups.
[4]Diet was not standardized according to the suggested treatment.
[5]Secondary objectives within the study.
[6]Analysis was performed within the same group (before–after treatment) despite the experimental design.

[7]Minimal hepatic encephalopathy reversion not measured. BDT: Block design test; CFF: Critical flicker frequency; DST: Digit symbol test; FCT: Figure connection test; FCT-A: Figure connection test A; ICT: Inhibitory Control Test; ITT: Intention to treat: LTT: Line tracing test; MHE: Minimal hepatic encephalopathy; NCT: Number connection test; NCT-A: Number connection test A; NCT-B: Number connection test B; NNT: Number Needed to Treat; OHE: Overt hepatic encephalopathy; PCT: Picture completion test; SDT: Serial dotting test.

by stimulating the growth and activity of the colon bacteria. The addition of insoluble fiber may potentiate the effects of disaccharides through acidification of the colonic lumen by the process of fermentation, which in turn, decreases the production of ammonia[42]. The increase in dietary fiber has been proposed as an alternative for the treatment of patients with MHE (Table 3); reversion of MHE has been reported in 50% of patients treated with symbiotics and fermentable fibers or simply fermentable fibers and in only 13% of patients treated with placebo[29].

Probiotics/symbiotics

The beneficial effects of the administration of probiotics and symbiotics on different variables related to MHE have been confirmed (Table 3). The mechanisms of action include displacement of gas-forming bacteria, inhibition of pathogenic bacteria adhesion, modulation of the immune response, and microbiota modification through the colonic acidification following fiber fermentation[43]. The colonization of the intestine by probiotics that compete against ammonia-producing microorganisms and the concomitant use of probiotics and prebiotics (symbiotics) may cause synergistic effects on the host by decreasing the colonic pH *via* fermentation and less colonization of ammonia-producing bacteria, resulting in decreased ammonemia[29].

Following a 60-d trial of consuming a probiotic yogurt, 70.58% of the patients with MHE reversed this condition, and none did in the control group. Despite having assessed treatment compliance in the probiotic yogurt group, this was not done in the control group nor were other dietary components controlled that could have affected the results[44]. The administration of VSL#3 led to reversion of MHE in 35% of patients and 10% in the placebo group after 3 mo of treatment[40]; after 8 wk of treatment, normalization of the CFF values were achieved in 8.68% of patients in the treatment group *vs* 2.28% in the placebo group[40].

The supplementation of symbiotics (*Pediacoccus pentoseceus* 5-33:3, *Leuconostoc mesenteroides* 32-77:1, *Lactobacillus paracasei* subspecies *paracasei* 19, and *Lactobacillus plantarum* 2592, and fermentable fiber; inulin, pectin, beta glucan, and resistant starch) for 1 mo decreased the mean ammonia 36.1% with respect to the mean basal concentrations, while treatment with fermentable fibers decreased this mean 34.7% in the same period; placebos only decreased this mean concentration in 3.14%[29]. Furthermore, when the basal values of endotoxins were considered in these three groups, a mean reduction was achieved in 24.7%, 38.89%, and 4.5%, respectively[29]. Similar results could be observed after 90 d of treatment with *Bifidobacterium longum* and fructooligosaccharides, where the mean decrease in ammonemia was 42.69% with respect to the basal concentration and 10.44% in the placebo group[45].

The importance of treating MHE underscores the possibility of avoiding a future OHE episode. In patients with liver cirrhosis without previous HE episodes and treated prophylactically with VSL#3 for a 3-mo period, the number needed to treat (NNT) for the group of patients with MHE was 5 *vs* 13 in those without MHE to avoid an OHE episode[46].

Non-absorbable disaccharides

In most of the studies on patients with MHE, the effects of lactulose have also been assessed (Table 4). The mechanisms of action of this synthetic disaccharide are mainly related to its cathartic effects, which allow for the reduction of intestinal transit, and therefore, the content of toxic compounds in its lumen and absorption. Along its colonic transit, lactulose is metabolized by bacteria, decreasing the colonic pH. This in turn, renders a hostile environment to urease-producing bacteria together with the increased growth of *Lactobacillae* and *Bifidobacteriae*, which reduce ammonia production in the colonic lumen and interfere with its absorption and that of other nitrogenous compounds produced in the intestine[45,47].

A 4-wk treatment with lactulose enables reversion of MHE in 54.2% of patients compared to 12% in the control group[48]; after 60 d of treatment, the reported reversion increased up to 69.5% in the lactulose group *vs* 21.4% in the placebo group in a per protocol analysis set[49]. Reversion after a 3-mo treatment with lactulose is estimated at 47.5% and 10% in the group receiving the placebo[39].

The treatment of MHE with lactulose also decreases the mean ammonia concentration an approximate 23.03%-32.25% after 1 mo of treatment and 28.66% with the concomitant use of probiotics and lactulose[48,50]. In another study where patients with MHE were followed for 2 mo, the mean decrease of ammonia was 22.1% considering the basal concentrations of the treated group; there was also an increase in 10.46% in the placebo group, $P < 0.001$[51]. Treatment of lactulose for 3 mo achieved a decrease between 23% and 79% considering the basal ammonia concentrations, rendering an average decrease of 63.16 μmol/L[49,52-54]. When the decrease was analyzed as a categoric variable, the improvement rate in the ammonia concentration in 14 patients was attested to in 6/7 of them (85.7%) when lactulose was administered *vs* 4/7 (57.4%) when patients were treated with lactose[55]. Another change observed after administration of lactulose in patients suffering from MHE has been the decrease in mean basal concentrations of inflammatory factors such as tumor necrosis factor alpha (17.29%), IL-6 (29.54%), IL-18 (32.14%), and endotoxins (36.76%) after a 3-mo treatment[52].

After the use of lactulose for 3 mo, positive changes in some variables related to sleeping such as total sleeping time, sleep efficiency, sleep latency, awake time, and latency rapid eye movement were found[54]. According to some tools that measure QOL such as the sickness impact profile, there is improvement in the overall score and within each of the categories after lactulose administration; however, the study does not describe if any of the patients had alterations in their QOL at the time of being included in the study[49,56].

When endpoints such as the prevention of an OHE episode were considered, patients with liver cirrhosis and no previous HE episode who were treated prophylactically with lactulose for a year showed a decreased incidence; however, the impact of the treatment was greater in those having MHE (NNT in no MHE patients: 50; NNT in MHE patients: 5)[57].

Certain characteristics of patients with MHE lead to lack of response to lactulose treatment. These include hyponatremia, which is prevalent among 50% of the non-responders, and a high basal ammonia concentration[58].

Some alterations that may be detected at the beginning of the treatment with lactulose are diarrhea, flatulence, abdominal bloating, distaste for lactulose, and nausea[11,48,55,59,60]. Diarrhea and flatulence may be reduced after adjusting the lactulose dose[60].

Non-absorbable antibiotics

Rifaximin is a non-absorbable antibiotic that modifies the abundance of bacteria and the intestinal bacterial metabolism. The administration of rifaximin in patients with MHE (Table 5) improves consciousness and decreases the degree of endotoxemia[61]. Rifaximin further reduces ammonemia and improves the psychometric tests more than non-absorbable disaccharides[59].

Use of rifaximin for 2 and 12 wk led to reversion of HE in 52.63% and 73.7%, respectively, while the group treated with lactulose during the same time period reverted it in 40.0% and 69.1% of cases[39]. Similar results were reported with rifaximin as a therapy in a different population of patients with MHE after 2 wk of follow-up. Reversion was reached in 57% of the patients, and when the follow-up was extended to 8 wk, reversion was observed in 75.5%. The group without this treatment reverted HE in 18% and 20% of the patients after 2 and 8 wk, respectively[56]. Response to an 8-wk treatment according to the normalization of the CFF values happened in 6.5% of the patients who underwent treatment *vs* 2.28% in patients included in the placebo group[40].

In a study including subjects with SIBO and MHE, a reversion was found in 42.3% of these patients after a week of rifaximin treatment; however, this result was not contrasted against a control group[27]. A reduction in the mean ammonemia of 24.22% with respect to the basal concentrations was found in a population with similar characteristics, while in patients with MHE and no SIBO there was a reversion in 18.5% after a week of treatment[27]. In yet another study in subjects where the SIBO status was not specified, ammonemia following an 8-wk treatment with rifaximin decreased 7% [61]. A quantitative improvement in terms of scores for QOL were also observed in patients with MHE treated with rifaximin[49,56].

Some studies that evaluated specific endpoints following the suspension of rifaximin and lactulose in patients originally treated for MHE did not find a difference on the rate of OHE development (7.14% *vs* 7.89%)[56,59,62].

Until now, the use of rifaximin has been associated with epigastric discomfort and vomiting in a few patients[56].

DISCUSSION

Prevention and treatment of MHE have been the subjects of many clinical trials. However, the lack of a standard of reference for its diagnosis, the impossibility of observing the impact of a simple maneuver over the reversion of MHE or surrogate variables, and the inclusion of multiple endpoint variables to evaluate the effects of these therapies represent challenges for their overall analysis[63]. Thus, result interpretation of each one of the studies should consider the diagnostic tool that was used, the heterogeneity of the patients regarding their demographic data, the level of schooling, history of previous decompensation, previous episodes of encephalopathy, the hepatic functional reserve, the dietary plan or the indication of concurrent treatments accompanying the experimental treatment, as well as the risk factors that predispose to MHE, such as hyponatremia, sarcopenia, hyperammonemia, the presence of SIBO or dysbiosis, because each one of these factors may potentially modify the novel treatment outcome[7,19,21,23,58].

Regarding the outcome, the assessment of the MHE reversion or the incidence of OHE, when performed through standardized tests, may report clinical utility. However, when results are referred as surrogate variables, such as the improvement in raw values of some psychometric tests, the decrease in blood ammonia concentrations, the changes in the prevalence of SIBO or qualitative data on microbiota in patients with MHE, the real impact on these variables are difficult to interpret. To date, few studies include outcomes related to changes in QOL and mortality.

CONCLUSION

There are currently different alternatives for the treatment of MHE. Prevention should probably be based on diets that provide adequate energy, protein, dietary fiber, and food sources for probiotics to enhance their therapeutic potential without causing the side effects of pharmacological therapies and avoiding the development of sarcopenia. The treatment for the reversion of MHE and its secondary prevention should be tailored for each individual and use pharmacological options of proven efficacy such as lactulose or rifaximin. The implementation of randomized clinical trials that establish the optimal duration of the treatment and its impact on the QOL and job performance as well as the analysis of the cost-benefit of every treatment for MHE are warranted.

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REFERENCES

1 **Amodio P**, Campagna F, Olianas S, Iannizzi P, Mapelli D, Penzo M, Angeli P, Gatta A. Detection of minimal hepatic encephalopathy: normalization and optimization of the Psychometric Hepatic Encephalopathy Score. A neuropsychological and quantified EEG study. *J Hepatol* 2008; **49**: 346-353 [PMID: 18602716 DOI: 10.1016/j.jhep.2008.04.022]

2 **Romero Gómez M**, Córdoba J, Jover R, del Olmo J, Fernández A, Flavià M, Compañy L, Poveda MJ, Felipo V; Red Nacional de Investigación en Encefalopatía Hepática. [Normality tables in the Spanish population for psychometric tests used in the diagnosis of minimal hepatic encephalopathy]. *Med Clin (Barc)* 2006; **127**: 246-249 [PMID: 16942726 DOI: 10.1157/13091264]

3 **Weissenborn K**, Ennen JC, Schomerus H, Rückert N, Hecker H. Neuropsychological characterization of hepatic encephalopathy. *J Hepatol* 2001; **34**: 768-773 [PMID: 11434627 DOI: 10.1016/S0168-8278(01)00026-5]

4 **Sharma P**, Sharma BC, Puri V, Sarin SK. Critical flicker frequency: diagnostic tool for minimal hepatic encephalopathy. *J Hepatol* 2007; **47**: 67-73 [PMID: 17459511 DOI: 10.1016/j.jhep.2007.02.022]

5 **Romero-Gómez M**, Córdoba J, Jover R, del Olmo JA, Ramírez M, Rey R, de Madaria E, Montoliu C, Nuñez D, Flavia M, Compañy L, Rodrigo JM, Felipo V. Value of the critical flicker frequency in

patients with minimal hepatic encephalopathy. *Hepatology* 2007; **45**: 879-885 [PMID: 17393525 DOI: 10.1002/hep.21586]

6 **Mina A**, Moran S, Ortiz-Olvera N, Mera R, Uribe M. Prevalence of minimal hepatic encephalopathy and quality of life in patients with decompensated cirrhosis. *Hepatol Res* 2014; **44**: E92-E99 [PMID: 24033755 DOI: 10.1111/hepr.12227]

7 **Merli M**, Giusto M, Lucidi C, Giannelli V, Pentassuglio I, Di Gregorio V, Lattanzi B, Riggio O. Muscle depletion increases the risk of overt and minimal hepatic encephalopathy: results of a prospective study. *Metab Brain Dis* 2013; **28**: 281-284 [PMID: 23224378 DOI: 10.1007/s11011-012-9365-z]

8 **Duarte-Rojo A**, Estradas J, Hernández-Ramos R, Ponce-de-León S, Córdoba J, Torre A. Validation of the psychometric hepatic encephalopathy score (PHES) for identifying patients with minimal hepatic encephalopathy. *Dig Dis Sci* 2011; **56**: 3014-3023 [PMID: 21461913 DOI: 10.1007/s10620-011-1684-0]

9 **Sharma P**, Sharma BC, Agrawal A, Sarin SK. Primary prophylaxis of overt hepatic encephalopathy in patients with cirrhosis: an open labeled randomized controlled trial of lactulose versus no lactulose. *J Gastroenterol Hepatol* 2012; **27**: 1329-1335 [PMID: 22606978 DOI: 10.1111/j.1440-1746.2012.07186.x]

10 **Les I**, Doval E, García-Martínez R, Planas M, Cárdenas G, Gómez P, Flavià M, Jacas C, Mínguez B, Vergara M, Soriano G, Vila C, Esteban R, Córdoba J. Effects of branched-chain amino acids supplementation in patients with cirrhosis and a previous episode of hepatic encephalopathy: a randomized study. *Am J Gastroenterol* 2011; **106**: 1081-1088 [PMID: 21326220 DOI: 10.1038/ajg.2011.9]

11 **Sharma BC**, Sharma P, Agrawal A, Sarin SK. Secondary prophylaxis of hepatic encephalopathy: an open-label randomized controlled trial of lactulose versus placebo. *Gastroenterology* 2009; **137**: 885-891, 891. e1 [PMID: 19501587 DOI: 10.1053/j.gastro.2009.05.056]

12 **Hartmann IJ**, Groeneweg M, Quero JC, Beijeman SJ, de Man RA, Hop WC, Schalm SW. The prognostic significance of subclinical hepatic encephalopathy. *Am J Gastroenterol* 2000; **95**: 2029-2034 [PMID: 10950053 DOI: 10.1111/j.1572-0241.2000.02265.x]

13 **Bajaj JS**, Saeian K, Schubert CM, Hafeezullah M, Franco J, Varma RR, Gibson DP, Hoffmann RG, Stravitz RT, Heuman DM, Sterling RK, Shiffman M, Topaz A, Boyett S, Bell D, Sanyal AJ. Minimal hepatic encephalopathy is associated with motor vehicle crashes: the reality beyond the driving test. *Hepatology* 2009; **50**: 1175-1183 [PMID: 19670416 DOI: 10.1002/hep.23128]

14 **Bajaj JS**, Pinkerton SD, Sanyal AJ, Heuman DM. Diagnosis and treatment of minimal hepatic encephalopathy to prevent motor vehicle accidents: a cost-effectiveness analysis. *Hepatology* 2012; **55**: 1164-1171 [PMID: 22135042 DOI: 10.1002/hep.25507]

15 **Ortiz M**, Jacas C, Córdoba J. Minimal hepatic encephalopathy: diagnosis, clinical significance and recommendations. *J Hepatol* 2005; **42** Suppl: S45-S53 [PMID: 15777572 DOI: 10.1016/j.jhep.2004.11.028]

16 **Rose CF**, Amodio P, Bajaj JS, Dhiman RK, Montagnese S, Taylor-Robinson SD, Vilstrup H, Jalan R. Hepatic encephalopathy: Novel insights into classification, pathophysiology and therapy. *J Hepatol* 2020; **73**: 1526-1547 [PMID: 33097308 DOI: 10.1016/j.jhep.2020.07.013]

17 **Han W**, Zhang H, Han Y, Duan Z. Cognition-tracking-based strategies for diagnosis and treatment of minimal hepatic encephalopathy. *Metab Brain Dis* 2020; **35**: 869-881 [PMID: 32495311 DOI: 10.1007/s11011-020-00539-w]

18 **Lockwood AH**, Yap EW, Wong WH. Cerebral ammonia metabolism in patients with severe liver disease and minimal hepatic encephalopathy. *J Cereb Blood Flow Metab* 1991; **11**: 337-341 [PMID: 1997506 DOI: 10.1038/jcbfm.1991.67]

19 **Sharma P**, Sharma BC. Predictors of minimal hepatic encephalopathy in patients with cirrhosis. *Saudi J Gastroenterol* 2010; **16**: 181-187 [PMID: 20616413 DOI: 10.4103/1319-3767.65189]

20 **Schulz C**, Schütte K, Reisener N, Voss J, Malfertheiner P. Prevalence of Helicobacter pylori Infection in Patients with Minimal Hepatic Encephalopathy. *J Gastrointestin Liver Dis* 2016; **25**: 191-195 [PMID: 27308650 DOI: 10.15403/jgld.2014.1121.252.hpy]

21 **Hanai T**, Shiraki M, Watanabe S, Kochi T, Imai K, Suetsugu A, Takai K, Moriwaki H, Shimizu M. Sarcopenia predicts minimal hepatic encephalopathy in patients with liver cirrhosis. *Hepatol Res* 2017; **47**: 1359-1367 [PMID: 28199774 DOI: 10.1111/hepr.12873]

22 **Zhang Y**, Feng Y, Cao B, Tian Q. The effect of small intestinal bacterial overgrowth on minimal hepatic encephalopathy in patients with cirrhosis. *Arch Med Sci* 2016; **12**: 592-596 [PMID: 27279853 DOI: 10.5114/aoms.2015.55675]

23 **Nardelli S**, Lattanzi B, Merli M, Farcomeni A, Gioia S, Ridola L, Riggio O. Muscle Alterations Are Associated With Minimal and Overt Hepatic Encephalopathy in Patients With Liver Cirrhosis. *Hepatology* 2019; **70**: 1704-1713 [PMID: 31038758 DOI: 10.1002/hep.30692]

24 **Agrawal A**, Gupta A, Chandra M, Koowar S. Role of Helicobacter pylori infection in the pathogenesis of minimal hepatic encephalopathy and effect of its eradication. *Indian J Gastroenterol* 2011; **30**: 29-32 [PMID: 21416318 DOI: 10.1007/s12664-011-0087-7]

25 **Gupta A**, Dhiman RK, Kumari S, Rana S, Agarwal R, Duseja A, Chawla Y. Role of small intestinal bacterial overgrowth and delayed gastrointestinal transit time in cirrhotic patients with minimal hepatic encephalopathy. *J Hepatol* 2010; **53**: 849-855 [PMID: 20675008 DOI: 10.1016/j.jhep.2010.05.017]

26 **Lunia MK**, Sharma BC, Sachdeva S. Small intestinal bacterial overgrowth and delayed orocecal

transit time in patients with cirrhosis and low-grade hepatic encephalopathy. *Hepatol Int* 2013; **7**: 268-273 [PMID: 26201641 DOI: 10.1007/s12072-012-9360-9]

27 **Zhang Y**, Feng Y, Cao B, Tian Q. Effects of SIBO and rifaximin therapy on MHE caused by hepatic cirrhosis. *Int J Clin Exp Med* 2015; **8**: 2954-2957 [PMID: 25932262]

28 **Bajaj JS**, Torre A, Fagan A, Rojas LM, Gavis E, De Leon O, Fuchs M, Escalona NI, White MB, Sikaroodi M, Gillevet PM. Gut microbiota are associated with minimal hepatic encephalopathy (MHE) in cirrhosis regardless of country of origin. *Gastroenterology* 2020; **158**: S-1352 [DOI: 10.1016/S0016-5085(20)34051-8]

29 **Liu Q**, Duan ZP, Ha DK, Bengmark S, Kurtovic J, Riordan SM. Synbiotic modulation of gut flora: effect on minimal hepatic encephalopathy in patients with cirrhosis. *Hepatology* 2004; **39**: 1441-1449 [PMID: 15122774 DOI: 10.1002/hep.20194]

30 **Shawcross DL**, Wright G, Olde Damink SW, Jalan R. Role of ammonia and inflammation in minimal hepatic encephalopathy. *Metab Brain Dis* 2007; **22**: 125-138 [PMID: 17260161 DOI: 10.1007/s11011-006-9042-1]

31 **Li W**, Li N, Wang R, Li Q, Wu H. Interferon gamma, interleukin-6, and -17a levels were correlated with minimal hepatic encephalopathy in HBV patients. *Hepatol Int* 2015; **9**: 218-223 [PMID: 25794550 DOI: 10.1007/s12072-015-9610-8]

32 **Montoliu C**, Piedrafita B, Serra MA, del Olmo JA, Urios A, Rodrigo JM, Felipo V. IL-6 and IL-18 in blood may discriminate cirrhotic patients with and without minimal hepatic encephalopathy. *J Clin Gastroenterol* 2009; **43**: 272-279 [PMID: 18562979 DOI: 10.1097/MCG.0b013e31815e7f58]

33 **Maharshi S**, Sharma BC, Sachdeva S, Srivastava S, Sharma P. Efficacy of Nutritional Therapy for Patients With Cirrhosis and Minimal Hepatic Encephalopathy in a Randomized Trial. *Clin Gastroenterol Hepatol* 2016; **14**: 454-460. quiz e33 [PMID: 26453952 DOI: 10.1016/j.cgh.2015.09.028]

34 **Bajaj JS**, Heuman DM, Hylemon PB, Sanyal AJ, Puri P, Sterling RK, Luketic V, Stravitz RT, Siddiqui MS, Fuchs M, Thacker LR, Wade JB, Daita K, Sistrun S, White MB, Noble NA, Thorpe C, Kakiyama G, Pandak WM, Sikaroodi M, Gillevet PM. Randomised clinical trial: Lactobacillus GG modulates gut microbiome, metabolome and endotoxemia in patients with cirrhosis. *Aliment Pharmacol Ther* 2014; **39**: 1113-1125 [PMID: 24628464 DOI: 10.1111/apt.12695]

35 **Egberts EH**, Schomerus H, Hamster W, Jürgens P. Branched chain amino acids in the treatment of latent portosystemic encephalopathy. A double-blind placebo-controlled crossover study. *Gastroenterology* 1985; **88**: 887-895 [PMID: 3882509 DOI: 10.1016/s0016-5085(85)80004-4]

36 **Ndraha S**, Hasan I, Simadibrata M. The effect of L-ornithine L-aspartate and branch chain amino acids on encephalopathy and nutritional status in liver cirrhosis with malnutrition. *Acta Med Indones* 2011; **43**: 18-22 [PMID: 21339541]

37 **Kato A**, Tanaka H, Kawaguchi T, Kanazawa H, Iwasa M, Sakaida I, Moriwaki H, Murawaki Y, Suzuki K, Okita K. Nutritional management contributes to improvement in minimal hepatic encephalopathy and quality of life in patients with liver cirrhosis: A preliminary, prospective, open-label study. *Hepatol Res* 2013; **43**: 452-458 [PMID: 22994429 DOI: 10.1111/j.1872-034X.2012.01092.x]

38 **Chatauret N**, Butterworth RF. Effects of liver failure on inter-organ trafficking of ammonia: implications for the treatment of hepatic encephalopathy. *J Gastroenterol Hepatol* 2004; **19**: S219-S223 [DOI: 10.1111/j.1440-1746.2004.03648.x]

39 **Mittal VV**, Sharma BC, Sharma P, Sarin SK. A randomized controlled trial comparing lactulose, probiotics, and L-ornithine L-aspartate in treatment of minimal hepatic encephalopathy. *Eur J Gastroenterol Hepatol* 2011; **23**: 725-732 [PMID: 21646910 DOI: 10.1097/MEG.0b013e32834696f5]

40 **Sharma K**, Pant S, Misra S, Dwivedi M, Misra A, Narang S, Tewari R, Bhadoria AS. Effect of rifaximin, probiotics, and l-ornithine l-aspartate on minimal hepatic encephalopathy: a randomized controlled trial. *Saudi J Gastroenterol* 2014; **20**: 225-232 [PMID: 25038208 DOI: 10.4103/1319-3767.136975]

41 **Kircheis G**, Nilius R, Held C, Berndt H, Buchner M, Görtelmeyer R, Hendricks R, Krüger B, Kuklinski B, Meister H, Otto HJ, Rink C, Rösch W, Stauch S. Therapeutic efficacy of L-ornithine-L-aspartate infusions in patients with cirrhosis and hepatic encephalopathy: results of a placebo-controlled, double-blind study. *Hepatology* 1997; **25**: 1351-1360 [PMID: 9185752 DOI: 10.1002/hep.510250609]

42 **Douglas LC**, Sanders ME. Probiotics and prebiotics in dietetics practice. *J Am Diet Assoc* 2008; **108**: 510-521 [PMID: 18313433 DOI: 10.1016/j.jada.2007.12.009]

43 **Rai R**, Saraswat VA, Dhiman RK. Gut microbiota: its role in hepatic encephalopathy. *J Clin Exp Hepatol* 2015; **5**: S29-S36 [PMID: 26041954 DOI: 10.1016/j.jceh.2014.12.003]

44 **Bajaj JS**, Saeian K, Christensen KM, Hafeezullah M, Varma RR, Franco J, Pleuss JA, Krakower G, Hoffmann RG, Binion DG. Probiotic yogurt for the treatment of minimal hepatic encephalopathy. *Am J Gastroenterol* 2008; **103**: 1707-1715 [PMID: 18691193 DOI: 10.1111/j.1572-0241.2008.01861.x]

45 **Malaguarnera M**, Greco F, Barone G, Gargante MP, Malaguarnera M, Toscano MA. Bifidobacterium longum with fructo-oligosaccharide (FOS) treatment in minimal hepatic encephalopathy: a randomized, double-blind, placebo-controlled study. *Dig Dis Sci* 2007; **52**: 3259-3265 [PMID: 17393330 DOI: 10.1007/s10620-006-9687-y]

46 **Lunia MK**, Sharma BC, Sharma P, Sachdeva S, Srivastava S. Probiotics prevent hepatic encephalopathy in patients with cirrhosis: a randomized controlled trial. *Clin Gastroenterol Hepatol*

2014; **12**: 1003-8. e1 [PMID: 24246768 DOI: 10.1016/j.cgh.2013.11.006]
47 **Prasad S**, Dhiman RK, Duseja A, Chawla YK, Sharma A, Agarwal R. Lactulose improves cognitive functions and health-related quality of life in patients with cirrhosis who have minimal hepatic encephalopathy. *Hepatology* 2007; **45**: 549-559 [PMID: 17326150 DOI: 10.1002/hep.21533]
48 **Ziada DH**, Soliman HH, El Yamany SA, Hamisa MF, Hasan AM. Can Lactobacillus acidophilus improve minimal hepatic encephalopathy? *Arab J Gastroenterol* 2013; **14**: 116-122 [PMID: 24206740 DOI: 10.1016/j.ajg.2013.08.002]
49 **Wang JY**, Bajaj JS, Wang JB, Shang J, Zhou XM, Guo XL, Zhu X, Meng LN, Jiang HX, Mi YQ, Xu JM, Yang JH, Wang BS, Zhang NP. Lactulose improves cognition, quality of life, and gut microbiota in minimal hepatic encephalopathy: A multicenter, randomized controlled trial. *J Dig Dis* 2019; **20**: 547-556 [PMID: 31448533 DOI: 10.1111/1751-2980.12816]
50 **Sharma P**, Sharma BC, Puri V, Sarin SK. An open-label randomized controlled trial of lactulose and probiotics in the treatment of minimal hepatic encephalopathy. *Eur J Gastroenterol Hepatol* 2008; **20**: 506-511 [PMID: 18467909 DOI: 10.1097/MEG.0b013e3282f3e6f5]
51 **Dhiman RK**, Sawhney MS, Chawla YK, Das G, Ram S, Dilawari JB. Efficacy of lactulose in cirrhotic patients with subclinical hepatic encephalopathy. *Dig Dis Sci* 2000; **45**: 1549-1552 [PMID: 11007104 DOI: 10.1023/a:1005556826152]
52 **Jain L**, Sharma BC, Srivastava S, Puri SK, Sharma P, Sarin S. Serum endotoxin, inflammatory mediators, and magnetic resonance spectroscopy before and after treatment in patients with minimal hepatic encephalopathy. *J Gastroenterol Hepatol* 2013; **28**: 1187-1193 [PMID: 23425082 DOI: 10.1111/jgh.12160]
53 **Singh J**, Sharma BC, Maharshi S, Puri V, Srivastava S. Spectral electroencephalogram in liver cirrhosis with minimal hepatic encephalopathy before and after lactulose therapy. *J Gastroenterol Hepatol* 2016; **31**: 1203-1209 [PMID: 26716736 DOI: 10.1111/jgh.13283]
54 **Singh J**, Sharma BC, Puri V, Sachdeva S, Srivastava S. Sleep disturbances in patients of liver cirrhosis with minimal hepatic encephalopathy before and after lactulose therapy. *Metab Brain Dis* 2017; **32**: 595-605 [PMID: 28070704 DOI: 10.1007/s11011-016-9944-5]
55 **Horsmans Y**, Solbreux PM, Daenens C, Desager JP, Geubel AP. Lactulose improves psychometric testing in cirrhotic patients with subclinical encephalopathy. *Aliment Pharmacol Ther* 1997; **11**: 165-170 [PMID: 9042989 DOI: 10.1046/j.1365-2036.1997.118289000.x]
56 **Sidhu SS**, Goyal O, Mishra BP, Sood A, Chhina RS, Soni RK. Rifaximin improves psychometric performance and health-related quality of life in patients with minimal hepatic encephalopathy (the RIME Trial). *Am J Gastroenterol* 2011; **106**: 307-316 [PMID: 21157444 DOI: 10.1038/ajg.2010.455]
57 **Agrawal A**, Sharma BC, Sharma P, Sarin SK. Secondary prophylaxis of hepatic encephalopathy in cirrhosis: an open-label, randomized controlled trial of lactulose, probiotics, and no therapy. *Am J Gastroenterol* 2012; **107**: 1043-1050 [PMID: 22710579 DOI: 10.1038/ajg.2012.113]
58 **Sharma P**, Sharma BC, Sarin SK. Predictors of nonresponse to lactulose for minimal hepatic encephalopathy in patients with cirrhosis. *Liver Int* 2009; **29**: 1365-1371 [PMID: 19555401 DOI: 10.1111/j.1478-3231.2009.02067.x]
59 **Sidhu SS**, Goyal O, Parker RA, Kishore H, Sood A. Rifaximin vs. lactulose in treatment of minimal hepatic encephalopathy. *Liver Int* 2016; **36**: 378-385 [PMID: 26201713 DOI: 10.1111/liv.12921]
60 **Morgan MY**, Alonso M, Stanger LC. Lactitol and lactulose for the treatment of subclinical hepatic encephalopathy in cirrhotic patients. A randomised, cross-over study. *J Hepatol* 1989; **8**: 208-217 [PMID: 2654285 DOI: 10.1016/0168-8278(89)90009-3]
61 **Bajaj JS**, Heuman DM, Sanyal AJ, Hylemon PB, Sterling RK, Stravitz RT, Fuchs M, Ridlon JM, Daita K, Monteith P, Noble NA, White MB, Fisher A, Sikaroodi M, Rangwala H, Gillevet PM. Modulation of the metabiome by rifaximin in patients with cirrhosis and minimal hepatic encephalopathy. *PLoS One* 2013; **8**: e60042 [PMID: 23565181 DOI: 10.1371/journal.pone.0060042]
62 **Goyal O**, Sidhu SS, Kishore H. Minimal hepatic encephalopathy in cirrhosis- how long to treat? *Ann Hepatol* 2017; **16**: 115-122 [PMID: 28051800 DOI: 10.5604/16652681.1226822]
63 **Saab S**, Suraweera D, Au J, Saab EG, Alper TS, Tong MJ. Probiotics are helpful in hepatic encephalopathy: a meta-analysis of randomized trials. *Liver Int* 2016; **36**: 986-993 [PMID: 26561214 DOI: 10.1111/liv.13005]
64 **Malaguarnera M**, Gargante MP, Cristaldi E, Vacante M, Risino C, Cammalleri L, Pennisi G, Rampello L. Acetyl-L-carnitine treatment in minimal hepatic encephalopathy. *Dig Dis Sci* 2008; **53**: 3018-3025 [PMID: 18357530 DOI: 10.1007/s10620-008-0238-6]
65 **Dhiman RK**, Rana B, Agrawal S, Garg A, Chopra M, Thumburu KK, Khattri A, Malhotra S, Duseja A, Chawla YK. Probiotic VSL#3 reduces liver disease severity and hospitalization in patients with cirrhosis: a randomized, controlled trial. *Gastroenterology* 2014; **147**: 1327-1337. e3 [PMID: 25450083 DOI: 10.1053/j.gastro.2014.08.031]
66 **Ahluwalia V**, Wade JB, Heuman DM, Hammeke TA, Sanyal AJ, Sterling RK, Stravitz RT, Luketic V, Siddiqui MS, Puri P, Fuchs M, Lennon MJ, Kraft KA, Gilles H, White MB, Noble NA, Bajaj JS. Enhancement of functional connectivity, working memory and inhibitory control on multi-modal brain MR imaging with Rifaximin in Cirrhosis: implications for the gut-liver-brain axis. *Metab Brain Dis* 2014; **29**: 1017-1025 [PMID: 24590688 DOI: 10.1007/s11011-014-9507-6]
67 **Bajaj JS**, Heuman DM, Wade JB, Gibson DP, Saeian K, Wegelin JA, Hafeezullah M, Bell DE, Sterling RK, Stravitz RT, Fuchs M, Luketic V, Sanyal AJ. Rifaximin improves driving simulator performance in a randomized trial of patients with minimal hepatic encephalopathy. *Gastroenterology*

2011; **140**: 478-487. e1 [PMID: 20849805 DOI: 10.1053/j.gastro.2010.08.061]

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MINIREVIEWS

COVID-19 and pediatric fatty liver disease: Is there interplay?

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Abstract

The rapid global spread of coronavirus disease 2019 (COVID-19) infection has become a major health issue with higher morbidity and mortality rates. Besides respiratory symptoms, a growing body of evidence indicates a variety of gastrointestinal manifestations including liver involvement. In this regard, several data supported an association between COVID-19 infection and liver injury in adults, while in children there is compelling but currently limited evidence. In particular, patients with COVID-19 have shown a higher risk of liver injury (mainly expressed as increased transaminase levels or hepatic steatosis). Conversely, a greater risk of more severe forms of COVID-19 infection has been observed in subjects with pre-existing chronic liver diseases. The dramatic interplay between COVID-19 and liver damage has been related to the inflammatory pathways chronically active in patients with nonalcoholic fatty liver disease and acutely in those affected by COVID-19, but other different pathogenic mechanisms have also been supposed. Of note, patients with previous metabolic comorbidities also had a higher risk of severe COVID-19 infection. This emphasizes the pathogenic interrelation of the inflammatory pathways with a dysregulated metabolic milieu in COVID-19 patients. Taking into account the prognostic role of fatty liver in COVID-19 patients and its intrinsic relationship with metabolic abnormalities even in childhood, a strict monitoring of this condition is recommended. We aimed to summarize the most recent evidence regarding the potential interplay between pediatric fatty liver and COVID-19.

Key Words: COVID-19; Children; Adults; Nonalcoholic fatty liver disease

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Core Tip: Both adult and pediatric data recently reported a liver involvement in

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coronavirus disease 2019 (COVID-19). Although several pathogenic mechanisms have been proposed, inflammatory pathways seem to play a pivotal role in the pathophysiology of liver damage in this viral infection. In particular, a complex and bidirectional relationship has been highlighted between fatty liver and COVID-19. Several data suggested this intriguing interplay by underscoring the need for an early close monitoring of this liver condition with an intrinsic greater cardiometabolic burden.

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INTRODUCTION

Since late 2019, the whole world has been suddenly disrupted by the coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). At the beginning of February 2021, this global acute pandemic produced more than 102 million worldwide confirmed cases and a total number of deaths to 2.2 million, representing an unprecedented healthcare challenge[1].

The pathogenic role of COVID-19 depends on binding of spike viral proteins to angiotensin I converting enzyme 2 (ACE-2) receptors facilitating the entry into the target cells[2-4]. Although mainly expressed in the respiratory tract, in particular in the nasopharynx epithelium, these receptors are also located in multiple sites such as the gastrointestinal tract and vascular endothelium[5-8]. Along with clinical respiratory symptoms, growing evidence supported a COVID-19 related gastrointestinal involvement[8-13]. COVID-19 positive adult patients might experience gastrointestinal symptoms due to the presence of ACE-2 receptors in the glandular cells of gastric, duodenal, and distal enterocytes that favor malabsorption, impaired intestinal secretion, and enteric nervous system activation[8,9,12,14]. The virus might also affect the liver through a direct viral translocation from gut or by an indirect mechanism involving systemic inflammation, pre-existing liver diseases, or drug-related liver damage[11,13-16].

Pediatric COVID-19 reports have shown both a milder course and a better prognosis of the disease in this population, probably due to the special immune response system pertaining to children. Gastrointestinal manifestations of the disease at this age are various and not uncommon, in particular in the early phase of the infection.

Nonalcoholic fatty liver disease (NAFLD) has become the most common worldwide chronic liver disease with an epidemic rate both in adults and children[17,18]. It has been largely recognized for its close relationship with insulin resistance and other metabolic features leading to an increased cardiometabolic risk from childhood to adulthood[19-23]. Nevertheless, NAFLD pathophysiology is still far from being fully clarified. To date, the "multiple hits" represents the most favored pathogenic hypothesis, including a role for several different factors (*e.g.*, inflammation, oxidative stress, gut axis, mitochondrial dysfunction, diet, hormones, and genetics)[24].

HOW CAN COVID-19 AFFECT THE LIVER?

The mechanism underlying the increase of liver enzymes seemed to be different between adult and pediatric patients, but further studies are needed for a better elucidation[25] .

In adults, two mechanisms for liver injury have been postulated such as a possible role of ACE-2 receptor binding of the virus to the epithelial cells of the bile ducts or a dysregulated hepatic innate immune response, which represents the most probable hypothesis. No evidence supported the involvement of ACE-2 receptor in pediatric liver injury. Moreover, both interleukin (IL)-6 and IL-10 levels did not significantly differ between infected children with and without elevated liver enzymes[25]. Of

interest, the presence of a protective mechanism against liver involvement in children accounting for the mild increase of aspartate aminotransferase and/or alanine aminotransferase (ALT) compared to adults has been suggested. Zhou *et al*[25] reported a higher prevalence of liver involvement (expressed as abnormal liver enzymes) in children aged 0-3 years than in those aged > 3 years, by speculating that this finding might be related to a more pronounced liver immaturity.

The exact mechanism linking COVID-19 to pediatric fatty liver remains poorly known[8,11,25] (Figure 1). In fact, the low incidence of COVID-19 infection in children and its relatively asymptomatic course at this age make it difficult to better understand the pathophysiology of this concern[8,11]. To complicate matters, one of the most likely hypotheses supporting the abnormal liver enzymes in adults such as the inflammatory cytokine storm (including IL-6-and IL-10) seemed to not play a central role in pediatric COVID-19 related liver damage[25]. In fact, studies in infected children have demonstrated that levels of these cytokines are linked to greater severity of the disease, but no statistical differences of both IL-6 and IL-10 serum concentrations have been detected between patients with and without increased transaminases[25]. Moreover, the pediatric clinical practice to first give paracetamol for fever management did not exclude the potential link between liver injury (expressed as abnormal transaminase levels) and drug administration[25].

However, all these studies suffered from some limitations. The first was relatively small sample sizes. Lack of a more accurate laboratory assessment and of longitudinal data represented another limitation. Finally, lack of previous data about comorbidities or drug administration before the hospitalization needed to be mentioned.

Nevertheless, given the growing amount of data regarding liver involvement during this pandemic, the American Association for the Study of Liver Diseases recommends to regularly monitor the mild increase of transaminase in patients with COVID-19 and (mainly in children) to further evaluate for potential underlying liver diseases because of the uncommon presentation and the current paucity of data at this age[26].

FATTY LIVER AND COVID-19

Evidence in adulthood

A large body of evidence has shown an association between COVID-19 and NAFLD in adults (Table 1). Findings from adult studies reported that the prevalence of steatosis (mainly detected by ultrasound abdominal scan or computed tomography) in the confirmed COVID-19 cases was significantly higher than in healthy subjects, even after adjustments for gender and age[27].

Additional data suggested that male patients aged > 60 years, with higher body mass index, underlying comorbidities, and NAFLD were at higher risk of COVID-19 progression[28]. Another study reported no difference in terms of admission to intensive care unit and mortality between NAFLD and non-NAFLD patients. Mortality was associated to male gender (similarly to non-NAFLD patients) and a pronounced inflammatory response (including high ferritin and early weaning score as main predictors). The latter might account for the presence of a dysregulated hepatic innate immune response, one of the most acclaimed mechanisms postulated for liver injury. In addition, NAFLD patients were significantly younger at presentation and showed an increased risk of severe COVID-19 in both genders, specifically in males[29].

Interestingly, taking into account the new definition of metabolic-associated fatty liver disease (MAFLD), authors investigated the potential relationship between COVID-19 infection and MAFLD patients[30-32]. MAFLD patients had a greater risk of disease progression, longer viral shedding times, and a higher probability of liver dysfunction from admission to discharge than patients without MAFLD[30-32]. Sharma *et al*[32] found that younger patients (aged < 60 years) with MAFLD showed an increased severity of COVID-19 disease. Overall, a significant association between MAFLD and younger presentation of COVID-19 was found[31,32].

Notably, as observed in NAFLD subjects, the direct relationship of MAFLD with COVID-19 infection emphasizes the relevance of the metabolic milieu in this viral condition, by pointing out the role of dysregulated hepatic innate immunity to trigger liver damage[29-32]. Moreover, overwhelming evidence supported the association of COVID-19 infection severity (defined as the occurrence of any of the following parameters: arterial $PaO_2/FiO_2 \leq 300$ mmHg, $SpO_2 \leq 93\%$, ≥ 30 breath per minute) with both NAFLD and/or MAFLD, even after full adjustment for common cardiometabolic

Table 1 Main findings regarding the association between fatty liver disease and coronavirus disease 2019 in adults

Ref.	Study design	Population	NAFLD	Main findings
Mahamid *et al*[34]	Retrospective case-control study, SZMC, Jerusalem	71 hospitalized patients with COVID-19 infection, both genders, age ≥ 18.0 yr (mean age 51.0 ± 21.7), 22 NAFLD, 49 non-NAFLD	CT within hospitalization or recently made	Significant association between NAFLD and severity of COVID-19 even after adjustments for obesity, hypertension, metabolic syndrome, diabetes, and smoking. This association was independent of metabolic syndrome and/or its components. NAFLD patients have an increased risk of severe COVID-19 in both genders, in particular in males (Male: P = 0.001 Female: P = 0.002)
Ji *et al*[28]	Retrospective case-control study. Patients of two COVID Hospital in China	202 patients with COVID-19 (hospitalized and follow-up within 12 mo of the diagnosis). Median age 44.5 (34.8-54.1), 163 patients with stable disease (37.6% of patients with NAFLD), 39 patients with progressive disease (25.8% of patients with NAFLD)	NAFLD defined as hepatic steatosis index: 8 × (ALT/AST) + BMI (+ 2 if type 2 diabetes, + 2 if female) > 36 and/or US	Male patients aged > 60 yr, with higher BMI, underlying comorbidities, and NAFLD were associated with COVID-19 progression. Patients with NAFLD had higher risk of disease progression, longer viral shedding times, and higher likelihood of abnormal liver function from admission to discharge than patients without NAFLD
Zhou *et al*[31]	Cohort study, Asian ethnicity	55 MAFLD patients with COVID-19 were 1:1 matched by age (± 5 yr), sex, and BMI (± 1 unit) to COVID-19 patients without MAFLD. Age < 60 yr	CT	The presence of MAFLD was associated with severity of COVID-19 even after full adjustment (age, sex, smoking status, obesity, diabetes, hypertension) and a trend to increased duration of hospitalization. MAFLD patients had higher levels of CRP, ALT, AST, GGT, fasting blood glucose, and triglycerides
Medeiros *et al*[27]	Retrospective case-control study. Radiology Departments of Hospital Beneficiencia Portuguesa, San Paolo-Brasil	316 patients clinically suspected of having COVID-19 infection: -n.204: RT-PCR positive; -n.112: RT-PCR and chest CT negative pattern. Age > 18 yr	CT: Attenuation value of ≤ 40 HU, measured in the region of interest (commonly in the right hepatic lobe) in non-enhanced phase	Higher prevalence of steatosis in affected patients, even after adjustments for sex and age
Forlano *et al*[29]	Retrospective cohort study. Imperial College Healthcare NHS Trust (London, United Kingdom)	193 hospitalized, adult patients with COVID-19 infection and CT imaging, NAFLD: 61 (31%); Non-NAFLD: 132 (66%), excluded: 5 (3%)	US or CT dated within 1 yr from the admission for COVID-19 or a known diagnosis of NAFLD. FIB-4 index for fibrosis	No difference in terms of admission to ICU and in mortality between NAFLD and non-NAFLD patients. NAFLD patients were significantly younger at presentation
Gao *et al*[30]	Cohort study. Four hospitals in China	130 nondiabetic patients with COVID-19: 65 MAFLD and 65 controls were 1:1 matched by age (± 5 yr) and sex	CT	MAFLD presence in nondiabetic patients was associated with a 4-fold increased risk of severe COVID-19, even after adjusting for age, sex, and coexisting comorbidities. The risk of severe COVID-19 increased with increasing numbers of metabolic risk factors
Targher *et al*[33]	Retrospective cohort study. Four hospitals in China	310 hospitalized, adult patients with COVID-19 infection	CT: FIB-4 index and NFS used to categorize liver fibrosis in low, intermediate, or high	In patients with MAFLD the presence of intermediate or high fibrosis (FIB-4 or NFS) was associated with a higher risk of severe COVID-19, even after adjusting for sex, obesity, and diabetes
Sharma *et al*[32]	Review	Adult patients	CT/FIB-4 index and NFS	Patients with MAFLD had higher risk of disease progression, longer viral shedding times, higher likelihood of abnormal liver function, and 4-6-fold increased risk of severe disease than patients with no MAFLD. Younger patients (age < 60 yr) were also at greater risk for increased severity of COVID-19

ALT/AST: Alanine aminotransferase/aspartate aminotransferase; BMI: Body mass index; COVID-19: Coronavirus disease 2019; CRP: C-reactive protein; CT: Computed tomography; FIB-4: Fibrosis-4; GGT: Gamma-glutamyl transferase; ICU: Intensive care unit; MAFLD: Metabolic-associated fatty liver disease; NAFLD: Nonalcoholic fatty liver disease; NFS: Nonalcoholic fatty liver disease fibrosis score; NHS: National Health Service; RT-PCR: Real-time reverse transcription polymerase chain reaction; US: Ultrasound.

risk factors such as obesity, hypertension, metabolic syndrome, diabetes, and smoking [30,33]. Hence, this association was found to be independent of metabolic syndrome and/or its components[33]. In particular, MAFLD presence in nondiabetic patients was associated with a 4-fold increased risk of severe COVID-19, even after adjusting for age, gender, and coexisting comorbidities, and the risk was even higher with increasing numbers of metabolic risk factors[30]. Moreover, the presence of intermediate or high fibrosis was also linked to a higher risk of severe COVID-19, even after adjusting for gender, obesity, and diabetes[34].

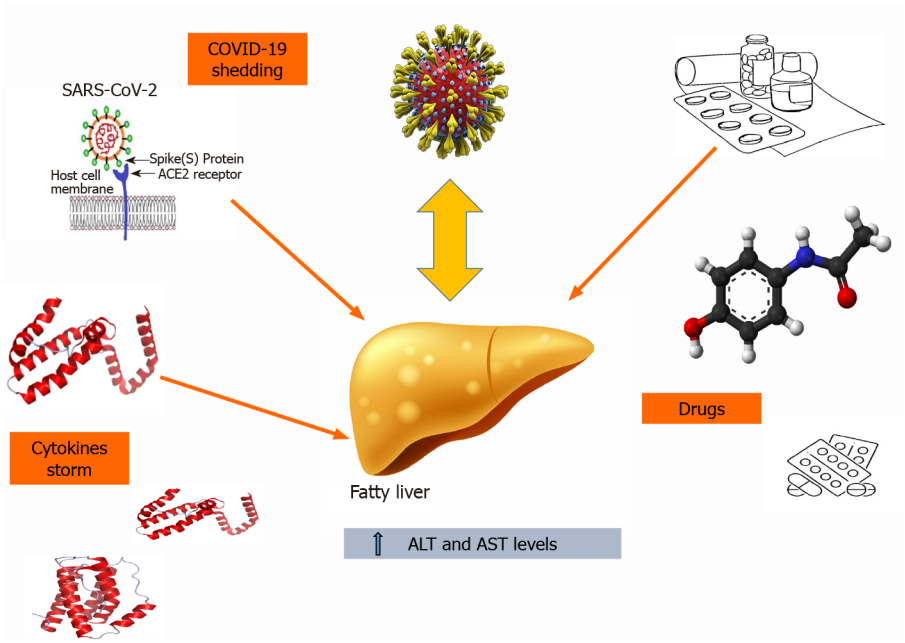


Figure 1 Potential mechanisms linking coronavirus disease 2019 to pediatric fatty liver. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; COVID-19: Coronavirus disease 2019.

Evidence in childhood

Compared to adult findings, evidence in children is currently limited but in line with the aforementioned association (Table 2). In fact, data from 6 pediatric case-series studies and a review reported that liver involvement, defined as an increase of ALT and/or aspartate aminotransferase, was rare and mild (< 2 × ULN)[35-41]. In contrast to adults, Zhou *et al*[25] found no gender difference in children with COVID-19 and elevated liver enzymes.

Pediatric studies examining different variables such as demographic data, clinical and laboratory features, chest imaging results, treatments, and clinical outcomes of the patients with COVID-19 showed a prevalence of possible liver involvement from 6% to 50%[25,35-41].

A retrospective analysis including eight severe or critically ill patients with COVID-19 (aged 2 mo-15 years) admitted to the intensive care unit of the Wuhan Children's Hospital reported a mild increase of ALT levels in half of the population, while total bilirubin levels in all patients were normal[36]. Similarly, a retrospective study of 20 pediatric patients with COVID-19 infection showed increased ALT levels (> 40 U/L) in 25% of cases[37]. A slight prevalence (6%) of elevated liver enzymes in confirmed-COVID-19 Chinese patients (aged 0-16 years) was also described in a cohort study[35]. In addition, it provided evidence for a minor liver involvement compared to adults with COVID-19 (18%), children with severe acute respiratory syndrome (48%), and children with influenza A (7%)[35].

It has been observed in children that both critically ill patients admitted to the intensive care unit and mild patients (defined as the presence of upper respiratory symptoms or asymptomatic infection, positive real-time reverse transcription polymerase chain reaction test for SARS-CoV-2, and no abnormal radiographic and septic presentation) rarely showed abnormal liver enzymes[35,36]. In these studies, all the patients underwent an antiviral treatment, therefore the possibility that transaminase abnormalities were drug-induced cannot be ruled out[35,36]. Moreover, it should be noted that the most common symptoms at admission were cough and fever, usually treated with paracetamol, of which an incorrect administration might also influence transaminase levels[41].

CONCLUSION

Gastrointestinal symptoms have been largely described in COVID-19 infected patients. Indeed, the wide presence of ACE-2 receptors through the entire gastrointestinal tract has been considered to be responsible for the spectrum of gastrointestinal manifest-

Table 2 Main findings regarding the association between fatty liver disease and coronavirus disease 2019 in children

Ref.	Study design	Population	NAFLD	Main findings
Qiu *et al*[36]	Cohort study. Three hospitals in Zhejiang Province, China	36 pediatric patients (aged 0-16 yr) with laboratory confirmed COVID-19 infection; mild cases (n = 17); moderate cases (n = 19) compared with adults with COVID-19 (n = 175); Children with SARS (n = 44); Children with H1N1 influenza (n = 167)	Increased liver enzymes	Elevated ALT in two patients (mild cases) and AST in three patients (two mild and one moderate case), 6% of cases with elevated liver enzymes, 18% of cases with elevated liver enzymes, 48% of cases with elevated liver enzymes, 17% of cases with elevated liver enzymes
Sun *et al*[37]	Retrospective analysis. Intensive Care Unit, Wuhan Children's Hospital	8 severe or critically ill COVID-19 patients admitted to the ICU. Age: 2 mo-15 yr	Increased liver enzymes	Elevated ALT levels in 4 out of 8 patients. Total bilirubin level in all patients was normal
Wang *et al*[39]	Retrospective study. Children from six provinces (autonomous region) in northern China.	31 cases of COVID-19. Age: 6 mo-17 yr	Increased liver enzymes	Elevation of liver enzyme (22%)
Xia *et al* [38]	Retrospective study. Wuhan Children's Hospital	20 COVID-19 pediatric patients. Age: 1 d-14 yr, 7 mo (median age: 2 yr and 1.5 mo)	No NAFLD screening. CT was performed for pneumonia	Elevated ALT (> 40 U/L) in 25% of cases
Jiehao *et al*[40]	Case series. Children's Hospital in Shanghai, Hainan, Hefei in Anhui province, and Qingdao in Shandong province	10 patients aged 3-131 mo (mean: 74 mo). Male: female 1:1.5	No NAFLD screening. CT was performed for pneumonia	Median ALT: 18.5 U/L, AST: 27.7 U/L. One patient had ALT: 100 U/L and AST: 142 U/L
Tan *et al*[41]	Retrospective study. North Hospital of Changsha First Hospital	10 children with confirmed COVID-19 infection. Mean age of 7 yr (1-12 yr)	Increased liver enzymes	Elevated AST in two patients

ALT/AST: Alanine aminotransferase/aspartate aminotransferase; CT: Computed tomography; COVID-19: Coronavirus disease 2019; H1N1: Influenza A; ICU: Intensive care unit; NAFLD: Nonalcoholic fatty liver disease; SARS: Severe acute respiratory syndrome.

ations including abdominal pain, nausea, vomiting, or less commonly fatty liver and acute hepatitis. In particular, liver involvement has been reported both in adult and pediatric affected patients with a higher prevalence in the former population[7,42,43]. Several pathogenic mechanisms have been hypothesized to explain liver damage, but its exact pathophysiology is still under debate[42,44]. First, it could be considered as a consequence of the "cytokine storm" resulting in an aberrant immune system reaction with an excessive inflammatory response, as observed in many affected subjects. Moreover, liver injury might represent a side effect of the administered drugs or a secondary effect of the hypoxia consequent to failure of other organs such as heart and lung. On the contrary, a direct viral effect is unlikely, as supported by the scarce evidence of an increase of the classical markers of biliary tract involvement such as gamma-glutamyl transferase and alkaline phosphatase in affected patients in the face of a large hepatic expression of ACE-2 on cholangiocyte surfaces[6,45].

Of concern, liver damage in patients affected by COVID-19 seems to be enhanced by the intrinsic chronic activation of inflammatory pathways in NAFLD, potentially worsening outcomes in subjects with previous coexisting metabolic diseases[7,8]. Moreover, patients with pre-existing chronic liver diseases have been found to be at higher risk to develop more severe forms of COVID-19 infection.

NAFLD expressed as hepatic steatosis and/or elevated liver enzymes represents one of the most common liver abnormalities reported during this pandemic, in particular in pediatric patients[7]. Studies reported an overall prevalence of mild liver enzyme increases of almost 25% in COVID-19 infected children. Although different pathogenic mechanisms than adults have been supposed, further studies are required to better clarify the pathophysiology of abnormal liver enzymes in children with COVID-19.

The potential prognostic role for NAFLD as well as its intrinsic close relationship with metabolic traits require a close monitoring of fatty liver in these patients.

REFERENCES

1 **World Health Organization**. Coronavirus Disease (COVID-2019) Situation Reports. Geneva:

WHO; 2020. [cited 5 February 2021] Available from: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation
2 **Li W**, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003; **426**: 450-454 [PMID: 14647384 DOI: 10.1038/nature02145]
3 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280. e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]
4 **Zou X**, Chen K, Zou J, Han P, Hao J, Han Z. Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Front Med* 2020; **14**: 185-192 [PMID: 32170560 DOI: 10.1007/s11684-020-0754-0]
5 **Jia HP**, Look DC, Shi L, Hickey M, Pewe L, Netland J, Farzan M, Wohlford-Lenane C, Perlman S, McCray PB Jr. ACE2 receptor expression and severe acute respiratory syndrome coronavirus infection depend on differentiation of human airway epithelia. *J Virol* 2005; **79**: 14614-14621 [PMID: 16282461 DOI: 10.1128/JVI.79.23.14614-14621.2005]
6 **Zhao Y**, Zhao Z, Wang Y, Zhou Y, Ma Y, Zuo W. Single-Cell RNA Expression Profiling of ACE2, the Receptor of SARS-CoV-2. *Am J Respir Crit Care Med* 2020; **202**: 756-759 [PMID: 32663409 DOI: 10.1164/rccm.202001-0179LE]
7 **Hamming I**, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol* 2004; **203**: 631-637 [PMID: 15141377 DOI: 10.1002/path.1570]
8 **Portincasa P**, Krawczyk M, Smyk W, Lammert F, Di Ciaula A. COVID-19 and non-alcoholic fatty liver disease: Two intersecting pandemics. *Eur J Clin Invest* 2020; **50**: e13338 [PMID: 32589264 DOI: 10.1111/eci.13338]
9 **Nobel YR**, Phipps M, Zucker J, Lebwohl B, Wang TC, Sobieszczyk ME, Freedberg DE. Gastrointestinal Symptoms and Coronavirus Disease 2019: A Case-Control Study From the United States. *Gastroenterology* 2020; **159**: 373-375. e2 [PMID: 32294477 DOI: 10.1053/j.gastro.2020.04.017]
10 **Liang W**, Feng Z, Rao S, Xiao C, Xue X, Lin Z, Zhang Q, Qi W. Diarrhoea may be underestimated: a missing link in 2019 novel coronavirus. *Gut* 2020; **69**: 1141-1143 [PMID: 32102928 DOI: 10.1136/gutjnl-2020-320832]
11 **Bangash MN**, Patel J, Parekh D. COVID-19 and the liver: little cause for concern. *Lancet Gastroenterol Hepatol* 2020; **5**: 529-530 [PMID: 32203680 DOI: 10.1016/S2468-1253(20)30084-4]
12 **Fu Y**, Zhu R, Bai T, Han P, He Q, Jing M, Xiong X, Zhao X, Quan R, Chen C, Zhang Y, Tao M, Yi J, Tian D, Yan W. Clinical Features of Patients Infected With Coronavirus Disease 2019 With Elevated Liver Biochemistries: A Multicenter, Retrospective Study. *Hepatology* 2020 [PMID: 32602604 DOI: 10.1002/hep.31446]
13 **Wu J**, Song S, Cao HC, Li LJ. Liver diseases in COVID-19: Etiology, treatment and prognosis. *World J Gastroenterol* 2020; **26**: 2286-2293 [PMID: 32476793 DOI: 10.3748/wjg.v26.i19.2286]
14 **Cha MH**, Regueiro M, Sandhu DS. Gastrointestinal and hepatic manifestations of COVID-19: A comprehensive review. *World J Gastroenterol* 2020; **26**: 2323-2332 [PMID: 32476796 DOI: 10.3748/wjg.v26.i19.2323]
15 **Li J**, Fan JG. Characteristics and Mechanism of Liver Injury in 2019 Coronavirus Disease. *J Clin Transl Hepatol* 2020; **8**: 13-17 [PMID: 32274341 DOI: 10.14218/JCTH.2020.00019]
16 **Méndez-Sánchez N**, Valencia-Rodríguez A, Qi X, Yoshida EM, Romero-Gómez M, George J, Eslam M, Abenavoli L, Xie W, Teschke R, Carrion AF, Keaveny AP. What Has the COVID-19 Pandemic Taught Us so Far? *J Clin Transl Hepatol* 2020; **8**: 0024 [PMID: 32309152 DOI: 10.14218/JCTH.2020.00024]
17 **Sahota AK**, Shapiro WL, Newton KP, Kim ST, Chung J, Schwimmer JB. Incidence of Nonalcoholic Fatty Liver Disease in Children: 2009-2018. *Pediatrics* 2020; **146** [PMID: 33214329 DOI: 10.1542/peds.2020-0771]
18 **Paul S**, Davis AM. Diagnosis and Management of Nonalcoholic Fatty Liver Disease. *JAMA* 2018; **320**: 2474-2475 [PMID: 30476962 DOI: 10.1001/jama.2018.17365]
19 **Morandi A**, Di Sessa A, Zusi C, Umano GR, El Mazloum D, Fornari E, Miraglia Del Giudice E, Targher G, Maffeis C. Nonalcoholic Fatty Liver Disease and Estimated Insulin Resistance in Obese Youth: A Mendelian Randomization Analysis. *J Clin Endocrinol Metab* 2020; **105** [PMID: 32841326 DOI: 10.1210/clinem/dgaa583]
20 **Di Bonito P**, Valerio G, Licenziati MR, Miraglia Del Giudice E, Baroni MG, Morandi A, Maffeis C, Campana G, Spreghini MR, Di Sessa A, Morino G, Crinò A, Chiesa C, Pacifico L, Manco M. High uric acid, reduced glomerular filtration rate and non-alcoholic fatty liver in young people with obesity. *J Endocrinol Invest* 2020; **43**: 461-468 [PMID: 31637675 DOI: 10.1007/s40618-019-01130-6]
21 **Targher G**, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; **363**: 1341-1350 [PMID: 20879883 DOI: 10.1056/NEJMra0912063]
22 **Targher G**. What's Past Is Prologue: History of Nonalcoholic Fatty Liver Disease. *Metabolites* 2020; **10** [PMID: 33049948 DOI: 10.3390/metabo10100397]
23 **Mantovani A**, Petracca G, Beatrice G, Tilg H, Byrne CD, Targher G. Non-alcoholic fatty liver disease and risk of incident diabetes mellitus: an updated meta-analysis of 501 022 adult individuals.

Gut 2020 [PMID: 32938692 DOI: 10.1136/gutjnl-2020-322572]

24 **Di Sessa A**, Umano GR, Cirillo G, Passaro AP, Verde V, Cozzolino D, Guarino S, Marzuillo P, Miraglia Del Giudice E. Pediatric non-alcoholic fatty liver disease and kidney function: Effect of *HSD17B13* variant. *World J Gastroenterol* 2020; **26**: 5474-5483 [PMID: 33024398 DOI: 10.3748/wjg.v26.i36.5474]

25 **Zhou YH**, Zheng KI, Targher G, Byrne CD, Zheng MH. Abnormal liver enzymes in children and infants with COVID-19: A narrative review of case-series studies. *Pediatr Obes* 2020; **15**: e12723 [PMID: 32875680 DOI: 10.1111/ijpo.12723]

26 **Fix OK**, Hameed B, Fontana RJ, Kwok RM, McGuire BM, Mulligan DC, Pratt DS, Russo MW, Schilsky ML, Verna EC, Loomba R, Cohen DE, Bezerra JA, Reddy KR, Chung RT. Clinical Best Practice Advice for Hepatology and Liver Transplant Providers During the COVID-19 Pandemic: AASLD Expert Panel Consensus Statement. *Hepatology* 2020; **72**: 287-304 [PMID: 32298473 DOI: 10.1002/hep.31281]

27 **Medeiros AK**, Barbisan CC, Cruz IR, de Araújo EM, Libânio BB, Albuquerque KS, Torres US. Higher frequency of hepatic steatosis at CT among COVID-19-positive patients. *Abdom Radiol (NY)* 2020; **45**: 2748-2754 [PMID: 32683613 DOI: 10.1007/s00261-020-02648-7]

28 **Ji D**, Qin E, Xu J, Zhang D, Cheng G, Wang Y, Lau G. Non-alcoholic fatty liver diseases in patients with COVID-19: A retrospective study. *J Hepatol* 2020; **73**: 451-453 [PMID: 32278005 DOI: 10.1016/j.jhep.2020.03.044]

29 **Forlano R**, Mullish BH, Mukherjee SK, Nathwani R, Harlow C, Crook P, Judge R, Soubieres A, Middleton P, Daunt A, Perez-Guzman P, Selvapatt N, Lemoine M, Dhar A, Thursz MR, Nayagam S, Manousou P. In-hospital mortality is associated with inflammatory response in NAFLD patients admitted for COVID-19. *PLoS One* 2020; **15**: e0240400 [PMID: 33031439 DOI: 10.1371/journal.pone.0240400]

30 **Gao F**, Zheng KI, Wang XB, Yan HD, Sun QF, Pan KH, Wang TY, Chen YP, George J, Zheng MH. Metabolic associated fatty liver disease increases coronavirus disease 2019 disease severity in nondiabetic patients. *J Gastroenterol Hepatol* 2021; **36**: 204-207 [PMID: 32436622 DOI: 10.1111/jgh.15112]

31 **Zhou YJ**, Zheng KI, Wang XB, Yan HD, Sun QF, Pan KH, Wang TY, Ma HL, Chen YP, George J, Zheng MH. Younger patients with MAFLD are at increased risk of severe COVID-19 illness: A multicenter preliminary analysis. *J Hepatol* 2020; **73**: 719-721 [PMID: 32348790 DOI: 10.1016/j.jhep.2020.04.027]

32 **Sharma P**, Kumar A. Metabolic dysfunction associated fatty liver disease increases risk of severe Covid-19. *Diabetes Metab Syndr* 2020; **14**: 825-827 [PMID: 32540736 DOI: 10.1016/j.dsx.2020.06.013]

33 **Mahamid M**, Nseir W, Khoury T, Mahamid B, Nubania A, Sub-Laban K, Schifter J, Mari A, Sbeit W, Goldin E. Nonalcoholic fatty liver disease is associated with COVID-19 severity independently of metabolic syndrome: a retrospective case-control study. *Eur J Gastroenterol Hepatol* 2020 [PMID: 32868652 DOI: 10.1097/MEG.0000000000001902]

34 **Targher G**, Mantovani A, Byrne CD, Wang XB, Yan HD, Sun QF, Pan KH, Zheng KI, Chen YP, Eslam M, George J, Zheng MH. Risk of severe illness from COVID-19 in patients with metabolic dysfunction-associated fatty liver disease and increased fibrosis scores. *Gut* 2020; **69**: 1545-1547 [PMID: 32414813 DOI: 10.1136/gutjnl-2020-321611]

35 **Zhong ZF**, Huang J, Yang X, Peng JL, Zhang XY, Hu Y, Fu N, Lin HL, Jiang B, Tian YY, Yao HY, Deng LP, Tang XQ, Zhou JC, Tang J, Xie X, Liu Q, Liu J, Dou CY, Dai RJ, Yan B, Yang XF. Epidemiological and clinical characteristics of COVID-19 patients in Hengyang, Hunan Province, China. *World J Clin Cases* 2020; **8**: 2554-2565 [PMID: 32607332 DOI: 10.12998/wjcc.v8.i12.2554]

36 **Qiu H**, Wu J, Hong L, Luo Y, Song Q, Chen D. Clinical and epidemiological features of 36 children with coronavirus disease 2019 (COVID-19) in Zhejiang, China: an observational cohort study. *Lancet Infect Dis* 2020; **20**: 689-696 [PMID: 32220650 DOI: 10.1016/S1473-3099(20)30198-5]

37 **Sun D**, Li H, Lu XX, Xiao H, Ren J, Zhang FR, Liu ZS. Clinical features of severe pediatric patients with coronavirus disease 2019 in Wuhan: a single center's observational study. *World J Pediatr* 2020; **16**: 251-259 [PMID: 32193831 DOI: 10.1007/s12519-020-00354-4]

38 **Xia W**, Shao J, Guo Y, Peng X, Li Z, Hu D. Clinical and CT features in pediatric patients with COVID-19 infection: Different points from adults. *Pediatr Pulmonol* 2020; **55**: 1169-1174 [PMID: 32134205 DOI: 10.1002/ppul.24718]

39 **Wang D**, Ju XL, Xie F, Lu Y, Li FY, Huang HH, Fang XL, Li YJ, Wang JY, Yi B, Yue JX, Wang J, Wang LX, Li B, Wang Y, Qiu BP, Zhou ZY, Li KL, Sun JH, Liu XG, Li GD, Wang YJ, Cao AH, Chen YN. [Clinical analysis of 31 cases of 2019 novel coronavirus infection in children from six provinces (autonomous region) of northern China]. *Zhonghua Er Ke Za Zhi* 2020; **58**: 269-274 [PMID: 32118389 DOI: 10.3760/cma.j.cn112140-20200225-00138]

40 **Jiehao C**, Jin X, Daojiong L, Zhi Y, Lei X, Zhenghai Q, Yuehua Z, Hua Z, Ran J, Pengcheng L, Xiangshi W, Yanling G, Aimei X, He T, Hailing C, Chuning W, Jingjing L, Jianshe W, Mei Z. A Case Series of Children With 2019 Novel Coronavirus Infection: Clinical and Epidemiological Features. *Clin Infect Dis* 2020; **71**: 1547-1551 [PMID: 32112072 DOI: 10.1093/cid/ciaa198]

41 **Tan YP**, Tan BY, Pan J, Wu J, Zeng SZ, Wei HY. Epidemiologic and clinical characteristics of 10 children with coronavirus disease 2019 in Changsha, China. *J Clin Virol* 2020; **127**: 104353 [PMID: 32302953 DOI: 10.1016/j.jcv.2020.104353]

42 **Mameli S**, Marcialis MA, Bassareo PP, Fanos V. COVID-19 and hepatic damage: what we know?

Panminerva Med 2021 [PMID: 33470582 DOI: 10.23736/S0031-0808.21.04239-7]

43 **Wijarnpreecha K**, Ungprasert P, Panjawatanan P, Harnois DM, Zaver HB, Ahmed A, Kim D. COVID-19 and liver injury: a meta-analysis. *Eur J Gastroenterol Hepatol* 2020 [PMID: 32639420 DOI: 10.1097/MEG.0000000000001817]

44 **Rohani P**, Ahmadi Badi S, Moshiri A, Siadat SD. Coronavirus disease 2019 (COVID-19) and pediatric gastroenterology. *Gastroenterol Hepatol Bed Bench* 2020; **13**: 351-354 [PMID: 33244377]

45 **Paizis G**, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, Shaw T, Warner FJ, Zuilli A, Burrell LM, Angus PW. Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. *Gut* 2005; **54**: 1790-1796 [PMID: 16166274 DOI: 10.1136/gut.2004.062398]

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ORIGINAL ARTICLE

Basic Study

Enhancer of zeste homolog 2 contributes to apoptosis by inactivating janus kinase 2/ signal transducer and activator of transcription signaling in inflammatory bowel disease

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Abstract

BACKGROUND

Inflammatory bowel disease (IBD) is a prevalent worldwide health problem featured by relapsing, chronic gastrointestinal inflammation. Enhancer of zeste homolog 2 (EZH2) is a critical epigenetic regulator in different pathological models, such as cancer and inflammation. However, the role of EZH2 in the IBD development is still obscure.

AIM

To explore the effect of EZH2 on IBD progression and the underlying mechanism.

METHODS

The IBD mouse model was conducted by adding dextran sodium sulfate (DSS), and the effect of EZH2 on DSS-induced colitis was assessed in the model. The function of EZH2 in regulating apoptosis and permeability was evaluated by Annexin V-FITC Apoptosis Detection Kit, transepithelial electrical resistance analysis, and Western blot analysis of related markers, including Zona occludens 1, claudin-5, and occludin, in NCM460 and fetal human colon (FHC) cells. The mechanical investigation was performed by quantitative reverse transcription-polymerase chain reaction, Western blot analysis, and chromatin immunoprecip-

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itation assays.

RESULTS

The colon length was inhibited in the DSS-treated mice and was enhanced by the EZH2 depletion in the system. DSS treatment caused a decreased histological score in the mice, which was reversed by EZH2 depletion. The inflammatory cytokines, such as tumor necrosis factor-α, interleukin-6, and interleukin-1β, were induced in the DSS-treated mice, in which the depletion of EZH2 could reverse this effect. Moreover, the tumor necrosis factor-α treatment induced the apoptosis of NCM460 and FHC cells, in which EZH2 depletion could reverse this effect in the cells. Moreover, the depletion of EZH2 attenuated permeability of colonic epithelial cells. Mechanically, the depletion of EZH2 or EZH2 inhibitor GSK343 was able to enhance the expression and the phosphorylation of janus kinase 2 (JK2) and signal transducer and activator of transcription in the NCM460 and FHC cells. Specifically, EZH2 inactivated JAK2 expression by regulating histone H3K27me3. JAK2 inhibitor TG101348 was able to reverse EZH2 knockdown-mediated colonic epithelial cell permeability and apoptosis.

CONCLUSION

Thus, we concluded that EZH2 contributed to apoptosis and inflammatory response by inactivating JAK2/ signal transducer and activator of transcription signaling in IBD. EZH2 may be applied as a potential target for IBD therapy.

Key Words: Inflammatory bowel disease; Apoptosis; Enhancer of zeste homolog 2; JAK2; Permeability; Inflammatory bowel disease therapy

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Core Tip: In this study, we discovered that enhancer of zeste homolog 2 contributed to apoptosis and inflammatory response by inactivating janus kinase 2/signal transducer and activator of transcription signaling in inflammatory bowel disease (IBD). Enhancer of zeste homolog 2 may be applied as a potential target for IBD therapy.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a worldwide health problem with an increasing incidence. IBD, including Crohn's disease and ulcerative colitis and IBD unclassified, is described as relapsing, chronic gastrointestinal inflammation[1,2]. IBD patients experience low life quality and a higher risk of colorectal cancer[3]. The critical feature of IBD is the unsolved inflammation of the intestinal field caused by a breakdown to shift from a pro-inflammatory situation to an anti-inflammatory situation[4]. Various immune cell populations, such as eosinophils, neutrophils, dendritic cells, and macrophages, are present in the intestinal mucosa, participating in inflammation during IBD[5,6]. These cells participate in inflammation by secreting chemokines, antimicrobial agents, and pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6[3], and IL-1β[7]. Therefore, the understanding of the molecular mechanism underlying the inflammatory regulation during IBD progression is urgently needed.

The fundamental function of epigenetic systems in determining T cell lineage fate decisions has been completely defined[8,9]. Nevertheless, the effect of the histone methyltransferase enhancer of zeste homolog 2 (EZH2) has been recently identified in the process[10,11]. EZH2 serves as a methylation of histone H3K27 to H3K27me3[12].

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It has been found that targeting EZH2 is able to alleviate intestinal inflammation in IBD[11]. Moreover, janus kinase 2 (JK2)/ signal transducer and activator of transcription 3 (STAT3) signaling has been identified to alleviate inflammation response during IBD[13]. However, the correlation of EZH2 with JAK2/STAT3 signaling in the IBD development remains unclear. In this study, we were interested in the molecular mechanism of EZH2-mediated IBD progression.

MATERIALS AND METHODS

Cell culture

The NCM460 and fetal human colon (FHC) cell lines were maintained in the lab and were incubated at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle Medium (GE, United States) containing fetal bovine serum (15%; GE Healthcare, Chicago, IL, United States), streptomycin (0.1 mg/mL), and penicillin (100 units/mL). The lentiviral plasmids carrying EZH2 short hairpin ribonucleic acid (shRNA) and the corresponding control shRNA were obtained (GenScript, Nanjing, China). The transfection in the cells was performed by Liposome 3000 (Invitrogen, Carlsbad, CA, United States). The EZH2 inhibitor GSK343 (Sigma, St. Louis, MO, United States) and JAK2 inhibitor TG101348 (Selleck, Houston, TX, United States) were used at the dose of 5 μmol/L. The TNF-α (Sigma) was used at the dose of 50 ng/mL.

Clinical IBD samples

The clinical IBD samples (n = 50) and non-IBD control cases (n = 50) were collected from The Third People's Hospital of Chengdu. The diagnosis of IBD was consistent with the standard combination of radiologic, histological, endoscopic, and clinical criteria. The application of the samples was under the approval of the patients and proved by the Ethics Committee of Sichuan Cancer Hospital & Institute. The patients provided their written informed consent to participate in this study.

IBD mouse model

C57BL/6 mice (female, 6–8 wk) were obtained from the Chinese Academy of Medical Sciences (Beijing, China). The mice were randomly divided into three groups: Water group; dextran sodium sulfate (DSS) group; DSS + shEZH2 group. The mice in DSS and DSS + shEZH2 groups were constructed by adding DSS (2.5%, MP Biomedicals, Santa Ana, CA, United States) to drinking water for 7 d, followed by normal drinking water for the remaining days. The mice in DSS + shEZH2 groups were intraperitoneally injected with lentiviral plasmids carrying EZH2 shRNA (GenScript). The mice received DSS (3%) to induce intestinal inflammation, and the natural death time of mice in the indicated groups within 15 d was recorded. The body weight was recorded at the indicated time. The colon length was measured and calculated using a ruler. The tissues were treated with 4% paraformaldehyde, and the decalcification was conducted in 5% nitric acid, followed by cutting into 5 μm sections. A set of alcohols was applied for the dehydration of the samples. Afterward, hematoxylin and eosin staining was performed in the slice samples of femoral head and then observed by microscope (BX-42; Olympus, Tokyo, Japan). The histologic score used to quantify the effect of EZH2 depletion in the mouse model by the pathologist was according to the criteria: Crypt architecture (normal, 0–severe crypt distortion with loss of entire crypts, 3), degree of inflammatory cell infiltration (normal, 0–dense inflammatory infiltrate, 3), muscle thickening (base of crypt sits on the muscularis mucosae, 0–marked muscle thickening, 3), crypt abscess (absent, 0–present, 1), and goblet cell depletion (absent, 0–present, 1)[1,2]. The levels of cytokines were measured by enzyme-linked immunosorbent assays (Sigma). Animal care was authorized by the Animal Ethics Committee. All experimental procedures with mice were performed in accordance and compliance with the regulations of the Laboratory Animal Welfare and Ethics Committee of Sichuan Cancer Hospital & Institute.

Analysis of cell apoptosis

About 2 × 10^5 NCM460 and FHC cells were plated on 6-well dishes. Cell apoptosis was assessed by employing the Annexin V-FITC Apoptosis Detection Kit (Sigma) using the manufacture's instruction. Shortly, about 2 × 10^5 collected and washed cells collected by binding buffer and were dyed at 25 °C, followed by the flow cytometry analysis.

Quantitative reverse transcription-polymerase chain reaction

Total RNAs were extracted using TRIZOL (Invitrogen). The first-strand complementary (c)DNA was manufactured as the manufacturer's instruction (TaKaRa, Kyoto, Japan). The quantitative reverse transcription-polymerase chain reaction was carried out by applying SYBR-Green (TaKaRa). The primer sequences are as follows: EZH2 forward: 5′-AATCAGAGTACATGCGACTGAGA-3′, reverse: 5′-GCTGTATCCTTCGCTGTTTCC; JAK2 forward: 5′-GCCTTCTTTCAGAGCCATCAT-3′, reverse: 5′-GTGTAGGATCCCGGTCTTCAA-3′; Glyceraldehyde-3-phosphate dehydrogenase forward: 5′-AACGGATTTGGTCGTATTGGG-3′, reverse: 5′-CCTGGAAGATGGTGATGGGAT-3′.

Western blot analysis

Total proteins were extracted from the cells using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, United States) and quantified using the BCA Protein Quantification Kit (Abbkine, Wuhan, China). The proteins at same concentration were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to poly(vinylidene fluoride) membranes (Millipore, Burlington, MA, United States), followed by the incubation with 5% milk and with the primary antibodies at 4 °C overnight. The corresponding secondary antibodies (Boster, Wuhan, China) were used for incubating the membranes 1 h at room temperature, followed visualization by using chemiluminescence detection kit (Beyotime, Beijing, China). The primary antibodies applied in this study comprised EZH2 (Abcam, Cambridge, United Kingdom), Zona occludens 1 (ZO-1) (Abcam), claudin-5 (Abcam), occludin (Abcam), H3K27me3 (Abcam), JAK2 (Abcam), STAT3 (Abcam), p-JAK2 (Abcam), p-STAT3 (Abcam), EZH2 (Abcam), caspase3, cleaved-caspase3 (Abcam), and β-actin (Abcam).

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) was performed using a SimpleChIP Enzymatic ChIP Kit (Cell Signaling Technology) according to the manufacturer's instruction. Chromatin prepared from the cells in a 15 cm dish was used to determine total DNA input and was incubated overnight with specific antibodies or normal rabbit immunoglobulin G. Then, the binding DNA was analyzed by quantitative-polymerase chain reaction assays; the primer sequences were shown as above.

Transepithelial electrical resistance measurements

Transepithelial electrical resistance (TEER) measurement was used to analyze the barrier function of the intestine. About 2×10^5 cells/mL cells were layered on collagen-covered polycarbonate penetrable support supplements (Corning, Corning, NY, United States). The medium was replaced every 2 d. The production of the cells polarized monolayer at 3 wk was settled by monitoring and morphology of TEER by employing Millicell-ERS2 (Merck-Millipore). Delta-toxin was used to polarized monolayer cells at 37 °C.

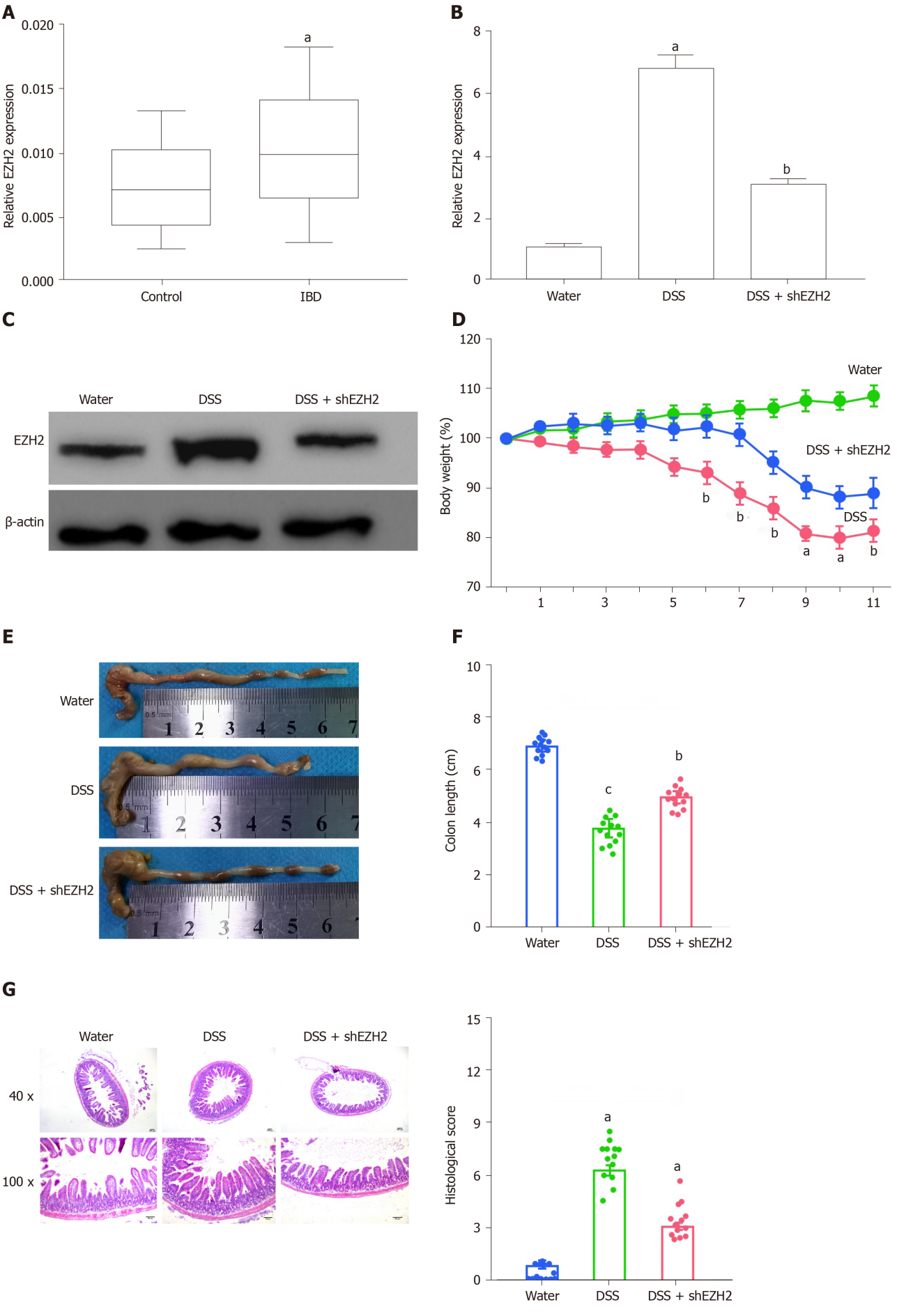
Statistical analysis

The data were normally distributed. Data were expressed as mean ± SD, and the statistical analysis was conducted using GraphPad prism 7 (San Diego, CA, United States). The unpaired Student's *t*-test was used to compare two groups, and the one-way analysis of variance was used to compare multiple groups. $P < 0.05$ was considered as statistically significant.

RESULTS

The depletion of EZH2 inhibits DSS-induced colitis in vivo

Firstly, we identified that the expression of EZH2 was enhanced in clinical IBD samples ($n = 50$) relative to healthy controls ($n = 50$) (Figure 1A). Meanwhile, we found that DSS enhanced EZH2 expression in mice, while the depletion of EZH2 by shRNA repressed the enhancement (Figure 1B and C). We then measured the effect of EZH2 on the DSS-induced colitis *in vivo*. We observed that the DSS treatment significantly resulted in a weight loss of the mice, which was attenuated by EZH2 depletion (Figure 1D). The colon length was reduced in the DSS-treated mice and was rescued by the EZH2 depletion in the system (Figure 1E and F). Meanwhile, the DSS treatment caused a decreased histological score in the mice, which was rescued by EZH2


A
Relative EZH2 expression
0.020
0.015
0.010
0.005
0.000
a
Control
IBD
B
Relative EZH2 expression
8
6
4
2
0
a
b
Water
DSS
DSS + shEZH2
C
Water
DSS
DSS + shEZH2
EZH2
β-actin
D
Body weight (%)
120
110
100
90
80
70
Water
DSS + shEZH2
DSS
b
a
1
3
5
7
9
11
E
Water
DSS
DSS + shEZH2
F
Colon length (cm)
10
8
6
4
2
0
c
b
Water
DSS
DSS + shEZH2
G
Water
DSS
DSS + shEZH2
40 x
100 x
Histological score
15
12
9
6
3
0
a
a
Water
DSS
DSS + shEZH2

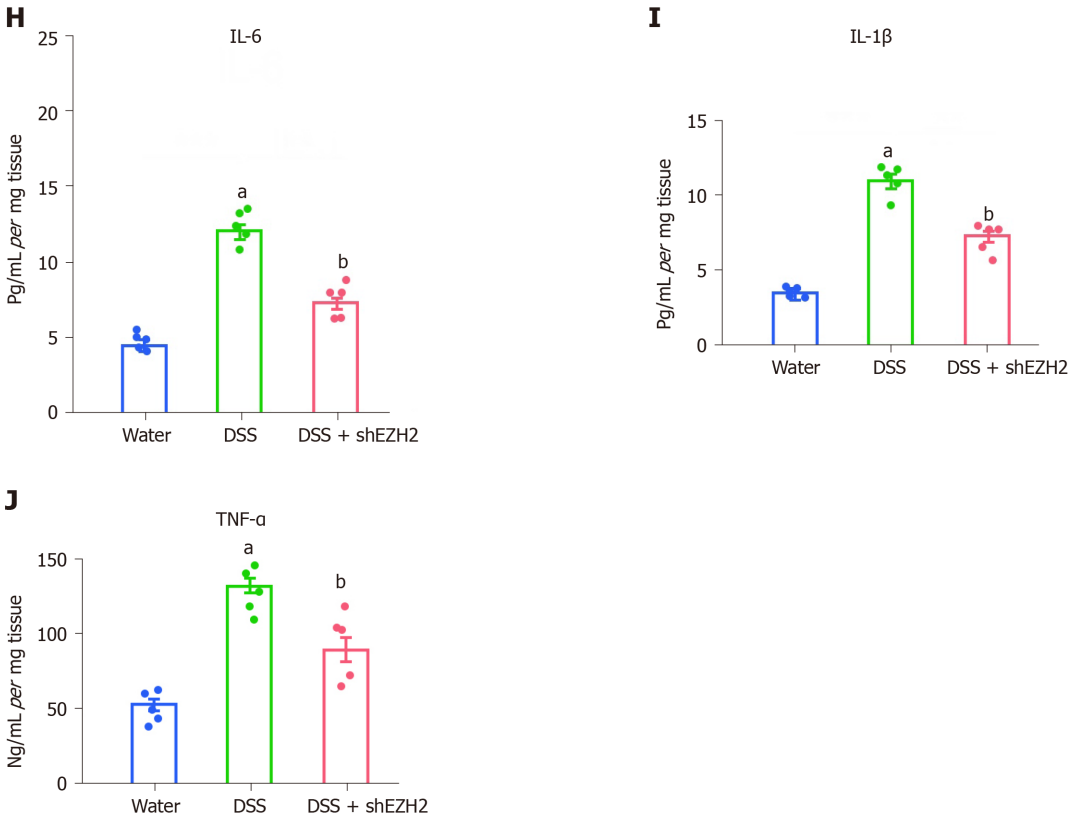


Figure 1 The depletion of enhancer of zeste homolog 2 inhibits dextran sodium sulfate-induced colitis *in vivo*. A: The expression of enhancer of zeste homolog 2 (EZH2) was measured by quantitative real-time polymerase chain reaction in clinical inflammatory bowel disease (IBD) samples (n = 50) and healthy controls (n = 50); B-J: The IBD mouse model was conducted by adding dextran sodium sulfate (DSS). The mice were intraperitoneally injected with lentiviral plasmids carrying EZH2 shRNA; B: The expression of EZH2 was detected by quantitative real-time polymerase chain reaction in the mice; C: The protein levels of EZH2 were analyzed by Western blot in the mice; D: The body weight in the mice; E and F: Colon length of the mice; G: representative hematoxylin and eosin staining of distal colon sections; H-J: Colonic inflammatory cytokines in the mice. n = 10, mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. TNF-α: Tumor necrosis factor-α; IL: Interleukin.

depletion (Figure 1G). Moreover, the inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, were enhanced in the DSS-treated mice, in which the depletion of EZH2 could reverse this effect (Figure 1H-J).

EZH2 depletion attenuates apoptosis of colonic epithelial cells

Then, the NCM460 and FHC colonic epithelial cells were treated with TNF-α or co-treated with TNF-α and EZH2 shRNA. We found that the expression of cleaved-caspase-3 was enhanced in the TNF-α-treated NCM460 and FHC cells, and the depletion of EZH2 was able to inhibit the enhancement in the cells (Figure 2A and B). Moreover, the TNF-α treatment induced the apoptosis of NCM460 and FHC cells, in which EZH2 knockdown could reverse this effect in the cells (Figure 2C and D).

The depletion of EZH2 attenuates permeability of colonic epithelial cells

Next, we further identified that the TEER was reduced by TNF-α treatment in the NCM460 and FHC cells, which was rescued by the depletion of EZH2 in the cells (Figure 3A and B). Consistently, the expression of permeability markers, including ZO-1, claudin-5, and occludin, was inhibited in the TNF-α-treated NCM460 and FHC cells, but the EZH2 depletion could rescue this phenotype in the cells (Figure 3C and D).

EZH2 inactivates JAK2/STAT3 signaling in colonic epithelial cells

Significantly, Western blot analysis showed that the depletion of EZH2 enhanced the expression and the phosphorylation of JAK2 and STAT3 in the NCM460 and FHC cells (Figure 4A and B). Meanwhile, the expression and the phosphorylation of JAK2 and STAT3 EZH2 inhibitor were induced by the EZH2 inhibitor GSK343 in the cells (Figure 4C and D).

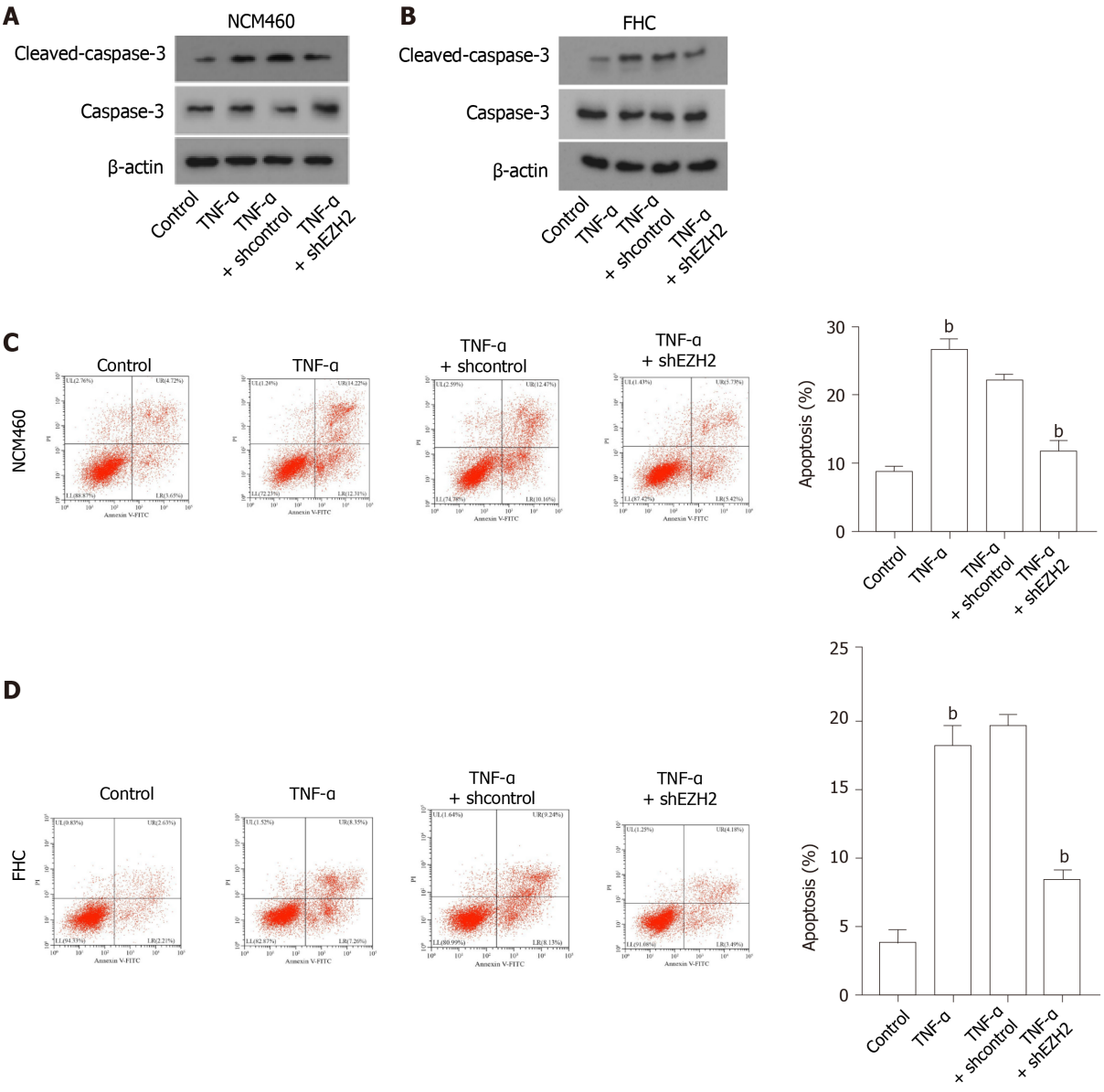


Figure 2 Enhancer of zeste homolog 2 depletion attenuates apoptosis of colonic epithelial cells. The NCM460 and fetal human colon (FHC) cells were treated with tumor necrosis factor-α (TNF-α), or co-treated with TNF-α and enhancer of zeste homolog 2 (EZH2) short hairpin RNA. A and B: Western blot analysis of the caspase3 and cleaved-caspase3 expression; C and D: Flow cytometry analysis of the cell apoptosis. n = 3, mean ± SD, $^{b}P < 0.01$.

EZH2 inactivates JAK2 expression by regulating histone H3K27me3

Furthermore, we observed that the H3K27me3 was reduced, along with the enhanced expression and the phosphorylation of JAK2 and STAT3, in the EZH2-knockdown NCM460 and FHC cells (Figure 5A and B). In addition, the depletion of EZH2 reduced the levels of H3K27me3 on the promoter of JAK2 in the cells (Figure 5C and D). Consistently, the mRNA expression of JAK2 was up-regulated by EZH2 depletion in the NCM460 and FHC cells (Figure 5E and F).

EZH2 promotes apoptosis and permeability by inactivating JAK2/STAT signaling in colonic epithelial cells

Next, we identified that EZH2 inhibitor GSK343 attenuated TNF-α-induced apoptosis of the NCM460 and FHC cells, and the JAK2 inhibitor TG101348 was able to rescue the phenotype in the cells (Figure 6A and B). Moreover, GSK343 rescued TNF-α-inhibited ZO-1, claudin-5, and occludin expression in the NCM460 and FHC cells, in which the co-treatment with TG101348 could reverse this effect in the system (Figure 6C and D).

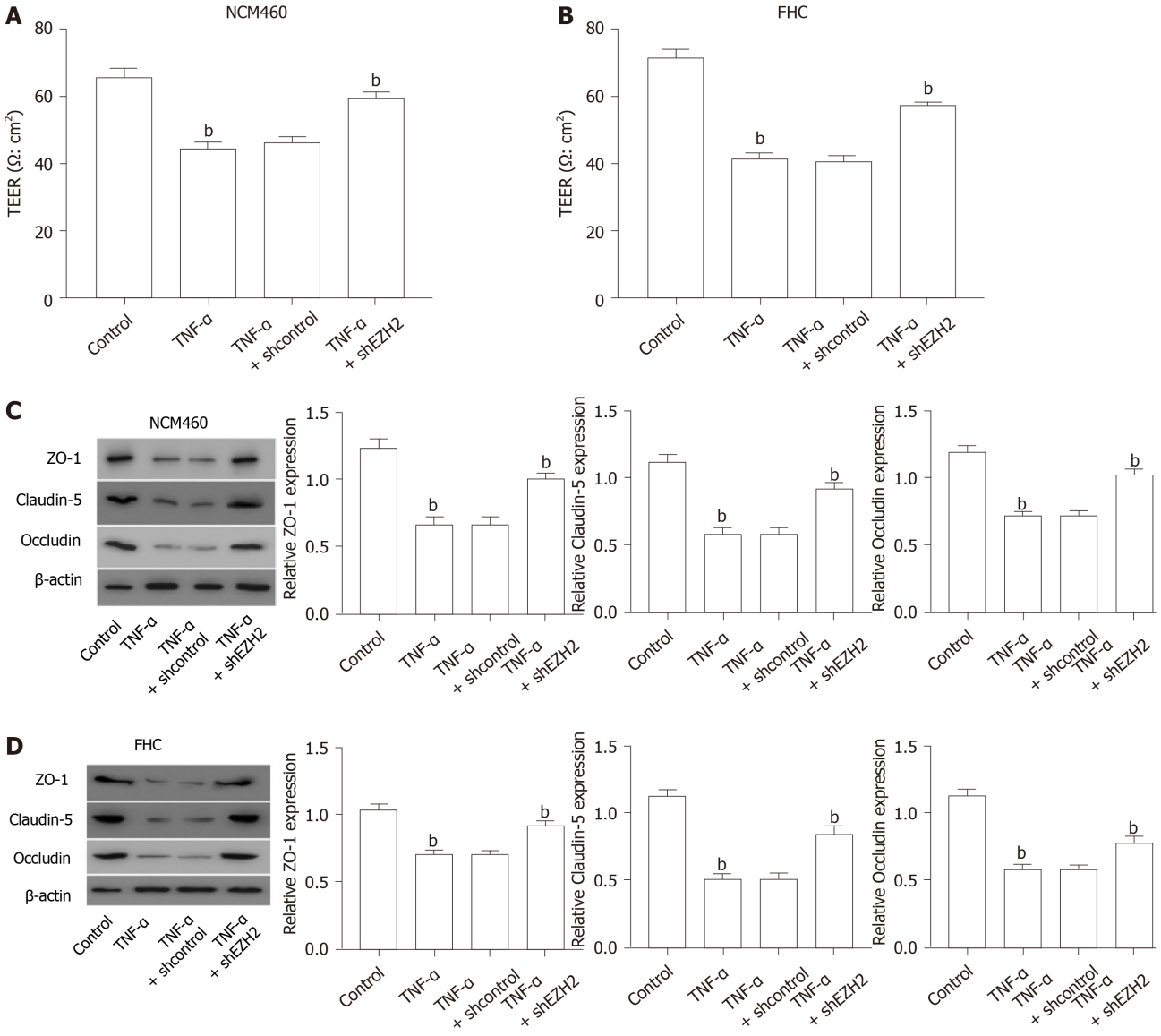


Figure 3 The depletion of enhancer of zeste homolog 2 attenuates permeability of colonic epithelial cells. The NCM460 and fetal human colon (FHC) cells were treated with tumor necrosis factor-α (TNF-α), or co-treated with TNF-α and enhancer of zeste homolog 2 (EZH2) short hairpin RNA. A and B: Transepithelial electrical resistance (TEER) measurement of the transepithelial electrical resistance levels; C and D: Western blot analysis of the Zona occludens 1 (ZO-1), claudin-5, and occludin expression. $n = 3$, mean ± SD, $^{b}P < 0.01$.

DISCUSSION

IBD is a prevalent inflammatory gastrointestinal disease with high incidence. As a critical epigenetic regulator, EZH2 has been identified to contribute to IBD progression, but the mechanism remains unclear. In this study, we found that EZH2 promoted apoptosis and inflammatory response by inactivating JAK2/STAT signaling in IBD.

Several previous investigations have shown the function of EZH2 in the modulation of IBD. It has been reported that EZH2 is involved in the pathobiological mechanism of IBD progression[14]. Inhibiting EZH2 attenuates intestinal inflammation of IBD development[11]. EZH2 is an epigenetic regulator in colitis by inhibiting TNF-α-regulated apoptosis and inflammation in IBD[15]. EZH2 modulates intestinal necroptosis and inflammation in intestinal epithelial cells by c-jun N-terminal kinase signaling[16]. Targeting EZH2 enhances cAMP response element-binding protein to inhibit the ulcerative colitis progression[17]. It has been well-recognized that EZH2 presents a critical biological significance of in autoimmune disorders and cancer, and thereby targeting EZH2 is a promising therapeutic strategy in these diseases[18,19]. In this study, we demonstrated that the depletion of EZH2 inhibited DSS-induced colitis *in vivo*. EZH2 depletion attenuated apoptosis and permeability of colonic epithelial cells. Our data identified a critical role of EZH2 in the IBD progression, providing

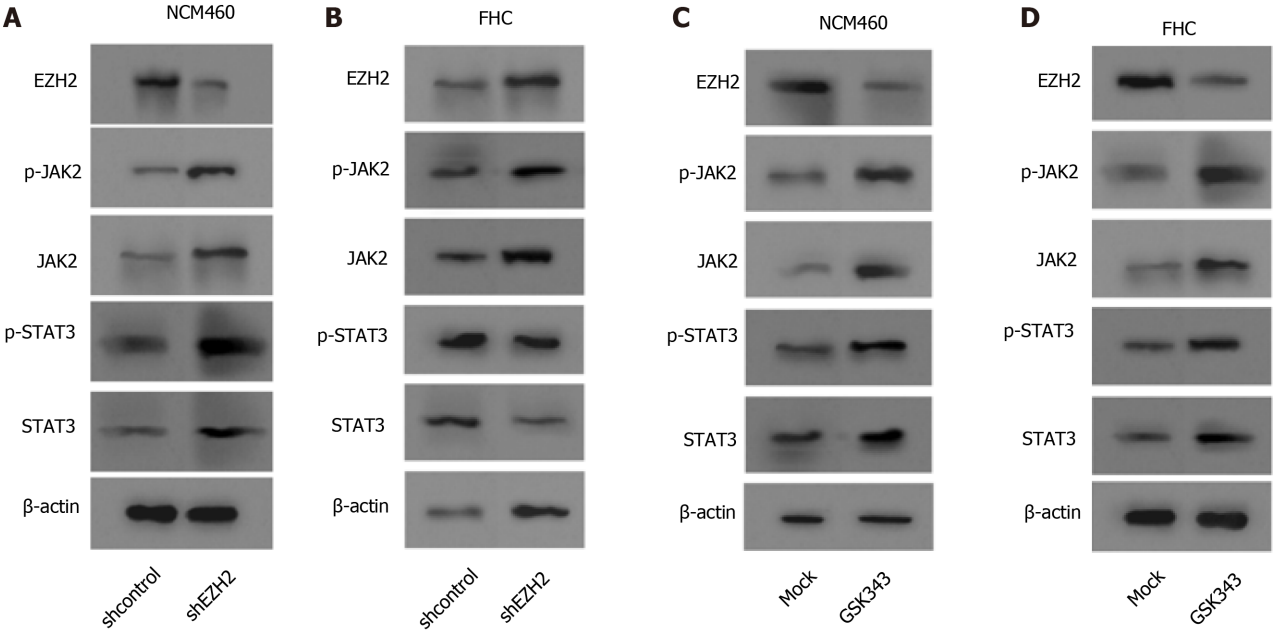


Figure 4 Enhancer of zeste homolog 2 inactivates janus kinase 2/ signal transducer and activator of transcription signaling in colonic epithelial cells. A and B: The NCM460 and fetal human colon (FHC) cells were treated with control shRNA or enhancer of zeste homolog 2 (EZH2) short hairpin RNA. Western blot analysis of the Janus kinase 2 (JAK2), Signal transducer and activator of transcription 3 (STAT3), and EZH2 expression and JAK2 and STAT3 phosphorylation; C and D: The NCM460 and FHC cells were treated with GSK343 (5 μM). Western blot analysis of the JAK2, STAT3, and EZH2 expression and JAK2 and STAT3 phosphorylation. *n* = 3.

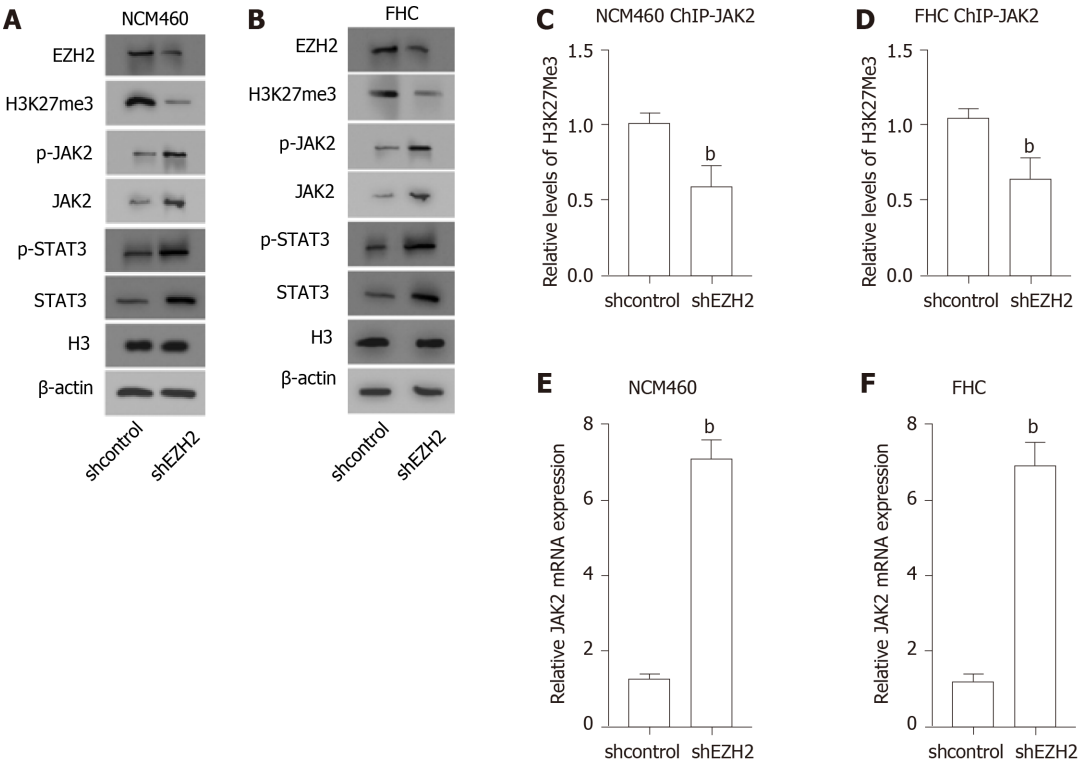


Figure 5 Enhancer of zeste homolog 2 inactivates janus kinase 2 expression by regulating histone H3K27me3. A-E: The NCM460 and fetal human colon (FHC) cells were treated with control short hairpin RNA or enhancer of zeste homolog 2 (EZH2) short hairpin RNA; A and B: Western blot analysis of the H3K27me3, Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), and EZH2 expression and JAK2 and STAT3 phosphorylation; C and D: Chromatin immunoprecipitation (ChIP) assays of the JAK2 promoter using H3K27me3 antibody; E and F: The quantitative real-time polymerase chain reaction assays of JAK2 messenger RNA expression. *n* = 3, mean ± SD, $^{b}P < 0.01$.

informative evidence of the function of EZH2 in modulating IBD development.

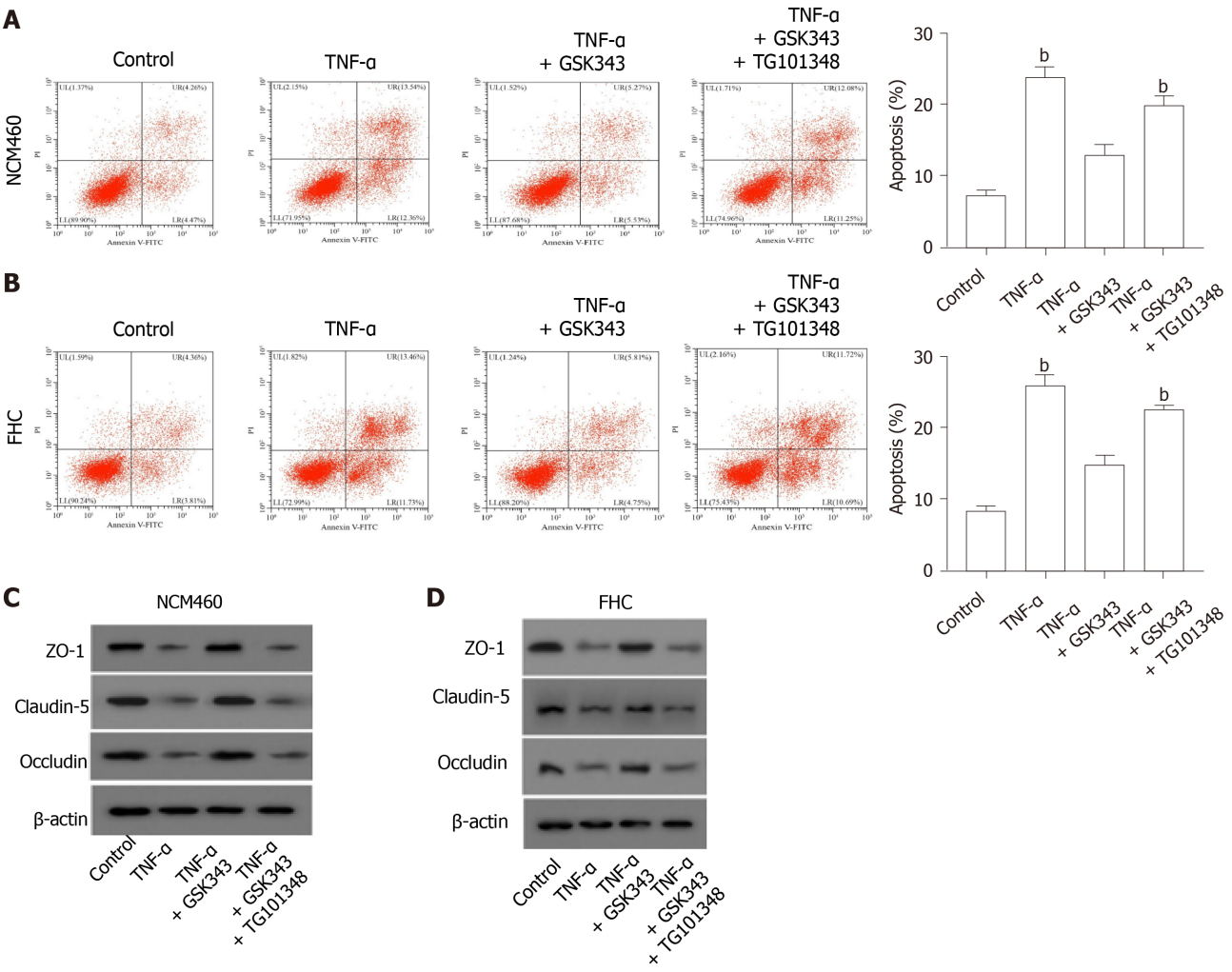


Figure 6 Enhancer of zeste homolog 2 promotes apoptosis and permeability by inactivating janus kinase 2/ signal transducer and activator of transcription signaling in colonic epithelial cells. The tumor necrosis factor-α (TNF-α)-treated NCM460 and fetal human colon (FHC) cells were treated with GSK343 (5 μM) or co-treated with GSK343 (5 μM) and TG101348 (5 μM). A and B: Flow cytometry analysis of the cell apoptosis; C and D: Western blot analysis of the Zona occludens 1 (ZO-1), claudin-5, and occludin expression. n = 3, mean ± SD. $^{b}P < 0.01$.

As essential cellular signaling in various pathological conditions, JAK2/STAT3 signaling plays critical role in IBD regulation. It has been found that V617F is able to up-regulate JAK2 expression in patients with IBD[20]. Sphk1 enhances ulcerative colitis by modulating JAK2/STAT3 signaling[21]. Renin-angiotensin system contributes to colonic inflammation by inducing helper T 17 activation through JAK2/STAT signaling[22]. A20 inhibits IBD *via* repressing nuclear factor-kappa B and STAT3 in mice[23]. Our mechanical studies showed that EZH2 inactivated JAK2 expression by regulating histone H3K27me3. EZH2 promoted apoptosis and permeability by inactivating JAK2/STAT signaling in colonic epithelial cells. These data discover an unreported association of EZH2 with JAK2/STAT signaling, elucidating a new mechanism involving EZH2 and JAK2/STAT in IBD pathogenesis.

CONCLUSION

In conclusion, we discovered that EZH2 contributed to apoptosis and inflammatory response by inactivating JAK2/STAT signaling in IBD. EZH2 is a promising target for treatment of IBD.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD) is a prevalent worldwide health problem featured by relapsing, chronic gastrointestinal inflammation. Enhancer of zeste homolog 2 (EZH2) is a critical epigenetic regulator in different pathological models, such as cancer and inflammation. However, the role of EZH2 in the IBD development is still obscure.

Research motivation

To identify the effect of EZH2 on the IBD progression and the underlying mechanism.

Research objectives

To explore the role of EZH2 in the IBD progression and the underlying mechanism.

Research methods

The IBD mouse model was conducted by adding dextran sodium sulfate (DSS) and examining the effect of EZH2 on DSS-induced colitis in the model. The function of EZH2 in regulating apoptosis and permeability was evaluated by Annexin V-FITC Apoptosis Detection Kit, transepithelial electrical resistance analysis, and Western blot analysis of related markers, including Zona occludens 1, claudin-5, and occludin, in NCM460 and fetal human colon (FHC) cells. The mechanical investigation was performed by quantitative reverse transcription-polymerase chain reaction, Western blot analysis, and chromatin immunoprecipitation assays.

Research results

The colon length was inhibited in the DSS-treated mice and was enhanced by the EZH2 depletion in the system. DSS treatment caused a decreased histological score in the mice, which was reversed by EZH2 depletion. The inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-6, and interleukin-1β, were induced in the DSS-treated mice, in which the depletion of EZH2 could reverse this effect. Moreover, the TNF-α treatment induced the apoptosis of NCM460 and FHC cells, in which EZH2 depletion could reverse this effect in the cells. Moreover, the depletion of EZH2 attenuated permeability of colonic epithelial cells. Mechanically, the depletion of EZH2 or EZH2 inhibitor GSK343 was able to enhance the expression and the phosphorylation of JAK2 and STAT3 in the NCM460 and FHC cells. Specifically, EZH2 inactivated JAK2 expression by regulating histone H3K27me3. JAK2 inhibitor TG101348 was able to reverse EZH2 knockdown-mediated colonic epithelial cell permeability and apoptosis.

Research conclusions

EZH2 contributes to apoptosis and inflammatory response by inactivating JAK2/STAT signaling in IBD.

Research perspectives

EZH2 may be applied as a potential target for IBD therapy.

REFERENCES

1 **Speca S**, Dubuquoy L. Chronic bowel inflammation and inflammatory joint disease: Pathophysiology. *Joint Bone Spine* 2017; **84**: 417-420 [PMID: 28062378 DOI: 10.1016/j.jbspin.2016.12.016]

2 **Sands BE**. Biomarkers of Inflammation in Inflammatory Bowel Disease. *Gastroenterology* 2015; **149**: 1275-1285.e2 [PMID: 26166315 DOI: 10.1053/j.gastro.2015.07.003]

3 **Franzosa EA**, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, Vatanen T, Hall AB, Mallick H, McIver LJ, Sauk JS, Wilson RG, Stevens BW, Scott JM, Pierce K, Deik AA, Bullock K, Imhann F, Porter JA, Zhernakova A, Fu J, Weersma RK, Wijmenga C, Clish CB, Vlamakis H, Huttenhower C, Xavier RJ. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat Microbiol* 2019; **4**: 293-305 [PMID: 30531976 DOI: 10.1038/s41564-018-0306-4]

4 **Shao BZ**, Wang SL, Pan P, Yao J, Wu K, Li ZS, Bai Y, Linghu EQ. Targeting NLRP3 Inflammasome in Inflammatory Bowel Disease: Putting out the Fire of Inflammation. *Inflammation* 2019; **42**: 1147-1159 [PMID: 30937839 DOI: 10.1007/s10753-019-01008-y]

5 **Graham DB**, Xavier RJ. Pathway paradigms revealed from the genetics of inflammatory bowel

disease. *Nature* 2020; **578**: 527-539 [PMID: 32103191 DOI: 10.1038/s41586-020-2025-2]
6 **Wright EK**, Ding NS, Niewiadomski O. Management of inflammatory bowel disease. *Med J Aust* 2018; **209**: 318-323 [PMID: 30257634 DOI: 10.5694/mja17.01001]
7 **Halfvarson J**, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, D'Amato M, Bonfiglio F, McDonald D, Gonzalez A, McClure EE, Dunklebarger MF, Knight R, Jansson JK. Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol* 2017; **2**: 17004 [PMID: 28191884 DOI: 10.1038/nmicrobiol.2017.4]
8 **Schmidl C**, Delacher M, Huehn J, Feuerer M. Epigenetic mechanisms regulating T-cell responses. *J Allergy Clin Immunol* 2018; **142**: 728-743 [PMID: 30195378 DOI: 10.1016/j.jaci.2018.07.014]
9 **Henning AN**, Roychoudhuri R, Restifo NP. Epigenetic control of $CD8^+$ T cell differentiation. *Nat Rev Immunol* 2018; **18**: 340-356 [PMID: 29379213 DOI: 10.1038/nri.2017.146]
10 **O'Meara MM**, Simon JA. Inner workings and regulatory inputs that control Polycomb repressive complex 2. *Chromosoma* 2012; **121**: 221-234 [PMID: 22349693 DOI: 10.1007/s00412-012-0361-1]
11 **Zhou J**, Huang S, Wang Z, Huang J, Xu L, Tang X, Wan YY, Li QJ, Symonds ALJ, Long H, Zhu B. Targeting EZH2 histone methyltransferase activity alleviates experimental intestinal inflammation. *Nat Commun* 2019; **10**: 2427 [PMID: 31160593 DOI: 10.1038/s41467-019-10176-2]
12 **Duan R**, Du W, Guo W. EZH2: a novel target for cancer treatment. *J Hematol Oncol* 2020; **13**: 104 [PMID: 32723346 DOI: 10.1186/s13045-020-00937-8]
13 **Polgar N**, Csongei V, Szabo M, Zambo V, Melegh BI, Sumegi K, Nagy G, Tulassay Z, Melegh B. Investigation of JAK2, STAT3 and CCR6 polymorphisms and their gene-gene interactions in inflammatory bowel disease. *Int J Immunogenet* 2012; **39**: 247-252 [PMID: 22269120 DOI: 10.1111/j.1744-313X.2012.01084.x]
14 **Sarmento OF**, Svingen PA, Xiong Y, Sun Z, Bamidele AO, Mathison AJ, Smyrk TC, Nair AA, Gonzalez MM, Sagstetter MR, Baheti S, McGovern DP, Friton JJ, Papadakis KA, Gautam G, Xavier RJ, Urrutia RA, Faubion WA. The Role of the Histone Methyltransferase Enhancer of Zeste Homolog 2 (EZH2) in the Pathobiological Mechanisms Underlying Inflammatory Bowel Disease (IBD). *J Biol Chem* 2017; **292**: 706-722 [PMID: 27909059 DOI: 10.1074/jbc.M116.749663]
15 **Liu Y**, Peng J, Sun T, Li N, Zhang L, Ren J, Yuan H, Kan S, Pan Q, Li X, Ding Y, Jiang M, Cong X, Tan M, Ma Y, Fu D, Cai S, Xiao Y, Wang X, Qin J. Epithelial EZH2 serves as an epigenetic determinant in experimental colitis by inhibiting TNFα-mediated inflammation and apoptosis. *Proc Natl Acad Sci USA* 2017; **114**: E3796-E3805 [PMID: 28439030 DOI: 10.1073/pnas.1700909114]
16 **Lou X**, Zhu H, Ning L, Li C, Li S, Du H, Zhou X, Xu G. EZH2 Regulates Intestinal Inflammation and Necroptosis Through the JNK Signaling Pathway in Intestinal Epithelial Cells. *Dig Dis Sci* 2019; **64**: 3518-3527 [PMID: 31273598 DOI: 10.1007/s10620-019-05705-4]
17 **Li K**, Yang J, Lei XF, Li SL, Yang HL, Xu CQ, Deng L. EZH2 inhibition promotes ANGPTL4/CREB1 to suppress the progression of ulcerative colitis. *Life Sci* 2020; **250**: 117553 [PMID: 32194081 DOI: 10.1016/j.lfs.2020.117553]
18 **Yang YX**, Shen HH, Cao F, Xie LY, Zhu GL, Sam NB, Wang DG, Pan HF. Therapeutic potential of enhancer of zeste homolog 2 in autoimmune diseases. *Expert Opin Ther Targets* 2019; **23**: 1015-1030 [PMID: 31747802 DOI: 10.1080/14728222.2019.1696309]
19 **Stazi G**, Zwergel C, Mai A, Valente S. EZH2 inhibitors: a patent review (2014-2016). *Expert Opin Ther Pat* 2017; **27**: 797-813 [PMID: 28394193 DOI: 10.1080/13543776.2017.1316976]
20 **Asadzadeh-Aghdaei H**, Mashayekhi K, Koushki K, Azimzadeh P, Rostami-Nejad M, Amani D, Chaleshi V, Haftcheshmeh SM, Sahebkar A, Zali MR. V617F-independent upregulation of JAK2 gene expression in patients with inflammatory bowel disease. *J Cell Biochem* 2019; **120**: 15746-15755 [PMID: 31069840 DOI: 10.1002/jcb.28844]
21 **Liu J**, Jiang B. Sphk1 promotes ulcerative colitis *via* activating JAK2/STAT3 signaling pathway. *Hum Cell* 2020; **33**: 57-66 [PMID: 31606874 DOI: 10.1007/s13577-019-00283-z]
22 **He L**, Du J, Chen Y, Liu C, Zhou M, Adhikari S, Rubin DT, Pekow J, Li YC. Renin-angiotensin system promotes colonic inflammation by inducing T_H17 activation *via* JAK2/STAT pathway. *Am J Physiol Gastrointest Liver Physiol* 2019; **316**: G774-G784 [PMID: 30995068 DOI: 10.1152/ajpgi.00053.2019]
23 **Lee SH**, Lee HR, Kwon JY, Jung K, Kim SY, Cho KH, Choi J, Lee HH, Lee BI, Jue DM, Cho ML. A20 ameliorates inflammatory bowel disease in mice *via* inhibiting NF-κB and STAT3 activation. *Immunol Lett* 2018; **198**: 44-51 [PMID: 29608924 DOI: 10.1016/j.imlet.2018.03.015]

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ORIGINAL ARTICLE

Retrospective Study

Quinone oxidoreductase 1 is overexpressed in gastric cancer and associated with outcome of adjuvant chemotherapy and survival

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Author contributions: Tang XW and Jiang ZN conceived the study; Ahmed SMU performed the experiments; Jiang ZN, Wang QC, Ahmed SM, Shi H, and Tang XW analyzed the data; Ahmed SM and Tang XW wrote the manuscript; all authors have read and approved the final manuscript; Jiang ZN and Ahmed SM contributed equally to this work.

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Institutional review board statement: The study was reviewed and approved by the Affiliated Sir Run Run Shaw Hospital, Zhejiang University School of Medicine Institutional Review Board [(Approval No. 20200917-32)].

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Abstract

BACKGROUND

Quinine oxidoreductase 1 (NQO1) plays a vital role in protecting normal cells against oxidative damage and electrophilic attack. It is highly expressed in many solid tumors, suggesting a role in cancer development and progression. However, the role of NQO1 in gastric cancer and its effect on cancer development and prognosis have not been fully investigated.

AIM

To investigate the clinical relevance of NQO1 protein expression in gastric cancer and to explore the potential of NQO1 to serve as a prognostic biomarker and therapeutic target.

METHODS

In this retrospective study, gastric cancer specimens of 175 patients who were treated between 1995 and 2011 were subjected to immunohistochemistry analyses for NQO1. The correlation of NQO1 expression with gastric cancer prognosis and

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clinical and pathological parameters was investigated.

RESULTS

NQO1 protein was overexpressed in 59.43% (104/175) of the analyzed samples. Overexpression of NQO1 was associated with a significantly inferior prognosis. In addition, multivariate analysis suggested that NQO1 overexpression, along with tumor stage and patient age, are prominent prognostic biomarkers for gastric cancer. Moreover, NQO1 overexpression was correlated to a better response to 5-fluorouracil (5-FU)-based adjuvant chemotherapy.

CONCLUSION

NQO1 overexpression is associated with a significantly poor prognosis and better response to 5-FU in patients with gastric cancer. These findings are relevant for improving therapeutic approaches for gastric cancer patients.

Key Words: Gastric cancer; Quinone oxidoreductase 1; Prognosis; Immunohistochemistry; Kaplan-Meier curves; Biomarker

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Core Tip: Quinone oxidoreductase 1 may be used as a useful prognostic biomarker and therapeutic target for the efficient management of patients with gastric cancer.

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INTRODUCTION

Gastric cancer (GC) is now the fourth most common cancer and the third most common cause of cancer-related deaths worldwide. It is estimated that 951600 new GC cases and 723100 deaths occurred in 2012. East Asia, including China, is among the areas with the highest incidence of GC, accounting for > 40% of all new cases[1,2]. Presently, the prevalence rates of GC are declining in most parts of the world owing to the establishment of mass screening and advanced treatment techniques. Despite this decline, the clinical outcomes of GC patients remain disappointing. Moreover, the prognosis of GC strongly depends on the TNM stage, biological factors, and clinical pathological factors[3,4]. Therefore, finding a new biomarker for the diagnosis and treatment of GC is of great value.

Quinine oxidoreductase 1 (NQO1) is a cytoplasmic flavoenzyme located on chromosome 16q22. It is also known as DT-diaphorase, menadione reductase, or quinone reductase 1. NQO1 directly reduces quinines to hyroquinones using reduced form of nicotinamide-adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor[5,6]. Numerous functions of NQO1 have been reported, such as xenobiotic detoxification, superoxide scavenging, and maintenance of endogenous antioxidant vitamins[7]. It is likely that NQO1 plays an important role in protecting normal cells against oxidative damage and electrophilic attack. Paradoxically, despite the suggestion that it exerts a protective action and plays an antioxidant role, disruption of an *NQO1* genetic polymorphism increases the risk of xenobiotic-induced toxicity and cancer[8,9]. High levels of NQO1 expression have been found in many human tumors, including breast cancer, melanoma, lung cancer, cholangiocarcinoma, and pancreatic cancer[10-13]. Garate *et al*[10] reported that NQO1 protein expression induces cell cycle progression and proliferation in melanoma cells. In a study by Lin *et al*[14], NQO1 expression in GC patients, non-cancer patients, and normal controls was quantified by immunohistochemistry (IHC). They reported that NQO1 played an important role in the progression of GC, and might be a potential prognostic biomarker and therapeutic target for GC. However,

the role of NQO1 in cancer progression remains controversial, and data on its expression in GC are scarce. In this study, we analyzed the expression of NQO1 and its potential association with survival and pathological parameters in primary GC. We aimed to investigate the associations between NQO1 protein expression status and responses to 5-fluorouracil (5-FU)-based adjuvant chemotherapy in patients with GC and thereby determine the potential of NQO1 to serve as a prognostic biomarker and therapeutic target.

MATERIALS AND METHODS

Western blot analysis

Unless otherwise stated, all chemicals were procured from Sigma-Aldrich Co. (Shanghai, China). Antibodies against rabbit NQO1 were used as described previously [15,16]. The human gastric cancer cell line AGS was obtained from the American Type Culture Collection (Manassas, VA, United States). Cells were maintained in RPMI-1640 growth medium (Shenggong, Shanghai, China) supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C[17]. Cell extracts were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and NQO1 immunoblotting was performed using a standard protocol with anti-NQO1 antibodies (dilution at 1:2000)[17,18].

Patients

A total of 175 samples of GC were used in this study. All patients were enrolled and treated between 1995 and 2011 at the Department of Surgical Oncology, Affiliated Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China. All patients received surgical treatment and were periodically followed for the determination of their survival status.

Immunohistochemistry

Tumor samples were assembled as multiple tissue arrays, as described in our previous study[19]. IHC staining for NQO1 in paraffin-embedded tumor sections was performed using standard procedures. In brief, to eliminate endogenous peroxidase activity, the slides were deparaffinized in xylene, rehydrated through a graded series of ethanol solutions, and incubated with 3% H_2O_2 in methanol for 15 min at room temperature. Antigen retrieval was performed by placing the slides in 0.01 M sodium citrate buffer (pH 6.0) at 121 °C for 15 min. Next, the slides were incubated with the NQO1 antibodies (1:5000) overnight at 4 °C. The immunoreactions were visualized with an ImmPRESS detection kit (Vector Laboratories), which uses a second antibody conjugated with horseradish peroxidase and a diaminobenzidine-based stain. Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Cytoplasmic NQO1 staining was considered as positive for NQO1 expression. NQO1 expression was semiquantitatively assessed by two experienced pathologists who were blinded to the clinical parameters. Analyses of NQO1 expression were based on both the staining intensity and proportion of positive cells. The staining intensity was scored from 0 to 3+ as follows: 0 for no staining, 1+ for weak staining, 2+ for moderate staining, and 3+ for strong staining[20]. The proportion of NQO1-positive cells was expressed as a percentage and divided into four grades: grade 0, 0% positive; grade 1, 1%-30% positive; grade 2, 31%-60% positive; and grade 3, > 60% positive cells. The total score was obtained by multiplying these two parameters (range 0-9); for statistical purposes, the samples were divided into two groups: Low expression level (0-4) and high expression level or overexpression (> 4) groups. As the median score of NQO1 expression in the GC samples was 4.5, a cut-off value of 4 was selected[20].

Data analysis

The correlation between the expression of NQO1 and clinical and pathological parameters was determined by statistical analysis using SSPS 16.0 for Windows (SPSS, incorporated, Chicago, IL, United States). The χ^2 or Fisher's exact test, Kaplan-Meier survival analysis, and Cox regression analysis were used as appropriate. P values < 0.05 were considered significant.

RESULTS

NQO1 antibody is specific and sensitive for IHC staining

Protein extracts from AGS human GC cells were separated by SDS-PAGE, and the proteins were detected by Western blot analysis. The results showed that NQO1 can be readily detected, and no other protein cross-reacted with the NQO1 antibodies (Figure 1). Therefore, these antibodies are sensitive and specific for IHC staining to detect NQO1 expression in paraffin-embedded tumor sections.

Patient characteristics

A total of 175 patients were enrolled in this study. The baseline characteristics are described in detail and summarized in Table 1. The patients ranged from 26 to 84 years of age. The study population consisted of 124 men and 51 women. The clinical and pathological parameters consisted of age, gender, tumor size, tumor location, tumor stage, tumor depth, lymph node metastasis, histological differentiation, and survival data. The number of patients with a tumor size < 5 cm and ≥ 5 cm was 63 and 99, respectively. The stage of GC was determined at the time of analysis, according to the American Joint Commission on Cancer system AJCC TNM 8th edition. Of the 175 samples, 69 cases were stage I-II and 102 were stage III-IV GC. Among all patients, 67 underwent total gastrectomy, 83 received distal subtotal gastrectomy, 1 received partial resection, and 14 received proximal subtotal gastrectomy. The information of the remaining 10 patients was missing. None of the patients received chemotherapy prior to surgery. A total of 84 patients were treated with 5-FU + platinum-based adjuvant chemotherapy. The patients were followed until the last follow-up date or death. Among the 175 patients, there were 75 death events and 65 recurrence events in total. The follow-up period ranged from 0.5-182 mo, and the median follow-up time was 22.4 mo.

Overexpression of NQO1 protein is associated with GC prognosis

IHC staining showed that the NQO1 protein was mainly localized in the cytoplasm of GC cells (Figure 2). The level of NQO1 protein expression was high in 49.43% of the samples, with an overall score of > 4, while 71 (40.57%) samples showed low NQO1 expression levels, with an overall score of ≤ 4. To evaluate the role of the NQO1 protein in GC progression, we analyzed the correlation between its expression and the clinical and pathological parameters (Table 2). The number of patients with high levels of NQO1 expression and lymph node metastasis was higher than that of patients with low NQO1 expression, but this difference was not significant (P = 0.06; Table 2). In addition, there were no significant correlations between NQO1 and other clinical parameters (Table 2). However, the follow-up results showed that the mean survival time for patients with low levels of NQO1 expression was 34 mo, while for those with NQO1 overexpression, it was 22 mo. Kaplan-Meier survival analysis revealed that patients with NQO1 overexpression had significantly poorer survival rates than those with low levels of NQO1 expression (P = 0.02; Figure 3).

We further used the Cox proportional hazard regression method to examine the correlations between various clinical indexes and survival (Table 3). Univariate analysis revealed significant associations of tumor size (P < 0.001), tumor stage (P < 0.001), and NQO1 expression (P = 0.01) with overall survival, whereas the other factors showed no significant association. However, multivariate analysis of these factors, including NQO1 expression, indicated that age, tumor stage, and NQO1 were independent prognostic factors for the overall survival of GC patients [hazard ratio (HR) = 1.90, P = 0.01; HR = 2.64, P < 0.001; and HR = 1.56, P = 0.05, respectively].

NQO1 overexpression predicts a favorable response to chemotherapy in GC patients

We also performed the survival analysis of patients from the various treatment subgroups based on the NQO1 expression. Among 84 patients treated with 5-FU-based chemotherapy, 52 (61.09%) had high levels of NQO1 expression and 32 (38.10%) had low levels of NQO1 expression. Similarly, high and low levels of NQO1 expression were found in 60% and 40% of patients who did not receive treatment, respectively. Patients with low levels of NQO1 expression showed poor response to adjuvant chemotherapy and similar survival, compared to those that underwent surgery alone (log rank P = 0.82) (Figure 4A). However, high levels of NQO1 expression indicated good response to adjuvant chemotherapy and longer overall survival compared with those in patients that underwent surgery alone (log rank P = 0.03) (Figure 4B). In addition, in the Kaplan-Meier analysis, we stratified NQO1

Table 1 Clinical characteristic of the gastric cancer patients

Clinical parameter	Number of cases (*n*)	(%)
Age group	Range (26-84, mean = 58.9)	
< 60 yr	83	47.43
≥ 60 yr	92	52.57
Sex		
Male	124	70.86
Female	51	29.14
Tumor size (cm)		
< 5	63	36.00
≥ 5	99	56.57
Unknown	13	7.43
Tumor location		
Fundus	40	22.86
Body	44	25.14
Antral	82	46.68
Whole	4	2.29
Unknown	5	2.85
TNM stage		
I II	69	39.43
III IV	102	58.29
Unknown	4	2.28
Tumor depth		
T1/T2	33	18.86
T3/T4	139	79.43
Unknown	3	1.71
Lymph node metastasis		
Yes	43	24.57
No	123	70.29
Unknown	9	5.14
Vascular invasion		
Yes	19	10.86
No	156	89.14
Histological Differentiation		
Poor	20	11.43
Moderate	44	25.14
Well	111	63.43
Surgical type		
Complete resection	67	38.29
Distal resection	83	47.43
Proximal resection	14	8.00
Partial resection	1	0.57
Unknown	10	5.71

Chemotherapy		
Adjuvant chemotherapy	84	48.00
Without chemotherapy	80	45.71
Unknown	11	6.29

Table 2 Association of quinine oxidoreductase 1 expression with clinical and pathological features of gastric patients

Clinical parameter	NQO1 expression		
	High (%)	Low (%)	*P* value
Age (yr)			0.92
< 60	34 (40.96)	49 (59.04)	
≥ 60	37 (40.22)	55 (59.78)	
Gender			
Male	48 (38.71)	76 (61.29)	
Female	23 (45.10)	28 (54.90)	
Tumor size (cm)			0.81
< 5	23 (36.51)	40 (63.49)	
≥ 5	38 (38.38)	61 (61.62)	
Tumor location			0.19
Fundus	18 (40.91)	26 (59.09)	
Body	11 (27.50)	29 (72.50)	
Antral	34 (41.46)	48 (58.54)	
Whole	3 (75.00)	1(25.00)	
Tumor staging			0.06
I/II	22 (31.88)	47 (68.12)	
III/IV	47 (46.08)	55 (53.92)	
Tumor depth			0.37
T1 T2	11 (33.33)	22 (66.67)	
T3 T4	58 (41.73)	81 (58.27)	
Lymph node metastasis			
Yes	12 (27.91)	31 (72.09)	
No	54 (43.90)	69 (56.10)	
Vascular invasion			0.72
Yes	7 (36.84)	12 (63.16)	
No	64 (41.03)	92 (58.97)	
Histological differentiation			0.57
Poor	8 (40.00)	12 (60.00)	
Moderate	15 (34.10)	29 (65.90)	
Well	48 (43.24)	63 (56.76)	

According to Table 1, the total case number is 162 based on tumor size, 170 based on tumor location, 171 based on tumor stage, and 172 based on tumor depth.

expression based on chemotherapy. Interestingly, the results showed that in patients who underwent surgery only, significant difference in overall survival was observed (log rank $P = 0.003$), whereas in patients who underwent surgery plus chemotherapy,

Table 3 Univariate and multivariate analyses of prognostic factors in gastric cancer patient

Clinical factor		[1]Univariate			Multivariate	
	P value	HR	95%CI	*P* value	HR	95%CI
Interval						
Age (< 60/≥ 60)						
	-	-	-	0.01	1.90	1.20-3.02
Gender (male/female)						
	-	-	-	0.96	1.03	0.62-1.66
Tumor size (< 5/≥ 5 cm)						
	< 0.001	1.75	1.10-2.87	0.40	1.00	0.76-2.25
Tumor location						
Proximal		1 (Reference)			1 (Reference)	
Body	0.67	1.13	0.65-1.94	0.82	1.06	0.59-1.95
Distal	0.09	0.7	0.43-1.15	0.11	0.69	0.41-1.19
Whole[2]	-	-	-	-	-	-
Tumor stage (III, IV/I, II)						
	< 0.001	3.11	1.96-5.09	< 0.001	2.64	1.56-4.61
Histological differentiation						
Well		1 (Reference)			1 (Reference)	
Moderate	0.3	1.58	0.68-4.30	0.62	1.26	0.53-3.48
Poor	0.07	2.03	0.96-5.23	0.15	1.79	0.83-4.67
NQO1 (high/low)						
	0.01	1.73	1.16-2.58	0.05	1.56	1.01-2.40

[1]Adjusted for age and gender.
[2]Patients in whole were not included due to too few cases (n = 4). NQO1: Quinine oxidoreductase 1; CI: Confidence interval; HR: Hazard ratio.

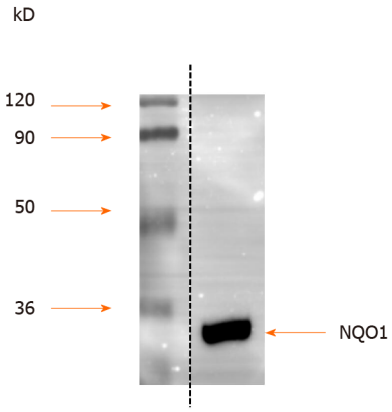


Figure 1 Western blot analysis of quinine oxidoreductase 1 expression in gastric carcinoma cell line AGS. Protein extracts from AGS human gastric cancer cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were detected by Western blot using anti-quinine oxidoreductase 1 antibodies. Molecular masses in kDa are indicated to the left. NQO1: Quinine oxidoreductase 1.

no difference was found (log rank P = 0.61) (Figure 4C and D). These findings indicated that GC patients with NQO1 overexpression may be suitable for adjuvant chemotherapy.

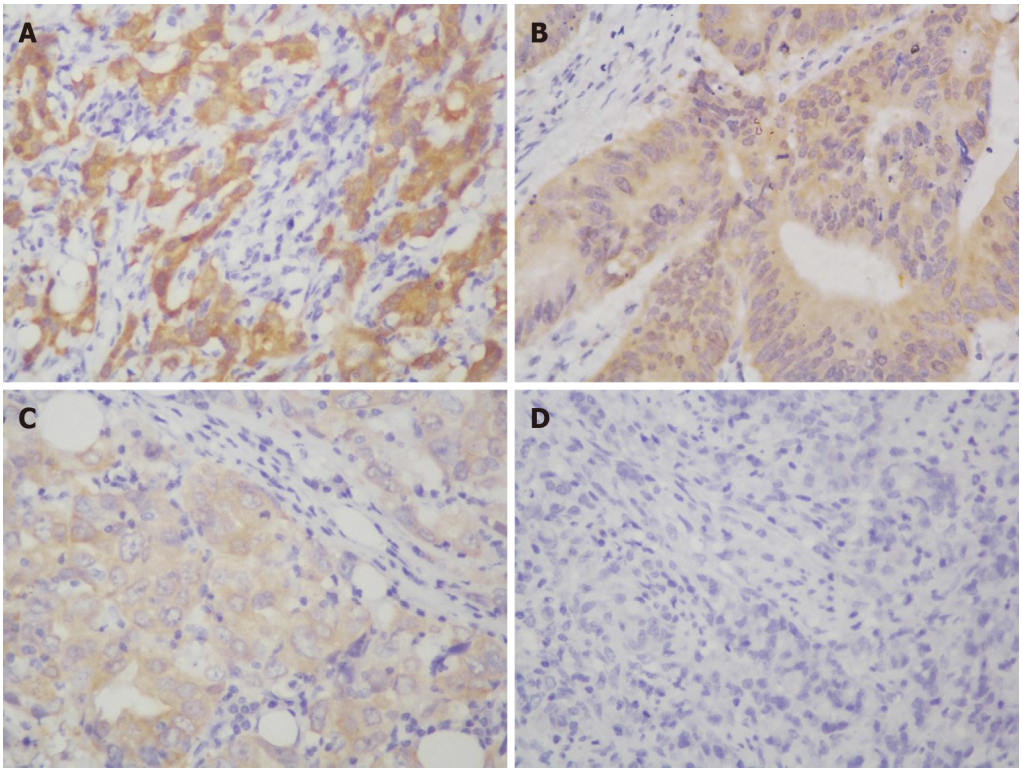


Figure 2 Representative images of immunohistochemical staining for quinine oxidoreductase 1 in gastric cancer samples. A: Strong (+++); B: Moderate (++); C: Weak (+); D: Negative staining (original magnification × 400).

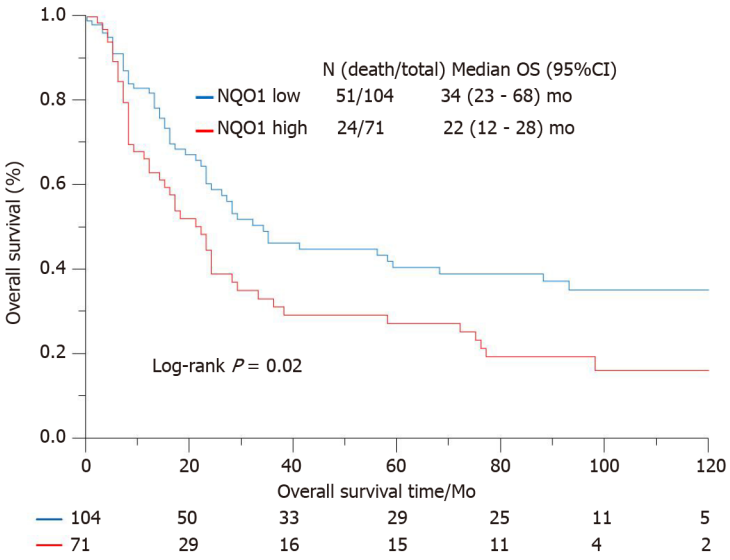


Figure 3 Kaplan-Meier survival curves of postoperative outcomes of gastric cancer patients according to quinine oxidoreductase 1 expression status. The correlation of high and low quinine oxidoreductase 1 expression with the overall survival rate was statistically significant (P = 0.02). NQO1: Quinine oxidoreductase 1; OS: Overall survival; CI: Confidence interval.

DISCUSSION

The catalytic properties of NQO1 were first reported by Ernster and Navazio[21] in 1958. NQO1 is a homodimeric flavoprotein that reduces quinines to hydroquinones in an obligatory two-electron reduction step[6]. After years of research, cumulative data have shown that NQO1 protects cells from exogenous substances, oxidants, and ultraviolet and ionizing radiation by inducing a series of protective reactions[10]. Consistent with this finding, recent research by Nagata *et al*[22] and Malik *et al*[23] showed that the C609T polymorphism in the *NQO1* gene might be linked to the risk of

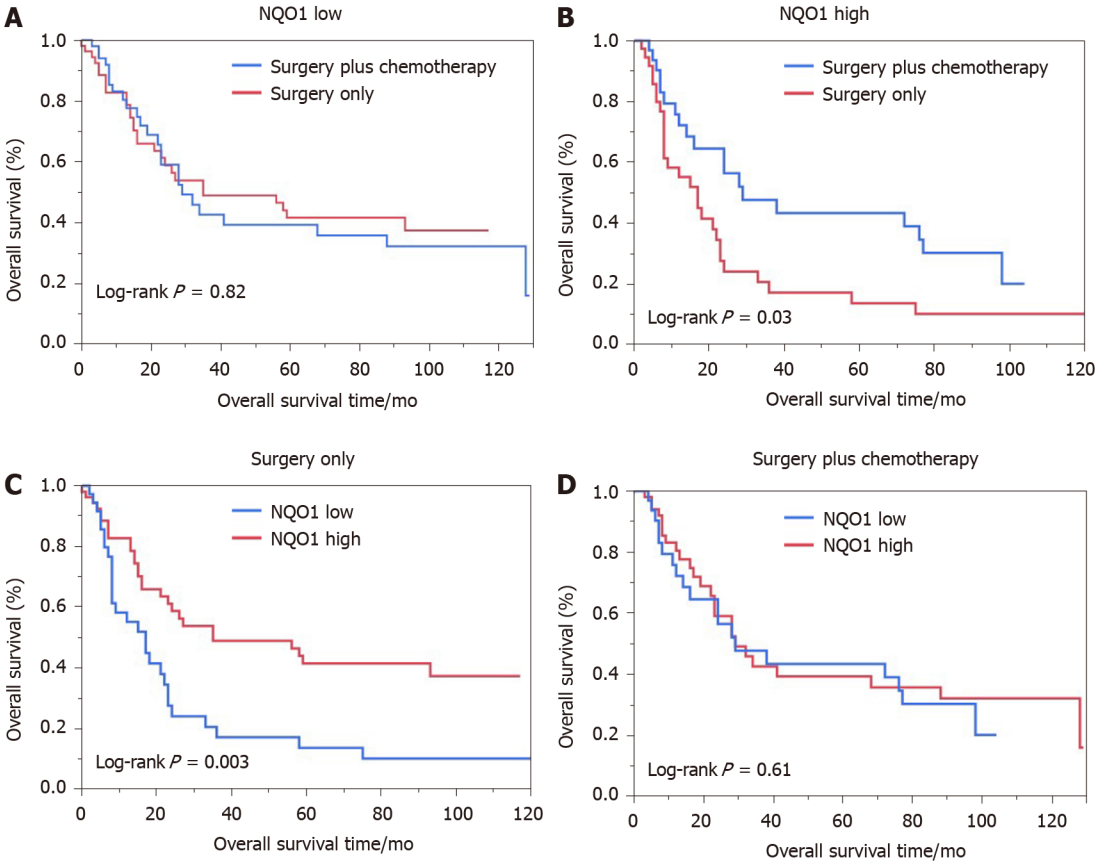


Figure 4 Kaplan-Meier analysis of overall survival rates in patients with gastric cancer. A: Low oxidoreductase 1 expression; B: High quinine oxidoreductase 1 expression; C: Surgery only; D: With adjuvant chemotherapy. NQO1: Quinine oxidoreductase 1.

cancer-associated death. In addition, an *NQO1* polymorphism that causes inactivation of the enzyme predicts a poor outcome in breast cancer[24]. Moreover, NQO1 plays a prominent role in regulating the stability of p53 by inhibiting its degradation and that of other labile proteins, such as ornithine decarboxylase[25,26]. Based on this evidence, NQO1 plays an important role in cancer progression.

Recent studies have reported that NQO1 protein expression is significantly elevated in the cytoplasm and nucleus in many human solid tumors[27]. For instance, Yang *et al*[28] showed that NQO1 is highly expressed in breast cancer tissues and may be associated with breast cancer progression. Cheng *et al*[29] demonstrated strong NQO1 expression in primary melanomas compared with that in dysplastic nevi, and that NQO1 may be involved in the initiation of melanoma development. Similarly, the expression of NQO1 in the cervical epithelium indicated that it might be responsible for cervical carcinogenesis[30]. Malkinson *et al*[31] reported elevated NQO1 expression in human lung cancer tissues. Li *et al*[32] showed that NQO1 is upregulated in non-small cell lung cancer and identified it as a biomarker of poor prognosis. In addition, the gene encoding NQO1 is thought to participate in the pathogenesis of lung cancer [33,34]. Consistently, Ji *et al*[35], Awadallah *et al*[13], and Lyn-Cook *et al*[36] demonstrated that elevated NQO1 expression is associated with pancreatic ductal adenocarcinoma and may be a potential prognostic biomarker for pancreatic cancer.

In the present study, elevated NQO1 expression was observed in cancer tissues. In addition, a higher rate of NQO1 protein overexpression in patients with lymph node metastasis than in those without metastasis was observed. This is partly consistent with the findings of Lin *et al*[14], which suggested that NQO1 upregulation contributes to tumorigenesis and progression of GC.

With regard to survival, we found that GC patients with overexpression of NQO1 protein had a significantly lower overall survival than those with low NQO1 expression. Buranrat *et al*[12] showed a significant correlation between overexpression of NQO1 protein and short overall survival in cholangiocarcinoma patients, enhancing the possibility of using NQO1 as a tumor biomarker. Univariate survival analysis revealed that tumor size, tumor location, tumor stage, histological differentiation, and NQO1 expression status were significantly associated with the overall survival of GC

patients. Likewise, multivariate survival analysis revealed that NQO1 was an independent prognostic biomarker, along with the tumor stage and patient age. Interestingly, it was also notable that patients with elevated NQO1 expression may be more sensitive to 5-FU-based chemotherapy than those with low NQO1 expression. Although the mechanism underlying this phenomenon remains unclear, the results indicated that NQO1 may serve as a biomarker for the early diagnosis and prognosis of GC, and as a potential molecular target for GC therapy. However, the role of NQO1 in GC has not been fully elucidated. Further manipulation of *NQO1* gene expression in different gastric cancer cell lines and investigation of the characteristics and mechanism of its effects on gastric cancer are needed in additional studies.

CONCLUSION

NQO1 is involved in the initiation and progression of GC. High NQO1 expression levels indicates a worse overall survival and better response to 5-FU therapy. NQO1 could be a useful prognostic biomarker and therapeutic target for the appropriate management of patients with GC.

ARTICLE HIGHLIGHTS

Research background

Quinone oxidoreductase 1 (NQO1) plays a cytoprotective role in normal tissues. It is overexpressed in many solid tumors, suggesting a role in cancer development and progression. However, the expression of NQO1 in gastric cancer and its influence on cancer development and prognosis have not been fully addressed.

Research motivation

Identifying molecular markers that can be used as an independent prognostic factor for gastric cancer is of great significance for the early diagnosis and targeted therapy of gastric cancer. The expression of NQO1 in gastric cancer and its influence on clinicopathological characteristics and prognosis have not previously been extensively determined.

Research objectives

The objective of this study was to discover novel effective biomarkers to classify patients with low or high survival. This would provide a guide to clinicians to design personalized therapeutic approaches for gastric cancer patients. In this study, we investigated NQO1 protein, which may determine patient management strategies.

Research methods

We used IHC to detect the expression of NQO1 in gastric cancer and adjacent tissues. Western blot analysis was performed to detect the expression of NQO1 in gastric cancer cell line. According to IHC scores, the patients were divided into NQO1 high expression group and NQO1 low expression group, and the relationship between the expression level and clinicalpathological data was analyzed.

Research results

We found no significant correlations between NQO1 and clinical parameters. However, the follow-up results showed that patients with NQO1 overexpression had a significantly poorer survival rates than those with low levels of NQO1.

Patients with low levels of NQO1 expression showed a poor response to adjuvant chemotherapy and similar survival, compared to those who underwent surgery alone. However, high levels of NQO1 expression indicated a good response to adjuvant chemotherapy and longer overall survival compared with those in patients who underwent surgery alone. In addition, in the Kaplan-Meier analysis, we stratified NQO1 expression based on chemotherapy. The results showed that in patients who underwent surgery only, a significant difference in overall survival was observed, whereas in patients who underwent surgery plus chemotherapy, no difference was found.

Research conclusions

This study found that high expression of NQO1 is closely related to a poor prognosis and better response to 5-fluorouracil in patients with gastric cancer.

Research perspectives

Our study found prognostic value of NQO1 protein expression in patients with gastric cancer. However, our results need to be validated by independent groups or cohorts in a prospective study.

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REFERENCES

1 **Torre LA**, Siegel RL, Ward EM, Jemal A. Global Cancer Incidence and Mortality Rates and Trends--An Update. *Cancer Epidemiol Biomarkers Prev* 2016; **25**: 16-27 [PMID: 26667886 DOI: 10.1158/1055-9965.EPI-15-0578]

2 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

3 **Warneke VS**, Behrens HM, Hartmann JT, Held H, Becker T, Schwarz NT, Röcken C. Cohort study based on the seventh edition of the TNM classification for gastric cancer: proposal of a new staging system. *J Clin Oncol* 2011; **29**: 2364-2371 [PMID: 21537040 DOI: 10.1200/JCO.2010.34.4358]

4 **Lagarde SM**, ten Kate FJ, Reitsma JB, Busch OR, van Lanschot JJ. Prognostic factors in adenocarcinoma of the esophagus or gastroesophageal junction. *J Clin Oncol* 2006; **24**: 4347-4355 [PMID: 16963732 DOI: 10.1200/JCO.2005.04.9445]

5 **Zhu CL**, Huang Q, Liu CH, Lin XS, Xie F, Shao F. NAD(P)H: quinone oxidoreductase 1 (NQO1) C609T gene polymorphism association with digestive tract cancer: a meta-analysis. *Asian Pac J Cancer Prev* 2013; **14**: 2349-2354 [PMID: 23725139 DOI: 10.7314/apjcp.2013.14.4.2349]

6 **Ross D**, Kepa JK, Winski SL, Beall HD, Anwar A, Siegel D. NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. *Chem Biol Interact* 2000; **129**: 77-97 [PMID: 11154736 DOI: 10.1016/s0009-2797(00)00199-x]

7 **Siegel D**, Gustafson DL, Dehn DL, Han JY, Boonchoong P, Berliner LJ, Ross D. NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger. *Mol Pharmacol* 2004; **65**: 1238-1247 [PMID: 15102952 DOI: 10.1124/mol.65.5.1238]

8 **Su XL**, Yan MR, Yang L; Qimuge-Suyila. NQO1 C609T polymorphism correlated to colon cancer risk in farmers from western region of Inner Mongolia. *Chin J Cancer Res* 2012; **24**: 317-322 [PMID: 23358185 DOI: 10.3978/j.issn.1000-9604.2012.08.01]

9 **Radjendirane V**, Joseph P, Lee YH, Kimura S, Klein-Szanto AJ, Gonzalez FJ, Jaiswal AK. Disruption of the DT diaphorase (NQO1) gene in mice leads to increased menadione toxicity. *J Biol Chem* 1998; **273**: 7382-7389 [PMID: 9516435 DOI: 10.1074/jbc.273.13.7382]

10 **Garate M**, Wani AA, Li G. The NAD(P)H:Quinone Oxidoreductase 1 induces cell cycle progression and proliferation of melanoma cells. *Free Radic Biol Med* 2010; **48**: 1601-1609 [PMID: 20226854 DOI: 10.1016/j.freeradbiomed.2010.03.003]

11 **Siegel D**, Franklin WA, Ross D. Immunohistochemical detection of NAD(P)H:quinone oxidoreductase in human lung and lung tumors. *Clin Cancer Res* 1998; **4**: 2065-2070 [PMID: 9748120]

12 **Buranrat B**, Chau-in S, Prawan A, Puapairoj A, Zeekpudsa P, Kukongviriyapan V. NQO1 expression correlates with cholangiocarcinoma prognosis. *Asian Pac J Cancer Prev* 2012; **13** Suppl: 131-136 [PMID: 23480754]

13 **Awadallah NS**, Dehn D, Shah RJ, Russell Nash S, Chen YK, Ross D, Bentz JS, Shroyer KR. NQO1 expression in pancreatic cancer and its potential use as a biomarker. *Appl Immunohistochem Mol Morphol* 2008; **16**: 24-31 [PMID: 18091324 DOI: 10.1097/PAI.0b013e31802e91d0]

14 **Lin L**, Qin Y, Jin T, Liu S, Zhang S, Shen X, Lin Z. Significance of NQO1 overexpression for prognostic evaluation of gastric adenocarcinoma. *Exp Mol Pathol* 2014; **96**: 200-205 [PMID: 24384455 DOI: 10.1016/j.yexmp.2013.12.008]

15 **Luo L**, Chen Y, Wu D, Shou J, Wang S, Ye J, Tang X, Jun Wang X. Differential expression patterns of Nqo1, AKR1B8 and Ho-1 in the liver and small intestine of C57BL/6 mice treated with sulforaphane. *Data Brief* 2015; **5**: 416-423 [PMID: 26958603 DOI: 10.1016/j.dib.2015.09.029]

16 **Luo L**, Chen Y, Wu D, Shou J, Wang S, Ye J, Tang X, Wang XJ. Butylated hydroxyanisole induces distinct expression patterns of Nrf2 and detoxification enzymes in the liver and small intestine of C57BL/6 mice. *Toxicol Appl Pharmacol* 2015; **288**: 339-348 [PMID: 26291391 DOI: 10.1016/j.taap.2015.08.006]

17 **Ahmed SM**, Wu X, Jin X, Zhang X, Togo Y, Suzuki T, Li Y, Kanematsu A, Nojima M, Yamamoto S, Sugimoto M, Kakehi Y. Synergistic induction of apoptosis by mapatumumab and anthracyclines in human bladder cancer cells. *Oncol Rep* 2015; **33**: 566-572 [PMID: 25483927 DOI: 10.3892/or.2014.3654]

18 **Tang X**, Wang H, Fan L, Wu X, Xin A, Ren H, Wang XJ. Luteolin inhibits Nrf2 Leading to negative regulation of the Nrf2/ARE pathway and sensitization of human lung carcinoma A549 cells to therapeutic drugs. *Free Radic Biol Med* 2011; **50**: 1599-1609 [PMID: 21402146 DOI: 10.1016/j.freeradbiomed.2011.03.008]

19 **Wang Q**, Shu X, Dong Y, Zhou J, Teng R, Shen J, Chen Y, Dong M, Zhang W, Huang Y, Xie S, Wei Q, Zhao W, Chen W, Yuan X, Qi X, Wang L. Tumor and serum gamma-glutamyl transpeptidase, new prognostic and molecular interpretation of an old biomarker in gastric cancer. *Oncotarget* 2017; **8**: 36171-36184 [PMID: 28404903 DOI: 10.18632/oncotarget.15609]

20 **Kawasaki Y**, Ishigami S, Arigami T, Uenosono Y, Yanagita S, Uchikado Y, Kita Y, Nishizono Y, Okumura H, Nakajo A, Kijima Y, Maemura K, Natsugoe S. Clinicopathological significance of nuclear factor (erythroid-2)-related factor 2 (Nrf2) expression in gastric cancer. *BMC Cancer* 2015; **15**: 5 [PMID: 25588809 DOI: 10.1186/s12885-015-1008-4]

21 **ERNSTER L**, LINDBERG O. Animal mitochondria. *Annu Rev Physiol* 1958; **20**: 13-42 [PMID: 13521742 DOI: 10.1146/annurev.ph.20.030158.000305]

22 **Nagata M**, Kimura T, Suzumura T, Kira Y, Nakai T, Umekawa K, Tanaka H, Matsuura K, Mitsuoka S, Yoshimura N, Oka T, Kudoh S, Hirata K. C609T Polymorphism of NADPH Quinone Oxidoreductase 1 Correlates Clinical Hematological Toxicities in Lung Cancer Patients Treated with Amrubicin. *Clin Med Insights Oncol* 2013; **7**: 31-39 [PMID: 23467445 DOI: 10.4137/CMO.S10839]

23 **Malik MA**, Zargar SA, Mittal B. Role of NQO1 609C>T and NQO2 -3423G>A gene polymorphisms in esophageal cancer risk in Kashmir valley and meta analysis. *Mol Biol Rep* 2012; **39**: 9095-9104 [PMID: 22736108 DOI: 10.1007/s11033-012-1781-y]

24 **Fagerholm R**, Hofstetter B, Tommiska J, Aaltonen K, Vrtel R, Syrjäkoski K, Kallioniemi A, Kilpivaara O, Mannermaa A, Kosma VM, Uusitupa M, Eskelinen M, Kataja V, Aittomäki K, von Smitten K, Heikkilä P, Lukas J, Holli K, Bartkova J, Blomqvist C, Bartek J, Nevanlinna H. NAD(P)H:quinone oxidoreductase 1 NQO1*2 genotype (P187S) is a strong prognostic and predictive factor in breast cancer. *Nat Genet* 2008; **40**: 844-853 [PMID: 18511948 DOI: 10.1038/ng.155]

25 **Asher G**, Lotem J, Cohen B, Sachs L, Shaul Y. Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1. *Proc Natl Acad Sci USA* 2001; **98**: 1188-1193 [PMID: 11158615 DOI: 10.1073/pnas.021558898]

26 **Asher G**, Bercovich Z, Tsvetkov P, Shaul Y, Kahana C. 20S proteasomal degradation of ornithine decarboxylase is regulated by NQO1. *Mol Cell* 2005; **17**: 645-655 [PMID: 15749015 DOI: 10.1016/j.molcel.2005.01.020]

27 **Siegel D**, Kepa JK, Ross D. NAD(P)H:quinone oxidoreductase 1 (NQO1) localizes to the mitotic spindle in human cells. *PLoS One* 2012; **7**: e44861 [PMID: 22984577 DOI: 10.1371/journal.pone.0044861]

28 **Yang Y**, Zhang Y, Wu Q, Cui X, Lin Z, Liu S, Chen L. Clinical implications of high NQO1 expression in breast cancers. *J Exp Clin Cancer Res* 2014; **33**: 14 [PMID: 24499631 DOI: 10.1186/1756-9966-33-14]

29 **Cheng Y**, Li J, Martinka M, Li G. The expression of NAD(P)H:quinone oxidoreductase 1 is increased along with NF-kappaB p105/p50 in human cutaneous melanomas. *Oncol Rep* 2010; **23**: 973-979 [PMID: 20204281 DOI: 10.3892/or_00000722]

30 **Ma Y**, Kong J, Yan G, Ren X, Jin D, Jin T, Lin L, Lin Z. NQO1 overexpression is associated with poor prognosis in squamous cell carcinoma of the uterine cervix. *BMC Cancer* 2014; **14**: 414 [PMID: 24912939 DOI: 10.1186/1471-2407-14-414]

31 **Malkinson AM**, Siegel D, Forrest GL, Gazdar AF, Oie HK, Chan DC, Bunn PA, Mabry M, Dykes DJ, Harrison SD. Elevated DT-diaphorase activity and messenger RNA content in human non-small cell lung carcinoma: relationship to the response of lung tumor xenografts to mitomycin Cł. *Cancer Res* 1992; **52**: 4752-4757 [PMID: 1324793]

32 **Li Z**, Zhang Y, Jin T, Men J, Lin Z, Qi P, Piao Y, Yan G. NQO1 protein expression predicts poor prognosis of non-small cell lung cancers. *BMC Cancer* 2015; **15**: 207 [PMID: 25880877 DOI: 10.1186/s12885-015-1227-8]

33 **Rosvold EA**, McGlynn KA, Lustbader ED, Buetow KH. Identification of an NAD(P)H:quinone oxidoreductase polymorphism and its association with lung cancer and smoking. *Pharmacogenetics* 1995; **5**: 199-206 [PMID: 8528266 DOI: 10.1097/00008571-199508000-00003]

34 **Heller G**, Zielinski CC, Zöchbauer-Müller S. Lung cancer: from single-gene methylation to methylome profiling. *Cancer Metastasis Rev* 2010; **29**: 95-107 [PMID: 20099008 DOI: 10.1007/s10555-010-9203-x]

35 **Ji M**, Jin A, Sun J, Cui X, Yang Y, Chen L, Lin Z. Clinicopathological implications of NQO1 overexpression in the prognosis of pancreatic adenocarcinoma. *Oncol Lett* 2017; **13**: 2996-3002 [PMID: 28521407 DOI: 10.3892/ol.2017.5821]

36 **Lyn-Cook BD**, Yan-Sanders Y, Moore S, Taylor S, Word B, Hammons GJ. Increased levels of NAD(P)H: quinone oxidoreductase 1 (NQO1) in pancreatic tissues from smokers and pancreatic adenocarcinomas: A potential biomarker of early damage in the pancreas. *Cell Biol Toxicol* 2006; **22**: 73-80 [PMID: 16532285 DOI: 10.1007/s10565-006-0156-3]

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ORIGINAL ARTICLE

Retrospective Study

Idiopathic mesenteric phlebosclerosis associated with long-term oral intake of geniposide

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Abstract

BACKGROUND

Idiopathic mesenteric phlebosclerosis (IMP) is a rare disease, and its etiology and risk factors remain uncertain.

AIM

To investigate the possible influence of Chinese herbal liquid containing geniposide on IMP.

METHODS

The detailed formula of herbal liquid prescriptions of all patients was studied, and the herbal ingredients were compared to identify the toxic agent as a possible etiological factor. Abdominal computed tomography (CT) and colonoscopy images were reviewed to determine the extent and severity of mesenteric phlebosclerosis and the presence of findings regarding colitis. The disease CT score was determined by the distribution of mesenteric vein calcification and colon wall thickening on CT images. The drinking index of medicinal liquor was calculated from the daily quantity and drinking years of Chinese medicinal liquor. Subsequently, Spearman's correlation analysis was conducted to evaluate the correlation between the drinking index and the CT disease score.

anonymous clinical data that were obtained after each patient agreed to treatment by written consent. Patient confidentiality was protected.

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RESULTS

The mean age of the 8 enrolled patients was 75.7 years and male predominance was found (all 8 patients were men). The patients had histories of 5-40 years of oral Chinese herbal liquids containing geniposide and exhibited typical imaging characteristics (*e.g.*, threadlike calcifications along the colonic and mesenteric vessels or associated with a thickened colonic wall in CT images). Calcifications were confined to the right-side mesenteric vein in 6 of the 8 patients (75%) and involved the left-side mesenteric vein of 2 cases (25%) and the calcifications extended to the mesorectum in 1 of them. The thickening of colon wall mainly occurred in the right colon and the transverse colon. The median disease CT score was 4.88 (n = 7) and the median drinking index was 5680 (n = 7). After Spearman's correlation analysis, the median CT score of the disease showed a significant positive correlation with the median drinking index (r = 0.842, P < 0.05).

CONCLUSION

Long-term oral intake of Chinese herbal liquid containing geniposide may play a role in the pathogenesis of IMP.

Key Words: Idiopathic mesenteric phlebosclerosis; Phlebosclerotic colitis; Chinese herbal liquid; Geniposide; Colonoscopy; Computed tomography

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Core Tip: Idiopathic mesenteric phlebosclerosis (IMP) is a rare entity that appears almost exclusively in Asian populations and is characterized by calcification of the mesenteric veins and thickening of the wall of the right hemicolon. Long-term and frequent ingestion of biochemicals and toxins are thought to be associated with the disease. Herbal ingredients present in the patients were compared to identify the toxic agent as a possible etiological factor, and the positive correlation of the computed tomography disease score and drinking index was explored. Furthermore, underlying disease (*e.g.*, diabetes, chronic nephritis, or malignancy) may be risk factors for IMP.

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INTRODUCTION

Idiopathic mesenteric phlebosclerosis (IMP) is a rare form of ischemic colitis that usually affects the right hemicolon. It is almost exclusively observed in Asian populations, is characterized by calcification of mesenteric veins and thickening of the wall of the right hemicolon. The etiology and pathogenesis remain unclear, but it is thought that long-term and frequent ingestion of biochemicals and toxins are associated with the disease[1,2]. As clarified by existing studies, long-term intake of herbal medicines or medicinal liquor containing geniposide is recognized as one of the major causes of IMP[2,3]. In this study, we describe 8 patients with mesenteric phlebosclerosis with long-term exposure to Chinese herbal medicines or medicinal liquor. The clinical manifestations and imaging features were summarized, and the relationship between the alcohol index and the severity of IMP observed by computed tomography (CT) were analyzed. This is an interesting article on a relatively rare disease and may present insights into the etiology of IMP.

MATERIALS AND METHODS

Ethical approval

This was a retrospective study, and the data originated from the medical records at Zhejiang Provincial People's Hospital from June 2016 to December 2020. The study was approved by the Institutional Review Board at Zhejiang Provincial People's Hospital. Informed consent was waived; patient confidentiality was protected.

Patient selection

The medical records of patients meeting the following inclusion criteria were retrospectively reviewed: (1) The clinical diagnosis of IMP was confirmed by abdominal CT, endoscopy, or pathology including at least one complete abdominal CT examination with or without intravenous contrast medium injection; and (2) Patients had complete clinical information.

Clinical data collection

Eight patients were identified and their clinical data, including symptoms, anamnesis, history of herbal medicines, and therapy, were collected. Patient herbal medicine history, herbal medicine names, contact time, and daily intake were highlighted. Of note, 7 patients consumed similar dosages of two medicines, Wu chia-pee liquor and Wanying die-da wine for a long time. Moreover, the degree of the two wines was similar. The drinking index was calculated as the daily intake (mL) × drinking duration (years).

Gastroscopy procedures

Gastroscopy was performed with an Olympus 290 colonoscope (Olympus, Tokyo, Japan). The endoscope was inserted to 5-10 cm from the terminal ileum. The color of mucosa, the vascular textures of the terminal ileum, ileocecal area, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum were noted, and the presence of congestion, edema, erosion and ulcer were verified. Multiple samples of the intestinal mucosa were collected for histopathological examination.

CT acquisition

CT scans were generated with a 64-channel multi-detector CT scanner (Somatom Definition Flash, Siemens Medical Solutions, Forchheim, Germany). The CT scanning parameters were: Detector collimation, 1 mm; pitch, 1.5:1; tube voltage, 120 kV; tube current, 120-250 mAs; rotation time, 0.33 s. Contrast-enhanced CT was performed with 80-90 mL of 370 mg I/mL iodinated contrast agent (Ultravist, Bayer Schering Pharma AG) injected in a peripheral vein with a dual high-pressure syringe at a flow rate of 2.2-3 mL/s. A bolus-tracking technique was used to obtain arterial- and venous-phase CT images with delays of 10 s and 50-65 s after a 100 Hounsfield unit threshold of the descending abdominal aorta. Image reconstruction was performed with a 1 mm slice thickness and a 1 mm slice interval with an Application Development Workstation (MM Reading, syngo.via, Version VB20A, Siemens Healthcare GmbH, Forchheim, Germany).

Assessment of the disease CT score

The CT imaging characteristics were assessed by 2 radiologists with 5- and 10-year of clinical experience. The severity of IMP was assessed with consideration of the calcification distribution at the tributaries of the portal vein and the range of thickening of the intestinal wall. Colonic wall thickening was defined as a lumen width exceeding 2 cm and wall thickness exceeding 5 mm. The CT scores of the IMP cases are listed in Table 1). A 4-grade calcification score was evaluated by the scope of mesenteric venous calcification of the colon[4]. Specifically, venous calcifications limited to the straight vein were scored as 1, and calcifications extending to the paracolic marginal vein were scored as 2. If the calcifications extended to the proximal part of main branch of the mesenteric vein, the score was 3. If the distal end of the main branch was involved, then the score was 4. Illustrations of calcification distribution are shown in Figure 1). The severity of colon wall thickening was classified by three scores depending on the extent of the lesion. Lesions confined to the ascending colon had a severity score of 1. Those extending to the traverse colon had a severity score of 2, and those involving the descending colon or distal segment had a severity score of 3. The calcification and colon wall thickening scores were summed to obtain the disease CT score.

Table 1 Imaging characteristics of lesions on endoscopy and computed tomography

No. case	Endoscopic findings				CT findings						
	Ileal	Ileocolic valve	Mucosa color	Bowel wall stiffening	Bowel wall thickening			Calcification dstribution[1]			Luminal narrowing
					Extended to A-colon	Extended to T-colon	Extended to L-colon	IMV	SMV	PV	
1	Not performed	Not performed	Not performed	Not performed	+	-	-	+++	-	-	No
2	Not involvement	Involvement	Dark blue	+	+	+	-	+++	-	-	No
3	Not involvement	Not involvement	Mild-blue	-	+	-	-	++	-	-	No
4	Not performed	Not performed	Not performed	Not performed	+	-	-	+++	-	-	No
5	Not involvement	Involvement	Dark purple	+	+	+	-	+++	-	-	Yes
6	Not performed	Not performed	Not performed	Not performed	+	+	-	+++	-	-	No
7	Not involvement	Involvement	Dark purple	+	+	+	+	+++	+++	-	No
8	Not performed	Not performed	Not performed	Not performed	-	-	-	++	++	-	No

+: Positive; -: Negative; CT: Computed tomography; A-colon: Ascending colon; IMV: Inferior mesenteric vein; L-colon: Left colon; PV: Portal vein; SMV: Superior mesenteric vein; T-colon: Transverse colon.

[1]Calcification scoring system.

Computed tomography score of idiopathic mesenteric phlebosclerosis = bowel wall thickening score + calcification score (+ = 1 point). +: Calcifications limited to straight vein of the colon; ++: Calcifications extended to the paracolic marginal vein; +++: Calcifications extended to the main branch of mesenteric vein; ++++: Calcifications included the trunk of the mesenteric vein; –: No calcifications.

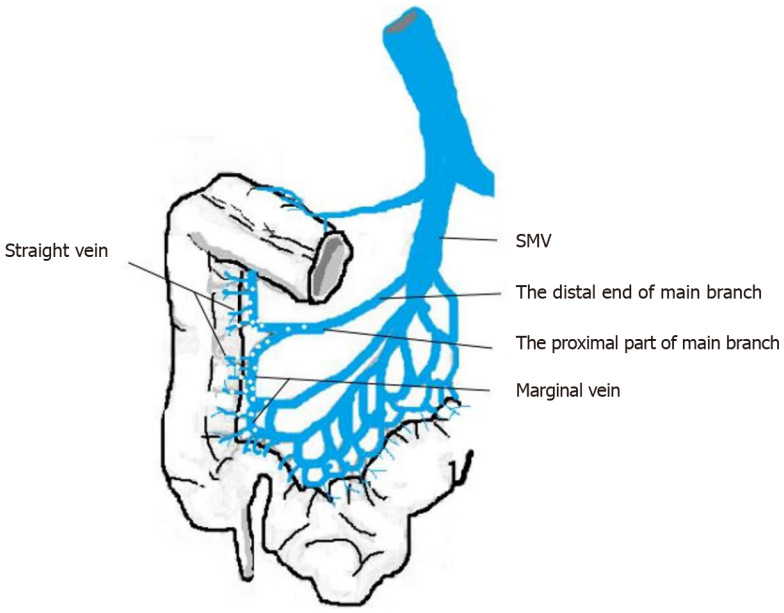


Figure 1 Graphical illustration of the distribution of calcification in the superior mesenteric vein. SMV: Superior mesenteric vein.

Statistical analysis

The statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS version 25.0, IBM Corp, Armonk NY, United States). Spearman's correlation analysis was used to assess the correlation between the drinking index and the disease CT score. Two-tailed *P* values of < 0.05 indicated statistical significance.

RESULTS

Eight male patients with an average age of 75.7 (range of 59–88) year were included. Five presented with abdominal symptoms (*e.g.*, abdominal pain, fullness, and diarrhea), one of whom had an intestinal obstruction. All 8 patients had histories of long-term use of Chinese herbal medicines or medicinal liquors. One had used an oral liquid for the treatment of chronic rhinitis. The 7 others had used Chinese medical liquors that contained gardenia, chuan xiong, and angelia dahurica. Tables 2 and 3 show the patient clinic characteristics and the ingredients that Chinese traditional medicines had in common. The patients all received conservative treatment.

All patients presented with punctate or linear calcification on CT images. Mesenteric venous calcification involved the ascending colon of all patients and extended to the transverse colon in 4 (Figures 1 and 2). In 2 of the 8 patients, the lesions extended to the descending colon. In 1 patient, the entire colon was involved (Figure 3). Calcification was limited to the right-side mesenteric vein in 6 of the 8 cases (75%). In 2 cases (25%), the left-side mesenteric vein was involved. Diffuse wall thickening in the affected region was observed in 7 patients. One patient presented with calcification without obvious thickening of the colon wall. The wall thickening was most often seen in the right and the transverse colon.

The median disease CT score was 4.88 (n = 7) and the median drinking index was 5680 (n = 7). The dispersion diagram in Figure 4 shows the relationship between the drinking index and the disease CT score. Spearman correlation analysis found a significant positive correlation between the alcohol drinking index and the disease CT score (r = 0.842, P < 0.05). In the 4 patients evaluated by of colonoscopy, blue or dark blue colored mucosa was the most characteristic variation. The colonoscopy revealed multiple erosions and ulcers in 1 patient (Figure 5). Table 1 lists the characteristic endoscopic view and CT findings. Histopathology of the biopsy samples showed the deposition of collagen fibers in the subepithelium and around the blood vessels. The vitreous deposits were negatively stained by Congo red and appeared blue following Masson-trichrome, staining, which indicated lamina propria hyalinization and fibrosis (Figure 6).

DISCUSSION

IMP, which is also known as phlebosclerotic colitis, is a rare intestinal ischemia syndrome with gradual onset and progression. It is characterized by thickening of the wall of the right hemicolon and calcification of mesenteric veins. Most cases have been reported in East Asian nations and regions, especially Japan and Taiwan. In 1991, Koyama *et al*[5] initially described the disease. To distinguish this disease from ischemic colitis associated with arterial diseases, it was termed as "phlebosclerotic colitis" by Yao *et al*[6] in 2000. In 2003, Iwashita *et al*[7] advocated the term "idiopathic mesenteric phlebosclerosis", as the affected site of this disease showed weak inflammatory changes. Most ischemic bowel diseases result from an insufficient arterial supply attributed to atherosclerosis, thrombosis, and embolus[8]. Disturbed venous return may also cause colitis, including IMP as described here. IMP is usually attributed to chronic ischemia of the colon resulting from calcification of the mesenteric venous system that causes venous congestion of the colon and even hemorrhagic infarction.

The disease incidence is low, with mostly chronic and insidious onset. Patients subject to IMP usually present with nonspecific symptoms (*e.g.*, abdominal pain, diarrhea, nausea, and vomiting). As the disease mostly involves the right colon, abdominal pain is more common in the right lower abdomen. Patients may be asymptomatic in the early stage of disease but may develop intestinal obstruction and even perforation in the advanced stage of the disease[9,10]. In this study, most of the 8 patients developed abdominal pain and diarrhea. One presented with intestinal obstruction as the first symptom, and another presented with gastrointestinal bleeding, which was basically consistent with existing reports.

The pathogenesis and etiology of IMP remain unclear. IMP is characterized by a defined area and endemic population distribution, and a relationship with a region-specific lifestyle stressed the etiology of this disease[11]. Long-term and frequent ingestion of biochemical substances and toxins is considered to be associated with the disease. Most reported cases of IMP have been associated with the use of herbal medicines and medicinal liquor, most of which contained gardenia fruits[12]. Gardenia fruit is the dried mature fruit of *Gardenia jasminoides* Ellis. It is a popular crude drug

Table 2 Clinical characteristics of patients with idiopathic mesenteric phlebosclerosis

No. case	Gender	Age	Symptom	Herb contact	Dosage of liquid medicine (mL/d)	Exposure time (yr)	Underlying disease	Treatment
1	M	59	Abdominal pain	Bi yuan-su oral liquid	30	10	Chronic rhinitis	Conservative
2	M	66	Diarrhea, positive stool occult blood	Wu chia-pee liquor	150	10	Diabetes	Conservative
3	M	69	Abdominal distention	Wu chia-pee liquor	150	10	None	Conservative
4	M	79	Positive stool occult blood	Wu chia-pee liquor	90	20	None	Conservative
5	M	80	Abdominal pain	Wu chia-pee liquor	200	30	Hypertension	Conservative
6	M	82	Abdominal pain, abdominal distention	Wu chia-pee liquor	200	35	Hypertension, hepatitis B, rheumatoid arthritis	Conservative
7	M	83	Dysuria	Wu chia-pee liquor	250	40	Hypertension, chronic nephritis	Conservative
8	M	88	Limbs numbness	Wanying die-da wine	120	5	Hypertension, prostatic cancer, arthrolithiasis	Conservative

M: Male.

Table 3 Composition of Chinese herbal liquid medicines

Name of liquid	Ingredient 1	Ingredient 2	Ingredient 3	Ingredient 4	Ingredient 5	Ingredient 6	Ingredient 7
Wu chia-pee liquor	Gardenia	Chuan xiong	Angelia dahurica	Cortex acanthopanacis	Clematis	Tetrandra root	Angelica sinensis
Wanying die-da wine	Gardenia	Chuan xiong	Angelia dahurica	Cortex acanthopanacis	Clematis	Tetrandra root	Angelica sinensis
Bi yuan-su oral liquid	Gardenia	Chuan xiong	Angelia dahurica	-	-	-	-

All 8 patients had taken one of the three Chinese herbal liquid medicines. The ingredients included in all the liquid formulations are listed.

used as a Chinese herb and has been extensively employed for treating cardiovascular, cerebrovascular diseases, hepatobiliary diseases, and diabetes. The main active ingredient of the gardenia fruit is geniposide. As deduced by some scholars, if patients take Chinese herbal drugs containing Gardenia for a long time, the geniposide can be hydrolyzed to genipin by bacteria in intestinal tract, and the absorbed genipin reacts with the protein in mesenteric vein plasma. In addition, collagen gradually accumulates under the mucosa, which subsequently progresses to hyperplastic myointima in the veins, accompanied by fibrosis/sclerosis. The changes ultimately result in venous occlusion[13]. As geniposide refers to one type of glycoside, orally administered geniposide is not directly absorbed after reaching the lower digestive tract. Geniposide is hydrolyzed only after entering the cecum and ascending colon and then transformed to its metabolite, genipin, which permeates the enterocyte membrane as impacted by numerous bacteria in the colon[12]. The transformation and absorption processes have been largely identified in the right colon and transverse colon, which explains the characteristic lesion site of mesenteric venous sclerosis.

As shown in Table 2, a clear male predominance was found among the patients. This trend is different from the female predominance described in existing reports from Japan and Taiwan, which might be explained as follows. In Japan and Taiwan, herbal prescriptions containing geniposide are commonly used and are thought to be effective for female-specific symptoms, which may account for the female predominance[14,15]. However, most of our patients had a history of taking Chinese medical liquors for a long time. Most were male, which may be the reason for male predominance in our study. Region-specific lifestyle may thus contribute to the understanding of the etiology of this disease. In this study, 7 patients had a history of taking the Chinese medical liquors named Wu chia-pee liquor and Wanying die-da wine, which consist of multiple Chinese herbs soaked in liquor and have various effects (*e.g.*,

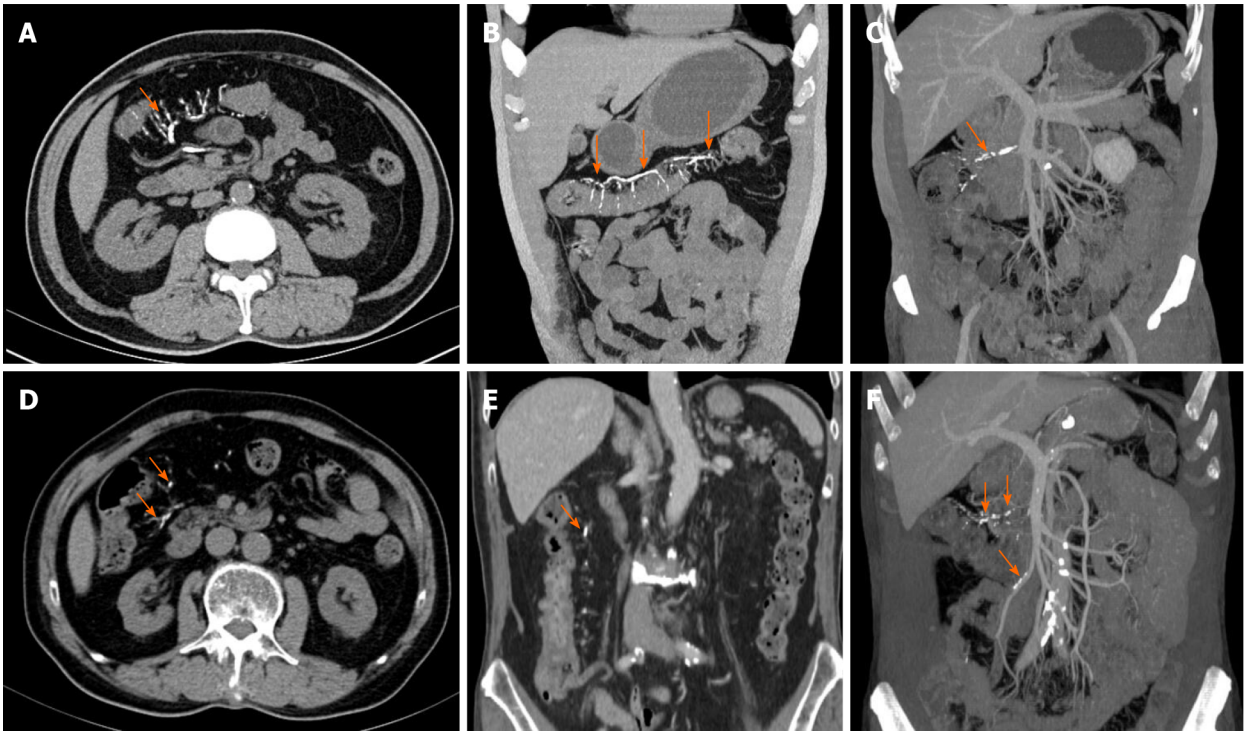


Figure 2 Abdominal computed tomography shows colonic wall thickenings with threadlike calcifications (arrow) of the superior mesenteric vein in the transverse colon and ascending colon. A-C: Case 2; D-F: Case 4. Three-dimensional maximum intensity projection reconstruction of computed tomography angiography effectively illustrates the extent of calcification along the mesenteric vein (C, F).

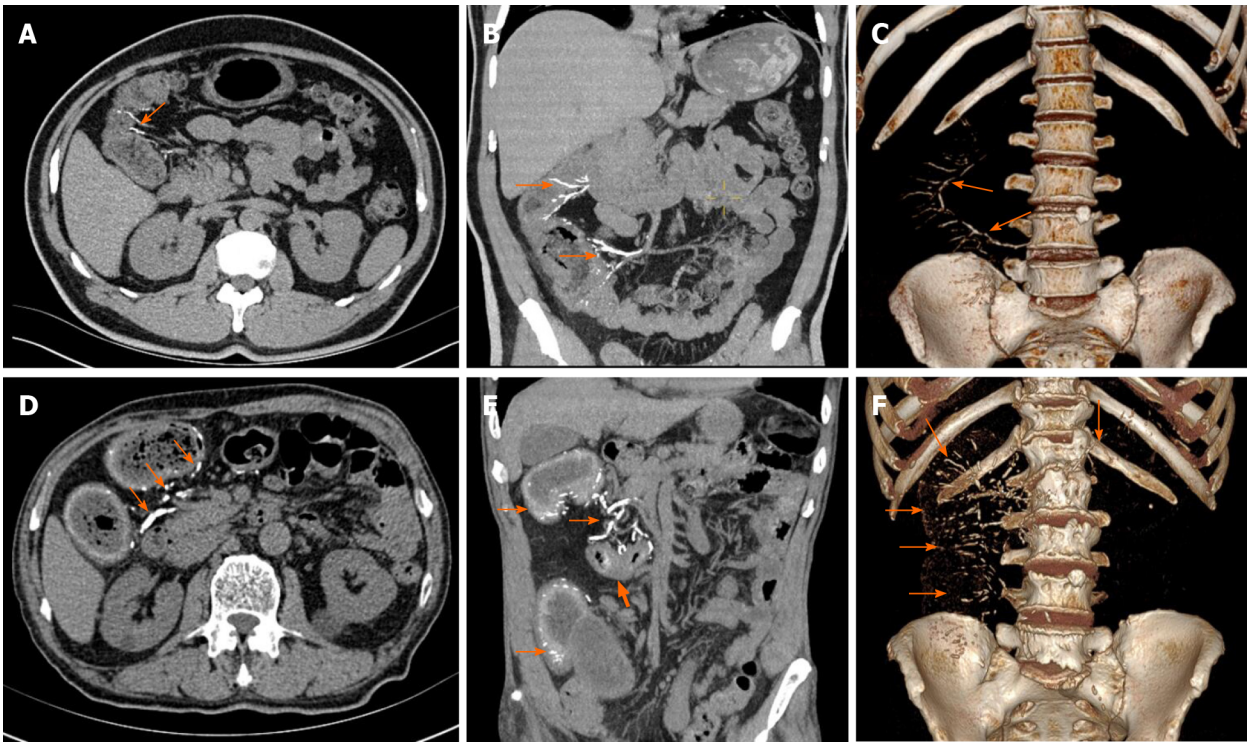


Figure 3 Abdominal computed tomography shows numerous linear and arc-like dense calcifications (arrow) distributed within the bowel wall of the ascending and hepatic flexure of the colon with thickening of the colon wall. A-C: Case 1; D-F: Case 5. Case 5 Local stenosis is seen in the transverse colon of case 5 (thick arrow). Volume rendering image shows that calcifications were more prominently distributed in the mesenteric veins in the right hemicolon (C, F).

enhancing fitness and optimizing immune responses)[3,14]. Another patient had no history of drinking alcohol and had been taking Biyuanshu oral liquid for a long time to treat chronic rhinitis. The medical liquids used by our 8 patients all contained geniposide, chuan xiong, and angelia dahurica (Table 3). A study by Hiramatsu *et al* [14] of 25 IMP patients in which geniposide was the only Chinese medicine common to all is further evidence the that Chinese herbal medicines containing geniposide are involved in the pathology of IMP. Nevertheless, whether geniposide is only factor directly involved in the pathogenesis of IMP, or whether it is accompanied by other Chinese medicine in the pathogenesis of IMP, needs to be further determined in larger datasets.

The clinical symptoms of IMP lack specificity, and the diagnosis is largely determined by the results of radiology and colonoscopy[9,16]. Abdominal CT scans show calcifications of the involved superior mesenteric vein and its branches, which are linear and follow the course of the blood vessels. The involved colon wall becomes swollen and thickened[17]. Endoscopy shows a blue or bluish purple mucosa at the lesion site[18], and the color might be attributed to chronic congestion with ischemia or toxins that stained the bowel mucosa[19]. Tortuous and irregular veins can be seen under the mucosa with poor light transmission. In severe cases, the vessels might disappear. Colonic mucosa was Hyperemia and edema of the colon are sometimes accompanied by erosion or ulcer. The lesions are continuous and the chronic course of disease involves spread from the ileocecal to the anal side. IMP mainly affects the right colon but may also involve the left colon and extend to the sigmoid colon, but generally does not involve the terminal ileum.

In this study, 2 patients had phlebosclerosis extending to the left colonic vein branch, 1 had chronic nephritis, and 1 had been treated 5 years previously with endocrine and radiotherapy for prostate cancer, which are rare in IMP[2,20]. The investigators speculated that poor renal function and long-term treatment of malignant tumors prolong the clearance of genipin, an active metabolite of geniposide, which allows genipin to accumulate in the branches of the veins following absorption, thus aggravating the severity of mesenteric venous sclerosis and reducing the absorption capacity of the colon. Genipin, that is not completely absorbed by the ascending and transverse colons, is absorbed from the left colon, leading to sclerosis and calcification of the left colon venous branch. In addition, systemic microvascular disease complicating diabetes and resulting in chronic hypoxia may increase the vulnerability of colon wall and colon vein[1]. Consequently, chronic nephritis, malignant tumors, and diabetes may increase the risk of the progression of IMP, as shown in Figure 6.

As shown in Figure 7, the severity of IMP is related to the drinking index, which reflects the daily intake of liquid medicine and the duration of contact. The effect on vessel wall is time- and dose-dependent, and that may be related to colonic flora and colonic absorption capacity. IMP has characteristic manifestations on both CT and endoscopy, which make it is relatively easy to diagnose. Conventional histopathology shows fibrosis and calcification of the vein wall and collagen deposition around the vessels. Because of the superficial location of the lesions, the pathological value of the specimens taken during colonoscopy for the diagnosis of IMP is limited, and the resected specimen may require in-depth observation. The treatment strategy for IMP can be determined on an individual basis. Patients with mild or asymptomatic symptoms can be treated conservatively, which will stop progress after the exposure to pathogenic ingredients is stopped (*e.g.*, the use of Chinese herbal medicine). Surgical treatment is necessary if severe complications such as colonic obstruction, necrosis, perforation, or massive intestinal bleeding occur[21]. However the presence of poor circulation may mean that colon surgery is not an appropriate treatment, and it must be chosen with care.

CONCLUSION

This study evidence supports geniposide as most likely to be involved in the pathology of IMP. Clinical conditions including chronic nephritis, malignant tumors, and diabetes mellitus, may be the risk factors of IMP. It is recommended that long-term use of Chinese herbs and medical liquors should be avoided, especially prescriptions or formulations containing gardenia. Both endoscopic and radiologic examinations can lead to a conclusive diagnosis even if biopsy results are insufficient or inconclusive.

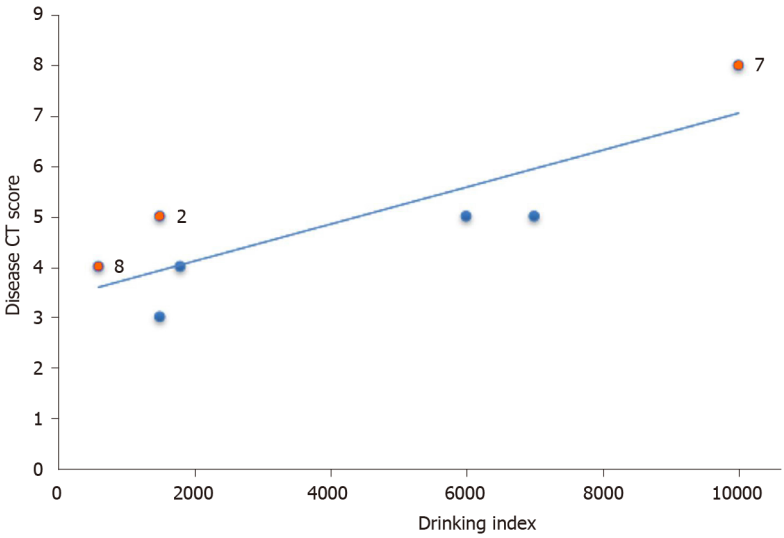


Figure 4 Dispersion diagram and best fitting line for the drinking index *vs* the disease computed tomography score. Patients two, seven, and eight had histories of diabetes, chronic nephritis, or prostate cancer are shown by red dots. CT: Computed tomography.

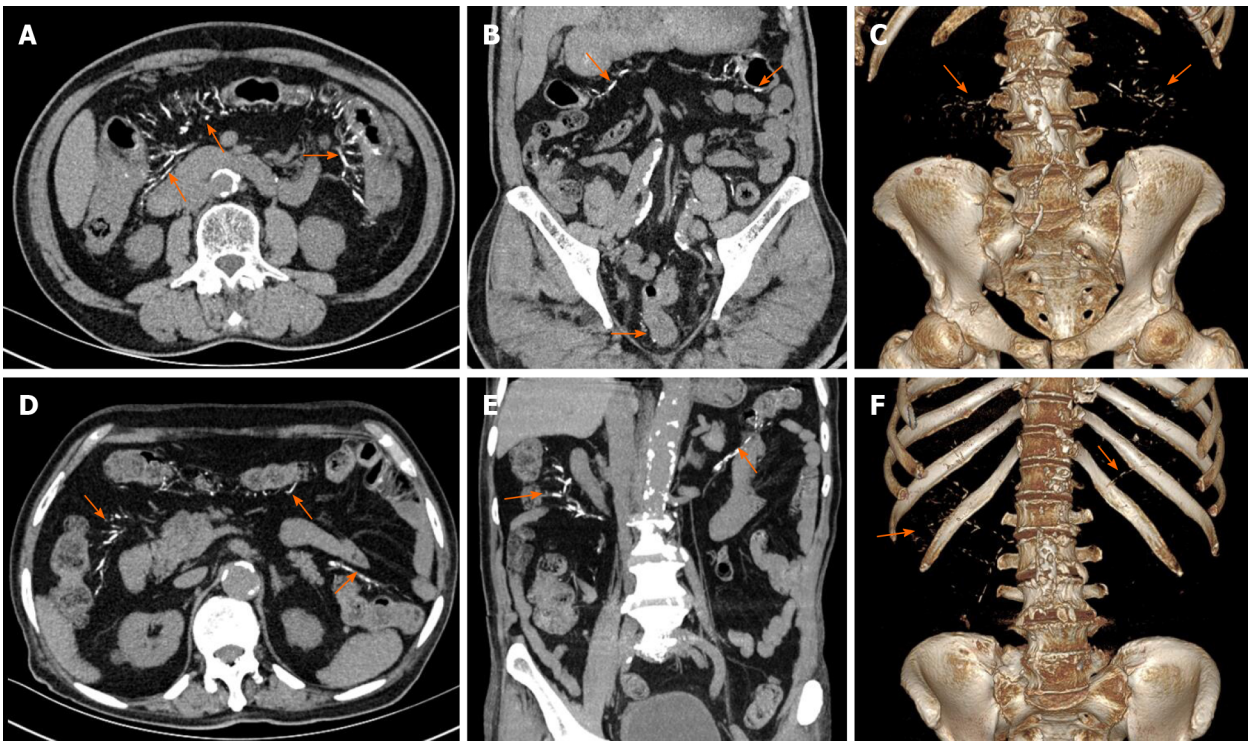


Figure 5 Abdominal computed tomography shows multiple threadlike calcifications within the colon wall and adjacent vein from the ileocecal junction to the descending colon (arrow). A-C: Case 6; D-F: Case 7. In case 6, calcifications of the mesenteric vein extended to the rectum; mild diffuse thickening of colon wall is seen. Volume rendering image illustrates the distribution of calcifications in the mesenteric veins, the inferior mesenteric vein with multifocal calcifications (C, F).

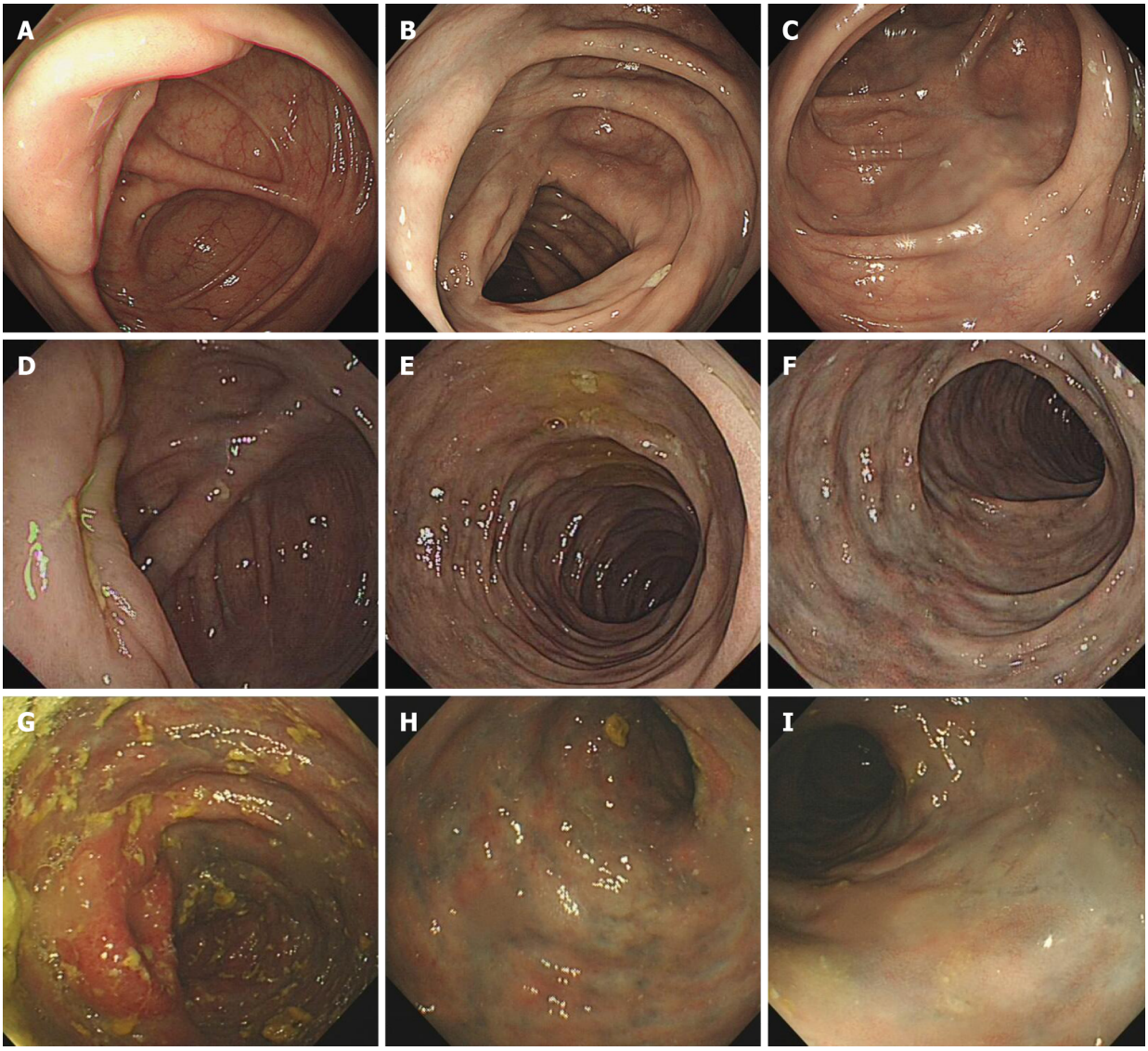


Figure 6 Representative endoscopic views. A-C: Colonoscopy in case 3 revealed light blue discoloration in the transverse colon; D-F: Colonoscopy case 2 showed edematous congested mucosa with pigmentation, and dark blue discoloration extending to the transverse colon; G-I: Colonoscopy of case 5 revealed edematous dark purple colonic mucosa and sclerotic changes of the colonic walls extending from the cecum to the splenic flexure of colon.

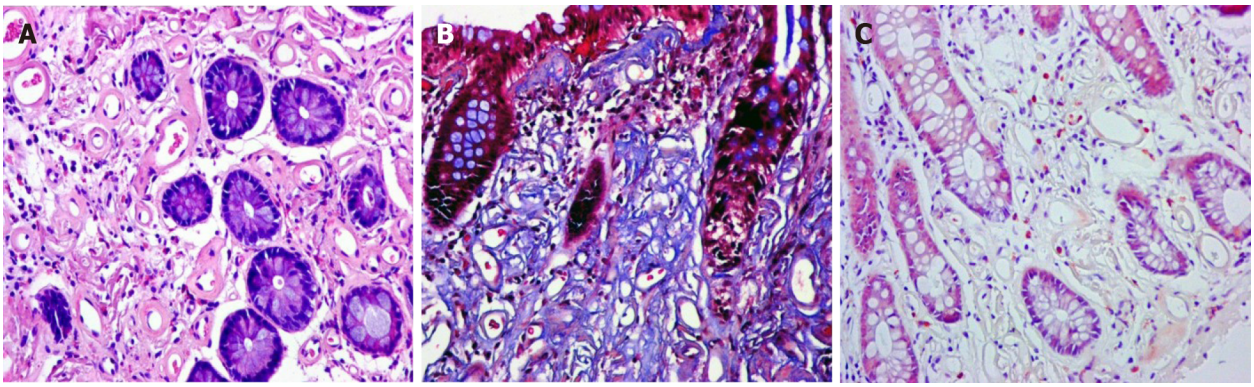


Figure 7 Histologic examination of the biopsy specimen of case 2. A: Hematoxylin and eosin staining revealed marked fibrosis of the deep layer of the mucosa wall thickening of the venules (× 40); B: Masson's trichrome stain indicated that the deposits were stained blue, suggesting that they were collagen fibers (× 40); C: Congo red staining revealed absence of vitreous deposition, excluding amyloidosis (× 40).

ARTICLE HIGHLIGHTS

Research background

Idiopathic mesenteric phlebosclerosis (IMP) is a rare disease, and its etiology and risk factors remain uncertain.

Research motivation

In terms of IMP etiology, the association with a region-specific lifestyle has been a concern. IMP is believed to be linked to chronic and frequent ingestion of biochemicals and toxins.

Research objectives

The objective was to explore the possible relationship between Chinese herbal liquid containing geniposide and IMP and to identify some clinical factors that may lead to disease progression.

Research methods

The disease computed tomography (CT) score was calculated from the distribution of mesenteric vein calcification and colon wall thickening on CT images. The drinking index of medicinal liquor was calculated from the daily intake and drinking years. The correlation between the drinking index and the disease CT score was analyzed by Spearman's correlation analysis. Comparison of the herbal ingredients included in the liquid prescriptions, allowed identification of possibly toxic agents as a pathogenic factor.

Research results

Geniposide was the only Chinese medicine in common with previous studies. Spearman's correlation analysis revealed that the median CT disease score was positively correlated with the median drinking index ($r = 0.842$, $P < 0.05$).

Research conclusions

Geniposide is most likely involved in the pathology of IMP, and its effect is time- and dose-dependent. Chronic nephritis, malignant tumors, diabetes mellitus, and other clinical symptoms may be risk factors for IMP.

Research perspectives

The number of cases in our retrospective study was relatively small, and the pathogenesis of IMP needs to be determined by further study with a larger data set.

REFERENCES

1 **Chang KM**. New histologic findings in idiopathic mesenteric phlebosclerosis: clues to its pathogenesis and etiology--probably ingested toxic agent-related. *J Chin Med Assoc* 2007; **70**: 227-235 [PMID: 17591581 DOI: 10.1016/S1726-4901(09)70364-8]

2 **Lee SM**, Seo JW. Phlebosclerotic colitis: case report and literature review focused on the radiologic findings in relation to the intake period of toxic material. *Jpn J Radiol* 2015; **33**: 663-667 [PMID: 26242772 DOI: 10.1007/s11604-015-0467-5]

3 **Guo F**, Zhou YF, Zhang F, Yuan F, Yuan YZ, Yao WY. Idiopathic mesenteric phlebosclerosis associated with long-term use of medical liquor: two case reports and literature review. *World J Gastroenterol* 2014; **20**: 5561-5566 [PMID: 24833888 DOI: 10.3748/wjg.v20.i18.5561]

4 **Yen TS**, Liu CA, Chiu NC, Chiou YY, Chou YH, Chang CY. Relationship between severity of venous calcifications and symptoms of phlebosclerotic colitis. *World J Gastroenterol* 2015; **21**: 8148-8155 [PMID: 26185388 DOI: 10.3748/wjg.v21.i26.8148]

5 **Koyama N**, Koyama H, Hanajima T, Matsubara N, Fujisaki J, Shimoda T. Chronic ischemic colitis causing stenosis: report of a case. *Stomach Intest* 1991; **26**: 455-460

6 **Yao T**, Iwashita A, Hoashi T, Matsui T, Sakurai T, Arima S, Ono H, Schlemper RJ. Phlebosclerotic colitis: value of radiography in diagnosis--report of three cases. *Radiology* 2000; **214**: 188-192 [PMID: 10644121 DOI: 10.1148/radiology.214.1.r00ja01188]

7 **Iwashita A**, Yao T, Schlemper RJ, Kuwano Y, Iida M, Matsumoto T, Kikuchi M. Mesenteric phlebosclerosis: a new disease entity causing ischemic colitis. *Dis Colon Rectum* 2003; **46**: 209-220 [PMID: 12576895 DOI: 10.1097/01.DCR.0000044720.43258.6E]

8 **Kusanagi M**, Matsui O, Kawashima H, Gabata T, Ida M, Abo H, Isse K. Phlebosclerotic colitis: imaging-pathologic correlation. *AJR Am J Roentgenol* 2005; **185**: 441-447 [PMID: 16037518 DOI:

10.2214/ajr.185.2.01850441]
9 **Chen W**, Zhu H, Chen H, Shan G, Xu G, Chen L, Dong F. Phlebosclerotic colitis: Our clinical experience of 25 patients in China. *Medicine (Baltimore)* 2018; **97**: e12824 [PMID: 30412073 DOI: 10.1097/MD.0000000000012824]
10 **Chang YY**, Lin HH, Lin CC. Phlebosclerotic colitis presenting as intestinal obstruction. *Clin Gastroenterol Hepatol* 2014; **12**: e81-e82 [PMID: 24561098 DOI: 10.1016/j.cgh.2014.02.018]
11 **Kitamura T**, Kubo M, Nakanishi T, Fushimi H, Yoshikawa K, Taenaka N, Furukawa T, Tsujimura T, Kameyama M. Phlebosclerosis of the colon with positive anti-centromere antibody. *Intern Med* 1999; **38**: 416-421 [PMID: 10397079 DOI: 10.2169/internalmedicine.38.416]
12 **Ohtsu K**, Matsui T, Nishimura T, Hirai F, Ikeda K, Iwashita A, Yorioka M, Hatakeyama S, Hoashi T, Koga Y, Sakurai T, Miyaoka M. [Association between mesenteric phlebosclerosis and Chinese herbal medicine intake]. *Nihon Shokakibyo Gakkai Zasshi* 2014; **111**: 61-68 [PMID: 24390259]
13 **Kang MJ**, Khanal T, Kim HG, Lee DH, Yeo HK, Lee YS, Ahn YT, Kim DH, Jeong HG, Jeong TC. Role of metabolism by human intestinal microflora in geniposide-induced toxicity in HepG2 cells. *Arch Pharm Res* 2012; **35**: 733-738 [PMID: 22553067 DOI: 10.1007/s12272-012-0418-y]
14 **Hiramatsu K**, Sakata H, Horita Y, Orita N, Kida A, Mizukami A, Miyazawa M, Hirai S, Shimatani A, Matsuda K, Matsuda M, Ogino H, Fujinaga H, Terada I, Shimizu K, Uchiyama A, Ishizawa S, Abo H, Demachi H, Noda Y. Mesenteric phlebosclerosis associated with long-term oral intake of geniposide, an ingredient of herbal medicine. *Aliment Pharmacol Ther* 2012; **36**: 575-586 [PMID: 22817400 DOI: 10.1111/j.1365-2036.2012.05221.x]
15 **Moschik EC**, Mercado C, Yoshino T, Matsuura K, Watanabe K. Usage and attitudes of physicians in Japan concerning traditional Japanese medicine (kampo medicine): a descriptive evaluation of a representative questionnaire-based survey. *Evid Based Complement Alternat Med* 2012; **2012**: 139818 [PMID: 22319543 DOI: 10.1155/2012/139818]
16 **Mathew RP**, Girgis S, Wells M, Low G. Phlebosclerotic Colitis - An Enigma Among Ischemic Colitis. *J Clin Imaging Sci* 2019; **9**: 18 [PMID: 31448169 DOI: 10.25259/JCIS-30-2019]
17 **Li YL**, Cheung KK. Thread like calcifications in mesenteric phlebosclerosis. *Abdom Radiol (NY)* 2018; **43**: 1504-1505 [PMID: 28879418 DOI: 10.1007/s00261-017-1311-4]
18 **Shibata H**, Nishikawa J, Sakaida I. Dark purple-colored colon: sign of idiopathic mesenteric phlebosclerosis. *Dig Endosc* 2014; **26**: 604-605 [PMID: 24720619 DOI: 10.1111/den.12299]
19 **Yamano T**, Tsujimoto Y, Noda T, Shimizu M, Ohmori M, Morita S, Yamada A. Hepatotoxicity of gardenia yellow color in rats. *Toxicol Lett* 1988; **44**: 177-182 [PMID: 3188076 DOI: 10.1016/0378-4274(88)90144-0]
20 **Chen MT**, Yu SL, Yang TH. A case of phlebosclerotic colitis with involvement of the entire colon. *Chang Gung Med J* 2010; **33**: 581-585 [PMID: 20979710]
21 **Washington C**, Carmichael JC. Management of ischemic colitis. *Clin Colon Rectal Surg* 2012; **25**: 228-235 [PMID: 24294125 DOI: 10.1055/s-0032-1329534]

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ORIGINAL ARTICLE

Retrospective Study

Early serum albumin changes in patients with ulcerative colitis treated with tacrolimus will predict clinical outcome

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Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Hamamatsu University School of Medicine (No. 20-356).

Informed consent statement: Informed written consent was obtained from the patient for publication of this report and any accompanying images.

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Abstract

BACKGROUND

Oral tacrolimus is a therapeutic agent for moderate to severe steroid-dependent or resistant ulcerative colitis (UC), but remission induction is difficult, and it is necessary to treat the patient while considering the next treatment.

AIM

To examine serum albumin (Alb) level as a prognostic factor for the therapeutic effect of tacrolimus in clinical practice.

METHODS

Forty-seven patients with UC treated with tacrolimus at our institution were divided into remission and failure groups (colectomy or switch to biologics), and the biological data at the start of observation and at weeks 1 and 2 were retrospectively examined. Kaplan-Meier and multivariate analyses were performed using Alb as a prognostic factor in UC treatment.

RESULTS

During the three months observed, 17 (36.2%) patients failed treatment with tacrolimus. A comparison between the failure and remission groups showed a

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significant difference only in Alb in week 2, and in the week 2/week 0 Alb ratio, which showed the rate of change in Alb. The cut-off value of the week 2/week 0 Alb ratio that predicted failure was 1, and its area under the curve was 0.751 (95%CI: 0.604-0.898). In the Kaplan-Meier analysis, a week 2/week 0 Alb ratio ≤ 1 had a significantly higher failure rate than that of > 1; Cox proportional hazard regression analysis also showed that a week 2/week 0 Alb ratio ≤ 1 was an independent prognostic factor for failure within 3 mo after the start of tacrolimus treatment.

CONCLUSION
A week 2/week 0 Alb ratio ≤ 1 predicts failure within 3 mo of tacrolimus administration for UC. High failure risk exists with week 2 Alb values ≤ 1 on admission.

Key Words: Ulcerative colitis; Tacrolimus; Albumin

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Core Tip: The usefulness of serum albumin as a predictor of the therapeutic effect of oral tacrolimus for ulcerative colitis was investigated. Lower albumin levels in week 2 than in week 0 after achieving trough levels of tacrolimus were shown to increase risk for failure, including colectomy and switch to biologics, within 3 mo. In these cases, changes to other treatment options, including surgical total colectomy, should be considered as soon as possible.

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INTRODUCTION

The incidence of ulcerative colitis (UC) is increasing worldwide[1]. Although corticosteroid treatment is the highest priority treatment option for patients with moderate to severe UC, there are patients with steroid dependence or resistance, and approximately 20%-30% of them do not improve following steroid treatment[2,3]. Although therapeutic agents such as biologics and calcineurin inhibitors are used as medical treatments to replace steroids, if none of these advanced treatments are successful, colectomy must be performed for these patients.

Treatment with oral tacrolimus, a calcineurin inhibitor, has been reported to be effective in patients with steroid-dependent and steroid-resistant UC[4,5]. Recently, in addition to studies on short-term results of tacrolimus for UC, studies on medium-to-long-term treatment results have also been reported[6-9]. Although the efficacy of tacrolimus has been demonstrated in these reports, there are patients who have failed to respond to this treatment and are forced to switch to other treatments, such as biologic therapy and colectomy. Continuing refractory treatment unnecessarily prolongs hospital stay and increases the risk of immunosuppressive and perioperative complications due to poor nutritional status[10,11]. When oral tacrolimus initially became available as a therapeutic agent for UC, only anti-tumor necrosis factor (TNF) α antibody agents were indicated for UC as biologics; however, various advanced treatments, including small-molecule Janus kinase inhibitors, anti-interleukin 12/23 antibodies, and anti-integrin antibodies are now available[12-14]. This means that we now have many alternative treatment options for tacrolimus refractory cases. Hence, the decision to switch to another treatment must be made as soon as possible and accurately in refractory cases.

Several studies have validated the therapeutic response to tacrolimus in UC. Nishida *et al*[15] proved that the neutrophil-to-lymphocyte ratio was an independent prognostic factor after the induction of tacrolimus therapy. Ban *et al*[16] reported that the factors predicting colectomy after the induction of calcineurin inhibitor were

cytomegalovirus antigenemia (C7HRP) positivity, a cumulative prednisolone dose of 10000 mg or more before the start of treatment, and a combination of hematocrit and the Onodera-prognostic nutritional index[16]. This study also demonstrated that the serum albumin (Alb) level before the induction of tacrolimus was useful as a predictor of early colectomy. Further, Herrlinger *et al*[17] reported that ABCB1 single-nucleotide polymorphisms predict the therapeutic response to tacrolimus.

In this study, we retrospectively examined the clinical course after the induction of remission by tacrolimus and the factors involved in its clinical outcome. As a prognostic factor that can be easily measured in clinical practice, changes in serum Alb levels before and after the induction of remission with tacrolimus were evaluated.

MATERIALS AND METHODS

Patients

Patients with UC who were treated with tacrolimus and had a medical record at the Hamamatsu University School of Medicine between August 2010 and January 2020 were enrolled in this study. The diagnosis of UC was made based on typical medical history and clinical features, as well as endoscopic and histological evaluation, in line with recent guidelines. Patients with inflammatory bowel disease (IBD) who were not diagnosed with UC, such as indeterminate colitis or unclassified IBD, were excluded. Patients were diagnosed with moderate to severe UC based on clinical activity index, biological data, and endoscopic findings.

Study design

This was a retrospective observational study conducted in a single center. The purpose of this study was to identify prognostic factors that may lead to clinical failure in patients with UC treated with tacrolimus. The primary outcome measure of this study was clinical failure in which a switch to colectomy or biologics was performed within 3 mo after tacrolimus induction. Secondary endpoints were comparison of biological data and clinical findings between the remission and failure groups.

Disease assessment

Enrolled patients were evaluated for clinical disease activity using the Rachmilewitz index[18]. The values of serum C-reactive protein (CRP), Alb, hemoglobin (Hb), and white blood cell (WBC) count and differential were measured in our facility on admission and more than twice per week.

Endoscopic assessment

Colonoscopy was performed on all enrolled patients before tacrolimus induction. UC mucosal status was assessed using the Mayo endoscopic subscore (MES)[19]. MES was assessed according to the following criteria: 0, normal or inactive disease; 1, mild disease with erythema, decreased vascular pattern, mild friability; 2, moderate disease with marked erythema, absence of vascular patterns, friability, and erosions; and 3, severe disease with spontaneous bleeding and ulceration. Mucosal healing was defined as MES 0 or 1.

Tacrolimus therapy and follow-up

The initial tacrolimus dose was started at 0.05 mg/kg twice daily. The blood trough levels of tacrolimus were measured 2 to 3 times a week, and tacrolimus doses were adjusted. The dose of tacrolimus was increased until a high trough level of 10-15 mg/mL was achieved. In this study, we defined day 1 as the day when a high trough level was reached after the start of oral tacrolimus administration. Oral administration of tacrolimus at a dose capable of maintaining a high trough level was continued for 2 wk, and then the dose was reduced to a low trough level (5-10 ng/mL). In this study, patients who underwent colectomy or switched to biologics within 3 mo after day 1 were defined as having clinical failure.

Statistical analysis

Statistical analyses of the data were performed using SPSS version 24 (IBM, Armonk, NY, United States) and SAS version 9.4 (SAS Institute, Cary, NC, United States) software. Differences between median values were compared using the Mann-Whitney U test and Fisher's exact test. Receiver operating characteristic (ROC) analysis was conducted to determine the optimal Alb cut-off value (week 2/week 0) for

predicting failure within 3 mo. The accuracy of the predicted failure ratio was evaluated using the area under the ROC curve. The cumulative remission rate was analyzed using Cox proportional hazard regression and Kaplan-Meier analysis, and was compared using the log-rank test. $P < 0.05$ was considered as statistically significant.

Ethical statement

This retrospective study protocol was reviewed and approved by the Ethics Committee of Hamamatsu University School of Medicine (number 20-356). This study was conducted in accordance with Good Clinical Practice principles in adherence to the Declaration of Helsinki.

RESULTS

Patient characteristics

The characteristics of forty-seven UC patients enrolled in this study are shown in Table 1. The mean patient age was 44.9 years, and the mean disease duration was 3.2 years. Patients with extensive colitis and left sided colitis numbered 42 and 5, respectively. On colonoscopy, 38 patients (80.9%) had MES 3, and nine patients (19.1%) had MES 2. Twelve patients (25.5%) had a history of treatment with biologics. The mean time to high-level trough with tacrolimus was 8.1 d.

Comparison of the remission and failure groups

During the 3-mo follow-up from the start of the study, 17 of 47 patients (36.2%) experienced clinical failure. On comparison of the remission and failure groups, as shown in Table 2, the failure group was older and tended to have a longer disease duration than the remission group, but this finding was not statistically significant. In addition, a history of treatment with biologics did not show a significant difference between the two groups.

Comparison of biological data between remission group and failure group

We compared biological data between the remission and failure groups before the start of treatment (week 0), 1 wk after the start of observation (week 1), and 2 wk after the start of observation (week 2) (Table 3). No significant difference was observed regarding the biological data before the start of treatment (week 0) between the two groups. There was no significant difference in biological data in week 1, and only serum Alb level in week 2 showed a significant difference between the remission and failure groups ($P = 0.015$).

In order to compare the changes in biological data before and after the start of treatment between the remission group and the failure group, the percentage of each value based on week 0 was calculated (Table 4). The week 1/week 0 ratio showed no significant difference in Alb, CRP, Hb, and WBC values. The week 2/week 0 ratio was significantly different only for Alb values between the remission and failure groups ($P = 0.003$).

Prediction of subsequent relapse using week 2 / week 0 Alb ratio

Since it was shown that the week 2/week 0 Alb ratio may be a factor predicting clinical failure for 3 mo, ROC analysis was performed using this ratio (Figure 1). The cut-off Alb ratio for week 2/week 0 was 1, and its sensitivity and specificity were 0.539 and 0.867, respectively. The AUC was 0.751 (95%CI: 0.604-0.898).

Furthermore, in order to investigate the reason for the cut-off value of 1 by week 2, we investigated the change in the Alb ratio (Figure 2). The Alb ratio increased significantly from week 0 to week 1 and showed a further increase from week 1 to week 2 ($P = 0.036$ and $P < 0.001$, respectively). In addition, when the Alb ratio was examined from the viewpoint of whether it was more or less than 1 in value, 40.4% of the patients in week 1 had an Alb ratio ≤ 1 (Figure 2). In contrast, at week 2, the number of patients with Alb ratio ≤ 1 decreased to 27.2%, and the number of patients with an Alb ratio > 1 increased to 72.8%.

Comparison of failure prediction using week 2 / week 0 Alb ratio

We compared the fractions of patients in remission between the week 2/week 0 Alb ratio > 1 group and week 2/week 0 Alb ratio ≤ 1 group using the Kaplan-Meier curve (Figure 3). There were 34 and 14 patients with week 2/week 0 Alb ratio > 1 and week

Table 1 Baseline characteristics of patients

Characteristics	*n* = 47
Age (yr), mean (range) ± SD	44.9 (16-78) ± 18.7
Male/female, *n* (%)	38/9 (80.9/10.6)
Disease duration (yr), mean (range) ± SD	3.2 (0.1-42) ± 7.0
Disease extent, *n* (%)	
Extensive colitis	42 (89.4)
Left sided colitis	5 (10.6)
CAI (Rachmilewitz index) mean (range) ± SD	11.0 (7-17) ± 2.7
MES	
MES 2	9 (19.1)
MES 3	38 (80.9)
Medication at study, *n* (%)	
Oral 5-ASA	21 (44.7)
Suppository 5-ASA	7 (14.9)
Systemic steroids	28 (59.6)
Immunomodulators	10 (21.3)
Biologics	11 (23.4)
History of biologics, *n* (%)	12 (25.5)
Time to high trough level (d), mean (range) ± SD	8.1 (0-46) ± 7.5

5-ASA: 5-aminosalicylic acid; CAI: Clinical activity index; MES: Mayo endoscopic subscore.

2/week 0 Alb ratio ≤ 1, respectively. Nine patients in both groups had clinical failures during the 3-mo follow-up. Patients with week 2/week 0 Alb ratio ≤ 1 had a significantly higher rate of clinical failure than patients with week 2/week 0 Alb ratio > 1 (log-rank test: $P = 0.002$). In addition, Cox proportional hazards regression analysis was performed for week 2/week 0 Alb ratio > 1 (Table 5). Week 2/week 0 Alb ratio ≤ 1 was shown by univariate and multivariate analyses to be an independent prognostic factor that resulted in increased rates of clinical failure within 3 mo of induction of tacrolimus treatment.

DISCUSSION

This study aimed to explore factors that could predict the prognosis of oral tacrolimus for UC. In particular, the analysis focused on biological data, which is a marker widely used in clinical practice. Tacrolimus is a calcineurin inhibitor used in patients with steroid-dependent or refractory UC and is indicated for moderate to severe cases. However, there are some cases in which colectomy is unavoidable due to ineffective treatment with tacrolimus. Approximately 6.1%-66.7% patients who were treated with tacrolimus underwent colectomy[6-8,20-26]. Therefore, the possibility of performing colectomy in the future should be considered, especially when starting treatment with tacrolimus for patients with very severe UC.

Conventionally, surgical treatment for IBD has many potential perioperative complications, and poor nutrition further increases the risk of postoperative complications[11,27]. Unnecessarily prolonging preoperative medical treatment will worsen the nutritional status of patients with UC, and surgical treatment in that malnourished state will further increase the risk of postoperative complications. It is extremely important to judge the effectiveness of treatment for UC at an early stage and to consider the next treatment before the general condition worsens. In this study, we searched for prognostic factors that could predict the efficacy of tacrolimus based on clinical laboratory data.

Table 2 Comparison between the failure and remission groups before tacrolimus induction

Characteristics	Remission (*n* = 30)	Failure (*n* = 17)	*P* value
Age (yr), mean ± SD	41.33 ± 16.8	51.1 ± 20.7	0.084
Male/female, *n* (%)	23/7 (76.7 / 23.3)	15/2 (88.2/11.8)	0.455
Disease duration (yr), mean ± SD	2.0 ± 2.8	5.46 ± 10.9	0.098
Disease extent, *n* (%)			
Extensive colitis	27 (90.0)	15 (88.2)	1
Left sided colitis	3 (10.0)	2 (11.8)	
CAI (Rachmilewitz index), mean ± SD	10.7 ± 2.4	11.7 ± 2.9	0.221
MES			
MES 2	6 (20.0)	3 (17.6)	1
MES 3	24 (80.0)	14 (82.4)	
Medication at study, *n* (%)			
Oral 5-ASA	16 (53.3)	5 (29.4)	0.138
Suppository 5-ASA	5 (16.7)	2 (11.8)	1
Systemic steroids	19 (63.3)	9 (52.9)	0.546
Immunomodulators	6 (20.0)	4 (23.5)	1
Biologics	6 (20.0)	5 (29.4)	0.493
History of biologics, *n* (%)	6 (20.0)	6 (35.3)	0.306
Time to high trough level (d), mean ± SD	7.9 ± 8.1	8.5 ± 6.4	0.773

5-ASA: 5-aminosalicylic acid; CAI: Clinical activity index; MES: Mayo endoscopic subscore.

Table 3 Comparison of biological data before and after tacrolimus induction

Biological data	Remission (*n* = 30)	Failure (*n* = 17)	*P* value
Serum Alb at week 0 (g/dL)	2.83 ± 0.68	2.81 ± 0.72	0.897
Serum Alb at week 1 (g/dL)	2.94 ± 0.61	2.85 ± 0.78	0.651
Serum Alb at week 2 (g/dL)	3.29 ± 0.57	2.81 ± 0.73	0.015
Serum CRP at week 0 (mg/dL)	5.58 ± 5.75	5.44 ± 5.45)	0.933
Serum CRP at week 1 (mg/dL)	0.88 ± 2.29	1.12 ± 1.51	0.696
Serum CRP at week 2 (mg/dL)	0.54 ± 1.50	1.17 ± 1.63	0.191
Hb at week 0 (g/dL)	11.2 ± 1.90	11.32 ± 2.06	0.871
Hb at week 1 (g/dL)	10.65 ± 1.60	11.15 ± 1.77	0.326
Hb at week 2 (g/dL)	10.78 ± 1.71	10.89 ± 1.88	0.837
WBC at week 0 (/μL)	8660.00 ± 4498.65	8846.47 ± 3876.21	0.887
WBC at week 1 (/μL)	5958.00 ± 2444.20	6618.24 ± 2201.22	0.362
WBC at week 2 (/μL)	5473.00 ± 2095.78	6672.35 ± 2154.37	0.069

Alb: Albumin; CRP: C-reactive protein; Hb: Hemoglobin; WBC: White blood cell. mean ± SD is shown.

In this study, we investigated the values of Alb, CRP, Hb, and WBC before the induction of tacrolimus (week 0) and at 1- and 2 wk after achieving high trough levels (week 1 and week 2, respectively). There was a significant difference in serum Alb levels at week 2 between the remission and failure groups, but there was no significant difference in CRP, a biomarker that reflects systemic or local inflammation. Since each

Table 4 Comparison of biological data ratio after tacrolimus induction

Rate of biological data	Remission (*n* = 30)	Failure (*n* = 17)	*P* value
Week 1/week 0 Alb ratio	1.05 ± 0.14	1.02 ± 0.13	0.429
Week 2/week 0 Alb ratio	1.19 ± 0.20	1.02 ± 0.16	0.003
Week 1/week 0 CRP ratio	0.28 ± 0.49	1.28 ± 3.80	0.159
Week 2/week 0 CRP ratio	0.16 ± 0.23	1.42 ± 4.28	0.112
Week 1/week 0 Hb ratio	0.96 ± 0.13	1.01 ± 0.18	0.315
Week 2/week 0 Hb ratio	0.97 ± 0.13	0.98 ± 0.14	0.892
Week 1/week 0 WBC ratio	0.77 ± 0.32	0.84 ± 0.33	0.520
Week 2/week 0 WBC ratio	0.72 ± 0.30	0.86 ± 0.40	0.194

Alb: Albumin; CRP: C-reactive protein; Hb: Hemoglobin; WBC: White blood cell. mean ± SD is shown.

Table 5 Multivariate analysis for predicting failure during 3 mo after tacrolimus induction

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Week 2/week 0 Alb ratio ≤ 1	3.632	1.396-9.445	0.008	7.195	2.011-25.74	0.002
Age	1.026	0.998-1.055	0.072	1.009	0.976-1.042	0.599
Sex (male)	2.139	0.489-9.362	0.313	2.420	0.435-13.45	0.313
Disease extent (extensive colitis)	1.062	0.243-4.640	0.937	4.665	0.554-39.30	0.157
MES 3	1.188	0.341-4.137	0.787	0.462	0.142-1.503	0.200
Medication						
Oral 5-ASA	0.450	0.158-1.277	0.133	0.578	0.191-1.744	0.331
Suppository 5-ASA	0.652	0.149-2.853	0.570	0.247	0.035-1.744	0.161
Systemic steroids	0.729	0.281-1.890	0.515	0.636	0.193-2.094	0.457
Immunomodulators	1.152	0.375-3.543	0.805	1.516	0.391-5.885	0.548
Biologics	1.400	0.493-3.978	0.527	0.924	0.173-4.920	0.926

5-ASA: 5-aminosalicylic acid; Alb: Albumin; HR: Hazard ratio; MES: Mayo endoscopic subscore.

Alb value before the start of treatment varies with each patient, the ratio of week 1 Alb value or week 2 Alb value divided by week 0 Alb value was calculated in this study. As a result, the week 2/week 0 Alb ratio showed a significant difference between the remission and failure groups. In the treatment of ulcerative colitis with tacrolimus, a sufficient therapeutic effect is exhibited, and mucosal healing is induced, by promptly reaching the effective range of blood concentration after the start of tacrolimus treatment. Therefore, in cases in which tacrolimus is effective, albumin is elevated because leakage of albumin from the intestinal tract is limited by mucosal healing. However, mucosal healing is not achieved in patients in whom tacrolimus is ineffective; therefore, it is thought that albumin loss from the intestinal tract persists and hypoalbuminemia does not improve. Although not shown in the data, the clinical activity index, which evaluated bloody stools and diarrhea, showed a greater improvement from week 0 to week 2 in the non-failure group than in the failure group.

We considered the factors leading to the significant difference in the Alb value by week 2 between groups; however, no significant difference was shown in Week 1. As shown in Figure 2, a significant increase in the Alb ratio was observed from Week 0 to Week 1, and a further significant increase was observed from Week 1 to Week 2 overall. Furthermore, as a result of examining the proportion of patients with an Alb ratio of 1 as the cut-off value in weeks 1 and 2, patients with an Alb ratio ≤ 1 (patients

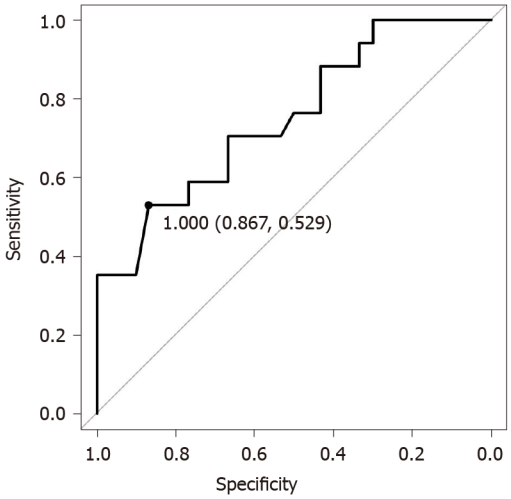


Figure 1 Receiver-operating characteristic analysis for prediction of failure during 3 mo after the reach of tacrolimus high trough level.

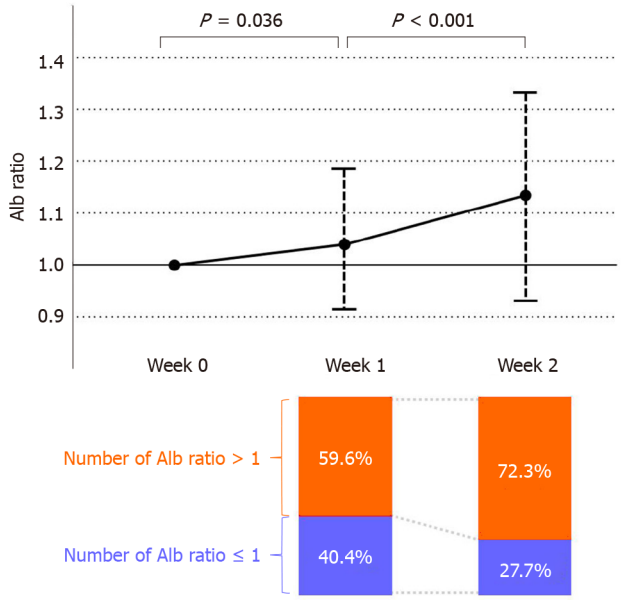


Figure 2 Changes in serum albumin ratio of each week and the percentage of the number of serum albumin ratio > 1 and ≤ 1. Serum albumin (Alb) ratio at week 1 and week 2 shows the week 1/week 0 and week 2/week 0 Alb ratio, respectively. The percentage of the number of Alb ratio > 1 and ≤ 1 at week 1 and week 2 are shown in the bar graph.

whose Alb value did not change or decrease in week 1 compared to week 0) comprised 40.4%, which was close to half. The reason for this may be that frequent diarrhea and bloody stools before the administration of tacrolimus may cause intravascular dehydration, resulting in higher Alb levels in blood. However, it was considered that after starting treatment, blood might have been diluted due to intravenous hydration in many patients, resulting in a temporary decrease in Alb levels. For this reason, the Alb level tends to decrease temporarily after the start of treatment, so it is too early to evaluate the Alb level by week 1, and judgment at week 2 was considered to be appropriate.

Lee *et al*[28] reported that determination of Alb levels at week 2 was important for anti-TNF treatment in UC, which is similar to our analysis. This report showed that the Alb value 2 wk after the start of anti-TNF treatment was associated with primary nonresponse, endoscopic outcomes, time-to-colectomy, and anti-TNF failure.

Several reports have attempted to predict the effect of tacrolimus on UC. Miyoshi *et al*[7] reported that colonoscopy evaluation 3 mo after remission induction with tacrolimus showed that groups with MES 0 and 1 had a better subsequent clinical course than the groups with MES 2 and 3. In addition to this report, there have been several other reports on the treatment responsiveness and prognosis of tacrolimus on

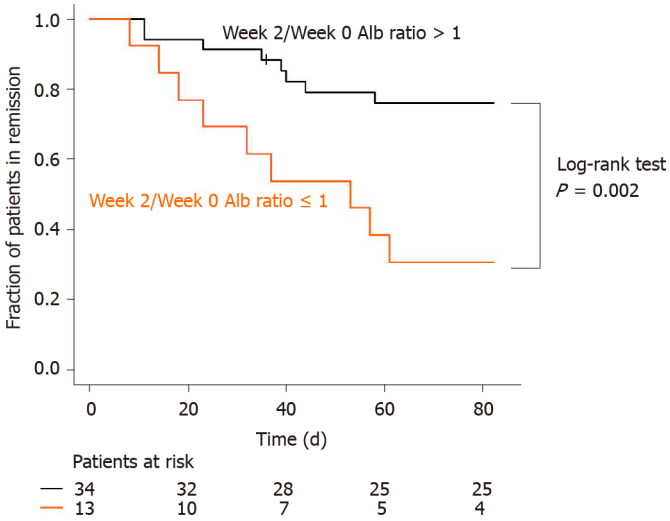


Figure 3 Kaplan-Meier time-to-relapse curve of patients with ulcerative colitis in relation to the week 2/week 0 albumin ratio > 1 and ≤ 1.

UC. In the report by Ban *et al*[16], an analysis focusing on Alb was performed similar to that in our study. Their study also examined patients with UC who received calcineurin inhibitors, including cyclosporine and tacrolimus, and who underwent colectomy within 60 d of their remission induction. The study reported that the mean Alb levels of the early colectomy group and the non-early colectomy group before tacrolimus treatment were 2.95 and 3.41, respectively, and that low Alb levels were a risk for early colectomy (P = 0.0256). However, in our study, the mean Alb levels before the start of tacrolimus in the failure and remission groups were 2.81 and 2.83, respectively, showing no significant difference. The mean Alb levels before the induction of tacrolimus in all cases were 3.3 and 2.82 in the study by Ban *et al*[16] and the present study, respectively; however, more patients had hypoalbuminemia before the start of treatment in our institution. This seems to be the reason for the difference in the results between the two clinical studies. In our study, 17 of 47 patients had clinical failure during a 3-mo follow-up, and the remission rate was 63.8%. Although there are differences in the observation period and the severity of enrolled patients, previous reports of the effects of tacrolimus on UC have reported a remission rate ranging from 9.4-75.6%[4-8,21-26,29].

In this study, even if the Alb level at week 0 was low, we could predict an effective remission if the albumin ratio was high in our study. Specifically, in the sub-group of Alb < 3 (n = 25), the week 2/week 0 Alb ratio of the failure group was significantly higher than that of the remission group (P = 0.003). In addition, a ROC analysis of the Alb < 3 sub-group showed a cut-off value of 1.235 and AUC of 0.853 with a 95%CI of 0.705-1.000. We considered that the AUC of Alb < 3 was higher than the AUC of all cases because low Alb levels at Week 0 could be slightly increased after treatment improved the condition of UC.

Limitations

This study has several limitations. First, this is a single-center retrospective study with few enrolled patients. Second, we did not evaluate certain fecal biomarkers that have recently become widely used. Fecal biomarkers used in UC not only reflect endoscopic scores, but can also predict clinical relapse. There have been several recent reports on the usefulness of fecal calprotectin and fecal immunochemical occult blood test [30-35]. However, it has not been verified whether these biomarkers are useful for evaluating the therapeutic effect and predicting prognosis in treatment using tacrolimus for UC, which is a subject for future research. In addition, this study evaluated Alb levels 2 wk after achieving high trough levels of tacrolimus as it is not beneficial for patients to postpone the decision to convert to surgical treatment beyond this time point. Indeed, early conversion to surgical treatment in steroid-refractory acute severe UC has been reported to improve postoperative prognosis[36,37]. Conversely, prolongation of preoperative medical treatment for refractory cases has been reported to worsen the postoperative prognosis of UC[38,39]. Alternatively, switching to other biologics may not improve the condition of UC patients, but rather increase the risk of complications. Therefore, we thought that the prediction of the

prognosis in moderate to severe UC *via* the Alb ratio, as shown in this study, could be a supportive factor in determining the next treatment.

CONCLUSION

In conclusion, a lower Alb value in week 2 than in week 0 after achieving trough level of tacrolimus predicts clinical failure in patients with UC. In these cases, changes to other treatment options, including surgical total colectomy, should be considered as soon as possible.

ARTICLE HIGHLIGHTS

Research background

There are cases of refractory ulcerative colitis in which tacrolimus does not induce remission.

Research motivation

A biomarker capable of predicting the induction effect of tacrolimus is desired.

Research objectives

The usefulness of serum albumin as a predictor of the therapeutic effect of oral tacrolimus for ulcerative colitis was investigated.

Research methods

Patients treated with tacrolimus at our hospital were divided into a remission group and a failure group, and data including serum albumin at the start of observation and at the first and second weeks were retrospectively examined.

Research results

Lower albumin levels in week 2 than in week 0 after achieving trough levels of tacrolimus were shown to increase risk for failure, including colectomy and switch to biologics, within 3 mo.

Research conclusions

Early serum albumin changes in patients with ulcerative colitis treated with tacrolimus predict clinical outcome.

Research perspectives

In cases where albumin levels in week 2 are lower than week 0, changes to other treatment options, including surgical total colectomy, should be considered as soon as possible.

REFERENCES

1 **Ungaro R**, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis. *Lancet* 2017; **389**: 1756-1770 [PMID: 27914657 DOI: 10.1016/S0140-6736(16)32126-2]
2 **Faubion WA Jr**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 2001; **121**: 255-260 [PMID: 11487534 DOI: 10.1053/gast.2001.26279]
3 **Turner D**, Walsh CM, Steinhart AH, Griffiths AM. Response to corticosteroids in severe ulcerative colitis: a systematic review of the literature and a meta-regression. *Clin Gastroenterol Hepatol* 2007; **5**: 103-110 [PMID: 17142106 DOI: 10.1016/j.cgh.2006.09.033]
4 **Ogata H**, Matsui T, Nakamura M, Iida M, Takazoe M, Suzuki Y, Hibi T. A randomised dose finding study of oral tacrolimus (FK506) therapy in refractory ulcerative colitis. *Gut* 2006; **55**: 1255-1262 [PMID: 16484504 DOI: 10.1136/gut.2005.081794]
5 **Ogata H**, Kato J, Hirai F, Hida N, Matsui T, Matsumoto T, Koyanagi K, Hibi T. Double-blind, placebo-controlled trial of oral tacrolimus (FK506) in the management of hospitalized patients with steroid-refractory ulcerative colitis. *Inflamm Bowel Dis* 2012; **18**: 803-808 [PMID: 21887732 DOI: 10.1002/ibd.21853]
6 **Yamamoto S**, Nakase H, Mikami S, Inoue S, Yoshino T, Takeda Y, Kasahara K, Ueno S, Uza N,

Kitamura H, Tamaki H, Matsuura M, Inui K, Chiba T. Long-term effect of tacrolimus therapy in patients with refractory ulcerative colitis. *Aliment Pharmacol Ther* 2008; **28**: 589-597 [PMID: 18549460 DOI: 10.1111/j.1365-2036.2008.03764.x]

7 **Miyoshi J**, Matsuoka K, Inoue N, Hisamatsu T, Ichikawa R, Yajima T, Okamoto S, Naganuma M, Sato T, Kanai T, Ogata H, Iwao Y, Hibi T. Mucosal healing with oral tacrolimus is associated with favorable medium- and long-term prognosis in steroid-refractory/dependent ulcerative colitis patients. *J Crohns Colitis* 2013; **7**: e609-e614 [PMID: 23680174 DOI: 10.1016/j.crohns.2013.04.018]

8 **Ikeya K**, Sugimoto K, Kawasaki S, Iida T, Maruyama Y, Watanabe F, Hanai H. Tacrolimus for remission induction in ulcerative colitis: Mayo endoscopic subscore 0 and 1 predict long-term prognosis. *Dig Liver Dis* 2015; **47**: 365-371 [PMID: 25682993 DOI: 10.1016/j.dld.2015.01.149]

9 **Endo K**, Onodera M, Shiga H, Kuroha M, Kimura T, Hiramoto K, Kakuta Y, Kinouchi Y, Shimosegawa T. A Comparison of Short- and Long-Term Therapeutic Outcomes of Infliximab- versus Tacrolimus-Based Strategies for Steroid-Refractory Ulcerative Colitis. *Gastroenterol Res Pract* 2016; **2016**: 3162595 [PMID: 26904108 DOI: 10.1155/2016/3162595]

10 **Markel TA**, Lou DC, Pfefferkorn M, Scherer LR 3rd, West K, Rouse T, Engum S, Ladd A, Rescorla FJ, Billmire DF. Steroids and poor nutrition are associated with infectious wound complications in children undergoing first stage procedures for ulcerative colitis. *Surgery* 2008; **144**: 540-5; discussion 545 [PMID: 18847637 DOI: 10.1016/j.surg.2008.07.005]

11 **Okita Y**, Araki T, Okugawa Y, Kondo S, Fujikawa H, Hiro J, Inoue M, Toiyama Y, Ohi M, Uchida K, Kusunoki M. The prognostic nutritional index for postoperative infectious complication in patients with ulcerative colitis undergoing proctectomy with ileal pouch-anal anastomosis following subtotal colectomy. *J Anus Rectum Colon* 2019; **3**: 91-97 [PMID: 31559374 DOI: 10.23922/jarc.2018-032]

12 **Sandborn WJ**, Su C, Sands BE, D'Haens GR, Vermeire S, Schreiber S, Danese S, Feagan BG, Reinisch W, Niezychowski W, Friedman G, Lawendy N, Yu D, Woodworth D, Mukherjee A, Zhang H, Healey P, Panés J; OCTAVE Induction 1, OCTAVE Induction 2, and OCTAVE Sustain Investigators. Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis. *N Engl J Med* 2017; **376**: 1723-1736 [PMID: 28467869 DOI: 10.1056/NEJMoa1606910]

13 **Sands BE**, Sandborn WJ, Panaccione R, O'Brien CD, Zhang H, Johanns J, Adedokun OJ, Li K, Peyrin-Biroulet L, Van Assche G, Danese S, Targan S, Abreu MT, Hisamatsu T, Szapary P, Marano C; UNIFI Study Group. Ustekinumab as Induction and Maintenance Therapy for Ulcerative Colitis. *N Engl J Med* 2019; **381**: 1201-1214 [PMID: 31553833 DOI: 10.1056/NEJMoa1900750]

14 **Sands BE**, Peyrin-Biroulet L, Loftus EV Jr, Danese S, Colombel JF, Törüner M, Jonaitis L, Abhyankar B, Chen J, Rogers R, Lirio RA, Bornstein JD, Schreiber S; VARSITY Study Group. Vedolizumab versus Adalimumab for Moderate-to-Severe Ulcerative Colitis. *N Engl J Med* 2019; **381**: 1215-1226 [PMID: 31553834 DOI: 10.1056/NEJMoa1905725]

15 **Nishida Y**, Hosomi S, Yamagami H, Sugita N, Itani S, Yukawa T, Otani K, Nagami Y, Tanaka F, Taira K, Kamata N, Tanigawa T, Watanabe T, Fujiwara Y. Pretreatment neutrophil-to-lymphocyte ratio predicts clinical relapse of ulcerative colitis after tacrolimus induction. *PLoS One* 2019; **14**: e0213505 [PMID: 30845259 DOI: 10.1371/journal.pone.0213505]

16 **Ban H**, Bamba S, Nishida A, Inatomi O, Shioya M, Takahashi KI, Imaeda H, Murata M, Sasaki M, Tsujikawa T, Andoh A. Prognostic factors affecting early colectomy in patients with moderate to severe ulcerative colitis treated with calcineurin inhibitors. *Exp Ther Med* 2016; **12**: 829-834 [PMID: 27446283 DOI: 10.3892/etm.2016.3341]

17 **Herrlinger KR**, Koc H, Winter S, Teml A, Stange EF, Fellermann K, Fritz P, Schwab M, Schaeffeler E. ABCB1 single-nucleotide polymorphisms determine tacrolimus response in patients with ulcerative colitis. *Clin Pharmacol Ther* 2011; **89**: 422-428 [PMID: 21289623 DOI: 10.1038/clpt.2010.348]

18 **Schroeder KW**, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987; **317**: 1625-1629 [PMID: 3317057 DOI: 10.1056/NEJM198712243172603]

19 **Rachmilewitz D**. Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis: a randomised trial. *BMJ* 1989; **298**: 82-86 [PMID: 2563951 DOI: 10.1136/bmj.298.6666.82]

20 **Högenauer C**, Wenzl HH, Hinterleitner TA, Petritsch W. Effect of oral tacrolimus (FK 506) on steroid-refractory moderate/severe ulcerative colitis. *Aliment Pharmacol Ther* 2003; **18**: 415-423 [PMID: 12940927 DOI: 10.1046/j.1365-2036.2003.01662.x]

21 **Benson A**, Barrett T, Sparberg M, Buchman AL. Efficacy and safety of tacrolimus in refractory ulcerative colitis and Crohn's disease: a single-center experience. *Inflamm Bowel Dis* 2008; **14**: 7-12 [PMID: 17879277 DOI: 10.1002/ibd.20263]

22 **Thin LW**, Murray K, Lawrance IC. Oral tacrolimus for the treatment of refractory inflammatory bowel disease in the biologic era. *Inflamm Bowel Dis* 2013; **19**: 1490-1498 [PMID: 23615528 DOI: 10.1097/MIB.0b013e318281f362]

23 **Schmidt KJ**, Herrlinger KR, Emmrich J, Barthel D, Koc H, Lehnert H, Stange EF, Fellermann K, Büning J. Short-term efficacy of tacrolimus in steroid-refractory ulcerative colitis - experience in 130 patients. *Aliment Pharmacol Ther* 2013; **37**: 129-136 [PMID: 23121200 DOI: 10.1111/apt.12118]

24 **Landy J**, Wahed M, Peake ST, Hussein M, Ng SC, Lindsay JO, Hart AL. Oral tacrolimus as maintenance therapy for refractory ulcerative colitis--an analysis of outcomes in two London tertiary centres. *J Crohns Colitis* 2013; **7**: e516-e521 [PMID: 23623737 DOI: 10.1016/j.crohns.2013.03.008]

25 **Kawakami K**, Inoue T, Murano M, Narabayashi K, Nouda S, Ishida K, Abe Y, Nogami K, Hida N, Yamagami H, Watanabe K, Umegaki E, Nakamura S, Arakawa T, Higuchi K. Effects of oral

tacrolimus as a rapid induction therapy in ulcerative colitis. *World J Gastroenterol* 2015; **21**: 1880-1886 [PMID: 25684955 DOI: 10.3748/wjg.v21.i6.1880]

26 **Saifuddin A**, Harris A. Tacrolimus therapy in moderate to subacute ulcerative proctocolitis: a large single-centre cohort study. *Frontline Gastroenterol* 2018; **9**: 148-153 [PMID: 29588844 DOI: 10.1136/flgastro-2017-100888]

27 **Gu J**, Stocchi L, Remzi F, Kiran RP. Factors associated with postoperative morbidity, reoperation and readmission rates after laparoscopic total abdominal colectomy for ulcerative colitis. *Colorectal Dis* 2013; **15**: 1123-1129 [PMID: 23627886 DOI: 10.1111/codi.12267]

28 **Lee SH**, Walshe M, Oh EH, Hwang SW, Park SH, Yang DH, Byeon JS, Myung SJ, Yang SK, Greener T, Weizman AV, Silverberg MS, Ye BD. Early Changes in Serum Albumin Predict Clinical and Endoscopic Outcomes in Patients With Ulcerative Colitis Starting Anti-TNF Treatment. *Inflamm Bowel Dis* 2020 [PMID: 33269403 DOI: 10.1093/ibd/izaa309]

29 **Hiraoka S**, Kato J, Moritou Y, Takei D, Inokuchi T, Nakarai A, Takahashi S, Harada K, Okada H, Yamamoto K. The earliest trough concentration predicts the dose of tacrolimus required for remission induction therapy in ulcerative colitis patients. *BMC Gastroenterol* 2015; **15**: 53 [PMID: 25925267 DOI: 10.1186/s12876-015-0285-3]

30 **Hiraoka S**, Kato J, Nakarai A, Takashima S, Inokuchi T, Takei D, Sugihara Y, Takahara M, Harada K, Okada H. Consecutive Measurements by Faecal Immunochemical Test in Quiescent Ulcerative Colitis Patients Can Detect Clinical Relapse. *J Crohns Colitis* 2016; **10**: 687-694 [PMID: 26802083 DOI: 10.1093/ecco-jcc/jjw025]

31 **Naganuma M**, Kobayashi T, Nasuno M, Motoya S, Kato S, Matsuoka K, Hokari R, Watanabe C, Sakamoto H, Yamamoto H, Sasaki M, Watanabe K, Iijima H, Endo Y, Ichikawa H, Ozeki K, Tanida S, Ueno N, Fujiya M, Sako M, Takeuchi K, Sugimoto S, Abe T, Hibi T, Suzuki Y, Kanai T. Significance of Conducting 2 Types of Fecal Tests in Patients With Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2020; **18**: 1102-1111. e5 [PMID: 31394288 DOI: 10.1016/j.cgh.2019.07.054]

32 **Nakarai A**, Hiraoka S, Takahashi S, Inaba T, Higashi R, Mizuno M, Takashima S, Inokuchi T, Sugihara Y, Takahara M, Harada K, Kato J, Okada H. Simultaneous Measurements of Faecal Calprotectin and the Faecal Immunochemical Test in Quiescent Ulcerative Colitis Patients Can Stratify Risk of Relapse. *J Crohns Colitis* 2018; **12**: 71-76 [PMID: 28961792 DOI: 10.1093/ecco-jcc/jjx118]

33 **Yamamoto T**, Shimoyama T, Umegae S, Matsumoto K. Endoscopic score vs. fecal biomarkers for predicting relapse in patients with ulcerative colitis after clinical remission and mucosal healing. *Clin Transl Gastroenterol* 2018; **9**: 136 [PMID: 29491393 DOI: 10.1038/s41424-018-0006-7]

34 **Urushikubo J**, Yanai S, Nakamura S, Kawasaki K, Akasaka R, Sato K, Toya Y, Asakura K, Gonai T, Sugai T, Matsumoto T. Practical fecal calprotectin cut-off value for Japanese patients with ulcerative colitis. *World J Gastroenterol* 2018; **24**: 4384-4392 [PMID: 30344422 DOI: 10.3748/wjg.v24.i38.4384]

35 **Buisson A**, Mak WY, Andersen MJ, Lei D, Kahn SA, Pekow J, Cohen RD, Zmeter N, Pereira B, Rubin DT. Faecal Calprotectin Is a Very Reliable Tool to Predict and Monitor the Risk of Relapse After Therapeutic De-escalation in Patients With Inflammatory Bowel Diseases. *J Crohns Colitis* 2019; **13**: 1012-1024 [PMID: 30726887 DOI: 10.1093/ecco-jcc/jjz023]

36 **Leeds IL**, Truta B, Parian AM, Chen SY, Efron JE, Gearhart SL, Safar B, Fang SH. Early Surgical Intervention for Acute Ulcerative Colitis Is Associated with Improved Postoperative Outcomes. *J Gastrointest Surg* 2017; **21**: 1675-1682 [PMID: 28819916 DOI: 10.1007/s11605-017-3538-3]

37 **Saha SK**, Panwar R, Kumar A, Pal S, Ahuja V, Dash NR, Makharia G, Sahni P. Early colectomy in steroid-refractory acute severe ulcerative colitis improves operative outcome. *Int J Colorectal Dis* 2018; **33**: 79-82 [PMID: 28920181 DOI: 10.1007/s00384-017-2903-8]

38 **Coakley BA**, Telem D, Nguyen S, Dallas K, Divino CM. Prolonged preoperative hospitalization correlates with worse outcomes after colectomy for acute fulminant ulcerative colitis. *Surgery* 2013; **153**: 242-248 [PMID: 23062652 DOI: 10.1016/j.surg.2012.08.002]

39 **Kim IK**, Park KJ, Kang GH, Im JP, Kim SG, Jung HC, Song IS, Kim JS. Risk factors for complications after total colectomy in ulcerative colitis. *Turk J Gastroenterol* 2012; **23**: 515-522 [PMID: 23161324 DOI: 10.4318/tjg.2012.0425]

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ORIGINAL ARTICLE

Observational Study

Preservation of superior rectal artery in laparoscopically assisted subtotal colectomy with ileorectal anastomosis for slow transit constipation

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Abstract

BACKGROUND

Slow transit constipation (STC) has traditionally been considered as a functional disorder. However, evidence is accumulating that suggests that most of the motility alterations in STC might be of a neuropathic etiology. If the patient does not meet the diagnosis of pelvic outlet obstruction and poorly response to conservative treatment, surgical intervention with subtotal colectomy may be effective. The most unwanted complication of the procedure is anastomotic leakage, however, preservation of the superior rectal artery (SRA) may reduce its incidence.

AIM

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To evaluate the preservation of the SRA in laparoscopically assisted subtotal colectomy with ileorectal anastomosis in STC patients.

METHODS

This was a single-center retrospective observational study. STC was diagnosed after a series of examinations which included a colonic transit test, anal manometry, a balloon expulsion test, and a barium enema. Eligible patients underwent laparoscopically assisted total colectomy with ileorectal anastomosis and were examined between January 2016 and January 2018. The operation time, blood loss, time to first flatus, length of hospital days, and incidence of minor or major complications were recorded.

RESULTS

A total of 32 patients (mean age, 42.6 years) who had received laparoscopic assisted subtotal colectomy with ileorectal artery anastomosis and preservation of the SRA. All patients were diagnosed with STC after a series of examinations. The mean operative time was 151 min and the mean blood loss was 119 mL. The mean day of first time to flatus was 3.0 d, and the mean hospital stay was 10.6 d. There were no any patients conversions to laparotomy. Post-operative minor complications including 1 wound infection and 1 case of ileus. There was no surgical mortality. No anastomosis leakage was noted in any of the patients.

CONCLUSION

Laparoscopically assisted subtotal colectomy with ileorectal anastomosis and preservation of the SRA can significantly improve bowel function with careful patient selection. Sparing the SRA may protect against anastomosis leakage.

Key Words: Slow transit constipation; Superior rectal artery; Anastomosis leakage; Laparoscopic-assisted colorectal surgery; Iliorectal anastomosis; Colonic transit time

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Core Tip: Slow transit constipation (STC) has traditionally been considered as a functional disorder. Surgical intervention with subtotal colectomy may be effective for STC. Laparoscopically assisted subtotal colectomy with ileorectal anastomosis and preservation of the superior rectal artery is tolerated and can have an excellent surgical outcome in reduce anastomosis leakage with significant improvement in bowel function under careful patient enrollment criteria.

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INTRODUCTION

Constipation means the incapacity to evacuate the bowel. Patients cannot evacuate completely and spontaneously at least three or more times *per* week although it is commonly one or fewer bowel movements *per* week. Most patients with constipation have one or more of the following symptoms: Hard infrequent stools, excessive straining, incomplete evacuation, excessive time attempting to evacuate or dissatisfaction with defecation[1-4].

Slow transit constipation (STC) has been traditionally considered and classified as a functional disorder. However, clinical and manometric evidence has been accumulating that suggests most of the motility abnormalities in STC might be neuropathic[5].

Radiopaque markers with a transit colon test showed delayed of the transpiration of the colon is used for making the diagnosis[2,3,6]. If the conservative treatment is failed

and pelvic outlet obstruction was ruled out, surgical procedure with a subtotal colectomy may be effective[7]. The most unwanted complication of the procedure is an anastomotic leak (AL). AL is a serious complication of colorectal surgery and can be defined as: 'the luminal contents leaking from a surgical connection between 2 hollow viscera'[8]. AL is responsible for up to 40% of the postoperative mortality rate, prolonged hospitalization, and an increase in the costs of healthcare due to the treatment of sepsis and the need for re-operation[9]. The main aim of this study is to make known a feasibility and functional outcome of laparoscopically assisted subtotal colectomy with the preservation of the superior rectal artery (SRA) in ileorectal anastomosis for STC.

MATERIALS AND METHODS

This investigation is an observational single-center study. The decision on whether to preserve the SRA was left to the discretion of the surgeon. Of the patients who were treated at the Division of Colon and Rectal Surgery at the Taiwan Adventist Hospital between January 2016 and January 2017, patients diagnosed with constipation (compatible with the Rome II criteria)[10] were tested with laboratory studies including a thyroid-function test, serum calcium, serum glucose, and complete blood count. All cases had clinical evaluation, such as a digital rectal examination and a psychological consultation. We excluded secondary causes (colonic obstruction, metabolic disorders, and drug-induced constipation).

The STC diagnostic workup includes a colonic transitional test, pressure measurement of anorectal, a balloon exploration test, and a barium enema. We defined a positive colonic transit test as patients who had greater than 20% of radiopaque marker stasis in the colorectum after 96 h. Anorectal pressure measurement and the balloon exploration test were arranged to assure there was no outlet obstructed defecation and to rule out pelvic floor dysfunction. Patients underwent a barium enema to ensure there were no mechanical obstruction problems, and this investigation showed redundant colon in all cases. Colonoscopy was normal and anal ultrasound showed no disruption of the external anal sphincter in all cases. Suitable patients were those in complete accord with the colonic transition test greater than 96 h, but who had normal results on the barium enema, balloon exploration test, anal pressure measurement, colonoscopy, and anal ultrasound. Informed consent was provided before all examinations. The operations were performed by the same team.

In all, 32 patients were diagnosed with STC and were treated by laparoscopically assisted subtotal colectomy with preservation of the SRA in ileorectal anastomosis between January 2016 and January 2017. Data on age, body mass index, preoperative laxative dependency, preoperative defecation duration, colonic transit time, operative time, estimated volume of blood loss, surgical complications, postoperative bowel movements, length of hospital stay, and functional outcome were recorded.

This study protocol was reviewed and approved by the Institutional Review Board of the Taiwan Adventist Hospital (TAHIRB No. 105-E-10). It was conducted in compliance with the Helsinki Declaration. The patients provided written informed consent before surgery and patient information was anonymized and de-identified before analysis.

Patient characteristics are summarized using total number, percentages, and mean ± SD. All statistical analyses were performed with IBM SPSS statistical software version 22 for Windows (IBM Corp., Armonk, NY, United States).

Surgical technique

All the laparoscopic surgical procedures for preservation of SRA in STC patients were performed by two experienced colorectal surgeons who have experience of more than 100 laparoscopic and open colorectal cancer procedures annually. In all cases, the planned procedure was a laparoscopic subtotal colectomy with ileorectal anastomosis. The patients were placed in the modified lithotomy position under general anesthesia. Five trocars were used with the insertion of the primary trocar with a 10 mm port to gain access to the peritoneal cavity and establish the pneumoperitoneum above the umbilicus for the 30° laparoscope. The other four working ports were a 12 mm port in the right iliac fossa, a 10 mm port in the left iliac fossa, and a 5 mm port was placed in each of the right and left upper quadrants of the abdomen. Mobilization of the colonic segments was started at the right side of the colon. Mobilization was performed with the Endoshears (LigaSure device).

After the colon was fully mobilized, the colon was resected to the level of the junction of the rectosigmoid with a laparoscopic linear stapler. The rectal stump was mobilized to facilitate the transanally inserted circular stapler or Hegar dilator. The SRA was preserved in all patients (Figure 1).

A 4-5 cm Pfannenstiel incision was made, and the mobilized bowel segment was brought out (Figure 2). The terminal ileum was divided a few centimeters proximal to the ileocecal valve with the anvil of a circular stapling device inserted into the lumen of the ileum. The ileum, with the anvil secured, was placed into the abdominal cavity. Finally, we performed the transanal end-to-end anastomosis using the circular stapling device. One Jackson Pratt drain was inserted into the pelvis, and the abdominal wall wounds were closed.

RESULTS

Thirty-two patients diagnosed with STC who had undergone laparoscopically assisted subtotal colectomy with preservation of the SRA in ileorectal anastomosis were included in the study. The preliminary data of these patients are presented in Table 1. Eighteen of them (56.2%) were women. The mean age of our patients was 42.6 ± 10.4 years. The mean body mass index was 26.6 ± 3.1 kg/m^2.

All patients had severe constipation, the mean defecation duration was 9.3 d and 3 (9.4%) patients had previous abdominal surgery. All patients complained of intermittent abdominal pain preoperatively. The mean operative time was 151 ± 29.9 min. The mean estimated blood loss was 119.1 ± 40.7 mL (Table 2).

The mean time to the first flatus was 3.0 ± 0.8 d, and the average time to the first stool was 2.5 ± 0.5 d. The average time to first oral intake was 2.8 ± 0.8 d. The mean time of discharge from the ward was 10.6 ± 2.9 d. The satisfaction of post-operative patients was excellent in 28 (90%) patients. The satisfaction was poor in 2 (5%) patients due to a wound infection and an incisional hernia.

There were no intraoperative complications. Post-operative complications included 1 wound infection (Pfannenstiel incision wound), prolonged ileus (over 5 d) in 2 patients and three patients developed a urinary tract infection. One patient had an incisional hernia (Table 3). All cases could be fed orally with a clear liquid diet on the 4th postoperative day. There was no major surgical complication and no patient required conversion to an exploratory laparotomy.

DISCUSSION

Constipation is a common condition. Most patients with constipation are treated with changes in their diet and the use of laxatives. If the condition proves recalcitrant, we should exclude secondary causes which may be underlying metabolic, organic or pharmacological factors. The mechanisms of slow transit include fewer colonic high-amplitude propagated contractions or a reduced colonic contractile response to a meal [1]. The cause for STC is, however, unclear.

STC means a prolonged time in the transit of stools through the colon without an underlying systemic metabolic or pathologic disorder or pelvic floor dysfunction[11]. Delays due to major dysfunction or innervation of colonic smooth muscle[12]. Patients with STC have an incorrect expression of serotonin receptors and incorrect responses to cholinesterase inhibitors[13,14]. The disease induced by a reduction in the interstitial cells of Cajal, which have a role in intestinal innervation and are situated in the submucosal and myenteric plexus of the bowel wall[15].

The enrollment criteria was done by the following steps. First, physiologic examinations for constipated patients are needed and include colonic transit studies with radiopaque markers, balloon tests, check anorectal pressure and defecation test[16]. The radiopaque marker test is very helpful. Physiologic examination should include anorectal manometry to rule out the problem of pelvic floor dysfunction. All of our patients had anorectal manometry which revealed no abnormal anorectal inhibitory reflexes. A colonoscopy or barium enema is a good tool to provide evidence of any pathologic disease or structural lesions[17,18].

The incidence of AL following gastrointestinal surgery varies according to the site of anastomosis (all resections: 4.3%-13%)[19]. Ischemia of the anastomotic region is one of the most significant factors leading to AL. Therefore, the preservation of the anatomical blood supply of the rectum is important. The effect of SRA-sparing on anastomotic healing and perioperative complications is poorly investigated. Sohn *et al*

Table 1 Preoperative variables

Variables	Mean
Age (yr)	43.0 ± 10.4
Body mass index (kg/m^2)	26.5 ± 2.9
Preoperative laxative dependence (yr)	13.7 ± 5.0
Preoperative defecation interval (d)	9.6 ± 3.9
Colonic transit time (h)	131 ± 13.4
Previous abdominal surgery	3 (9.4)
Preoperative abdominal pain	31 (96.9)

Values are given as mean ± SD or *n* (%).

Table 2 Surgical and postoperative variables

Variables	mean ± SD
Operation time (min)	151.0 ± 29.9
Estimated blood loss (mL)	119.1 ± 40.7
Time to first flatus (d)	3.0 ± 0.8
Time to first stool passing (d)	2.5 ± 0.5
Time to oral intake (d)	2.4 ± 0.5
Dose of demerol used (mg)	87.5 ± 40.15
Duration of hospital stay (d)	10.6 ± 2.9
Postoperative bowel frequency (*per* day)	2.2 ± 1.0

Values are given as mean ± SD or *n* (%).

Table 3 Intraoperative and postoperative complications

Characteristics	Number of subjects, *n* (%)
Intraoperative complications	
None	32 (100)
Postoperative complications	
None	25 (78.1)
Urinary tract infection	3 (9.3)
Ileus (over 5 d)	2 (6.2)
Incisional hernia	1 (3.1)
Wound infection	1 (3.1)
Anastomosis leakage	0 (0)
Satisfaction, excellent/good/poor	28/2/2 (90%/5%/5%)

Values are given as mean ± SD or *n* (%).

[20] have demonstrated that preservation of the SRA seems to be associated with a reduction of the leak rate in patients undergoing laparoscopic sigmoid resection for diverticular disease[20]. Beyond AL, an SRA sparing technique leads to preservation of the hypogastric nerves and thereby could potentially improve functional outcomes [20]. However, no studies have focused on the preservation of SRA with subtotal

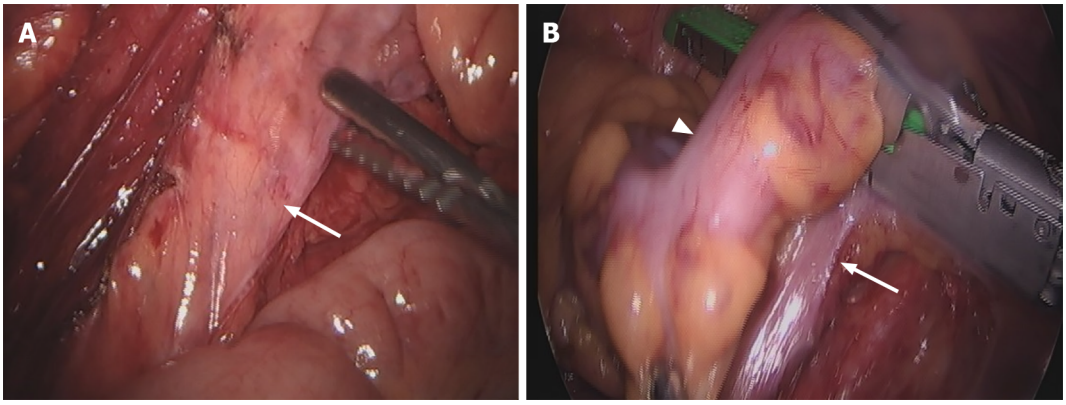


Figure 1 Colon. A: Preservation of superior rectal artery (arrow) in laparoscopically assisted subtotal colectomy with ileorectal anastomosis; B: Sigmoid-rectal junction (arrowhead) and sparing of the superior rectal artery (arrow).



Figure 2 Specimen after laparoscopic-assisted subtotal colectomy.

colectomy in STC patients. We observed no AL in our 32 STC patients who underwent an SRA-sparing operation. Although our study had a small sample size, the preservation of the SRA seems to have an excellent surgical outcome.

Laparoscopic techniques have been successfully used in colon surgery, thereby reducing morbidity, mortality and length of hospital stay[21,22]. The length of hospital stay is in the range of 7 to 13 d in STC patients[13,23,24], with postoperative ileus complicating the treatment course in many of them[7]. In our study, the mean hospital stay was 10.6 ± 2.9 d, and the mean number of days to the first flatus was 3.0 ± 0.8 d.

Abdominal distention with pain and vomiting all improved after surgery[25]. In a study by FitzHarris *et al*[26], although subtotal colectomy for slow-transit constipation increased bowel-movement frequency, the persistence of abdominal pain and diarrhea or postoperative incontinence adversely affected the quality of life following the surgery[26].

Preoperative counseling and an integrated post-operative functional and rehabilitational follow-up is important in patient underwent anterior rectal resection[26]. In a study by Sturiale *et al*[27], patients with defecation disorders, should receive an adequate re-educational process which often results in relevant clinical improvement for the patient[27]. Biofeedback therapy also shows effective treatment for fecal incontinence patient after anorectal operation[28].

A key limitation of the present study is its retrospective character. Moreover, technical aspects could have influenced the dissection technique. Thus, sparing of the mesosigmoid and the mesorectum can be performed much more easily today than at

the beginning of the laparoscopic era, constituting a possible bias. This also explains the late publication of our data. Despite these limitations, and the bias involved through missing randomization, small sample size and surgeon preference of technique, we report the largest patient sample with vascular preservation in STC patients.

CONCLUSION

In conclusion, laparoscopically assisted subtotal colectomy with ileorectal anastomosis and preservation of the SRA is tolerated and can have better results with significant improvement in bowel function with careful patient enrollment criteria. No anastomosis leakage was noted in any of our patients; preservation of the SRA appears to result in excellent surgical outcomes.

ARTICLE HIGHLIGHTS

Research background

Slow transit constipation (STC) has traditionally been considered as a functional disorder. However, evidence is accumulating that suggests that most of the motility alterations in STC might be of a neuropathic etiology. If the patient's condition responds poorly to non-surgical treatment, operative intervention with subtotal colectomy may be effective.

Research motivation

However, preservation of the superior rectal artery (SRA) may reduce its incidence.

Research objectives

STC patients underwent laparoscopically assisted total colectomy with ileorectal anastomosis and were examined between January 2016 and January 2017.

Research methods

This was a single-center retrospective observational study. STC was diagnosed after a series of examinations which included a colonic transit test, anal manometry, a balloon expulsion test, and a barium enema. Eligible patients underwent laparoscopically assisted total colectomy with ileorectal anastomosis and were examined between January 2016 and January 2017. The main outcome include operation time, estimated blood loss, time to first flatulence and incidence of complications.

Research results

A total of 32 patients (mean age, 42.6 years) underwent laparoscopically assisted subtotal colectomy with ileorectal anastomosis and preservation of the SRA. All patients were diagnosed with STC after a series of examinations. The mean day of first time to flatus was 3.0 d, and the mean hospital stay was 10.6 d. There were no conversions to laparotomy. Post-operative complications included 1 wound infection and 1 case of ileus. No anastomosis leakage was noted in any of the patients.

Research conclusions

Laparoscopically assisted subtotal colectomy with ileorectal anastomosis and preservation of the SRA can significantly improve bowel function with careful patient selection. Sparing the SRA may protect against anastomosis leakage.

Research perspectives

Laparoscopically assisted subtotal colectomy with ileorectal anastomosis and preservation of the SRA can have well results with improvement in bowel function with careful patient enrollment criteria. No anastomosis leakage was noted in any of our patients; preservation of the SRA appears to result in excellent surgical outcomes.

REFERENCES

1 **Bassotti G**, de Roberto G, Castellani D, Sediari L, Morelli A. Normal aspects of colorectal motility and abnormalities in slow transit constipation. *World J Gastroenterol* 2005; **11**: 2691-2696 [PMID: 15884105 DOI: 10.3748/wjg.v11.i18.2691]

2 **Lembo A**, Camilleri M. Chronic constipation. *N Engl J Med* 2003; **349**: 1360-1368 [DOI: 10.1016/j.rchipe.2015.06.017]

3 **Pfeifer J**, Agachan F, Wexner SD. Surgery for constipation: a review. *Dis Colon Rectum* 1996; **39**: 444-460 [DOI: 10.1007/BF02054062]

4 **Drossman DA**. The functional gastrointestinal disorders and the Rome II process. *Gut* 1999; **45** Suppl 2: II1-II5 [PMID: 10457038 DOI: 10.1136/gut.45.2008.ii1]

5 **Bassotti G**, Villanacci V. Slow transit constipation: a functional disorder becomes an enteric neuropathy. *World J Gastroenterol* 2006; **12**: 4609-4613 [PMID: 16937428 DOI: 10.3748/wjg.v12.i29.4609]

6 **Lane WA**. Remarks ON THE RESULTS OF THE OPERATIVE TREATMENT OF CHRONIC CONSTIPATION. *Br Med J* 1908; **1**: 126-130 [PMID: 20763645 DOI: 10.1136/bmj.1.2455.126]

7 **Barkley KT**. John C. Reeve, M.D., pioneer Dayton physician (1826-1920). *Ohio State Med J* 1978; **74**: 187-188, 190 [PMID: 345166]

8 **Veyrie N**, Ata T, Muscari F, Couchard AC, Msika S, Hay JM, Fingerhut A, Dziri C; French Associations for Surgical Research. Anastomotic leakage after elective right versus left colectomy for cancer: prevalence and independent risk factors. *J Am Coll Surg* 2007; **205**: 785-793 [PMID: 18035262 DOI: 10.1016/j.jamcollsurg.2007.06.284]

9 **Boccola MA**, Buettner PG, Rozen WM, Siu SK, Stevenson AR, Stitz R, Ho YH. Risk factors and outcomes for anastomotic leakage in colorectal surgery: a single-institution analysis of 1576 patients. *World J Surg* 2011; **35**: 186-195 [PMID: 20972678 DOI: 10.1007/s00268-010-0831-7]

10 **Thompson WG**, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Müller-Lissner SA. Functional bowel disorders and functional abdominal pain. *Gut* 1999; **45** Suppl 2: II43-II47 [PMID: 10457044 DOI: 10.1136/gut.45.2008.ii43]

11 **Preston DM**, Lennard-Jones JE. Severe chronic constipation of young women: 'idiopathic slow transit constipation'. *Gut* 1986; **27**: 41-48 [PMID: 3949236 DOI: 10.1136/gut.27.1.41]

12 **Knowles CH**, Martin JE. Slow transit constipation: a model of human gut dysmotility. Review of possible aetiologies. *Neurogastroenterol Motil* 2000; **12**: 181-196 [PMID: 10877606 DOI: 10.1046/j.1365-2982.2000.00198.x]

13 **Bassotti G**, Chiarioni G, Imbimbo BP, Betti C, Bonfante F, Vantini I, Morelli A, Whitehead WE. Impaired colonic motor response to cholinergic stimulation in patients with severe chronic idiopathic (slow transit type) constipation. *Dig Dis Sci* 1993; **38**: 1040-1045 [PMID: 8508698 DOI: 10.1007/BF01295719]

14 **Zhao RH**, Baig MK, Thaler KJ, Mack J, Abramson S, Woodhouse S, Tamir H, Wexner SD. Reduced expression of serotonin receptor(s) in the left colon of patients with colonic inertia. *Dis Colon Rectum* 2003; **46**: 81-86 [PMID: 12544526 DOI: 10.1007/s10350-004-6500-x]

15 **Hotta T**, Yamaue H. Laparoscopic surgery for rectal cancer: review of published literature 2000-2009. *Surg Today* 2011; **41**: 1583-1591 [PMID: 21969189 DOI: 10.1007/s00595-010-4555-y]

16 **Hsiao KC**, Jao SW, Wu CC, Lee TY, Lai HJ, Kang JC. Hand-assisted laparoscopic total colectomy for slow transit constipation. *Int J Colorectal Dis* 2008; **23**: 419-424 [PMID: 18193433 DOI: 10.1007/s00384-007-0431-7]

17 **Thompson WG**. Constipation: a physiological approach. *Can J Gastroenterol* 2000; **14** Suppl D: 155D-162D [PMID: 11110630 DOI: 10.1155/2000/391807]

18 **Lahr SJ**, Lahr CJ, Srinivasan A, Clerico ET, Limehouse VM, Serbezov IK. Operative management of severe constipation. *Am Surg* 1999; **65**: 1117-21; discussion 1122 [PMID: 10597057]

19 **Khan AA**, Wheeler JM, Cunningham C, George B, Kettlewell M, Mortensen NJ. The management and outcome of anastomotic leaks in colorectal surgery. *Colorectal Dis* 2008; **10**: 587-592 [PMID: 18070185 DOI: 10.1111/j.1463-1318.2007.01417.x]

20 **Sohn M**, Schlitt HJ, Hornung M, Zülke C, Hochrein A, Moser C, Agha A. Preservation of the superior rectal artery: influence of surgical technique on anastomotic healing and postoperative morbidity in laparoscopic sigmoidectomy for diverticular disease. *Int J Colorectal Dis* 2017; **32**: 955-960 [PMID: 28378155 DOI: 10.1007/s00384-017-2792-x]

21 **Franklin ME Jr**, Rosenthal D, Abrego-Medina D, Dorman JP, Glass JL, Norem R, Diaz A. Prospective comparison of open vs. laparoscopic colon surgery for carcinoma. Five-year results. *Dis Colon Rectum* 1996; **39**: S35-S46 [PMID: 8831545 DOI: 10.1007/BF02053804]

22 **Lacy AM**, García-Valdecasas JC, Piqué JM, Delgado S, Campo E, Bordas JM, Taurá P, Grande L, Fuster J, Pacheco JL. Short-term outcome analysis of a randomized study comparing laparoscopic vs open colectomy for colon cancer. *Surg Endosc* 1995; **9**: 1101-1105 [PMID: 8553212 DOI: 10.1007/BF00188996]

23 **Piccirillo MF**, Reissman P, Wexner SD. Colectomy as treatment for constipation in selected patients. *Br J Surg* 1995; **82**: 898-901 [PMID: 7648101 DOI: 10.1002/bjs.1800820713]

24 **Wexner SD**, Daniel N, Jagelman DG. Colectomy for constipation: physiologic investigation is the key to success. *Dis Colon Rectum* 1991; **34**: 851-856 [PMID: 1914716 DOI: 10.1007/BF02049695]

25 **Vergara-Fernandez O**, Mejía-Ovalle R, Salgado-Nesme N, Rodríguez-Dennen N, Pérez-Aguirre J, Guerrero-Guerrero VH, Sánchez-Robles JC, Valdovinos-Díaz MA. Functional outcomes and quality

of life in patients treated with laparoscopic total colectomy for colonic inertia. *Surg Today* 2014; **44**: 34-38 [PMID: 23686591 DOI: 10.1007/s00595-012-0464-6]

26 **FitzHarris GP**, Garcia-Aguilar J, Parker SC, Bullard KM, Madoff RD, Goldberg SM, Lowry A. Quality of life after subtotal colectomy for slow-transit constipation: both quality and quantity count. *Dis Colon Rectum* 2003; **46**: 433-440 [PMID: 12682533 DOI: 10.1007/s10350-004-6576-3]

27 **Sturiale A**, Martellucci J, Zurli L, Vaccaro C, Brusciano L, Limongelli P, Docimo L, Valeri A. Long-term functional follow-up after anterior rectal resection for cancer. *Int J Colorectal Dis* 2017; **32**: 83-88 [PMID: 27695976 DOI: 10.1007/s00384-016-2659-6]

28 **Brusciano L**, Gambardella C, Tolone S, Del Genio G, Terracciano G, Gualtieri G, Schiano di Visconte M, Docimo L. An imaginary cuboid: chest, abdomen, vertebral column and perineum, different parts of the same whole in the harmonic functioning of the pelvic floor. *Tech Coloproctol* 2019; **23**: 603-605 [PMID: 31065825 DOI: 10.1007/s10151-019-01996-x]

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ORIGINAL ARTICLE

Prospective Study

High fecal calprotectin levels are associated with SARS-CoV-2 intestinal shedding in COVID-19 patients: A proof-of-concept study

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Abstract

BACKGROUND

One third of coronavirus disease 2019 (COVID-19) patients have gastrointestinal symptoms. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA has been detected in stool samples of approximately 50% of COVID-19 individuals. Fecal calprotectin is a marker of gastrointestinal inflammation in the general population.

AIM

To investigate if fecal calprotectin correlates with SARS-CoV-2 intestinal shedding in COVID-19 patients with pneumonia.

METHODS

Patients with SARS-CoV-2 pneumonia admitted to the Infectious Disease Unit (University Hospital of Trieste, Italy) from September to November 2020 were

approved by the Ethics Committee (Unique Regional Ethical Committee, Friuli Venezia-Giulia 16 April 2020), No. CEUR 2020-OS-072.

Clinical trial registration statement: This study did not involve clinical trials.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: All study participants provided written consent prior to study enrollment.

CONSORT 2010 statement: CONSORT not applicable.

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consecutively enrolled in the study. Fecal samples were collected and analyzed for quantification of fecal calprotectin (normal value < 50 mg/kg) and SARS-CoV-2 RNA presence by polymerase chain reaction (PCR). Inter-group differences were determined between patients with and without diarrhea and patients with and without detection of fecal SARS-CoV-2.

RESULTS

We enrolled 51 adults (40 males) with SARS-CoV-2 pneumonia. Ten patients (20%) presented with diarrhea. Real-time-PCR of SARS-CoV-2 in stools was positive in 39 patients (76%), in all patients with diarrhea (100%) and in more than two thirds (29/41, 71%) of patients without diarrhea. Obesity was one of the most common comorbidities (13 patients, 25%); all obese patients (100%) (P = 0.021) tested positive for fecal SARS-CoV-2. Median fecal calprotectin levels were 60 mg/kg [interquartile range (IQR) 21; 108]; higher fecal calprotectin levels were found in the group with SARS-CoV-2 in stools (74 mg/kg, IQR 29; 132.5) compared to the group without SARS-CoV-2 (39 mg/kg, IQR 14; 71) (P < 0.001).

CONCLUSION

High fecal calprotectin levels among COVID-19 patients correlate with SARS-CoV-2 detection in stools supporting the hypothesis that this virus can lead to bowel inflammation and potentially to the 'leaky gut' syndrome.

Key Words: COVID-19; SARS-CoV-2; Obesity; Fecal calprotectin; Gut; Viral shedding

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Core Tip: In this prospective study of 51 hospitalized patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pneumonia, whether fecal calprotectin correlated with SARS-CoV-2 intestinal shedding was investigated. We found that high fecal calprotectin level is a common finding among hospitalized coronavirus disease 2019 (COVID-19) patients, especially those with SARS-CoV-2 fecal shedding. Obese COVID-19 patients showed high fecal viral shedding.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) expresses a high affinity to human angiotensin-converting enzyme 2 (ACE2) receptors. High ACE2 expression was identified within the oral cavity, type II pulmonary alveolar cells, ileal and colonic enterocytes, myocardial cells, vascular endothelium, proximal tubule, and bladder urothelial cells[1].

One-third of coronavirus disease 2019 (COVID-19) patients have gastrointestinal symptoms, with diarrhea being the most common symptom[2]. Moreover, critically ill COVID-19 patients often develop intestinal complications. Ileus, gastrointestinal bleeding, and bowel ischemia are the most common[3,4]. Spontaneous intestinal perforations among COVID-19 patients are also increasingly seen in clinical practice [4].

SARS-CoV-2 RNA has been detected in stool samples of approximately 50% COVID-19 individuals[5]. Often, viral detection in stools persists after viral clearance from respiratory samples[6], with a mean duration of fecal viral shedding of 17 d[7].

It is likely that SARS-CoV-2 can also be transmitted *via* the fecal–oral route and that the potential of this mode of transmission has been widely underestimated[8].

The pathogenesis of gastrointestinal symptoms caused by SARS-CoV-2 is likely

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multifactorial, including disruption of the intestinal mechanical barrier integrity, alteration of the gut microbiome, increased translocation of bacteria and their metabolites, and systemic inflammatory response to the virus, which in critically ill patients could be disproportionate, with uncontrolled production and release of cytokines[1].

Calprotectin is a protein derived principally from neutrophils. Upon neutrophil activation or death, calprotectin is released extracellularly, where it has a role within the innate immune response with direct antimicrobial effects. It is present in many body fluids, in proportion to the degree of inflammation. The concentration of calprotectin in feces is about six times that in plasma, and its measurement is used as a surrogate marker of gastrointestinal inflammation[1,9].

Fecal calprotectin in COVID-19 patients was found to be a marker of intestinal inflammation, both in patients with and without gastrointestinal symptoms[10,11]. Ojetti *et al*[12] also found a significant correlation between the development of pneumonia among COVID-19 patients and a high level of fecal calprotectin[11,12].

Serum calprotectin has been proposed as a severe COVID-19 progression marker, supporting innate immunity as a potential perpetrator of inflammation in COVID-19. It should be kept in mind that neutropenia seen in the peripheral blood of COVID-19 patients should in part reflect neutrophil migration to the tissues[12-14] and complement activation in different organs of COVID-19 patients[15] contributes to tissue damage and to the recruitment of neutrophils.

Given these premises, we aimed to investigate if fecal calprotectin correlates with SARS-CoV-2 intestinal shedding in hospitalized patients with COVID-19 pneumonia.

MATERIALS AND METHODS

We performed a prospective monocentric study enrolling consecutive adults (aged >18 years) with SARS-CoV-2 pneumonia admitted to the Infectious Diseases Unit of Trieste University Hospital, Italy, from September to November 2020. Patients with inflammatory bowel diseases, gastrointestinal malignancy, and other known gastrointestinal disorders were categorically excluded.

SARS-CoV-2 detection in stool samples was determined by real-time polymerase chain reaction (RT-PCR) (LightMix® Modular SARS and Wuhan CoV E-gene and RdRp kit-TIB Molbiol, Berlin, Germany, with LightCycler Multiplex RNA Virus Master-Roche, Basel, Switzerland). According to the manufacturer's specifications, fecal calprotectin levels were tested with the LIAISON®-Calprotectin (Diasorin, Vercelli, Italy) (normal value < 50 mg/kg). These tests were performed on hospital admission, regardless of gastrointestinal signs or symptoms.

The following data were collected on admission: Age, gender, comorbidities, gastrointestinal signs/symptoms, blood cell count, biochemical parameters, and clinical outcomes. In addition, administered drugs prior to and during hospitalization were collected. Diarrhea and obesity were defined as loose stools ≥ three times/day and a body mass index ≥ 30, respectively.

According to the size of our sample, the Shapiro-Wilk test was performed to verify the normal distribution of variables. Inter-group differences (patients with diarrhea *vs* patients without diarrhea and patients with fecal SARS-CoV-2 *vs* patients without fecal SARS-CoV-2) were determined with the Mann-Whitney U test for continuous variables and the Pearson's Chi Square Test for discrete variables. For all analyses, two-sided statistical significance was defined as a $P < 0.05$. Data were analyzed using SPSS (Statistical Package for Social Science) version 25.0 (IBM SPSS Statistics for MAC OS. Armonk NY: IBM Corp.).

This study was conducted according to the declaration of Helsinki and approved by the Ethics Committee (Unique Regional Ethical Committee, Friuli Venezia-Giulia 16 April 2020), No. CEUR 2020-OS-072.

RESULTS

We enrolled 51 consecutive adults with SARS-CoV-2 pneumonia. Patient age ranged from 28 to 87 years [median 64 years, interquartile range (IQR) 57; 71] and 40 (78%) were males. The most common comorbidities were: Hypertension (34 patients, 67%), diabetes mellitus (5, 25%), obesity (5, 25%), heart disease (5, 25%), chronic kidney disease (5, 10%) and chronic obstructive pulmonary disease (5, 10%). Two (4%) patients were active smokers. Ten (20%) patients had diarrhea.

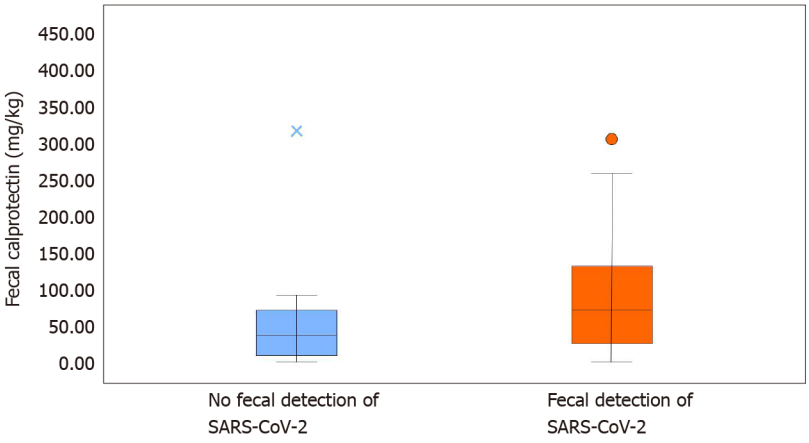


Figure 1 Fecal calprotectin content detected in stool with or without severe acute respiratory syndrome coronavirus 2 virus. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Median fecal calprotectin levels were 60 mg/kg (IQR 21; 108). RT-PCR of SARS-CoV-2 in stools was positive in 39 patients (76%) (Figure 1). The clinical features and biochemical parameters of enrolled patients are reported in Table 1.

No statistically significant differences in the compared variables between the two groups (patients with diarrhea *vs* patients without diarrhea) were found (Table 1). Both groups showed increased fecal calprotectin levels: 58 mg/kg (IQR 31; 75) in the diarrhea group and 64 mg/kg (IQR 18; 108) in those without diarrhea. Fecal SARS-CoV-2 was detected in all patients with diarrhea and in more than two thirds (29/41, 71%) of patients without diarrhea.

Comparing patients with and without fecal SARS-CoV-2 shedding (Table 2), we found higher fecal calprotectin levels in the former (74 mg/kg, IQR 29; 132.5) compared to the latter group (39 mg/kg, IQR 14; 71) ($P < 0.001$) (Figure 2). None of the patients without SARS-CoV-2 shedding had diarrhea. Neutrophil count was higher in patients with fecal SARS-CoV-2 shedding ($P = 0.035$), as well as D-dimer levels ($P = 0.011$) and white blood cell count ($P = 0.038$). C-reactive protein was higher in patients without fecal SARS-CoV-2 shedding ($P = 0.029$). Fecal SARS-CoV-2 was found in all obese patients ($P = 0.021$).

DISCUSSION

SARS-CoV-2 fecal shedding is a common finding among COVID-19 patients irrespective of gastrointestinal symptoms[16,17]. Our results confirmed that fecal SARS-CoV-2 was present in approximately three quarters of our patients with COVID-19 pneumonia.

Fecal calprotectin was found to be a marker of intestinal inflammation, both in COVID-19 patients and in the general population[10,11]. Our work demonstrates that hospitalized COVID-19 patients with pneumonia have high fecal calprotectin levels, regardless of gastrointestinal symptoms.

To our knowledge this is the first study to investigate whether fecal calprotectin correlates with SARS-CoV-2 intestinal shedding in COVID-19 patients. In our study, calprotectin levels were significantly higher in those with SARS-CoV-2 fecal shedding. This finding supports the hypothesis that bowel inflammation can lead to the "leaky gut" syndrome with potential distribution of the virus to other organs[18].

While the detection of SARS-CoV-2 in feces does not necessarily lead to more gastrointestinal symptoms, the presence of SARS-CoV-2 in gastrointestinal tissue generally correlates with more severe symptoms[19].

High fecal calprotectin in COVID-19 patients is likely secondary to increased neutrophil activation in the intestinal tract. In fact, SARS-CoV-2 can activate neutrophil extracellular traps and increase levels of intracellular reactive oxygen species[20].

In our cohort, all obese patients had SARS-CoV-2 RNA detected in stools. Obesity is one of the main risk factors for severe COVID-19 and poor clinical outcomes[20,21], and is associated with a strong inflammatory response both in the general population and in COVID-19 patients[22]. This could justify a more prolonged viral shedding in

Table 1 Clinical and biochemical characteristics of coronavirus disease 2019 patients with and without diarrhea, *n* (%)

	Overall, *n* = 51	Patients with diarrhea, *n* = 10	Patients without diarrhea, *n* = 41	Significance
Gender, male	40 (78.4)	8 (80)	32 (78)	NS
Age	64 (57; 71)	63 (56; 70)	65 (57; 71)	NS
Hypertension	34 (66.6)	6 (60)	28 (68.3)	NS
Heart disease	13 (25.5)	4 (40)	9 (21.9)	NS
Diabetes	13 (25.5)	2 (20)	11 (26.8)	NS
COPD	5 (9.8)	1 (10)	4 (9.75)	NS
CKD	5 (9.8)	1 (10)	4 (9.75)	NS
Active smokers	2 (3.9)	1 (10)	1 (2.4)	NS
Obesity	13 (25.5)	2 (20)	11 (26.8)	NS
WBC (cell count/μL)	6730 (5256; 9110)	6595 (4672; 7420)	6730 (5420; 9480)	NS
Neutrophils (cell count/μL)	5455 (3700; 7760)	5310 (3112; 6527)	5310 (3112; 6725)	NS
CRP (mg/L)	74 (27; 131)	73 (25; 161)	74 (29; 127)	NS
D-dimer (ng/mL FEU)	720 (545; 1061)	710 (560; 1511)	720 (540; 1060)	NS
Fecal calprotectin(mg/kg)	60 (21.5; 108)	58 (31; 75)	64 (18; 108)	NS
Fecal-RNA detection (*n* = positive)	39 (76.5)	10 (100)	29 (70.8)	NS
Deceased	3 (5.9)	1 (10)	2 (4.8)	NS
Hospital stay	5 (2.5; 8)	5 (2.75; 6.75)	5 (3; 9.25)	NS

COPD: Chronic obstructive pulmonary disease; CKD: Chronic kidney disease; CRP: C-reactive protein; NS: Not significant; WBC: White blood cells.

this subgroup of patients.

Our work has two main limitations. First, this was a monocentric study with a small sample of patients. Second, SARS-CoV-2 was detected in stools by PCR. This technique does not discriminate between live virus and non-infectious viral particles. Only a few studies have reported live SARS-CoV-2 in the stools of COVID-19 patients[23-26]. There is also limited evidence on the duration of fecal viral shedding[7].

Our study demonstrates that higher fecal calprotectin levels correlate with SARS-CoV-2 fecal shedding in hospitalized COVID-19 patients with pneumonia. Our results support the role of neutrophils in SARS-CoV-2 pathogenesis.

CONCLUSION

In conclusion, our study provides two main results: (1) Fecal SARS-CoV-2 is present in approximately three quarters of hospitalized patients with COVID-19 pneumonia and in all patients with diarrhea; interestingly, all obese COVID-19 patients show fecal viral shedding; and (2) High fecal calprotectin levels are a common finding among hospitalized COVID-19 patients, especially those with SARS-CoV-2 fecal shedding. We believe that our results could strengthen the hypothesis that SARS-CoV-2-induced intestinal damage mediated by innate immunity (complement activation and consequent activated neutrophil migration) could contribute to COVID-19 pathogenesis.

Table 2 Clinical and biochemical characteristics of coronavirus disease 2019 patients with and without severe acute respiratory syndrome coronavirus 2 fecal detection, *n* (%)

	Overall, *n* = 51	Patients without fecal SARS-CoV-2, *n* = 12	Patients with fecal SARS-CoV-2, *n* = 39	Significance
Gender, male	40 (78.4)	12 (100)	28 (71.8)	NS
Age	64 (57; 71)	62 (57; 68)	65 (56; 72)	NS
Hypertension	34 (66.6)	6 (50)	28 (71.8)	NS
Heart disease	13 (25.5)	1 (8.3)	12 (30.8)	NS
Diabetes	13 (25.5)	2 (16.6)	11 (28.2)	NS
COPD	5 (9.8)	0 (0)	5 (12.8)	NS
CKD	5 (9.8)	1 (8.3)	4 (10.25)	NS
Active smokers	2 (3.9)	1 (8.3)	1 (2.6)	NS
Obesity	13 (25.5)	0 (0)	13 (33.3)	$P = 0.021$
WBC (cell count/μL)	6730 (5256; 9110)	6105 (4870; 8400)	7110 (5525; 9115)	$P = 0.038$
Neutrophils (cell count/μL)	5455 (3700; 7760)	4390 (3485; 7417)	5550 (3965; 8110)	$P = 0.035$
CRP (mg/L)	74 (27; 131)	113 (88; 150)	58 (24.5; 125)	$P = 0.029$
D-dimer (ng/Ml FEU)	720 (545; 1061)	580 (402; 791)	723 (562; 1157)	$P = 0.011$
Fecal calprotectin (mg/kg)	60 (21.5; 108)	39 (14; 71)	74 (29; 132.5)	$P < 0.001$
Diarrhea	41 (80.4)	0 (0)	29 (74.35)	$P < 0.001$
Deceased	3 (5.9)	0 (0)	3 (7.7)	NS
Hospital stay	5 (2.5; 8)	3 (2; 5)	5 (3; 9.75)	NS

COPD: Chronic obstructive pulmonary disease; CKD: Chronic kidney disease; CRP: C-reactive protein; NS: Not significant; WBC: White blood cells; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

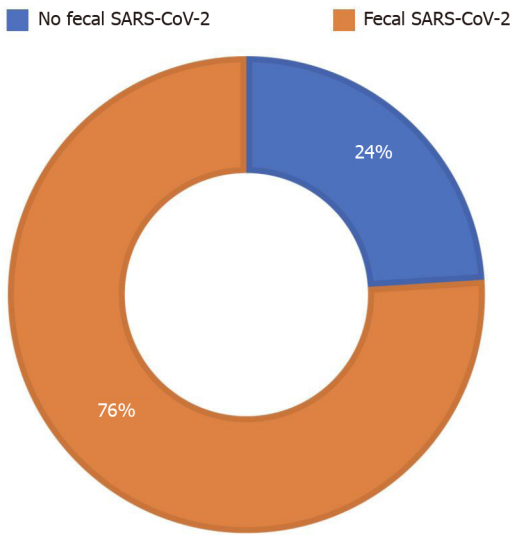


Figure 2 Comparison of patients with and without fecal severe acute respiratory syndrome coronavirus 2 shedding. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

ARTICLE HIGHLIGHTS

Research background

One third of coronavirus disease 2019 (COVID-19) patients have gastrointestinal symptoms. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA has been detected in stool samples of approximately 50% of COVID-19 individuals. Fecal calprotectin is a marker of gastrointestinal inflammation.

Research motivation

The pathogenesis of gastrointestinal symptoms caused by SARS-CoV-2 is multifactorial and little evidence is available on this topic.

Research objectives

To investigate whether fecal calprotectin correlates with SARS-CoV-2 intestinal shedding in COVID-19 patients with pneumonia.

Research methods

Fecal samples from patients with SARS-CoV-2 pneumonia were collected and analyzed for quantification of fecal calprotectin and SARS-CoV-2 RNA presence using polymerase chain reaction (PCR).

Research results

Real-time-PCR of SARS-CoV-2 in the stools of 51 patients with pneumonia was positive in 39 patients (76%), in all patients with diarrhea (100%) and in more than two thirds (29/41, 71%) of those without diarrhea. Higher fecal calprotectin levels were found in the group with SARS-CoV-2 in stools [74 mg/kg, interquartile range (IQR) 29; 132.5] compared to the group without SARS-CoV-2 (39 mg/kg, IQR 14; 71) ($P < 0.001$).

Research conclusions

High fecal calprotectin levels in COVID-19 patients correlates with SARS-CoV-2 detection in stools.

Research perspectives

Our results support the hypothesis that SARS-CoV-2-induced intestinal damage mediated by innate immunity could contribute to COVID-19 pathogenesis.

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REFERENCES

1 **Syed A**, Khan A, Gosai F, Asif A, Dhillon S. Gastrointestinal pathophysiology of SARS-CoV2 - a literature review. *J Community Hosp Intern Med Perspect* 2020; **10**: 523-528 [PMID: 33194122 DOI: 10.1080/20009666.2020.1811556]

2 **Ye L**, Yang Z, Liu J, Liao L, Wang F. Digestive system manifestations and clinical significance of coronavirus disease 2019: A systematic literature review. *J Gastroenterol Hepatol* 2020 [PMID: 33150978 DOI: 10.1111/jgh.15323]

3 **Kaafarani HMA**, El Moheb M, Hwabejire JO, Naar L, Christensen MA, Breen K, Gaitanidis A, Alser O, Mashbari H, Bankhead-Kendall B, Mokhtari A, Maurer L, Kapoen C, Langeveld K, El Hechi MW, Lee J, Mendoza AE, Saillant NN, Parks J, Fawley J, King DR, Fagenholz PJ, Velmahos GC. Gastrointestinal Complications in Critically Ill Patients With COVID-19. *Ann Surg* 2020; **272**: e61-e62 [PMID: 32675498 DOI: 10.1097/SLA.0000000000004004]

4 **Giuffrè M**, Bozzato AM, Di Bella S, Occhipinti AA, Martingano P, Cavallaro MFM, Luzzati R, Monica F, Cova MA, Crocè LS. Spontaneous Rectal Perforation in a Patient with SARS-CoV-2 Infection. *J Pers Med* 2020; **10** [PMID: 33049924 DOI: 10.3390/jpm10040157]

5 **Gupta S**, Parker J, Smits S, Underwood J, Dolwani S. Persistent viral shedding of SARS-CoV-2 in faeces - a rapid review. *Colorectal Dis* 2020; **22**: 611-620 [PMID: 32418307 DOI: 10.1111/codi.15138]

6 **Cheung KS**, Hung IFN, Chan PPY, Lung KC, Tso E, Liu R, Ng YY, Chu MY, Chung TWH, Tam AR, Yip CCY, Leung KH, Fung AY, Zhang RR, Lin Y, Cheng HM, Zhang AJX, To KKW, Chan KH, Yuen KY, Leung WK. Gastrointestinal Manifestations of SARS-CoV-2 Infection and Virus Load

in Fecal Samples From a Hong Kong Cohort: Systematic Review and Meta-analysis. *Gastroenterology* 2020; **159**: 81-95 [PMID: 32251668 DOI: 10.1053/j.gastro.2020.03.065]

7 **Cevik M**, Tate M, Lloyd O, Maraolo AE, Schafers J, Ho A. SARS-CoV-2, SARS-CoV, and MERS-CoV viral load dynamics, duration of viral shedding, and infectiousness: a systematic review and meta-analysis. *Lancet Microbe* 2021; **2**: e13-e22 [PMID: 33521734 DOI: 10.1016/S2666-5247(20)30172-5]

8 **Xiao F**, Sun J, Xu Y, Li F, Huang X, Li H, Zhao J, Huang J. Infectious SARS-CoV-2 in Feces of Patient with Severe COVID-19. *Emerg Infect Dis* 2020; **26**: 1920-1922 [PMID: 32421494 DOI: 10.3201/eid2608.200681]

9 **Ayling RM**, Kok K. Fecal Calprotectin. *Adv Clin Chem* 2018; **87**: 161-190 [PMID: 30342711 DOI: 10.1016/bs.acc.2018.07.005]

10 **Effenberger M**, Grabherr F, Mayr L, Schwaerzler J, Nairz M, Seifert M, Hilbe R, Seiwald S, Scholl-Buergi S, Fritsche G, Bellmann-Weiler R, Weiss G, Müller T, Adolph TE, Tilg H. Faecal calprotectin indicates intestinal inflammation in COVID-19. *Gut* 2020; **69**: 1543-1544 [PMID: 32312790 DOI: 10.1136/gutjnl-2020-321388]

11 **Giuffrè M**, Di Bella S, Sambataro G, Zerbato V, Cavallaro M, Occhipinti AA, Palermo A, Crescenti A, Monica F, Luzzati R, Crocè LS. COVID-19-Induced Thrombosis in Patients without Gastrointestinal Symptoms and Elevated Fecal Calprotectin: Hypothesis Regarding Mechanism of Intestinal Damage Associated with COVID-19. *Trop Med Infect Dis* 2020; **5** [PMID: 32947803 DOI: 10.3390/tropicalmed5030147]

12 **Ojetti V**, Saviano A, Covino M, Acampora N, Troiani E, Franceschi F; GEMELLI AGAINST COVID-19 group. COVID-19 and intestinal inflammation: Role of fecal calprotectin. *Dig Liver Dis* 2020; **52**: 1231-1233 [PMID: 33060042 DOI: 10.1016/j.dld.2020.09.015]

13 **Shi H**, Zuo Y, Yalavarthi S, Gockman K, Zuo M, Madison JA, Blair C, Woodward W, Lezak SP, Lugogo NL, Woods RJ, Lood C, Knight JS, Kanthi Y. Neutrophil calprotectin identifies severe pulmonary disease in COVID-19. *J Leukoc Biol* 2021; **109**: 67-72 [PMID: 32869342 DOI: 10.1002/JLB.3COVCRA0720-359R]

14 **Bauer W**, Diehl-Wiesenecker E, Ulke J, Galtung N, Havelka A, Hegel JK, Tauber R, Somasundaram R, Kappert K. Outcome prediction by serum calprotectin in patients with COVID-19 in the emergency department. *J Infect* 2020 [PMID: 33217473 DOI: 10.1016/j.jinf.2020.11.016]

15 **Li Q**, Chen Z. An update: the emerging evidence of complement involvement in COVID-19. *Med Microbiol Immunol* 2021; 1-9 [PMID: 33811541 DOI: 10.1007/s00430-021-00704-7]

16 **van Doorn AS**, Meijer B, Frampton CMA, Barclay ML, de Boer NKH. Systematic review with meta-analysis: SARS-CoV-2 stool testing and the potential for faecal-oral transmission. *Aliment Pharmacol Ther* 2020; **52**: 1276-1288 [PMID: 32852082 DOI: 10.1111/apt.16036]

17 **Patel KP**, Patel PA, Vunnam RR, Hewlett AT, Jain R, Jing R, Vunnam SR. Gastrointestinal, hepatobiliary, and pancreatic manifestations of COVID-19. *J Clin Virol* 2020; **128**: 104386 [PMID: 32388469 DOI: 10.1016/j.jcv.2020.104386]

18 **Synowiec A**, Szczepański A, Barreto-Duran E, Lie LK, Pyrc K. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): a Systemic Infection. *Clin Microbiol Rev* 2021; **34** [PMID: 33441314 DOI: 10.1128/CMR.00133-20]

19 **Lin L**, Jiang X, Zhang Z, Huang S, Fang Z, Gu Z, Gao L, Shi H, Mai L, Liu Y, Lin X, Lai R, Yan Z, Li X, Shan H. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. *Gut* 2020; **69**: 997-1001 [PMID: 32241899 DOI: 10.1136/gutjnl-2020-321013]

20 **Jayawardena R**, Jeyakumar DT, Misra A, Hills AP, Ranasinghe P. Obesity: A potential risk factor for infection and mortality in the current COVID-19 epidemic. *Diabetes Metab Syndr* 2020; **14**: 2199-2203 [PMID: 33395781 DOI: 10.1016/j.dsx.2020.11.001]

21 **Hoong CWS**, Hussain I, Aravamudan VM, Phyu EE, Lin JHX, Koh H. Obesity is Associated with Poor Covid-19 Outcomes: A Systematic Review and Meta-Analysis. *Horm Metab Res* 2021; **53**: 85-93 [PMID: 33395706 DOI: 10.1055/a-1326-2125]

22 **McNeill JN**, Lau ES, Paniagua SM, Liu EE, Wang JK, Bassett IV, Selvaggi CA, Lubitz SA, Foulkes AS, Ho JE. The role of obesity in inflammatory markers in COVID-19 patients. *Obes Res Clin Pract* 2021; **15**: 96-99 [PMID: 33390322 DOI: 10.1016/j.orcp.2020.12.004]

23 **Lui RN**. Safety in endoscopy for patients and healthcare workers during the COVID-19 pandemic. *Tech Innov Gastrointest Endosc* 2020 [PMID: 33103130 DOI: 10.1016/j.tige.2020.10.004]

24 **Wang W**, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. *JAMA* 2020; **323**: 1843-1844 [PMID: 32159775 DOI: 10.1001/jama.2020.3786]

25 **Zhang Y**, Cao C, Shuangli Z. Isolation of SARs-CoV-2 from stool specimen of a confirmed case of COVID-19. [cited 16 January 2021]. Available from: https://www.cebm.net/study/isolation-of-sars-cov-2-from-a-stool-specimen-of-confirmed-case-of-covid-19/

26 **Li L**, Tan C, Zeng J, Luo C, Hu S, Peng Y, Li W, Xie Z, Ling Y, Zhang X, Deng E, Xu H, Wang J, Xie Y, Zhou Y, Zhang W, Guo Y, Liu Z. Analysis of viral load in different specimen types and serum antibody levels of COVID-19 patients. *J Transl Med* 2021; **19**: 30 [PMID: 33413461 DOI: 10.1186/s12967-020-02693-2]

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CORRECTION

Helicobacter pylori promotes invasion and metastasis of gastric cancer by enhancing heparanase expression

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Abstract

Correction to "Liu LP, Sheng XP, Shuai TK, Zhao YX, Li B, Li YM. *Helicobacter pylori* promotes invasion and metastasis of gastric cancer by enhancing heparanase expression. *World J Gastroenterol* 2018; 24: 4565-4577 [PMID: 30386106 DOI: 10.3748/wjg.v24.i40.4565]." In this article, we have identified some of the images in Figure 2A, C, E, G, and I are identical to the images in Figures 1B, 2A, 3B, 3E, and 3G of another paper entitled "Liu L, Zhao Y, Fan G, Shuai T, Li B, Li Y. Helicobacter pylori infection enhances heparanase leading to cell proliferation *via* mitogenactivated protein kinase signalling in human gastric cancer cells.", which was published by us in the *Molecular Medicine Reports* in December, 2018 [PMID: 30320396 DOI: 10.3892/mmr.2018.9558]. The reason why we asked to replace the pictures was that when we were simultaneously preparing to submit our two different articles to the *World Journal of Gastroenterology* (*WJG*) and *Molecular Medicine Reports*, we uploaded the wrong pictures to the *WJG*, which were same as those submitted to the *Molecular Medicine Reports*. We apologize for this negligence and any inconvenience that this may cause. We would be grateful if

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Key Words: Correction; Replace the wrong pictures; Gastric cancer; *Helicobacter pylori*

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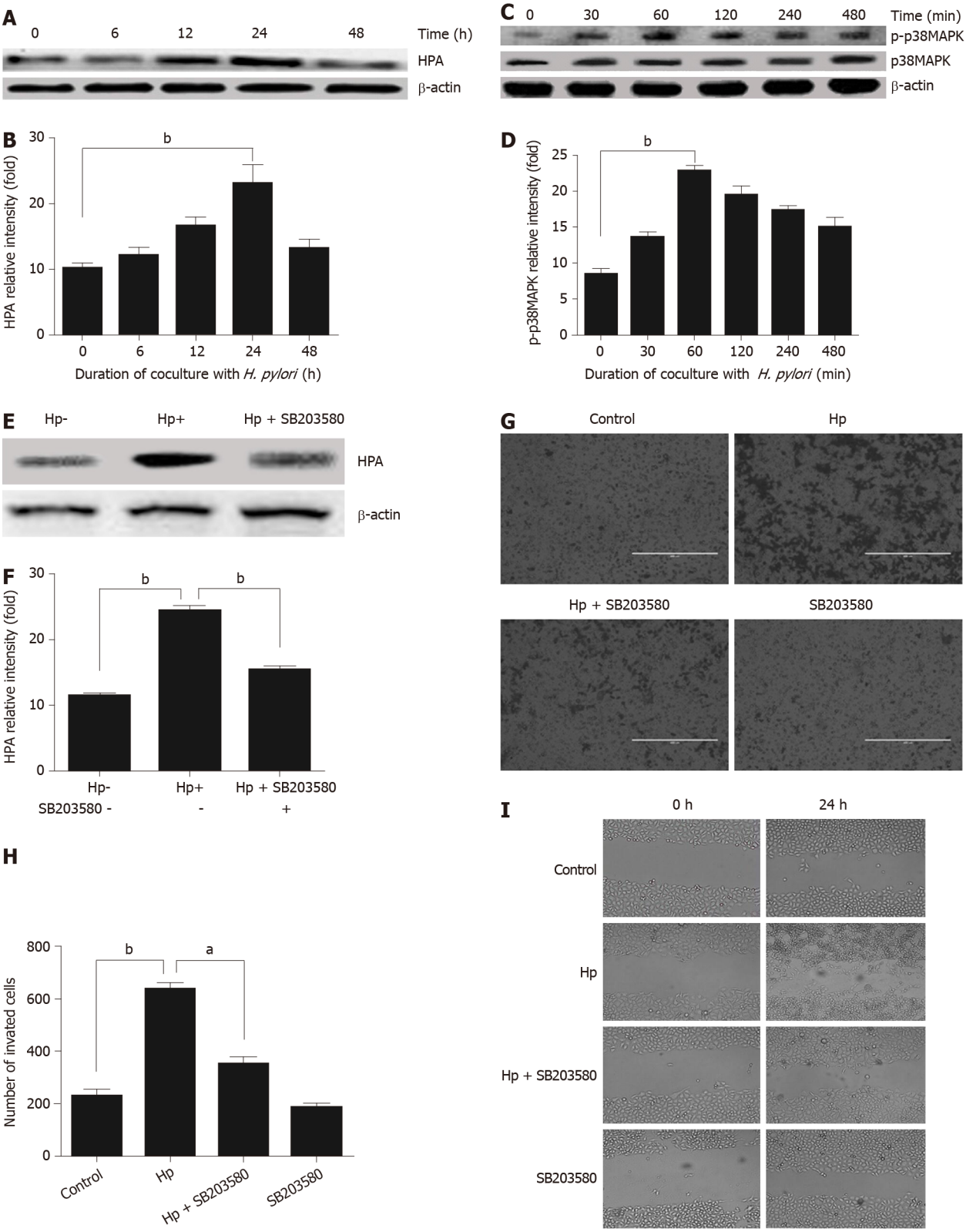
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A
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48
Time (h)
HPA
β-actin
C
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30
60
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480
Time (min)
p-p38MAPK
p38MAPK
β-actin
B
HPA relative intensity (fold)
b
Duration of coculture with H. pylori (h)
D
p-p38MAPK relative intensity (fold)
b
Duration of coculture with H. pylori (min)
E
Hp-
Hp+
Hp + SB203580
HPA
β-actin
F
HPA relative intensity (fold)
b
b
Hp-
Hp+
Hp + SB203580
SB203580 -
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+
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Control
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Hp + SB203580
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Number of invated cells
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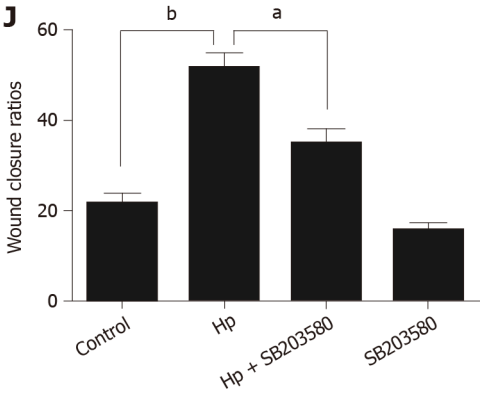


Figure 1 Heparanase protein expression following Helicobacter pylori infection in MKN-45 gastric cancer cells *via* the mitogen-activated protein kinase signaling pathway. A: Heparanase (HPA) expression was determined by Western blot at 0, 6, 12, 24, and 48 h after *Helicobacter pylori* (*H. pylori*) infection; B: Quantitative Western blot results of HPA; C: p-p38 mitogen-activated protein kinase (MAPK) expression was determined by Western blot at 0, 30, 60, 120, and 480 min after *H. pylori* infection; D: Quantitative Western blot results of p-p38MAPK; E: HPA expression when the MAPK inhibitor SB203580 was given to MKN-45 cells before *H. pylori* infection; F: Quantitative Western blot results of HPA when the MAPK inhibitor SB203580 was given. $^{b}P < 0.01$ compared with the value at 0 h; G and H: Cell invasion rates in the three groups detected using a Transwell invasion assay; I and J: Migration rates in the three groups detected using a scratch migration assay. $^{a}P < 0.05$, $^{b}P < 0.01$. HPA: Heparanase; MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*. Figure 2 in the original manuscript: Liu LP, Sheng XP, Shuai TK, Zhao YX, Li B, Li YM. *Helicobacter pylori* promotes invasion and metastasis of gastric cancer by enhancing heparanase expression. *World J Gastroenterol* 2018; 24: 4565-4577 [PMID: 30386106 DOI: 10.3748/wjg.v24.i40.4565].

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REFERENCES

1 **Liu LP**, Sheng XP, Shuai TK, Zhao YX, Li B, Li YM. *Helicobacter pylori* promotes invasion and metastasis of gastric cancer by enhancing heparanase expression. *World J Gastroenterol* 2018; **24**: 4565-4577 [PMID: 30386106 DOI: 10.3748/wjg.v24.i40.4565]

2 **Liu L**, Zhao Y, Fan G, Shuai T, Li B, Li Y. Helicobacter pylori infection enhances heparanase leading to cell proliferation *via* mitogenactivated protein kinase signalling in human gastric cancer cells. *Mol Med Rep* 2018; **18**: 5733-5741 [PMID: 30320396 DOI: 10.3892/mmr.2018.9558]

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EDITORIAL

Pain management in chronic pancreatitis incorporating safe opioid practices: Challenge accepted

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Abstract

Patients with chronic pancreatitis often experience severe, unrelenting abdominal pain, which can significantly impact their quality of life. Pain control, therefore, remains central to the overall management of chronic pancreatitis. Most of the strategies aimed at treating the pain of chronic pancreatitis are based on expert opinion and vary from one institution to another, as there are no uniform guidelines to direct a stepwise approach towards achieving this goal. In this editorial, we comment on best practice strategies targeted towards pain control in chronic pancreatitis, specifically highlighting the use of opioid medications in this patient population. We discuss various safe and efficacious prescription monitoring practices in this article.

Key Words: Chronic pancreatitis; Chronic pain; Pain management; Opioid use disorder; Prescription opioid misuse; Prescription opioid abuse

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Core Tip: Pain management in chronic pancreatitis is complex; collaboration with local pain specialists maybe necessary to provide optimal care.

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INTRODUCTION

Chronic pancreatitis (CP) is a fibro-inflammatory disorder characterized by irreversible parenchymal and ductal injury of the pancreas caused by a variety of genetic, metabolic and environmental factors. Globally, the prevalence of CP ranges between 36 and 125 per 100000 persons[1]. Patients with CP almost universally present with abdominal pain. Although the pathophysiology of the pain is poorly understood, it seems to be related to various factors such as pancreatic inflammation, duct obstruction and nerve damage[2-6].

With abdominal pain being the predominant symptom, pain management is central to the treatment of CP. Lifestyle modifications such as alcohol and tobacco cessation along with frequent consumption of small meals remain the first-line treatment for CP [7,8]. Efficacy of pancreatic enzymes has been debated based on results from multiple randomized controlled clinical trials, and the current consensus is that uncoated preparations containing large amounts of pancreatic enzymes can be used for pain control in selected patients, in addition to treating symptoms of pancreatic insufficiency[2,9]. Similarly, some studies have shown that antioxidants with or without other analgesics could be beneficial in treating pain *via* suppression of oxidative stress which would reduce pancreatic inflammation[10,11].

PAIN MANAGEMENT STRATEGIES

Several experts have recommended following the World Health Organization (WHO) pain relief ladder while choosing the appropriate analgesic in CP[2,3,5,12]. Acetaminophen continues to remain the analgesic of choice. This is followed by neuromodulators such as gabapentin, pregabalin and tricyclic antidepressants. Antispasmodics and muscle relaxants such as baclofen and hyoscyamine have also been used as adjunctive therapies. Oftentimes, endoscopic intervention with sphincterotomy and stent placement is employed in patients with distal pancreatic duct obstruction. Surgical techniques aimed at ductal decompression or parenchymal resection or both, albeit limited by their invasive nature, are often employed in patients who have pain that is refractory to other treatment measures. In the United States, total pancreatectomy with islet auto transplantation has shown promising results, although there have been mixed results on long term diabetes control and insulin dependence[13-16]. Denervation techniques such as celiac plexus blocks are also used when other treatments fail[17]. Use of neurostimulation techniques such as spinal cord stimulation and transcranial magnetic stimulation have shown promising results, although rarely employed in standard practice owing to paucity of literature [18,19]. When these strategies fail, providers are often left with opioids for pain management although there is limited published literature outlining their use in patients with CP. Knowing about the devastating nature of the current opioid epidemic that has been prevalent for more than 25 years, it is important to have a mindful and cautious approach while effectively using opioids to treat pain in such patients[20,21].

Opioid use disorder (OUD) has evolved into a major health emergency over the last few years, contributing to more than 600000 deaths just in the United States, mandating judicious opioid use and close prescription monitoring in an attempt to prevent opioid misuse[22]. Although potentially effective in pain management, opioid use has serious consequences including narcotic bowel syndrome and opioid-induced hyperalgesia[23]. It has been shown that about 13%-50% patients with inflammatory bowel disease and irritable bowel syndrome use chronic opioids, and similarly, up to 66% patients of CP are known to use opioids[24-27]. Over the last two decades, opioid use among several gastrointestinal conditions with chronic pain has steadily continued to increase with an 88% increase in inflammatory bowel disease and a 57.6% increase in CP[27].

With the increased use of opioids, there remains a high level of concern among gastroenterologists and other providers for the risk of opioid dependence and abuse along with increased healthcare utilization. In CP, opioid use has limited data and uniform guidelines are lacking to direct prescription practices, which can lead to further apprehension and confusion among providers when deciding upon an ideal approach. Although the WHO pain relief ladder has been recommended by some experts while choosing an appropriate analgesic in patients with CP, it should be noted that this stepwise approach was originally designed for achieving pain control primarily in cancer patients, and the feasibility of its use has not been studied in CP[2,

12]. Absence of a uniform stepwise approach towards analgesic use in CP, may further result in irregular and scattered methods of opioid prescription among various providers, further adding to opioid overuse and associated adverse outcomes.

Curbing the overuse of opioids and regulating a streamlined opioid-prescription system, is a critical task for providers caring for patients with chronic pancreatitis. There is a need for treatment approaches that are successful in minimizing pain while minimizing the risk of developing OUD. Several pain societies have developed prescription guidelines for patients with non-cancer pain and thus it is important to partner with experienced pain clinics who can provide appropriate attention to patients requiring narcotic therapy[28-31].

Selection of patients who would benefit from opioid therapy and predicting their risk of misuse potential should be carefully executed. Several validated screening tools for OUD can be used for this purpose including the Current Opioid Misuse Measure, which has been studied in nonalcoholic CP[32-35]. Clinicians should be vigilant for commonly encountered psychosocial risk factors that may be associated with a higher risk of abuse potential such as active or prior concurrent substance abuse, alcohol use, polypharmacy with sedating medications such as benzodiazepines, mental health disorders, *etc*.[36]. Since CP patients could often have comorbid substance abuse disorder, close attention should be paid to either completely avoiding or at least minimizing opioid prescriptions in patients with these risk factors[35,36].

While there is limited data on the opioid prescription guidelines in patients with CP, most experts recommend a one-to-four-week course of low dose opioids (less than 40 morphine milliequivalents) before committing to a long-term opioid therapy for patients with non-cancer pain. This is because higher doses of more than 60 to 90 morphine milliequivalents have been associated with increased risk of dependence and adverse outcomes[30,31,37]. Furthermore, there is a dearth of evidence to suggest better efficacy and safety profile of any one opioid over another[30]. Priority should be given to weaker opioids such as tramadol and extended-release formulations should be avoided due to their higher risk of overdose[30,37]. Transdermal opioids such as fentanyl should only considered when patients are unable to tolerate oral medications [30].

Prior to initiating long term opioid therapy, it is important to extensively educate the patient about its abuse potential and its associated consequences. Many states have mandated opioid treatment agreements that should be signed by both the prescriber and the patient and renewed on a regular basis[38-40]. Once long-term opioid therapy has been initiated, it is important to closely monitor for opioid misuse *via* various physician-based and state-mandated tools. Frequent office visits should be scheduled, no less than once in every 90 d[41]. During each visit, *via* means of a comprehensive history taking and physical exam, an assessment should carefully made to determine need for continuing opioid therapy. If continuing opioid therapy is deemed necessary, an attempt should be made to assess if the opioid dose can be progressively reduced to the minimum effective dose. Additionally, many experts recommend urine drug testing (UDT) prior to starting opioids and eventually for regular monitoring in an effort to curb opioid misuse[28,30]. The United States has mandated prescription monitoring programs (PMP) to track controlled prescriptions which is an efficient tool that can be regularly used by providers to reduce misuse of opioids and other controlled substances[28,30,31,37]. Close attention can thus be paid to patients with frequent emergency room visits who may be receiving multiple overlapping prescriptions of short course opioids. At any point, if lack of response, adverse events or opioid misuse are encountered, opioids may need to be discontinued[37]. Our approach is summarized in Figure 1.

CONCLUSION

Abdominal pain is the central symptom for most patients with CP resulting in high healthcare utilization. While several non-opioid therapies exist, unfortunately patients remain symptomatic and thus opioids may be necessary to treat ongoing abdominal pain. With the ongoing opioid epidemic and elevated risk of OUD, several strategies should be incorporated while using opioids for the treatment of pain in patients with CP. These include robust screening measures to predetermine the risk of OUD, safe prescription practices using tools such as UDT and PMP, close clinical follow up and frequent reassessment to assess the need for continuing opioids. Finally, a multidisciplinary approach and coordination with pain management physicians is recommended to adequately cater to each patient's individual needs. Further studies and guidelines

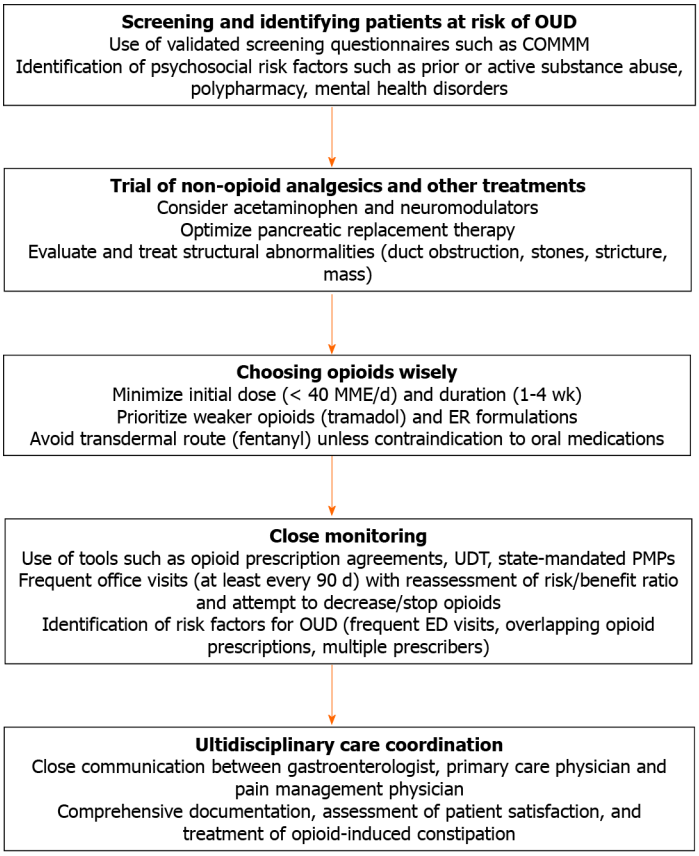


Figure 1 Stepwise approach to management of pain in chronic pancreatitis. OUD: Opioid use disorder; COMMM: Current opioid misuse measure; ER: Extended release; UDT: Urine drug testing; PMP: Prescription monitoring program; ED: Emergency Department.

are needed to address opioid use in CP.

REFERENCES

1 **Machicado JD**, Dudekula A, Tang G, Xu H, Wu BU, Forsmark CE, Yadav D. Period prevalence of chronic pancreatitis diagnosis from 2001-2013 in the commercially insured population of the United States. *Pancreatology* 2019; **19**: 813-818 [PMID: 31350077 DOI: 10.1016/j.pan.2019.07.003]

2 **Drewes AM**, Bouwense SAW, Campbell CM, Ceyhan GO, Delhaye M, Demir IE, Garg PK, van Goor H, Halloran C, Isaji S, Neoptolemos JP, Olesen SS, Palermo T, Pasricha PJ, Sheel A, Shimosegawa T, Szigethy E, Whitcomb DC, Yadav D; Working group for the International (IAP – APA – JPS – EPC) Consensus Guidelines for Chronic Pancreatitis. Guidelines for the understanding and management of pain in chronic pancreatitis. *Pancreatology* 2017; **17**: 720-731 [PMID: 28734722 DOI: 10.1016/j.pan.2017.07.006]

3 **Whitcomb DC**, Shimosegawa T, Chari ST, Forsmark CE, Frulloni L, Garg P, Hegyi P, Hirooka Y, Irisawa A, Ishikawa T, Isaji S, Lerch MM, Levy P, Masamune A, Wilcox CM, Windsor J, Yadav D, Sheel A, Neoptolemos JP; Working Group for the International (IAP – APA – JPS – EPC) Consensus Guidelines for Chronic Pancreatitis. International consensus statements on early chronic Pancreatitis. Recommendations from the working group for the international consensus guidelines for chronic pancreatitis in collaboration with The International Association of Pancreatology, American Pancreatic Association, Japan Pancreas Society, PancreasFest Working Group and European Pancreatic Club. *Pancreatology* 2018; **18**: 516-527 [PMID: 29793839 DOI: 10.1016/j.pan.2018.05.008]

4 **Drewes AM**, Olesen AE, Farmer AD, Szigethy E, Rebours V, Olesen SS. Gastrointestinal pain. *Nat Rev Dis Primers* 2020; **6**: 1 [PMID: 31907359 DOI: 10.1038/s41572-019-0135-7]

5 **Beyer G**, Habtezion A, Werner J, Lerch MM, Mayerle J. Chronic pancreatitis. *Lancet* 2020; **396**: 499-512 [PMID: 32798493 DOI: 10.1016/S0140-6736(20)31318-0]

6 **Löhr JM**, Dominguez-Munoz E, Rosendahl J, Besselink M, Mayerle J, Lerch MM, Haas S, Akisik F, Kartalis N, Iglesias-Garcia J, Keller J, Boermeester M, Werner J, Dumonceau JM, Fockens P, Drewes A, Ceyhan G, Lindkvist B, Drenth J, Ewald N, Hardt P, de Madaria E, Witt H, Schneider A, Manfredi R, Brøndum FJ, Rudolf S, Bollen T, Bruno M; HaPanEU/UEG Working Group. United European Gastroenterology evidence-based guidelines for the diagnosis and therapy of chronic pancreatitis

(HaPanEU). *United European Gastroenterol J* 2017; **5**: 153-199 [PMID: 28344786 DOI: 10.1177/2050640616684695]
7 **Singhvi A**, Yadav D. Myths and realities about alcohol and smoking in chronic pancreatitis. *Curr Opin Gastroenterol* 2018; **34**: 355-361 [PMID: 29965868 DOI: 10.1097/MOG.0000000000000466]
8 **Edderkaoui M**, Thrower E. Smoking and Pancreatic Disease. *J Cancer Ther* 2013; **4**: 34-40 [PMID: 24660091 DOI: 10.4236/jct.2013.410A005]
9 **Warshaw AL**, Banks PA, Fernández-Del Castillo C. AGA technical review: treatment of pain in chronic pancreatitis. *Gastroenterology* 1998; **115**: 765-776 [PMID: 9721175 DOI: 10.1016/s0016-5085(98)70157-x]
10 **Zhou D**, Wang W, Cheng X, Wei J, Zheng S. Antioxidant therapy for patients with chronic pancreatitis: A systematic review and meta-analysis. *Clin Nutr* 2015; **34**: 627-634 [PMID: 25035087 DOI: 10.1016/j.clnu.2014.07.003]
11 **Talukdar R**, Lakhtakia S, Nageshwar Reddy D, Rao GV, Pradeep R, Banerjee R, Gupta R, Ramchandani M, Tandan M, Murthy HV. Ameliorating effect of antioxidants and pregabalin combination in pain recurrence after ductal clearance in chronic pancreatitis: Results of a randomized, double blind, placebo-controlled trial. *J Gastroenterol Hepatol* 2016; **31**: 1654-1662 [PMID: 26945817 DOI: 10.1111/jgh.13332]
12 **Jadad AR**, Browman GP. The WHO analgesic ladder for cancer pain management. Stepping up the quality of its evaluation. *JAMA* 1995; **274**: 1870-1873 [PMID: 7500538]
13 **Kesseli SJ**, Smith KA, Gardner TB. Total pancreatectomy with islet autologous transplantation: the cure for chronic pancreatitis? *Clin Transl Gastroenterol* 2015; **6**: e73 [PMID: 25630865 DOI: 10.1038/ctg.2015.2]
14 **Bellin MD**, Abu-El-Haija M, Morgan K, Adams D, Beilman GJ, Chinnakotla S, Conwell DL, Dunn TB, Freeman ML, Gardner T, Kirchner VA, Lara LF, Long-Simpson L, Nathan JD, Naziruddin B, Nyman JA, Pruett TL, Schwarzenberg SJ, Singh VK, Smith K, Steel JL, Wijkstrom M, Witkowski P, Hodges JS; POST study consortium. A multicenter study of total pancreatectomy with islet autotransplantation (TPIAT): POST (Prospective Observational Study of TPIAT). *Pancreatology* 2018; **18**: 286-290 [PMID: 29456124 DOI: 10.1016/j.pan.2018.02.001]
15 **Gardner TB**, Smith KD. Total Pancreatectomy and Islet Autotransplant in the Treatment of Chronic Pancreatitis: Tread Very, Very Carefully. *Am J Gastroenterol* 2018; **113**: 322-323 [PMID: 29134966 DOI: 10.1038/ajg.2017.419]
16 **Bellin MD**, Whitcomb DC, Abberbock J, Sherman S, Sandhu BS, Gardner TB, Anderson MA, Lewis MD, Alkaade S, Singh VK, Baillie J, Banks PA, Conwell D, Cote GA, Guda NM, Muniraj T, Tang G, Brand RE, Gelrud A, Amann ST, Forsmark CE, Wilcox CM, Slivka A, Yadav D. Patient and Disease Characteristics Associated With the Presence of Diabetes Mellitus in Adults With Chronic Pancreatitis in the United States. *Am J Gastroenterol* 2017; **112**: 1457-1465 [PMID: 28741615 DOI: 10.1038/ajg.2017.181]
17 **Sachdev AH**, Gress FG. Celiac Plexus Block and Neurolysis: A Review. *Gastrointest Endosc Clin N Am* 2018; **28**: 579-586 [PMID: 30241645 DOI: 10.1016/j.giec.2018.06.004]
18 **Kapural L**, Cywinski JB, Sparks DA. Spinal cord stimulation for visceral pain from chronic pancreatitis. *Neuromodulation* 2011; **14**: 423-426 [PMID: 21854493 DOI: 10.1111/j.1525-1403.2011.00381.x]
19 **Fregni F**, Potvin K, Dasilva D, Wang X, Lenkinski RE, Freedman SD, Pascual-Leone A. Clinical effects and brain metabolic correlates in non-invasive cortical neuromodulation for visceral pain. *Eur J Pain* 2011; **15**: 53-60 [PMID: 20822942 DOI: 10.1016/j.ejpain.2010.08.002]
20 **Singh VK**, Yadav D, Garg PK. Diagnosis and Management of Chronic Pancreatitis: A Review. *JAMA* 2019; **322**: 2422-2434 [PMID: 31860051 DOI: 10.1001/jama.2019.19411]
21 **Stoicea N**, Costa A, Periel L, Uribe A, Weaver T, Bergese SD. Current perspectives on the opioid crisis in the US healthcare system: A comprehensive literature review. *Medicine (Baltimore)* 2019; **98**: e15425 [PMID: 31096439 DOI: 10.1097/MD.0000000000015425]
22 **Gostin LO**, Hodge JG Jr, Noe SA. Reframing the Opioid Epidemic as a National Emergency. *JAMA* 2017; **318**: 1539-1540 [PMID: 28832871 DOI: 10.1001/jama.2017.13358]
23 **Szigethy E**, Knisely M, Drossman D. Opioid misuse in gastroenterology and non-opioid management of abdominal pain. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 168-180 [PMID: 29139482 DOI: 10.1038/nrgastro.2017.141]
24 **Cross RK**, Wilson KT, Binion DG. Narcotic use in patients with Crohn's disease. *Am J Gastroenterol* 2005; **100**: 2225-2229 [PMID: 16181373 DOI: 10.1111/j.1572-0241.2005.00256.x]
25 **Nusrat S**, Yadav D, Bielefeldt K. Pain and opioid use in chronic pancreatitis. *Pancreas* 2012; **41**: 264-270 [PMID: 21792080 DOI: 10.1097/MPA.0b013e318224056f]
26 **Ahmed A**, Yakah W, Freedman SD, Kothari DJ, Sheth SG. Evaluation of Opioid Use in Acute Pancreatitis in Absence of Chronic Pancreatitis: Absence of Opioid Dependence an Important Feature. *Am J Med* 2020; **133**: 1209-1218 [PMID: 32272099 DOI: 10.1016/j.amjmed.2020.03.010]
27 **Bilal M**, Chatila A, Siddiqui MT, Al-Hanayneh M, Shah AR, Desai M, Wadhwa V, Parupudi S, Casey BW, Krishnan K, Hernandez-Barco YG. Rising Prevalence of Opioid Use Disorder and Predictors for Opioid Use Disorder Among Hospitalized Patients With Chronic Pancreatitis. *Pancreas* 2019; **48**: 1386-1392 [PMID: 31688606 DOI: 10.1097/MPA.0000000000001430]
28 **Manchikanti L**, Abdi S, Atluri S, Balog CC, Benyamin RM, Boswell MV, Brown KR, Bruel BM, Bryce DA, Burks PA, Burton AW, Calodney AK, Caraway DL, Cash KA, Christo PJ, Damron KS, Datta S, Deer TR, Diwan S, Eriator I, Falco FJ, Fellows B, Geffert S, Gharibo CG, Glaser SE, Grider

JS, Hameed H, Hameed M, Hansen H, Harned ME, Hayek SM, Helm S 2nd, Hirsch JA, Janata JW, Kaye AD, Kaye AM, Kloth DS, Koyyalagunta D, Lee M, Malla Y, Manchikanti KN, McManus CD, Pampati V, Parr AT, Pasupuleti R, Patel VB, Sehgal N, Silverman SM, Singh V, Smith HS, Snook LT, Solanki DR, Tracy DH, Vallejo R, Wargo BW; American Society of Interventional Pain Physicians. American Society of Interventional Pain Physicians (ASIPP) guidelines for responsible opioid prescribing in chronic non-cancer pain: Part 2--guidance. *Pain Physician* 2012; **15**: S67-116 [PMID: 22786449 DOI: 10.36076/ppj.2012/15/s67]

29 **Chou R**, Fanciullo GJ, Fine PG, Adler JA, Ballantyne JC, Davies P, Donovan MI, Fishbain DA, Foley KM, Fudin J, Gilson AM, Kelter A, Mauskop A, O'Connor PG, Passik SD, Pasternak GW, Portenoy RK, Rich BA, Roberts RG, Todd KH, Miaskowski C; American Pain Society-American Academy of Pain Medicine Opioids Guidelines Panel. Clinical guidelines for the use of chronic opioid therapy in chronic noncancer pain. *J Pain* 2009; **10**: 113-130 [PMID: 19187889 DOI: 10.1016/j.jpain.2008.10.008]

30 **Dowell D**, Haegerich TM, Chou R. CDC Guideline for Prescribing Opioids for Chronic Pain--United States, 2016. *JAMA* 2016; **315**: 1624-1645 [PMID: 26977696 DOI: 10.1001/jama.2016.1464]

31 **Black E**, Khor KE, Demirkol A. Responsible Prescribing of Opioids for Chronic Non-Cancer Pain: A Scoping Review. *Pharmacy (Basel)* 2020; **8**: 150 [PMID: 32825483 DOI: 10.3390/pharmacy8030150]

32 **Butler SF**, Budman SH, Fernandez K, Jamison RN. Validation of a screener and opioid assessment measure for patients with chronic pain. *Pain* 2004; **112**: 65-75 [PMID: 15494186 DOI: 10.1016/j.pain.2004.07.026]

33 **Cheatle MD**, Compton PA, Dhingra L, Wasser TE, O'Brien CP. Development of the Revised Opioid Risk Tool to Predict Opioid Use Disorder in Patients with Chronic Nonmalignant Pain. *J Pain* 2019; **20**: 842-851 [PMID: 30690168 DOI: 10.1016/j.jpain.2019.01.011]

34 **Butler SF**, Budman SH, Fernandez KC, Houle B, Benoit C, Katz N, Jamison RN. Development and validation of the Current Opioid Misuse Measure. *Pain* 2007; **130**: 144-156 [PMID: 17493754 DOI: 10.1016/j.pain.2007.01.014]

35 **Barth KS**, Balliet W, Pelic CM, Madan A, Malcolm R, Adams D, Morgan K, Owczarski S, Borckardt JJ. Screening for current opioid misuse and associated risk factors among patients with chronic nonalcoholic pancreatitis pain. *Pain Med* 2014; **15**: 1359-1364 [PMID: 24716629 DOI: 10.1111/pme.12403]

36 **Vowles KE**, Witkiewitz K, Pielech M, Edwards KA, McEntee ML, Bailey RW, Bolling L, Sullivan MD. Alcohol and Opioid Use in Chronic Pain: A Cross-Sectional Examination of Differences in Functioning Based on Misuse Status. *J Pain* 2018; **19**: 1181-1188 [PMID: 29758355 DOI: 10.1016/j.jpain.2018.04.013]

37 **Manchikanti L**, Kaye AM, Knezevic NN, McAnally H, Slavin K, Trescot AM, Blank S, Pampati V, Abdi S, Grider JS, Kaye AD, Manchikanti KN, Cordner H, Gharibo CG, Harned ME, Albers SL, Atluri S, Aydin SM, Bakshi S, Barkin RL, Benyamin RM, Boswell MV, Buenaventura RM, Calodney AK, Cedeno DL, Datta S, Deer TR, Fellows B, Galan V, Grami V, Hansen H, Helm Ii S, Justiz R, Koyyalagunta D, Malla Y, Navani A, Nouri KH, Pasupuleti R, Sehgal N, Silverman SM, Simopoulos TT, Singh V, Solanki DR, Staats PS, Vallejo R, Wargo BW, Watanabe A, Hirsch JA. Responsible, Safe, and Effective Prescription of Opioids for Chronic Non-Cancer Pain: American Society of Interventional Pain Physicians (ASIPP) Guidelines. *Pain Physician* 2017; **20**: S3-S92 [PMID: 28226332 DOI: 10.36076/ppj.2017.s92]

38 **Straub M**, Pashkova AA. Controlled Substance Agreements and Other Best Opioid Prescription Practices. *Otolaryngol Clin North Am* 2020; **53**: 877-883 [PMID: 32684286 DOI: 10.1016/j.otc.2020.05.017]

39 **Kraus CN**, Baldwin AT, Curro FA, McAllister RG Jr. Clinical implications of patient-provider agreements in opioid prescribing. *Curr Drug Saf* 2015; **10**: 159-164 [PMID: 25986037 DOI: 10.2174/1574886309666140922095844]

40 **McAuliffe Staehler TM**, Palombi LC. Beneficial opioid management strategies: A review of the evidence for the use of opioid treatment agreements. *Subst Abus* 2020; **41**: 208-215 [PMID: 31900073 DOI: 10.1080/08897077.2019.1692122]

41 **Kaye AD**, Jones MR, Kaye AM, Ripoll JG, Galan V, Beakley BD, Calixto F, Bolden JL, Urman RD, Manchikanti L. Prescription Opioid Abuse in Chronic Pain: An Updated Review of Opioid Abuse Predictors and Strategies to Curb Opioid Abuse: Part 1. *Pain Physician* 2017; **20**: S93-S109 [PMID: 28226333 DOI: 10.36076/ppj.2017.s111]

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EVIDENCE REVIEW

Pancreatitis and pancreatic cancer: A case of the chicken or the egg

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Abstract

Acute pancreatitis (AP), chronic pancreatitis (CP) and pancreatic cancer are three distinct pancreatic diseases with different prognoses and treatment options. However, it may be difficult to differentiate between benign and malignant disease. AP may be a first symptom of pancreatic cancer, particularly in patients between the ages of 56 and 75 with presumed idiopathic AP who had a concomitant diagnosis of new-onset diabetes mellitus or patients who present with CP at diagnosis of AP. In these patients, additional imaging is warranted, preferably by endoscopic ultrasonography. CP may lead to pancreatic cancer through oncogenic mutations, mostly in patients with hereditary CP, and in patients in whom risk factors for pancreatic cancer (*e.g.*, nicotine and alcohol abuse) are also present. Patients with *PRSS1*-mediated CP and patients with a history of autosomal dominant hereditary CP without known genetic mutations may be considered for surveillance for pancreatic cancer. Pancreatic inflammation may mimic pancreatic cancer by appearing as a focal mass-forming lesion on imaging. Differentiation between the above mentioned benign and malignant disease may be facilitated by specific features like the duct-penetrating sign and the duct-to-parenchyma ratio. Research efforts are aimed towards developing a superior discriminant between pancreatitis and pancreatic cancer in the form of imaging modalities or biomarkers. This may aid clinicians in timely diagnosing pancreatic cancer in a potentially curable stage.

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Core Tip: It is essential to distinguish acute pancreatitis and chronic pancreatitis (CP) from pancreatic ductal adenocarcinoma (PDAC), as these diseases have different treatment options and prognoses. Idiopathic acute pancreatitis may be a first symptom of PDAC, and endoscopic ultrasonography should therefore be considered. CP may obscure PDAC, and long-standing inflammation may cause PDAC, especially in hereditary CP and patients with a history of nicotine and alcohol abuse. Patients should be counselled in cessation of tobacco and alcohol use. Pancreatitis may mimic PDAC by presenting as a mass on imaging. Specific imaging features can aid in the differentiation between benign and malignant disease.

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INTRODUCTION

Acute pancreatitis (AP) and chronic pancreatitis (CP) are inflammatory diseases of the pancreas. While AP is sudden and transient, CP is a longstanding inflammation of the pancreas characterized by pancreatic calcifications and exocrine pancreatic insufficiency. In CP, bouts of so-called acute-on-chronic episodes of pancreatitis may also occur.

Both AP and CP are benign diseases that are, in different ways, associated with pancreatic ductal adenocarcinoma (PDAC). Although these are three separate disease entities, inflammation and cancer can co-exist, and inflammation may cause cancer while cancer may also cause inflammation. This entails they are not always easy to distinguish from each other. As the treatment and prognosis of AP, CP and PDAC are completely different, it is important to understand the nature of the association between these diseases and be able to take the proper diagnostic steps to gather the correct diagnosis and subsequent treatment options.

In this review, we will focus on the relationship between AP, CP and PDAC and how to distinguish between these three disease entities.

CLINICAL CASE: A 64-YEAR-OLD MAN WITH ABDOMINAL PAIN

A 64-year-old man visited the emergency department with acute abdominal pain in the epigastric region. Laboratory tests revealed an amylase level of 2100 U/L (normal < 107 U/L), a lipase level of 5548 U/L (normal 13-60 U/L) and normal liver enzymes, calcium and triglycerides. Computed tomography (CT) showed inflammation of the head of the pancreas (Figure 1). The patient was admitted with AP, received fluid resuscitation therapy and analgesics. Transabdominal ultrasound showed no cause for the AP episode. After 3 d, the patient was discharged with a diagnosis of idiopathic AP.

Approximately 1.5 years later, this patient was readmitted with a recurrent AP episode. CT of the abdomen revealed inflammation of the pancreas with a splenorenal fluid collection and slight dilatation of the pancreatic duct in the tail of the pancreas (Figure 2). Additional magnetic resonance imaging (MRI) showed a hypo-intense lesion in the pancreatic tail, causing a stenosis of the pancreatic duct (Figure 3). Endoscopic ultrasonography (EUS) with biopsy confirmed the diagnosis of PDAC (Figure 4). The patient underwent a pancreatic tail and spleen resection, and pathology revealed a radically resected T3N0 PDAC.

AP AND PANCREATIC CANCER

The incidence of AP is rising, with a median increase of 3.4% per year[1]. Reported incidence rates ranges from 9.8 to 100 per 100000 in Europe and rise with age[1-3]. AP is often caused by alcohol or gallstone disease, although the incidence of these etiologies differ geographically[1].

A strong association between AP and PDAC has been established through multiple studies. A recent systematic review and meta-analysis by Liu *et al*[4] included 11 studies reporting on the risk of PDAC after an episode of AP and found an effect estimate of 2.07 [95% confidence interval (CI): 1.36-2.78] during a follow-up time exceeding 10 years. In a subgroup analysis of 103961 AP patients and 1442158 control subjects from five prospective studies in this systematic review, a relative risk of 7.81 (95%CI: 5.00-12.19) for PDAC was observed in patients with AP as compared to healthy control subjects.

Multiple hypotheses have been raised to explain this apparent association between AP and PDAC. Firstly, it has been suggested that risk factors for AP are similar to risk factors for PDAC. For instance, alcohol abuse is a prevalent etiology of AP and also a known risk factor for pancreatic cancer. Although the etiological value of nicotine abuse in AP is still a topic of discussion, it is an established risk factor for recurrent AP, while also being a strong risk factor for PDAC[5-7]. Secondly, in recurrent AP, the accumulating inflammatory states of the pancreas are thought to increase the chance of oncogenic mutations in pancreatic cells, leading to carcinogenesis in a similar fashion as CP, as elaborated below in the section on CP[8].

However, the most prevailing hypothesis has been that a single episode of AP cannot cause PDAC but that PDAC may cause AP. Although this is rare, it has been acknowledged that PDAC can cause an episode of AP by ductal obstruction before the PDAC has even been diagnosed[9]. Temporal data on the association of PDAC and AP endorse this hypothesis. In the previously mentioned meta-analysis by Liu *et al*[4], the strongest association between AP and PDAC was found in the first year after AP [effect estimate 23.47 (95%CI: 3.26-43.68)], while this association diminished over a course of 2 years after AP [effect estimate 9.82 (95%CI: 3.01-16.64)], 5 years [effect estimate 2.47 (95%CI: 1.93-3.02)], 10 years [effect estimate 1.69 (95%CI: 1.26; 2.11)] and over 10 years after AP [effect estimate 1.17 (95%CI: 0.78-1.57)]. A Danish matched-cohort study, which was included in this meta-analysis, also adjusted for alcohol- and smoking-related conditions and the Charlson Comorbidity Index score and excluded patients who developed CP or other exocrine pancreatic disease during follow-up[10]. This well-designed study found that a presumed idiopathic etiology appeared to be associated with the highest risk of PDAC [adjusted hazard ratio of 2.52 (95%CI: 1.83-3.47)]. This finding was supported by a recent cohort study, which found that biliary AP and a history of alcohol-related disease were predictors for no underlying PDAC [11]. In this study of 28231 patients with AP, 283 received the diagnosis PDAC during follow-up (overall risk 1.0%). They found several predictors for PDAC in patients with AP, including age between 56 and 75 at diagnosis of AP (risk approximately 1.6%), new-onset diabetes mellitus (DM) and new-onset CP [risk 2.0% (95%CI: 1.1-3.3%)] at diagnosis of AP. Interestingly, severe AP, smoking-related diseases, venous thromboembolism and previous malignancies were not associated with a higher risk for PDAC. A Swedish matched-cohort study of 49749 AP patients and 138750 matched subjects also identified recurrent pancreatitis as a risk factor for PDAC [hazard ratio 4.44 (95%CI: 1.181-10.89) after censoring for diagnosis of CP)][12].

Management

It has become more and more apparent that PDAC is a noteworthy etiology of AP, particularly in patients with presumed idiopathic etiology after standard diagnostic work-up. In these patients with idiopathic AP, primarily in patients aged 56-75, it is imperative to exclude the presence of an occult pancreatic tumor by providing additional imaging. Commonly, standard diagnostic work-up includes a personal history, laboratory tests (including liver enzymes, calcium and triglycerides) and transabdominal ultrasonography. EUS has been proven to be more sensitive for the detection of PDAC than CT (98 *vs* 74%) and transabdominal ultrasonography (94% *vs* 67%), especially for small lesions[13]. A meta-analysis comparing the diagnostic yield of EUS *vs* magnetic resonance cholangiopancreatography (MRCP) showed that EUS is superior to MRCP in detecting all etiologies in idiopathic AP[14]. Thus, mainly in patients aged 56-75 with idiopathic AP, recurring AP or new-onset DM or CP concomitant with their AP diagnosis, additional imaging is warranted, preferably using EUS.

CP AND PANCREATIC CANCER

After a first episode of AP, 17% of patients have at least one recurrent attack, and 8% develop CP[5]. CP is a progressive inflammatory disease that often leads to pain, exocrine pancreatic insufficiency and DM. It is characterized by a pathologic response to pancreatic injury leading to irreversible parenchymal damage[15]. Estimates for global CP incidence range from 5.0 to 10.0 cases per 100000 person-years[16]. In the majority of cases, CP develops after a history of recurrent AP in patients with multiple environmental and genetic risk factors[17]. Alcohol and nicotine are the most important environmental risk factors for CP[5]. Genetic risk factors include mutations in the protease serine 1 (*PRSS1*) gene, which cause hereditary CP, and in the serine protease inhibitor Kazal-type 1 (*SPINK1*) and cystic fibrosis chymotrypsinogen C (*CFTR*) genes[18].

Lowenfels *et al*[19] were the first to describe the link between CP and progression to PDAC. In this study including 2015 CP patients, a cumulative risk for PDAC was observed in 1.8% (95%CI: 1.0-2.6) and 4.0% (95%CI: 2.0-5.9) 10 and 20 years after diagnosis, respectively[19]. To date, the increased risk of PDAC in CP patients has been confirmed by several meta-analyses[20-22]. However, the exact risk remains debated, mainly because different factors contribute to PDAC progression.

The risk of PDAC is much higher for patients with hereditary CP from *PRSS1* mutations [relative risk 69.0 (95%CI: 56.4-84.4)] and tropical CP [relative risk 100 (95%CI: 37.0-218)], when compared to patients with sporadic CP [relative risk 5.1 (95%CI: 3.5-7.3)][21,23-25]. Additionally, alcohol consumption and smoking, both well-known risk factors for PDAC, form important confounding factors as patients with CP often have a history of alcohol- and nicotine abuse[26]. Other risk factors are older age at disease onset, obesity, concurrent DM and pancreatic duct dilatation[27,28].

Even though many contributing factors play a role in the association between CP and PDAC, evidence suggests that a causal relationship is plausible. The pathway from CP to PDAC is not yet entirely understood. The prevailing hypothesis is that the long-standing inflammatory state of the pancreas associated with CP leads to oncogenic mutations. Several studies demonstrated that the pancreas of a notable proportion of CP patients harbor downregulating mutations of the tumor suppressing *p16*, *p53* and *SMAD4* genes and upregulating mutations of the oncogenic K-ras, tumor necrosis factor-α and nuclear factor κ B genes, which are also present in the majority of PDAC[8,29-31]. These mutations could subsequently facilitate carcinogenesis in patients who may already have a genetic disposition or environmental risk factors (*e.g.*, nicotine abuse) for developing PDAC.

Additional to the risk of progression to PDAC in CP, there is a risk of PDAC being misdiagnosed as CP because discrimination between these diseases can be difficult. A retrospective study found that 5% of PDAC patients were misdiagnosed with CP, while actually having PDAC[32]. This finding has been supported by the temporal data of a recent meta-analysis of 13 studies[20]. This meta-analysis found a decrease in PDAC risk between a 2-year lag-period [effect estimate 16.16 (95%CI: 12.59-20.73)] and a 5- and 9-year lag-period [effect estimate 7.90 (95%CI: 4.26-14.66) and 3.53 (95%CI: 1.69-7.38), respectively][20]. The peak of diagnosis of PDAC in the first years after diagnosis of CP suggests that these were either patients with PDAC who were initially wrongfully diagnosed with CP or patients with CP and concomitant PDAC in whom the PDAC was not recognized at diagnosis of CP.

Management

Special attention should be paid to the presence of PDAC in newly diagnosed CP patients, given the high risk of PDAC in the first years after CP diagnosis. Although the discussion is ongoing, international guidelines currently only consider surveillance of PDAC justified in patients with *PRSS1*-mediated CP and patients with a history of autosomal dominant hereditary CP without known genetic mutations, from the age of 40 until the patient is no longer suitable for (surgical) intervention. As small tumor lesions may be obscured by inflammation, fibrosis and calcification on EUS, it is advised to screen using CT imaging or MRI, although high quality evidence on the accuracy of different imaging modalities in detection of DPAC in CP patients is sparse [33]. Other contributing factors for PDAC progression that justify screening in patients with CP should be further clarified.

Additionally, time and effort should be invested in reducing environmental risk factors for PDAC, such as nicotine and alcohol abuse. These risk factors are prevalent among patients with CP and counselling and support should be provided to patients willing to quit smoking or drinking.

DIAGNOSTICS: PANCREATITIS OR PANCREATIC CANCER?

Inflammatory diseases of the pancreas can sometimes mimic PDAC by the appearance of a focal, mass-forming pancreatitis. Although diffuse forms of pancreatitis are more common, some patients present with a focal pancreatitis, in particular autoimmune pancreatitis, groove pancreatitis and CP. Clinical symptoms of mass-forming pancreatitis often overlap with symptoms related to PDAC, including abdominal pain, weight loss and jaundice. In patients undergoing surgery for suspected (periampullary) cancer, up to 23% turn out to be chronic inflammatory lesions[34].

Imaging

Similar to PDAC, focal CP can lead to focal parenchymal atrophy or enlargement and appears as a hypovascular mass on imaging, accompanied by upstream ductal dilation and atrophy[35]. Imaging features that point towards an inflammatory cause include an unobstructed or smooth tapering pancreatic duct through the hypovascular mass (duct-penetrating sign), while PDAC usually demonstrates an abrupt or irregular obstruction (Figure 2)[36-38]. The duct-penetrating sign is highly specific for an inflammatory mass (96%), with a sensitivity of 85%, and can be best visualized during MRCP[39]. In addition, collateral duct dilation or side-branch dilation in the upstream pancreas favors CP, due to traction effect of parenchymal fibrosis on the upstream healthy parenchyma[39,40]. In contrast, side-branches adjacent to malignancy are usually obliterated owing to mass-effect[39,41]. The presence of (pseudo)cysts and parenchymal or intraductal calcifications may suggest an inflammatory cause, although these findings can also sometimes be found in intraductal papillary mucinous neoplasms, especially with main duct involvement[39,42]. Peripheral displacement of calcifications can be seen in patients with a malignant mass in the background of CP[43]. Another imaging feature that can be used to differentiate between malignancy and inflammation is the extent of ductal dilation and parenchymal atrophy, expressed by the pancreatic duct-to-parenchyma ratio. A duct-to-parenchyma ratio ≥ 0.34 on EUS, indicating pronounced ductal dilation and parenchymal atrophy, is highly suggestive for PDAC instead of CP, with a diagnostic accuracy of 97%[44]. Signs that are more often observed in advanced PDAC compared to pancreatitis are vascular encasement by attenuating soft-tissue, caliber narrowing and occlusion[39]. Interestingly, a superior mesenteric artery-to-superior mesenteric vein ratio ≥ 1.0 favors the diagnosis of an malignancy[45]. Although not well-understood, enlargement of the superior mesenteric artery is probably caused by an increased resistance to blood flow in the pancreas of patients with PDAC, while in inflammatory conditions an increase in the diameter of the superior mesenteric vein can be observed secondary to the release of pancreatitis-induced vasoactive agents [39]. Imaging findings are summarized in Table 1.

Even though secondary imaging features can provide valuable information for the differentiation between pancreatitis and PDAC, none of them are 100% specific or sensitive. The two conditions may not only have overlapping clinical and imaging features but can also coexist, and the diagnostic performance of imaging modalities remains operator-dependent. Recent advances in machine learning-based diagnostic models may overcome part of these limitations. Preliminary studies using machine-learning methods reported promising results in differentiating inflammatory from malignant lesions on EUS but need to be further evolved before implementation in clinical practice[46,47].

Biomarkers

Serum carbohydrate antigen 19-9 (CA 19-9) is a widely used diagnostic tool for PDAC diagnosis. However, patients with CP often have elevation of serum CA 19-9 that is not indicative of malignancy but a result of pancreatic inflammation[48]. Therefore, the international consensus guideline do not recommend CA 19-9 as a diagnostic tool for PDAC in the CP population[33]. Over the last decades, several studies have investigated the use of other early predictive biomarkers (*i.e.* K-ras, interleukins, microRNAs and proteomics) in CP patients, however, no accurate biomarker is yet available for clinical use[49].

Many questions regarding the exact relative risk estimates of PDAC in CP patients remain, but most importantly, we need an adequate strategy for differentiation between CP and PDAC. Future research should devise the most optimal imaging methods and early detection biomarker to prevent misdiagnosis of PDAC as CP and, hence, delay of cancer diagnosis. As this delay compromises diagnosis of early-stage tumors and consequently resectable treatment options, optimizing diagnostic methods

Table 1 Imaging findings favoring an inflammatory or malignant cause[39]

Inflammatory cause	Malignant cause
Penetrating duct sign	Abrupt cutoff of the pancreatic duct
Collateral or side branch dilation	Upstream obliteration of side branches
Duct-to-parenchyma ratio < 0.34	Duct-to-parenchyma ratio ≥ 0.34
AIP: occasionally perivascular involvement	Vessel encasement and caliber changes
SMA-to-SMV ratio < 1.0	SMA-to-SMV ratio ≥ 1.0

AIP: Autoimmune pancreatitis; SMA: Superior mesenteric artery; SMV: Superior mesenteric vein.

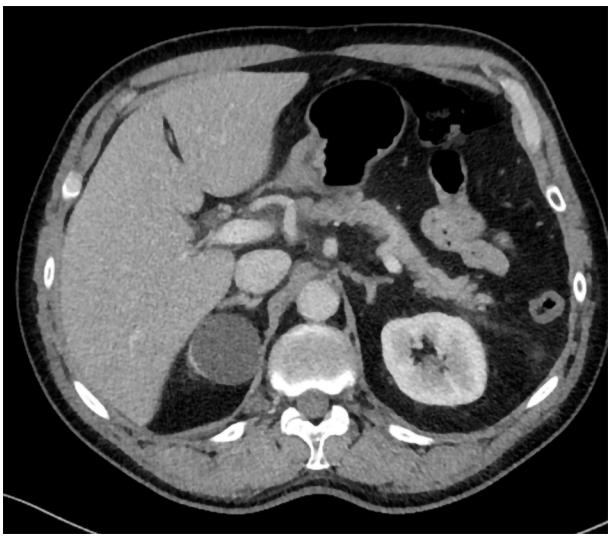


Figure 1 Abdominal computed tomography imaging of acute pancreatitis. Inflammation is present around the head of the pancreas.

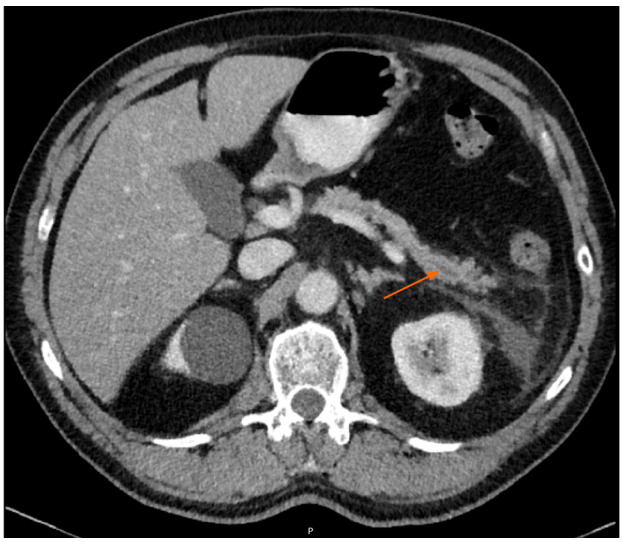


Figure 2 Abdominal computed tomography imaging of acute pancreatitis. In the pancreatic tail, a dilatation of the pancreatic duct can be observed.

is clinically relevant.

CONCLUSION

AP can be a first symptom of underlying PDAC, especially in patients with presumed idiopathic AP, between the ages of 56 and 75, and those who had a diagnosis of new-onset DM or CP. Additional imaging to exclude PDAC in these patients should at least

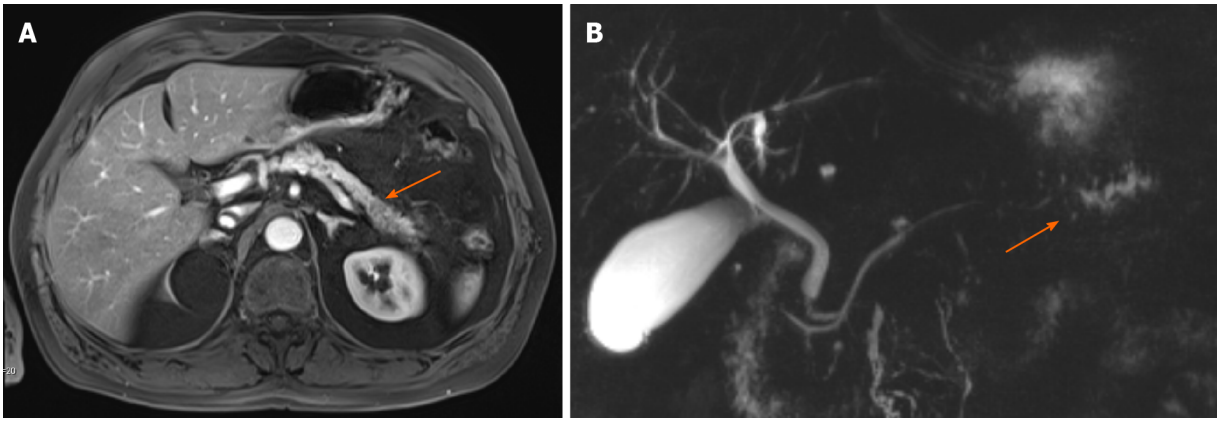


Figure 3 Abdominal magnetic resonance imaging of the pancreas and magnetic resonance cholangiopancreatography. A: Abdominal magnetic resonance imaging of the pancreas; B: Magnetic resonance cholangiopancreatography. A hypo-intense lesion (A, arrow) is causing a pancreatic duct stenosis with upstream dilatation of the pancreatic duct (B, arrow).

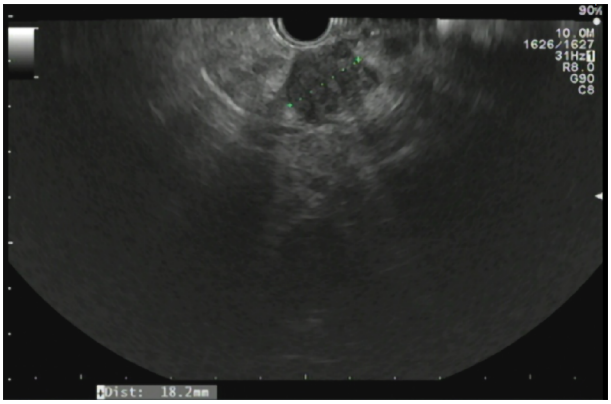


Figure 4 Endoscopic ultrasonography of the pancreas. A hypo-echoic lesion measuring 18.2 mm is present. Biopsy of this lesion revealed a pancreatic ductal adenocarcinoma.

be considered. EUS seems to be the preferred imaging modality.

CP, particularly hereditary CP, may lead to PDAC through oncogenic mutations caused by long-standing pancreatic inflammation, and CP patients may be exposed to overlapping risk factors for CP and PDAC. In patients with *PRSS1*-mediated CP or a history of autosomal dominant hereditary CP without known mutations, surveillance for PDAC can be considered, although the efficacy and modalities of surveillance are still up for debate.

Chronic pancreatic inflammation may present as a focal mass on imaging. Specific findings, such as the duct-penetrating sign (MRCP) and the duct-to-parenchyma ratio (EUS), may aid in the differentiation between pancreatitis and PDAC.

Currently, considerable effort is focused on finding a biomarker or machine-learning methods as a superior discriminant between CP and PDAC. Unfortunately, no clinically useful technique has yet emerged. Improving the possibility to differentiate between CP and PDAC, as well as identifying patients at risk of underlying or future PDAC, may give clinicians the opportunity to enhance the diagnostic process.

REFERENCES

1 **Roberts SE**, Morrison-Rees S, John A, Williams JG, Brown TH, Samuel DG. The incidence and aetiology of acute pancreatitis across Europe. *Pancreatology* 2017; **17**: 155-165 [PMID: 28159463 DOI: 10.1016/j.pan.2017.01.005]

2 **Lankisch PG**, Karimi M, Bruns A, Maisonneuve P, Lowenfels AB. Temporal trends in incidence and severity of acute pancreatitis in Lüneburg County, Germany: a population-based study. *Pancreatology* 2009; **9**: 420-426 [PMID: 19468248 DOI: 10.1159/000209217]

3 **Gluszek S**, Kozieł D. Prevalence and progression of acute pancreatitis in the Świętokrzyskie

Voivodeship population. *Pol Przegl Chir* 2012; **84**: 618-625 [PMID: 23399628]
4 **Liu J**, Wang Y, Yu Y. Meta-analysis reveals an association between acute pancreatitis and the risk of pancreatic cancer. *World J Clin Cases* 2020; **8**: 4416-4430 [PMID: 33083401 DOI: 10.12998/wjcc.v8.i19.4416]
5 **Ahmed Ali U**, Issa Y, Hagenaars JC, Bakker OJ, van Goor H, Nieuwenhuijs VB, Bollen TL, van Ramshorst B, Witteman BJ, Brink MA, Schaapherder AF, Dejong CH, Spanier BW, Heisterkamp J, van der Harst E, van Eijck CH, Besselink MG, Gooszen HG, van Santvoort HC, Boermeester MA; Dutch Pancreatitis Study Group. Risk of Recurrent Pancreatitis and Progression to Chronic Pancreatitis After a First Episode of Acute Pancreatitis. *Clin Gastroenterol Hepatol* 2016; **14**: 738-746 [PMID: 26772149 DOI: 10.1016/j.cgh.2015.12.040]
6 **Iodice S**, Gandini S, Maisonneuve P, Lowenfels AB. Tobacco and the risk of pancreatic cancer: a review and meta-analysis. *Langenbecks Arch Surg* 2008; **393**: 535-545 [PMID: 18193270 DOI: 10.1007/s00423-007-0266-2]
7 **Lynch SM**, Vrieling A, Lubin JH, Kraft P, Mendelsohn JB, Hartge P, Canzian F, Steplowski E, Arslan AA, Gross M, Helzlsouer K, Jacobs EJ, LaCroix A, Petersen G, Zheng W, Albanes D, Amundadottir L, Bingham SA, Boffetta P, Boutron-Ruault MC, Chanock SJ, Clipp S, Hoover RN, Jacobs K, Johnson KC, Kooperberg C, Luo J, Messina C, Palli D, Patel AV, Riboli E, Shu XO, Rodriguez Suarez L, Thomas G, Tjønneland A, Tobias GS, Tong E, Trichopoulos D, Virtamo J, Ye W, Yu K, Zeleniuch-Jacquette A, Bueno-de-Mesquita HB, Stolzenberg-Solomon RZ. Cigarette smoking and pancreatic cancer: a pooled analysis from the pancreatic cancer cohort consortium. *Am J Epidemiol* 2009; **170**: 403-413 [PMID: 19561064 DOI: 10.1093/aje/kwp134]
8 **Kolodecik T**, Shugrue C, Ashat M, Thrower EC. Risk factors for pancreatic cancer: underlying mechanisms and potential targets. *Front Physiol* 2013; **4**: 415 [PMID: 24474939 DOI: 10.3389/fphys.2013.00415]
9 **Minato Y**, Kamisawa T, Tabata T, Hara S, Kuruma S, Chiba K, Kuwata G, Fujiwara T, Egashira H, Koizumi K, Saito I, Endo Y, Koizumi S, Fujiwara J, Arakawa T, Momma K, Kurata M, Honda G. Pancreatic cancer causing acute pancreatitis: a comparative study with cancer patients without pancreatitis and pancreatitis patients without cancer. *J Hepatobiliary Pancreat Sci* 2013; **20**: 628-633 [PMID: 23494612 DOI: 10.1007/s00534-013-0598-y]
10 **Kirkegård J**, Cronin-Fenton D, Heide-Jørgensen U, Mortensen FV. Acute Pancreatitis and Pancreatic Cancer Risk: A Nationwide Matched-Cohort Study in Denmark. *Gastroenterology* 2018; **154**: 1729-1736 [PMID: 29432727 DOI: 10.1053/j.gastro.2018.02.011]
11 **Kirkegård J**, Mortensen FV, Heide-Jørgensen U, Cronin-Fenton D. Predictors of underlying pancreatic cancer in patients with acute pancreatitis: a Danish nationwide cohort study. *HPB (Oxford)* 2020; **22**: 553-562 [PMID: 31521499 DOI: 10.1016/j.hpb.2019.08.013]
12 **Sadr-Azodi O**, Oskarsson V, Discacciati A, Videhult P, Askling J, Ekbom A. Pancreatic Cancer Following Acute Pancreatitis: A Population-based Matched Cohort Study. *Am J Gastroenterol* 2018; **113**: 1711-1719 [PMID: 30315287 DOI: 10.1038/s41395-018-0255-9]
13 **Kitano M**, Yoshida T, Itonaga M, Tamura T, Hatamaru K, Yamashita Y. Impact of endoscopic ultrasonography on diagnosis of pancreatic cancer. *J Gastroenterol* 2019; **54**: 19-32 [PMID: 30406288 DOI: 10.1007/s00535-018-1519-2]
14 **Wan J**, Ouyang Y, Yu C, Yang X, Xia L, Lu N. Comparison of EUS with MRCP in idiopathic acute pancreatitis: a systematic review and meta-analysis. *Gastrointest Endosc* 2018; **87**: 1180-1188. e9 [PMID: 29225082 DOI: 10.1016/j.gie.2017.11.028]
15 **Löhr JM**, Dominguez-Munoz E, Rosendahl J, Besselink M, Mayerle J, Lerch MM, Haas S, Akisik F, Kartalis N, Iglesias-Garcia J, Keller J, Boermeester M, Werner J, Dumonceau JM, Fockens P, Drewes A, Ceyhan G, Lindkvist B, Drenth J, Ewald N, Hardt P, de Madaria E, Witt H, Schneider A, Manfredi R, Brøndum FJ, Rudolf S, Bollen T, Bruno M; HaPanEU/UEG Working Group. United European Gastroenterology evidence-based guidelines for the diagnosis and therapy of chronic pancreatitis (HaPanEU). *United European Gastroenterol J* 2017; **5**: 153-199 [PMID: 28344786 DOI: 10.1177/2050640616684695]
16 **Lévy P**, Domínguez-Muñoz E, Imrie C, Löhr M, Maisonneuve P. Epidemiology of chronic pancreatitis: burden of the disease and consequences. *United European Gastroenterol J* 2014; **2**: 345-354 [PMID: 25360312 DOI: 10.1177/2050640614548208]
17 **Etemad B**, Whitcomb DC. Chronic pancreatitis: diagnosis, classification, and new genetic developments. *Gastroenterology* 2001; **120**: 682-707 [PMID: 11179244 DOI: 10.1053/gast.2001.22586]
18 **Whitcomb DC**. Value of genetic testing in the management of pancreatitis. *Gut* 2004; **53**: 1710-1717 [PMID: 15479696 DOI: 10.1136/gut.2003.015511]
19 **Lowenfels AB**, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andrén-Sandberg A, Domellöf L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. *N Engl J Med* 1993; **328**: 1433-1437 [PMID: 8479461 DOI: 10.1056/NEJM199305203282001]
20 **Kirkegård J**, Mortensen FV, Cronin-Fenton D. Chronic Pancreatitis and Pancreatic Cancer Risk: A Systematic Review and Meta-analysis. *Am J Gastroenterol* 2017; **112**: 1366-1372 [PMID: 28762376 DOI: 10.1038/ajg.2017.218]
21 **Raimondi S**, Lowenfels AB, Morselli-Labate AM, Maisonneuve P, Pezzilli R. Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection. *Best Pract Res Clin Gastroenterol* 2010; **24**: 349-358 [PMID: 20510834 DOI: 10.1016/j.bpg.2010.02.007]

22 **Tong GX**, Geng QQ, Chai J, Cheng J, Chen PL, Liang H, Shen XR, Wang DB. Association between pancreatitis and subsequent risk of pancreatic cancer: a systematic review of epidemiological studies. *Asian Pac J Cancer Prev* 2014; **15**: 5029-5034 [PMID: 24998582 DOI: 10.7314/apjcp.2014.15.12.5029]

23 **Lowenfels AB**, Maisonneuve P, DiMagno EP, Elitsur Y, Gates LK Jr, Perrault J, Whitcomb DC. Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group. *J Natl Cancer Inst* 1997; **89**: 442-446 [PMID: 9091646 DOI: 10.1093/jnci/89.6.442]

24 **Howes N**, Lerch MM, Greenhalf W, Stocken DD, Ellis I, Simon P, Truninger K, Ammann R, Cavallini G, Charnley RM, Uomo G, Delhaye M, Spicak J, Drumm B, Jansen J, Mountford R, Whitcomb DC, Neoptolemos JP; European Registry of Hereditary Pancreatitis and Pancreatic Cancer (EUROPAC). Clinical and genetic characteristics of hereditary pancreatitis in Europe. *Clin Gastroenterol Hepatol* 2004; **2**: 252-261 [PMID: 15017610 DOI: 10.1016/s1542-3565(04)00013-8]

25 **Chari ST**, Mohan V, Pitchumoni CS, Viswanathan M, Madanagopalan N, Lowenfels AB. Risk of pancreatic carcinoma in tropical calcifying pancreatitis: an epidemiologic study. *Pancreas* 1994; **9**: 62-66 [PMID: 8108373 DOI: 10.1097/00006676-199401000-00009]

26 **Yadav D**, Lowenfels AB. The epidemiology of pancreatitis and pancreatic cancer. *Gastroenterology* 2013; **144**: 1252-1261 [PMID: 23622135 DOI: 10.1053/j.gastro.2013.01.068]

27 **Korpela T**, Udd M, Mustonen H, Ristimäki A, Haglund C, Seppänen H, Kylänpää L. Association between chronic pancreatitis and pancreatic cancer: A 10-year retrospective study of endoscopically treated and surgical patients. *Int J Cancer* 2020; **147**: 1450-1460 [PMID: 32162688 DOI: 10.1002/ijc.32971]

28 **Jeon CY**, Chen Q, Yu W, Dong EY, Chung J, Pandol SJ, Yadav D, Conwell DL, Wu BU. Identification of Individuals at Increased Risk for Pancreatic Cancer in a Community-Based Cohort of Patients With Suspected Chronic Pancreatitis. *Clin Transl Gastroenterol* 2020; **11**: e00147 [PMID: 32352677 DOI: 10.14309/ctg.0000000000000147]

29 **Lüttges J**, Diederichs A, Menke MA, Vogel I, Kremer B, Klöppel G. Ductal lesions in patients with chronic pancreatitis show K-ras mutations in a frequency similar to that in the normal pancreas and lack nuclear immunoreactivity for p53. *Cancer* 2000; **88**: 2495-2504 [PMID: 10861425 DOI: 10.1002/1097-0142(20000601)88:11<2495::aid-cncr10>3.0.co;2-b]

30 **Maire F**, Micard S, Hammel P, Voitot H, Lévy P, Cugnenc PH, Ruszniewski P, Puig PL. Differential diagnosis between chronic pancreatitis and pancreatic cancer: value of the detection of KRAS2 mutations in circulating DNA. *Br J Cancer* 2002; **87**: 551-554 [PMID: 12189555 DOI: 10.1038/sj.bjc.6600475]

31 **Kong X**, Sun T, Kong F, Du Y, Li Z. Chronic Pancreatitis and Pancreatic Cancer. *Gastrointest Tumors* 2014; **1**: 123-134 [PMID: 26674754 DOI: 10.1159/000365306]

32 **Munigala S**, Kanwal F, Xian H, Agarwal B. New diagnosis of chronic pancreatitis: risk of missing an underlying pancreatic cancer. *Am J Gastroenterol* 2014; **109**: 1824-1830 [PMID: 25286967 DOI: 10.1038/ajg.2014.318]

33 **Greenhalf W**, Lévy P, Gress T, Rebours V, Brand RE, Pandol S, Chari S, Jørgensen MT, Mayerle J, Lerch MM, Hegyi P, Kleeff J, Castillo CF, Isaji S, Shimosegawa T, Sheel A, Halloran CM, Garg P, Takaori K, Besselink MG, Forsmark CE, Wilcox CM, Maisonneuve P, Yadav D, Whitcomb D, Neoptolemos J; Working group for the International (IAP – APA – JPS – EPC) Consensus Guidelines for Chronic Pancreatitis. International consensus guidelines on surveillance for pancreatic cancer in chronic pancreatitis. Recommendations from the working group for the international consensus guidelines for chronic pancreatitis in collaboration with the International Association of Pancreatology, the American Pancreatic Association, the Japan Pancreas Society, and European Pancreatic Club. *Pancreatology* 2020; **20**: 910-918 [PMID: 32624419 DOI: 10.1016/j.pan.2020.05.011]

34 **Kennedy T**, Preczewski L, Stocker SJ, Rao SM, Parsons WG, Wayne JD, Bell RH, Talamonti MS. Incidence of benign inflammatory disease in patients undergoing Whipple procedure for clinically suspected carcinoma: a single-institution experience. *Am J Surg* 2006; **191**: 437-441 [PMID: 16490563 DOI: 10.1016/j.amjsurg.2005.10.051]

35 **Gandhi NS**, Feldman MK, Le O, Morris-Stiff G. Imaging mimics of pancreatic ductal adenocarcinoma. *Abdom Radiol (NY)* 2018; **43**: 273-284 [PMID: 29038855 DOI: 10.1007/s00261-017-1330-1]

36 **Ichikawa T**, Sou H, Araki T, Arbab AS, Yoshikawa T, Ishigame K, Haradome H, Hachiya J. Duct-penetrating sign at MRCP: usefulness for differentiating inflammatory pancreatic mass from pancreatic carcinomas. *Radiology* 2001; **221**: 107-116 [PMID: 11568327 DOI: 10.1148/radiol.2211001157]

37 **Raman SP**, Salaria SN, Hruban RH, Fishman EK. Groove pancreatitis: spectrum of imaging findings and radiology-pathology correlation. *AJR Am J Roentgenol* 2013; **201**: W29-W39 [PMID: 23789694 DOI: 10.2214/AJR.12.9956]

38 **Lekkerkerker SJ**, Nio CY, Issa Y, Fockens P, Verheij J, Busch OR, van Gulik TM, Rauws EA, Boermeester MA, van Hooft JE, Besselink MG. Clinical outcomes and prevalence of cancer in patients with possible groove pancreatitis. *J Gastroenterol Hepatol* 2016; **31**: 1895-1900 [PMID: 26997497 DOI: 10.1111/jgh.13376]

39 **Wolske KM**, Ponnatapura J, Kolokythas O, Burke LMB, Tappouni R, Lalwani N. Chronic Pancreatitis or Pancreatic Tumor? *Radiographics* 2019; **39**: 1965-1982 [PMID: 31584860 DOI: 10.1148/rg.2019190011]

40 **Lin E**, Alexander D. Focal Chronic Pancreatitis vs Pancreatic Cancer. In: Lin EC, Escott EJ, Garg KD, Bleicher AG, Alexander D. Practical Differential Diagnosis for CT and MRI. New York: Thieme, 2008: 212

41 **Guarise A**, Faccioli N, Morana G, Megibow AJ. Chronic Pancreatitis vs Pancreatic Tumors. In: Balthazar EJ, Megibow AJ, Pozzi Mucelli R. Imaging of the Pancreas. Berlin, Germany: Springer, 2009: 329-369

42 **Perez-Johnston R**, Narin O, Mino-Kenudson M, Ingkakul T, Warshaw AL, Fernandez-Del Castillo C, Sahani VD. Frequency and significance of calcification in IPMN. *Pancreatology* 2013; **13**: 43-47 [PMID: 23395569 DOI: 10.1016/j.pan.2012.11.306]

43 **Schima W**, Böhm G, Rösch CS, Klaus A, Függer R, Kopf H. Mass-forming pancreatitis *vs* pancreatic ductal adenocarcinoma: CT and MR imaging for differentiation. *Cancer Imaging* 2020; **20**: 52 [PMID: 32703312 DOI: 10.1186/s40644-020-00324-z]

44 **Eloubeidi MA**, Luz LP, Tamhane A, Khan M, Buxbaum JL. Ratio of pancreatic duct caliber to width of pancreatic gland by endosonography is predictive of pancreatic cancer. *Pancreas* 2013; **42**: 670-679 [PMID: 23271395 DOI: 10.1097/MPA.0b013e31827305b8]

45 **Elmas N**, Oran I, Oyar O, Ozer H. A new criterion in differentiation of pancreatitis and pancreatic carcinoma: artery-to-vein ratio using the superior mesenteric vessels. *Abdom Imaging* 1996; **21**: 331-333 [PMID: 8661577 DOI: 10.1007/s002619900075]

46 **Săftoiu A**, Vilmann P, Gorunescu F, Gheonea DI, Gorunescu M, Ciurea T, Popescu GL, Iordache A, Hassan H, Iordache S. Neural network analysis of dynamic sequences of EUS elastography used for the differential diagnosis of chronic pancreatitis and pancreatic cancer. *Gastrointest Endosc* 2008; **68**: 1086-1094 [PMID: 18656186 DOI: 10.1016/j.gie.2008.04.031]

47 **Săftoiu A**, Vilmann P, Gorunescu F, Janssen J, Hocke M, Larsen M, Iglesias-Garcia J, Arcidiacono P, Will U, Giovannini M, Dietrich CF, Havre R, Gheorghe C, McKay C, Gheonea DI, Ciurea T; European EUS Elastography Multicentric Study Group. Efficacy of an artificial neural network-based approach to endoscopic ultrasound elastography in diagnosis of focal pancreatic masses. *Clin Gastroenterol Hepatol* 2012; **10**: 84-90. e1 [PMID: 21963957 DOI: 10.1016/j.cgh.2011.09.014]

48 **Furuya N**, Kawa S, Hasebe O, Tokoo M, Mukawa K, Maejima S, Oguchi H. Comparative study of CA242 and CA19-9 in chronic pancreatitis. *Br J Cancer* 1996; **73**: 372-376 [PMID: 8562344 DOI: 10.1038/bjc.1996.63]

49 **Ballehaninna UK**, Chamberlain RS. Biomarkers for pancreatic cancer: promising new markers and options beyond CA 19-9. *Tumour Biol* 2013; **34**: 3279-3292 [PMID: 23949878 DOI: 10.1007/s13277-013-1033-3]

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REVIEW

Pancreatic adenocarcinoma: A review of recent paradigms and advances in epidemiology, clinical diagnosis and management

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Abstract

Pancreatic cancer is one of the dreaded malignancies for both the patient and the clinician. The five-year survival rate of pancreatic adenocarcinoma (PDA) is as low as 2% despite multimodality treatment even in the best hands. As *per* the Global Cancer Observatory of the International Agency for Research in Cancer estimates of pancreatic cancer, by 2040, a 61.7% increase is expected in the total number of cases globally. With the widespread availability of next-generation sequencing, the entire genome of the tumors is being sequenced regularly, providing insight into their pathogenesis. As invasive PDA arises from pancreatic intraepithelial neoplasia and mucinous neoplasm and intraductal papillary neoplasm, screening for them can be beneficial as the disease is curable with resection at an early stage. Routine preoperative biliary drainage has no role in patients suffering from PDA with obstructive jaundice. If performed, metallic stents are preferred over plastic ones. Minimally invasive procedures are preferred to open procedures as they have less morbidity. The duct-to-mucosa technique for pancreaticojejunostomy is presently widely practiced. The role of intraperitoneal drains after surgery for PDA is controversial. Neoadjuvant chemoradiotherapy has been proven to have a significant role both in locally advanced as well as in resectable PDA. Many new regimens and drugs have been added in the arsenal of chemoradiotherapy for metastatic disease. The roles of immunotherapy and gene therapy in PDA are being investigated. This review article is intended to improve the understanding of the readers with respect to the latest updates of PDA, which may help to trigger new research ideas and make better management decisions.

Key Words: Pancreatic adenocarcinoma; Pancreatic cancer; Chemotherapy; Chemoradiotherapy; Pancreaticoduodenectomy; Distal pancreatectomy

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Core Tip: Pancreatic cancer is one of the dreaded malignancies for both patients and clinicians. This narrative review highlights the newer trends and achievements in the epidemiology, etiopathogenesis, screening, diagnosis and management of pancreatic adenocarcinoma (PDA). It is intended to improve the readers' understanding of the latest updates of PDA, which may help to trigger new research ideas and make better management decisions. Newer screening and diagnostic techniques will help in diagnosing the patients in early stages and prognosticate them better. The newer discoveries in drugs and management protocols will help increase the survival and quality of life of the patients.

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INTRODUCTION

Pancreatic cancer is one of the dreaded malignancies for both patients and clinicians. For patients, it is associated with a poor survival rate and decreased quality of life due to local invasion and complications, and for the clinician, it is challenging to diagnose at an early stage and treat.

Pancreatic cancer is the third leading cause of cancer deaths in the United States of America and the seventh leading cause worldwide as *per* the 2018 GLOBACON data[1,2]. It may arise from either the exocrine or the endocrine pancreas with the former being far more common than the latter. Pancreatic adenocarcinoma (PDA) and its subtypes constitute more than 90% of pancreatic tumors[3]. Most patients with PDA present at an advanced stage, which makes curative treatment virtually impossible[4]. The five-year survival rate of PDA is as low as 2% despite multimodality treatment even in the best hands[5]. The high mortality and morbidity associated with PDA have stimulated researchers all over the world to intensify the search for better diagnostic and treatment protocols.

Clinically, patients with PDA present to the healthcare facility with symptoms only in the advanced stage[6]. Early lesions have a good prognosis but are clinically silent. Diagnosis of an early-stage PDA is rare as there are no effective and reliable screening tools or investigations at present[7]. Surgery is the mainstay of treatment if therapy is planned with curative intention in PDA patients[8]. Patients with PDA from the surgical point of view are classified into resectable, borderline resectable, unresectable and metastatic disease categories at diagnosis. Chemotherapy and radiotherapy remain the backbone of the treatment for PDA with almost all patients requiring some form of chemoradiotherapy for curative or palliative purposes[9-11].

In view of the above facts, in the present narrative review, the authors have reviewed the latest trends in the epidemiology, diagnosis and management of PDA based on the published English literature so far. We have searched the literature using the keyword "PDA". This review article is intended to improve the understanding of the readers regarding the latest updates of PDA, which may help to trigger new research ideas and promote better management decisions.

PARADIGMS IN EPIDEMIOLOGY

In the last decade, there has been a steady increase in the incidence and mortality of PDA across the globe for both males and females (Figure 1)[12,13]. Its incidence was higher among males when compared to females and continues to be so[1,12-14]. PDA constitutes about 2.5% of the total cancers diagnosed worldwide. The 2020 estimated crude incidence rate and age-standardized rate (ASR) of pancreatic cancer are 6.4% and 4.9%, respectively[1]. The ASR of pancreatic cancer is the highest in North America (8%) and Europe (7.8%) and lowest in Africa (2.3%)[1]. The incidence rate is also very high in countries with a high human development index (HDI) when

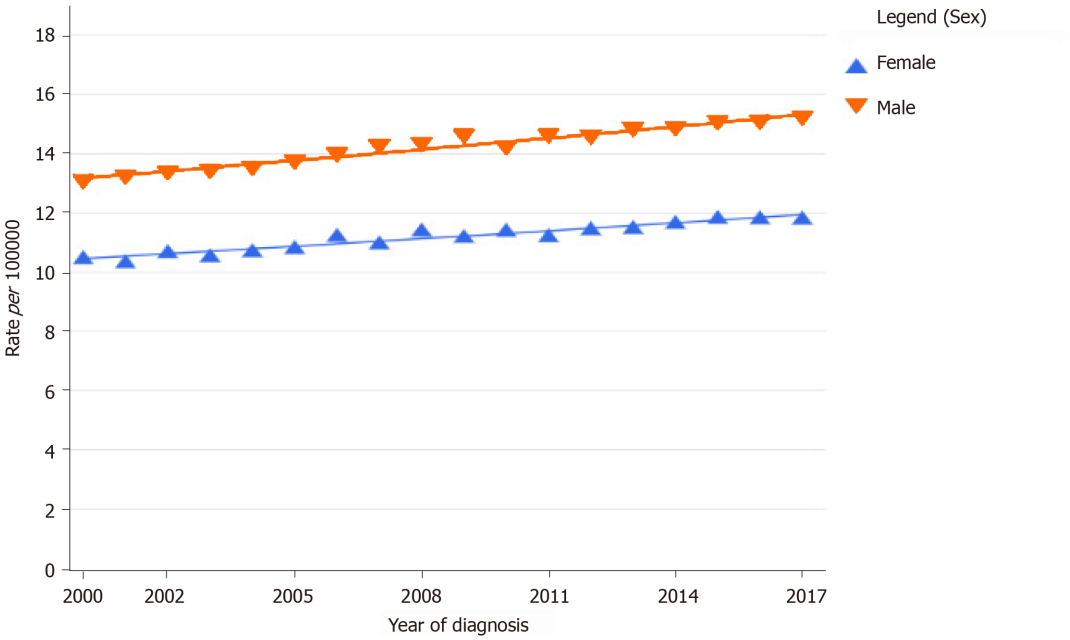


Figure 1 Pancreas cancer-recent trends in surveillance, epidemiology, and end results age-adjusted incidence rates, 2000-2017[12]**.** Used with permission from National Cancer Institute.

compared to countries with low HDI (Figure 2)[1]. This difference can be attributed to the variation in the tobacco smoking, alcohol consumption and obesity rates among different countries[15-18].

The Surveillance, Epidemiology, and End Results Program of the National Cancer Institute reveals an age-specific trend in the increase of the incidence rate of pancreatic cancer in the age groups of 20-29 years and > 80 years in the United States[19]. The increase also varied with the stage of the disease at diagnosis. The last decade has shown a higher increase in the age-adjusted incidence rates of early and localized pancreatic cancer when compared to advanced disease. The age-adjusted incidence rate of metastatic disease has declined[12] (Figure 3). When the American Joint Committee on Cancer Tumor-Node-Metastasis staging was compared with the age-adjusted incidence rate, there was an increase in the stage I and II patients and a simultaneous decline in the stage III and IV patients at diagnosis, indicating that the evolving techniques of screening and diagnosis of pancreatic cancer are showing some result[14].

As *per* the Global Cancer Observatory of the International Agency for Research in Cancer estimates of pancreatic cancer, by 2040, a 61.7% increase is expected in the total number of cases globally. The most notable trend expected is the rapid increase in the number of cases from Africa (an increase of 100.1%) followed by Asia (81.5%). The expected increase from Europe and North America is less (Table 1)[1]. The mortality rates also follow the incidence rates with the expected increase in the mortality due to pancreatic cancer highest in Africa (Figure 4). There is an expected regional variation in the incidence among male and female sex in various continents[1,13]. The temporal trends of incidence and mortality of pancreatic cancer in various continents could be attributed to the temporal trends in tobacco smoking[15,20,21]. Anti-tobacco measures, which are being strongly advocated in developed countries, could be one of the main reasons for declining incidence rates[22]. On the other hand, the rise in the incidence and mortality rates in the developing and under-developed countries is a cause of concern and may be attributed to the lifestyle changes adopted as well as the impact of socioeconomic conditions.

Based on the present and expected trends of pancreatic cancer, there must be a widespread endorsement of healthy lifestyle practices and strong anti-tobacco laws. If not, the developing countries will have to bear the major brunt of the disease in the near future as the diagnostic and treatment services are still under-developed in these countries.

NEWER CONCEPTS IN ETIOPATHOGENESIS

PDA is recently being called a genetic disease. With the widespread availability of next-generation sequencing, the entire genome of the tumors is being sequenced

Table 1 The expected increase in the number of new cases among various continents by 2040[1]

Population	Number of new cases		Change in number of cases	Change in number of cases due to population
	2020	2040		
Africa	17070	34165	+ 100.1%	+ 100.1%
Asia	233701	424138	+ 81.5%	+ 81.5%
Europe	140116	178438	+ 27.4%	+ 27.4%
Latin America and Caribbean	37352	67836	+ 81.6%	+ 81.6%
Northern America	62643	89124	+ 42.3%	+ 42.3%
Oceania	4891	7933	+ 62.2%	+ 62.2%
Totals	495773	801634	+ 61.7%	+ 61.7%

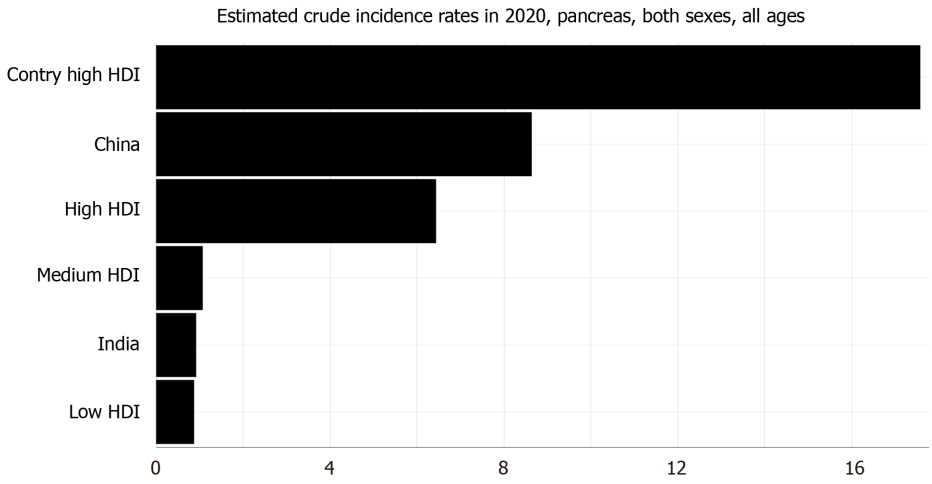


Figure 2 Estimated crude incidence rates in 2020, pancreas, both sexes, all ages as *per* human development index[1]. Used with permission from International Agency for Research on Cancer. HDI: Human development index.

regularly, providing insight into its pathogenesis. PDA has about 60 genetic alterations *per* tumor. The most important finding differentiating PDA from other cancers is the heterogeneity of the genome of each patient, meaning that each patient has a tumor with a specific genomic signature[23,24]. Four predominant genes have been identified in PDA. They are *K-ras, CDKN2A, TP53* and *SMAD4*. There are many other genes identified, but they are mutated at a lower frequency (less than 10%)[23].

The *K-ras* oncogene is the most common gene mutation in PDA with an incidence of more than 90%[25]. Due to its high frequency of mutation, it is believed that the tumor pathogenesis revolves predominantly around the molecular pathways regulated by this gene and forms the basis for research on *K-ras* inhibitors. However, *K-ras* inhibitors were associated with high in-vivo toxicity[26]. *CDKN2A* gene is involved in the regulation of *RB1* and plays an important role in the G1/S checkpoint inhibition of the cell cycle[27]. *TP53* is the predominant DNA repair pathway gene and induces cell cycle arrest at G1 or G2 checkpoint[28]. *SMAD4* gene is a part of the transforming growth factor β pathway and regulates the G1/S checkpoint of cell cycle[28]. The group of genes involved in the genome maintenance DNA repair pathway (*BRCA2, PALB2, FANCC, FANCG*) constitute less than 10% of the mutated genes in PDA but are important as tumors deficient in these genes can be targeted with DNA damaging agents and poly ADP-ribose pathway inhibitor therapy[23,24,29]. Other gene mutations are involved in the regulation of pathways such as *Ras*, cell cycle regulators, WNT pathway and NOTCH pathway[23,24].

Invasive PDA arises from pancreatic intraepithelial neoplasia (PanIN). The lesions of PanIN progress from PanIN1 to PanIN3 by acquiring progressive mutations as shown in Table 2[30]. PanINs are also associated with lobulocentric acinar atrophy and local pancreatic inflammation secondary to obstruction of small duct secretions[31]. The time required from the onset of PanIN till progression to invasive carcinoma is about 10 years suggesting a significant lead time if PanIN are screened and detected

Table 2 The histological features and genetic alterations in pancreatic intraepithelial neoplasia

	PanIN-1	PanIN-2	PanIN-3
Histology	Columnar epithelial cells with basally oriented uniform and round nuclei	More nuclear changes such as loss of nuclear polarity, pleomorphism, hyperchromasia and nuclear pseudostratification	Cribriform pattern, budding cells into lumen and nuclear changes
Genetic changes	*K-Ras* mutations	*CDKN2A* mutations	*TP53* loss
	Telomere shortening		*SMAD4* loss
			BRCA2 loss

PanIN: Pancreatic intraepithelial neoplasia.

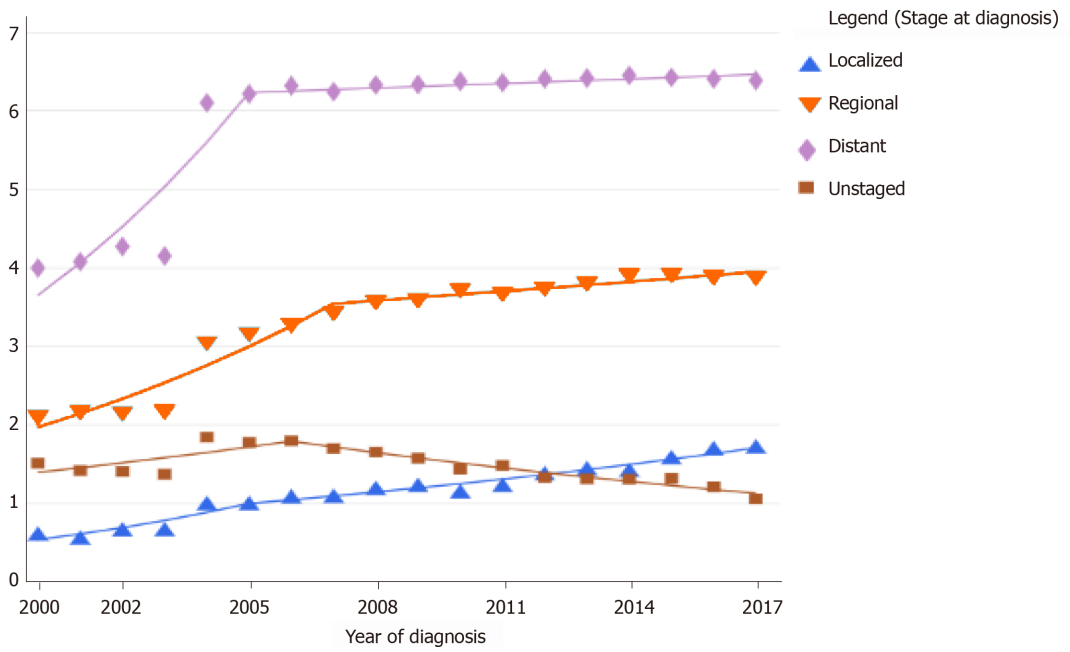


Figure 3 Pancreas cancer-recent trends in surveillance, epidemiology, and end results age-adjusted incidence rates as *per* stage at diagnosis, 2000-2017[12]**.** Used with permission from National Cancer Institute.

early. The proteins expressed by PanIN and invasive carcinoma are similar, which can be used for screening these lesions[32-35]. The high-grade PanINs express mucins such as MUC1, MUC4, MUC5AC, and MUC6[36-38]. These can be used for screening and in the treatment of PanIN[39,40].

Based on the transcriptome analysis, PDA is divided into molecular subtypes[41]. The three subtypes named initially were classical, quasimesenchymal and exocrine-like, which have been modified as progenitor, squamous and aberrantly differentiated endocrine exocrine types, respectively, by the International Cancer Genome Consortium study[41]. An immunogenic subtype was also identified. The squamous subtype was associated with a poor prognosis similar to the basal subtype carcinomas of other organs and has a poor response to chemoradiotherapy and also exhibits *TP53* mutations more frequently[41]. Recently, RNA-based sequencing of PDA revealed the heterogeneity of the tumor at the molecular level[42]. RNA-based subgrouping may add further insights into the pathogenesis and tumor progression. The desmoplastic stroma is the predominant component of the PDA. Recent transcriptome analysis of the stromal cells revealed two subtypes similar to PDA cells–normal subtype and activated subtype[43]. The normal subtype resembles pancreatic stellate cells, and the inflammatory subtype has immunogenic signatures. The gene expression is also different for the two subtypes leading to the inference of the role of stromal and neoplastic cell interaction in determining the tumor heterogeneity[44].

The neoplastic cells of PDA are acclimatized to survive in a microenvironment of depleted oxygen and nutrients due to the poor vascularity and intense desmoplastic stroma of the tumor. The *K-ras* mutation up-regulates several metabolic pathways such

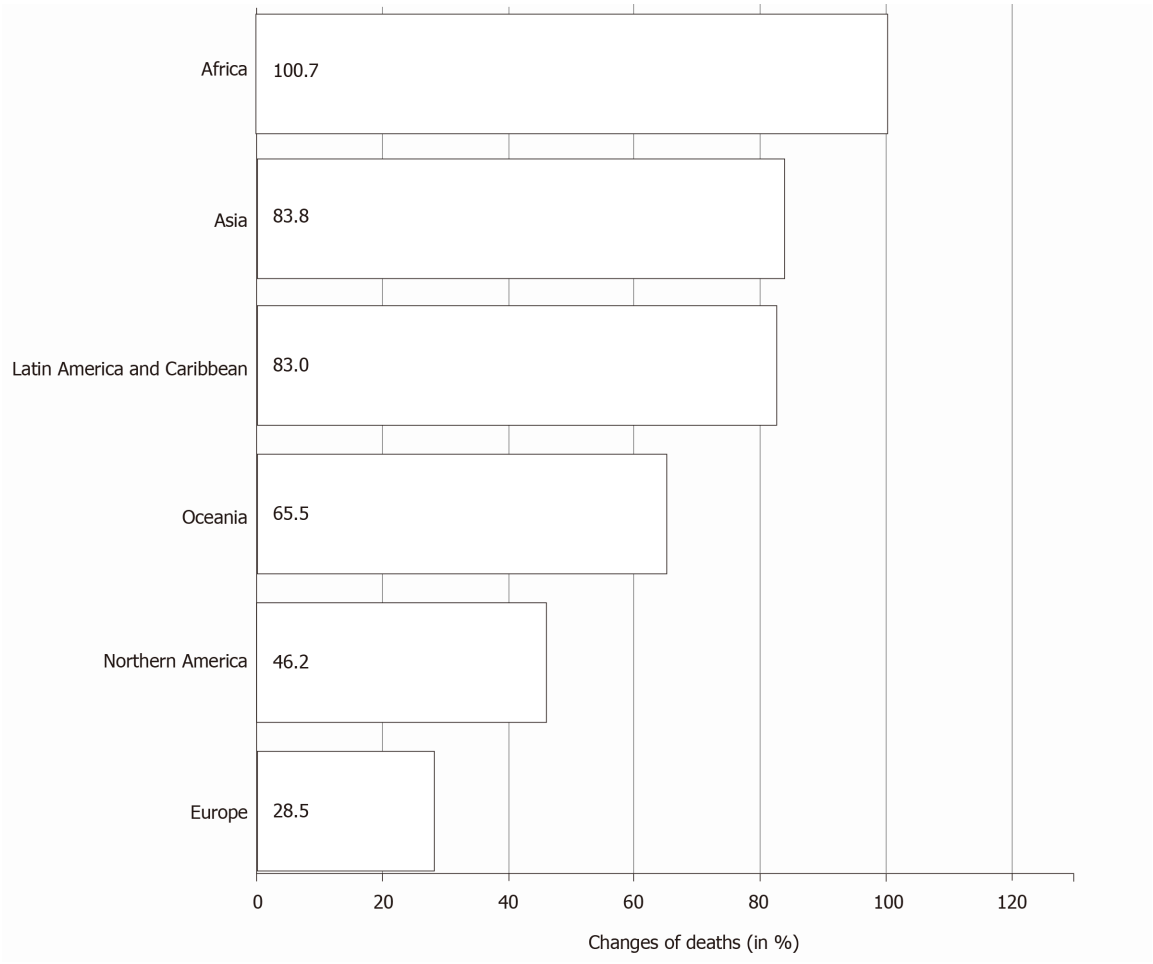


Figure 4 Projected Changes of deaths due to pancreatic cancer from 2020 to 2040, both sexes, age (0-85+)[1]**.** Used with permission from International Agency for Research on Cancer.

as glucose uptake and glycolysis[23]. Apart from the metabolic adaptations, cells survive by autophagy, mitophagy and macropinocytosis stimulated by the *K-ras* gene [24]. Genetic or pharmacological inhibition of autophagy leads to decreased tumor growth as seen in mouse models. Co-targeting with mitogen-activated protein kinase kinase/extracellular signal-regulated kinase inhibitors is an area of intense research in the treatment of pancreatic cancer[45,46]. Furthermore, the deregulation of these metabolic pathways is one of the reasons for resistance to chemotherapy in PDA[47-49].

RISK FACTORS

Risk factors for PDA can be divided into modifiable and non-modifiable. Smoking has been proven beyond doubt to be the main modifiable risk factor. The risk is approximately twice in smokers, and they are at risk even after smoking cessation for about 20 years[50,51]. Alcohol is an additional risk factor in smokers but not in non-smokers [52]. Obesity is clearly associated with an increased risk of PDA as well as mortality [53]. Dietary factors such as consumption of red meat and processed foods are associated with an increased risk of PDA while consumption of fresh fruits and folate is protective[54,55]. Occupational exposure to nickel, cadmium and chlorinated biphenyls is associated with an increased risk[56].

Among the nonmodifiable risk factors, male sex, increasing age and African-American ethnicity are associated with an increased risk[13]. Genetic factors play an important role as 10% of the PDAs have a family history[57,58]. Mutations in the genes such as *BRCA2, PALB2, STK11, CDKN2A, APC*, Lynch syndrome genes, *ATM, FANCC* and *FANCG* are responsible for the familial causes of PDA[59]. Chronic pancreatitis is a risk factor for PDA, particularly chronic pancreatitis due to hereditary pancreatitis (*PRSS1/SPINK1* gene mutation)[60]. Diabetes type 1 and 2, particularly recent-onset

diabetes, are associated with an increased risk of PDA. However, the causal association was not proved and is a matter of debate as diabetes may be a manifestation of PDA[61]. The risk of PDA decreases with an increased duration of diabetes [62]. The association of *Helicobacter pylori* infection and PDA was proved in a meta-analysis[63]. There is also growing evidence of association of PDA with chronic diseases such as hepatitis B and C[64]. Recent studies have also proved the association between non-O blood group and PDA[64,65].

RECENT ADVANCES IN SCREENING AND DIAGNOSIS

As invasive PDA arises from PanIN and from mucinous neoplasm and intraductal papillary neoplasm (IPMN) screening for these lesions can be beneficial as the disease can be cured with resection at an early stage. However, till now there is no approved and reliable screening test for PDA[66,67]. PDA is a cancer of comparatively low prevalence but with high mortality. Due to the non-availability of any standard, economical and reliable screening test, screening the entire general population for PDA is not possible. However, patients with a family history of pancreatic cancer have an increased risk of PDA and benefit from screening[66]. Screening is particularly recommended for those with at least two first-degree relatives with PDA or in patients with known familial syndromes. Even though IPMNs are visible on conventional imaging, PanIN are very small lesions of size less than 5 mm and are not identified on routine imaging studies[66]. Hence, we need to rely on biomarkers alone or in combination with imaging studies for screening.

The most commonly used biomarker is carbohydrate antigen (CA) 19-9, which is sialylated Lewis blood group antigen on MUC1 expressed by neoplastic cells of PDA and also by the normal cells of the pancreaticobiliary system, stomach, colon, endometrium and salivary glands[68]. It is elevated in only 65% of resectable pancreatic cancers and hence of low sensitivity[69]. It is also elevated in benign diseases of the biliary tract, biliary obstruction and also in malignancies arising from other organs, and hence it is also less specific[69,70]. CA19-9 cannot be used as a tumor marker in populations who do not express Lewis antigen (4%-15%)[66,69,70]. Therefore, it is primarily used to assess the response to treatment and in the follow-up of patients diagnosed with PDA[69,70]. Carcinoembryonic antigen (CEA) in the pancreatic juice can also be used to screen pancreatic cancer with reasonable accuracy [71,72]. However, the main limiting factor of CEA is the low sensitivity although specificity is high[71,72].

PAM4 is an anti MUC1 antibody, which is directed specifically against an epitope of MUC1 secreted by the pancreatic cancer cells absent in normal pancreas and other tissues and therefore found to be more specific and sensitive than CA19-9 in differentiating pancreatitis and pancreatic cancer[73]. Patients with advanced disease had higher values of PAM4 compared to early stages[74]. PAM4 can also be used to screen early lesions of PDA such as PanINs and IPMNs as the expression begins at an early stage of the disease and continues throughout[75]. The role of several new biomarkers such as CA494, CA50, CA242, CEA -related cell adhesion molecule 1, CAM 17.1-Ab, parathyroid hormone-related oncoprotein and serum beta-human chorionic gonadotropin in diagnosing early pancreatic lesions is growingly evident[70,76-80]. In a meta-analysis of seven studies evaluating the role of tumor M2-pyruvate kinase in screening pancreatic cancer, the conclusion was that the efficacy of tumor M2-pyruvate kinase was similar to CA19-9[81]. SPan-1 is also one of the markers studied for the diagnosis of exocrine pancreatic cancer, but it did not improve the rates when combined with CA19-9[82].

Various genetic and epigenetic mutations can be detected in the pancreatic juice obtained by endoscopic ultrasound (EUS) and endoscopic retrograde cholangiopancreticography[83]. *K-ras* mutations and *TP53* mutations identified in the pancreatic juice are associated with low specificity and sensitivity even though they are mutated in most of the PDAs[84,85]. DNA methylation abnormalities of a panel of genes were associated with a sensitivity of 82% and a specificity of 100% in identifying pancreatic cancer in a study[86]. Identification of mitochondrial mutations in the pancreatic juice is also being studied in diagnosing pancreatic cancer[87]. The main drawback of the above-mentioned tests is the requirement of invasive intervention to obtain the sample. Micro-RNAs are being intensely investigated in diagnosing various human cancers including pancreatic cancer[88]. Circulating tumor cells are also present in 47% of patients with PDA and are also associated with early lesions.

Multi-detector computerized tomography (MDCT) is presently the gold standard for the diagnosis of pancreatic lesions[89,90]. It is the virtual eye of a surgeon to assess the resectability of the tumor and also to accurately stage the disease[91,92]. The major drawback is the low sensitivity of MDCT in identifying lesions less than 2 cm and negligible sensitivity for identifying the pre-invasive lesions. Also, the routine use of MDCT for screening purposes in high-risk patients may increase the risk of radiation-induced secondary tumors. Hence, MDCT is preferably not used for the purpose of screening high-risk individuals and is comparatively inferior to EUS for the same[93]. Magnetic resonance imaging (MRI) is a non-ionizing investigation, which can image the entire abdomen as opposed to EUS. MRCP can provide very accurate details of the biliary and pancreatic ductal system and can identify small cystic lesions such as IPMNs. In fact, MRCP is proven to be superior to MDCT in detecting these lesions[93, 94]. In a prospective study evaluating the role of MRI in the screening of patients with *P16* mutations, MRI was able to identify early lesions[95].

EUS plays an important role in the screening and diagnosis of PDA. EUS was shown to be superior to MDCT, MRI and positron emission tomography (PET) in detecting small lesions and lymph node involvement[96]. It was able to detect twice the number of lesions compared to MDCT and MRI when used for screening[94]. It can also be used for biopsying a suspected lesion. The major drawback is the invasiveness of the procedure and its operator dependence. It may also be associated with rare but severe complications such as iatrogenic gastrointestinal perforation. EUS is usually performed in sequence with biomarker tests (in those with elevated CA19-9) or after a basic imaging test rather than as a first option[66,97].

PET scan is one of the valuable investigations in PDA with good sensitivity but an average specificity. In two meta-analyses from Tang *et al*[98] and Wu *et al*[99], the pooled sensitivities were 90.1% and 87%, respectively, and pooled specificities were 80.1% and 83%, respectively[98,99]. The low specificity is because of the inability of the PET scan to differentiate between inflammatory lesions and neoplastic lesions[100]. The role of the PET scan in determining the T stage is limited and is surpassed by MDCT[101]. Even though the PET scan was able to pick up positive lymph nodes in other malignancies, the sensitivity of PET in accurately determining the N stage of PDA is limited[100,102]. However, a PET scan is invaluable in diagnosing metastatic PDA, which can alter the management of the patient[103].

ADVANCES IN SURGICAL MANAGEMENT OF PDA

Preoperative preparation

Surgical resection of PDA either pancreaticoduodenectomy (PD) or distal pancreatectomy (DP) with splenectomy is associated with high morbidity and mortality. Hence, thorough preoperative preparation is essential to avoid complications. The preoperative preparation is similar to any other major surgery except for two unique complications encountered in PDA: Obstructive jaundice and nutritional deficiencies. Obstructive jaundice is common in patients with carcinoma involving the head of the pancreas.

There were many studies evaluating the role of preoperative biliary drainage (PBD) *vs* surgery alone. Preoperative drainage is associated with improvement in the general condition of the patient and liver function. However, it is associated with significant side effects such as the introduction of infection into the biliary tree[104]. Furthermore, preoperative drainage will make the surgery challenging due to inflammation and fibrosis induced by the common bile duct (CBD) stents and decreased diameter of the CBD making anastomosis difficult. In a meta-analysis by Sewnath *et al*[105], the lack of benefit of PBD over direct surgery in patients of periampullary carcinoma with obstructive jaundice was proven[105], further confirmed by a Cochrane review and in a meta-analysis by Wang *et al*[106] and Fang *et al*[107]. It was proven in various studies that PBD and delayed surgery to improve the general condition were not associated with any improvement in survival[108]. The only indications accepted for PBD before surgery at present include patients with cholangitis, poor general condition and poor performance status precluding surgery and in whom neoadjuvant treatment is planned. PBD can be undertaken with the help of CBD stents or percutaneous transhepatic biliary drainage. Among the CBD stents, metallic or plastic ones can be selected. In a meta-analysis of five studies by Crippa *et al*[109], the rate of re-intervention and post-operative biliary fistula was shown to be lower in the case of metallic stents compared to plastic stents[109]. Hence, at present, the available evidence supports the use of metallic stents over plastic stents, in both unresectable

PDA and for PBD.

Surgery

With the mortality and morbidity rates of pancreatic surgery improving over several decades, focus has been shifted to performing the surgeries using minimally invasive techniques. In a meta-analysis by Venkat *et al*[110] evaluating laparoscopic DP with open technique, they inferred that the laparoscopic technique was associated with lower blood loss, shorter hospital stay and lower overall postoperative complications compared to the open technique with no difference in the margin status and operating time[110]. Similar results were obtained in the meta-analysis by Jin *et al*[111].

The results from studies comparing laparoscopic PD (LPD) and open PD (OPD) are also encouraging. A summary of the outcomes of few recent studies in this regard is presented in Table 3. LPD was associated with lower intra-operative blood loss, faster recovery and shorter hospital stay with similar rate of post-operative complications and oncological outcomes[112-118]. However, in most of the studies, LPD was associated with greater operating times compared to OPD[112-118]. Robotic surgery has the added advantage of increased degrees of freedom and better images without motion artifacts compared to conventional laparoscopic surgery. In a retrospective study by Nassour *et al*[119], 428 minimally invasive PD surgeries were analyzed, and the 30-d complication rate was found to be the same between robotic and laparoscopic groups[119]. In a meta-analysis of 44 studies by Kamarajah *et al*[120] comparing robotic and conventional laparoscopic PD, the conclusion was that the robotic group was associated with lower conversion rates compared to the laparoscopic group[120]. No significant difference was noted in the operating times and blood loss between the two groups. The robotic surgery group had a shorter hospital stay compared to the laparoscopic group.

With the expertise of vascular reconstructions, venous involvement is no longer an absolute contraindication for resection of PDA with the criteria for resectability being updated regularly[121]. Venous reconstructions are widely practiced, and the present limiting factor seems to be arterial involvement. Hence, the approach has been shifted to the artery-first approach to determine the resectability of the tumor at the initial phase of the surgery itself. These include the posterior approach, medial uncinate approach, inferior infracolic approach, left posterior approach, inferior supracolic approach and superior approaches, which have become popular[122]. In a meta-analysis of 22 studies by Yu *et al*[123], PD combined with portal-superior mesenteric vein synchronous resection (PSMVR) was found to be associated with similar post-operative morbidity and mortality compared to the group without resection[123]. However, only the group with R0 resection had a significant improvement in survival [123]. In a similar meta-analysis by Bell *et al*[124], it was concluded that PSMVR was associated with a higher R1 rate, poor 5-year survival rate and was not cost effective [124]. In a meta-analysis of 26 studies by Mollberg *et al*[125] comparing pancreatectomy with arterial resection (AR) and without AR, pancreatectomy with AR was associated with an increased peri-operative mortality and poor 1-year and 2-year survival[125].

Post-operative pancreatic fistula (POPF) is one of the most dreaded complications of PD[126]. Many methods have been advocated to reduce its incidence, beginning with the type of pancreaticoenteric anastomosis. Several randomized control trial (RCTs) and meta-analysis have shown that pancreaticogastrostomy was associated with less incidence of POPF[127-129]. However, pancreatojejunostomy (PJ) is the mostly widely practiced technique because it is more physiological and is associated with lower long-term complications than pancreaticogastrostomy[130]. Several techniques of PJ have been compared in trials for the incidence of POPF. Among the duct-to-mucosa anastomoses, the Blumgart technique was found to be better compared to the Cattell-Warren technique[131]. Both the Blumgart and Kakita techniques were associated with similar results in many studies[132,133].

The continuous suturing technique was associated with a lower incidence of POPF compared to the interrupted suturing technique in several studies[134,135]. It is hypothesized that continuous sutures lead to a uniform distribution of tension along the suture line compared to intermittent sutures. A brief interest was sparked in the invagination techniques after the introduction of the jejunal eversion with binding PJ technique by Peng *et al*[136] and the end-to-side invagination technique by Berger *et al* [137], which showed better results with respect to POPF compared to the duct-to-mucosa technique[136,137]. However, the results were not reproduced in other trials [138]. In a study by Kojima *et al*[139], it was concluded that the Blumgart technique of PJ when combined with the tight dressings of the wound and drain sites (complete packing method) was associated with less incidence of POPF[139].

Table 3 The outcomes of various latest studies comparing laparoscopic pancreaticoduodenectomy with open procedure

Ref.	Study	Comparison	Outcome
Nickel *et al*[112], 2020	Meta-analysis of 3 RCTs	LPD and OPD	90-d mortality, post-operative complications and oncological outcomes were similar in both groups
			Blood loss was less for LPD
			Operating time was more for LPD
Yoo *et al*[113], 2020	Retrospective cohort study 359 patients	LPD and OPD	Post-operative complications and hospital stay were shorter for LPD
			Operative time was longer for LPD
			Recurrence free outcomes andoverall survival rates were similar
Chen *et al*[114], 2020	Meta-analysis of 6 cohort studies	LPD and OPD for PDA	Number of lymph nodes harvested, number of positive lymph nodes, rate of adjuvant therapy, time to adjuvant therapy, 1 yr survival and 2 yr survival are same for both the groups
Zhou *et al*[115], 2019	Retrospective cohort study	LPD and OPD	Overall complications and survival were similar between the two groups
Chen *et al*[116], 2018	Retrospective cohort study of 102 patients	LPD and OPD	Intra-operative blood loss, post-operative recovery and hospital stay were shorter for LPD
			Operative time was longer for LPD
			Post operative complications were similar in both the groups
Dang *et al*[117], 2020	Retrospective cohort study	LPD and OPD	Intra-operative blood loss, operating time and hospital stay for shorter for LPD
			30 d and 90 d mortality rates were better for LPD
			Long term survival rates were similar
Palanivelu *et al*[118], 2017	RCT of 68 patients with periampullary carcinoma	LPD and OPD	Intra-operative blood loss and hospital stay for shorter for LPD
			Operative time was longer for LPD
			Post-operative complications were similar in both the groups

RCT: Randomized control trial; LPD: Laparoscopic pancreaticoduodenectomy; OPD: Open pancreaticoduodenectomy.

In a move to reduce the incidence of POPF, stenting of the PJ was evaluated in various trials. Stenting is hypothesized to prevent the pancreatic enzymes from coming in contact with the anastomosis, thereby promoting healing of the anastomosis. Studies comparing internal stents and no stents revealed no significant difference in the rate of POPF[140-142]. In trials comparing external stenting and no stenting, few trials have demonstrated the benefit of external stenting[142-145]. However, these stents are associated with complications such as tube-related complications, digestive enzyme loss and possible peritonitis during tube removal[141]. Moreover, no difference was observed between the internal and external stents in preventing the POPF[146]. Most of the high-volume centers do not stent the PJ at present.

In the case of DP, stump closure using hand-sewn or stapler techniques was evaluated with respect to POPF. There was no significant difference in the POPF rates between the two techniques, and the stapler technique is commonly used by most surgeons[147,148]. In a meta-analysis evaluating bare metallic staplers and reinforced staplers using bioabsorbable materials, the superiority of the reinforced staplers was not proven even though the rate of POPF was less in reinforced staplers[149]. Pancreaticoenteric anastomosis of the distal stump has been shown to reduce the rate of POPF but increased the rate of post–operative hemorrhage[150,151].

Topical application of fibrin sealants over pancreatic anastomosis has no effect on POPF incidence in various studies[152,153]. Similarly, omental wrapping around the pancreatic anastomosis has no effect on the POPF or post-operative hemorrhage[154]. Covering the distal pancreatic stump with a teres ligament patch has shown to reduce the rate of reoperations and readmissions compared to simple closure even though the rate of POPF was not significantly different between the two groups in a randomized control trial[155].

The role of prophylactic intraperitoneal drains following pancreatectomy is controversial. In case of DP, there is no role of prophylactic intraperitoneal drains as concluded in various studies[156,157]. Moreover, prophylactic drain placement

increases hospital stay. The PANDRA trial randomized 395 patients with PD with or without drains and concluded that there was no need for routine prophylactic intraperitoneal drainage[158]. However, other studies have reported increased mortality in patients who underwent PD without drains[159,160]. However, in low-risk patients, drains can be safely avoided[160]. If drains are placed, they must be removed as early as possible once their purpose is served as their prolonged placement may lead to intra-abdominal infections and increase the risk of POPF[161].

The role of somatostatin analogues in preventing POPF is unclear. Earlier RCTs revealed the efficacy of octreotide in preventing POPF. However, newer RCTs proved that there is no role of octreotide in preventing POPF[162-164]. Pasireotide was found to reduce the rate of POPF in one RCT by Allen *et al*[165], but not in other RCTs[165-167]. At present, the use of somatostatin analogues cannot be recommended due to inconsistent results in clinical trials.

ADVANCES IN SYSTEMIC THERAPY

Neoadjuvant therapy

Borderline and locally advanced lesions are started on chemotherapy ± chemoradiation as *per* the present guidelines[168,169]. The current preferred regimen is 5-Flurouracil, irinotecan and oxaliplatin (FOLFIRINOX) ± chemoradiation, if the patient has good performance status[169-172]. Alternatively, gemcitabine and nab-paclitaxel ± chemoradiation can also be used[170-172]. In patients with known mutations in the *BRCA* gene, substituting paclitaxel with cisplatin may provide added benefit[170]. Patients with poor performance status can be started on single-agent chemotherapy or provided with palliative care. The likelihood of resection depends upon the response to neoadjuvant therapy. As *per* the latest studies, the response should be measured by falling CA19-9 Levels and absence of disease progression while on neoadjuvant therapy rather than by assessing the radiological regression[173-176].

Most of the patients with PDA in the long term, even after complete resection, develop distant metastasis. This points to the notion of micrometastasis even in a resectable localized cancer at the time of presentation. Also, due to the morbidity associated with surgery, a significant proportion of patients are unable to receive adjuvant therapy or have a delay. The encouraging results of neoadjuvant and perioperative chemoradiation therapies in esophageal, gastric and rectal carcinoma have stimulated research on the role of neoadjuvant therapy in resectable lesions of PDA. It also improves the rate of R0 resections. Multiple trials have been conducted to assess the role of neoadjuvant chemotherapy and chemoradiation in resectable PDA (Table 4). The results of many of the latest trials support neoadjuvant chemotherapy therapy in resectable PDA[177,178]. The results of ongoing trails such as NEPAFOX, NorPACT-1, NEOPAC and NCT02562716 are eagerly awaited[179-183]. The role of neoadjuvant chemoradiotherapy in resectable PDA is proved in several trials[184-186].

Adjuvant therapy

The trials of GITSG and EORTC 40891 have clearly proven the role of adjuvant chemoradiotherapy in PDA[187,188]. The ESPAC-1 trial has shown the beneficial effects of chemotherapy over chemoradiotherapy[189]. Even though the opinion on the role of adjuvant chemoradiotherapy is different among practitioners in Europe and the United States of America, most of them have a common opinion on the role of adjuvant chemotherapy. The CONKO-1 trial after showcasing the efficacy of gemcitabine in adjuvant chemotherapy has shifted the adjuvant chemotherapy regimens from 5-flurouracil (5-FU) to gemcitabine-based regimens[190]. The ESPAC-3 trial has shown no significant difference between the 5-FU and gemcitabine regimen [191]. The ESPAC-4 trial has proved the increased efficacy of the gemcitabine and capecitabine combination over gemcitabine alone and is now the recommended regimen for adjuvant therapy[192]. The success of the FOLFIRINOX regimen in the metastatic setting has led to the evaluation of its role in the adjuvant setting. The PRODIGE 24 trial has proven the efficacy of FOLFIRINOX over gemcitabine, but its use is limited to patients with a good performance status[193]. In the Japanese trial JASPAC-01, S1 was proven superior to gemcitabine, but it is still not widely used outside Japan[194]. The CONKO-05 and CONKO-06 trials did not prove the efficacy of gemcitabine + erlotinib and gemcitabine +sorafenib, respectively, over gemcitabine alone[195,196].

Table 4 Studies showing the role of neoadjuvant chemotherapy and chemoradiotherapy in resectable pancreatic adenocarcinoma

Ref.	Type of study	Type of neoadjuvant therapy	Drugs	Results
Tajima *et al* [177], 2012	Retrospective pilot study	Chemotherapy	Gemcitabine and S1	The 3 yr survival rates of NACT group (55.6%) was higher than control group (29.6%)
O'Reilly *et al* [178], 2014	Phase II trial non randomized	Chemotherapy	Gemcitabine and oxalipaltin	Resectability was 71% Overall survival was 21.7 mo
Motoi *et al* [179], 2013	RCT- NACT *vs* direct surgery	Chemotherapy	Gemcitabine and S1	Results awaited
Scott *et al*[180], 2017	RCT- NACT *vs* direct surgery	Chemotherapy	FOLFIRINOX	Results awaited
Labori *et al* [181], 2017	RCT- NACT *vs* direct surgery	Chemotherapy	FOLFIRINOX	Results awaited
Heinrich *et al* [182], 2011	RCT- NACT *vs* direct surgery	Chemotherapy	Gemcitabine and oxalipaltin	Results awaited
Sohal *et al* [183], 2017	RCT-FOLFIRINOX *vs* GnP	Chemotherapy	FOLFIRINOX *vs* Gemcitabine and nab paclitaxel	Results awaited
Turrini *et al* [184], 2009	Prospective study	Chemoradiotherapy	5-Flurouracil and cisplatin with radiotherpay	Respectability rate is 82.6% Median overall survival for resected patients is 23 mo
Golcher *et al* [185], 2015	RCT- NACRT *vs* direct surgery	Chemoradiotherapy	Gemcitabine and Cisplatin with radiotherpay	R0 resection rate (52%) and median overall survival after tumor resection (27 mo) was greater NACRT arm
Okano *et al* [186], 2017	Prospective study	Chemoradiotherapy	S-1 with radiotherapy	1-yr and 2-yr survival rates are 91% and 83% in resectable group

RCT: Randomized control trial; NACT: Neoadjuvant chemotherapy; NACRT: Neoadjuvant chemoradiotherapy; FOLFIRINOX: 5-Flurouracil, irinotecan and oxaliplatin.

Combination chemotherapy regimens are now being recommended over single agent gemcitabine for patients with metastatic disease with good performance status. The ACCORD trial has laid the ground for the FOLFIRINOX regimen as the preferred regimen over gemcitabine[197]. Several new second-line regimens were added to the arsenal after the success of the MPACT and NAPOLI-1 trials[198,199]. The trial comparing extracellular matrix degrader pegvorhyaluronidase alfa (PEGPH20) was stopped as its primary end-point was not met[200]. The results of the clinical trial AVENGER 50 comparing modified FOLFIRINOX with or without CPI-613 are awaited [201].

Studies on the role of immunotherapy in cancers are encouraging, but that is not the case with PDA[202]. The highly desmoplastic stroma and absence of any effector cells in the tumor microenvironment seem to be the predominant reason for the failure of immunotherapy[203]. Even though pembrolizumab is approved in patients with microsatellite instability, the latest results from the KEYNOTE-158 study are disappointing[204]. Two new targeted therapy drugs have been approved. Larotrectinib was approved in patients with tropomyosin receptor kinase (TRK) fusion-positive tumors[205]. Entrectinib, a multiple tyrosine kinase inhibitor, was also approved in patients with *NTRK1/2/3, ROS1*, or *ALK* gene fusions[206]. Cisplatin-based regimens are recommended in patients with germline mutations in *BRAC2* [207]. Based on the results of the POLO trial, olaparib is approved as maintenance therapy in patients with metastatic PDA and *BRCA2* mutation and with a good performance status[208].

Gene therapy for pancreatic cancer is being widely studied. A number of clinical trials and ongoing studies have been conducted in this regard[209]. Even though the results of gene therapy in phase 1 trials are encouraging, the same is not being replicated in the phase 2 studies in comparison with standardized treatment. The role of miRNAs such as *miR-4516* is being studied, which may be the future target for therapies[210]. The role of intra-operative chemotherapy in PDA is being studied in the combiCaRe trial[211].

CONCLUSION

This narrative review highlights the newer trends and achievements in the epidemiology, etiopathogenesis, screening, diagnosis and management of PDA. The newer trends in epidemiology will help us to predict the population and countries at risk in the near future. The newer concepts in the field of etiopathogenesis will help us to understand this stubborn disease better. Newer screening and diagnostic techniques will help in diagnosing the patients at an early stage and prognosticate better. The newer discoveries in drugs and management protocols will help the physicians and surgeons increase the survival and quality of life of the patients.

REFERENCES

1 **International Agency for Research on Cancer**. Global cancer observatory: cancer today. Lyon, France: International Agency for Research on Cancer. [cited 22 December 2020]. Available from: https://gco.iarc.fr/today

2 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

3 **Feldmann G**, Beaty R, Hruban RH, Maitra A. Molecular genetics of pancreatic intraepithelial neoplasia. *J Hepatobiliary Pancreat Surg* 2007; **14**: 224-232 [PMID: 17520196 DOI: 10.1007/s00534-006-1166-5]

4 **Luchini C**, Capelli P, Scarpa A. Pancreatic Ductal Adenocarcinoma and Its Variants. *Surg Pathol Clin* 2016; **9**: 547-560 [PMID: 27926359 DOI: 10.1016/j.path.2016.05.003]

5 **McGuigan A**, Kelly P, Turkington RC, Jones C, Coleman HG, McCain RS. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J Gastroenterol* 2018; **24**: 4846-4861 [PMID: 30487695 DOI: 10.3748/wjg.v24.i43.4846]

6 **Vareedayah AA**, Alkaade S, Taylor JR. Pancreatic Adenocarcinoma. *Mo Med* 2018; **115**: 230-235 [PMID: 30228728]

7 **Poruk KE**, Firpo MA, Adler DG, Mulvihill SJ. Screening for pancreatic cancer: why, how, and who? *Ann Surg* 2013; **257**: 17-26 [PMID: 22895395 DOI: 10.1097/SLA.0b013e31825ffbfb]

8 **Buanes TA**. Role of surgery in pancreatic cancer. *World J Gastroenterol* 2017; **23**: 3765-3770 [PMID: 28638216 DOI: 10.3748/wjg.v23.i21.3765]

9 **White RR**, Reddy S, Tyler DS. The role of chemoradiation therapy in locally advanced pancreatic cancer. *HPB (Oxford)* 2005; **7**: 109-113 [PMID: 18333172 DOI: 10.1080/13651820510016506]

10 **Pathy S**, Chander S. Chemoradiotherapy in pancreatic carcinoma. *Indian J Med Paediatr Oncol* 2009; **30**: 55-60 [PMID: 20596304 DOI: 10.4103/0971-5851.60049]

11 **Suker M**, Beumer BR, Sadot E, Marthey L, Faris JE, Mellon EA, El-Rayes BF, Wang-Gillam A, Lacy J, Hosein PJ, Moorcraft SY, Conroy T, Hohla F, Allen P, Taieb J, Hong TS, Shridhar R, Chau I, van Eijck CH, Koerkamp BG. FOLFIRINOX for locally advanced pancreatic cancer: a systematic review and patient-level meta-analysis. *Lancet Oncol* 2016; **17**: 801-810 [PMID: 27160474 DOI: 10.1016/S1470-2045(16)00172-8]

12 **SEER*Explorer**. An interactive website for SEER cancer statistics [Internet]. Surveillance Research Program, National Cancer Institute. [cited 14 September 2020]. Available from: https://seer.cancer.gov/explorer/

13 **Rawla P**, Sunkara T, Gaduputi V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J Oncol* 2019; **10**: 10-27 [PMID: 30834048 DOI: 10.14740/wjon1166]

14 **Pei X**, Song F, Wang Z. Emerging incidence trends and application of curative treatments of pancreatic cancer in the USA. *Medicine (Baltimore)* 2019; **98**: e17175 [PMID: 31860944 DOI: 10.1097/MD.0000000000017175]

15 **Ezzati M**, Henley SJ, Lopez AD, Thun MJ. Role of smoking in global and regional cancer epidemiology: current patterns and data needs. *Int J Cancer* 2005; **116**: 963-971 [PMID: 15880414 DOI: 10.1002/ijc.21100]

16 **Willett WC**. Diet and cancer. *Oncologist* 2000; **5**: 393-404 [PMID: 11040276 DOI: 10.1634/theoncologist.5-5-393]

17 **Genkinger JM**, Spiegelman D, Anderson KE, Bernstein L, van den Brandt PA, Calle EE, English DR, Folsom AR, Freudenheim JL, Fuchs CS, Giles GG, Giovannucci E, Horn-Ross PL, Larsson SC, Leitzmann M, Männistö S, Marshall JR, Miller AB, Patel AV, Rohan TE, Stolzenberg-Solomon RZ, Verhage BA, Virtamo J, Willcox BJ, Wolk A, Ziegler RG, Smith-Warner SA. A pooled analysis of 14 cohort studies of anthropometric factors and pancreatic cancer risk. *Int J Cancer* 2011; **129**: 1708-1717 [PMID: 21105029 DOI: 10.1002/ijc.25794]

18 **Jarosz M**, Sekuła W, Rychlik E. Influence of diet and tobacco smoking on pancreatic cancer incidence in poland in 1960-2008. *Gastroenterol Res Pract* 2012; **2012**: 682156 [PMID: 23319943 DOI: 10.1155/2012/682156]

19 **Wu W**, He X, Yang L, Wang Q, Bian X, Ye J, Li Y, Li L. Rising trends in pancreatic cancer incidence and mortality in 2000-2014. *Clin Epidemiol* 2018; **10**: 789-797 [PMID: 30022856 DOI: 10.2147/CLEP.S160018]

20 **Parkin DM**, Boyd L, Walker LC. 16. The fraction of cancer attributable to lifestyle and environmental factors in the UK in 2010. *Br J Cancer* 2011; **105** Suppl 2: S77-S81 [PMID: 22158327 DOI: 10.1038/bjc.2011.489]

21 **West R**. Tobacco smoking: Health impact, prevalence, correlates and interventions. *Psychol Health* 2017; **32**: 1018-1036 [PMID: 28553727 DOI: 10.1080/08870446.2017.1325890]

22 **Kuroczycki-Saniutycz S**, Grzeszczuk A, Zwierz ZW, Kołodziejczyk P, Szczesiul J, Zalewska-Szajda B, Ościłowicz K, Waszkiewicz N, Zwierz K, Szajda SD. Prevention of pancreatic cancer. *Contemp Oncol (Pozn)* 2017; **21**: 30-34 [PMID: 28435395 DOI: 10.5114/wo.2016.63043]

23 **Ying H**, Dey P, Yao W, Kimmelman AC, Draetta GF, Maitra A, DePinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev* 2016; **30**: 355-385 [PMID: 26883357 DOI: 10.1101/gad.275776.115]

24 **Yao W**, Maitra A, Ying H. Recent insights into the biology of pancreatic cancer. *EBioMedicine* 2020; **53**: 102655 [PMID: 32139179 DOI: 10.1016/j.ebiom.2020.102655]

25 **Waters AM**, Der CJ. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harb Perspect Med* 2018; **8** [PMID: 29229669 DOI: 10.1101/cshperspect.a031435]

26 **Bollag G**, Zhang C. Drug discovery: Pocket of opportunity. *Nature* 2013; **503**: 475-476 [PMID: 24256732 DOI: 10.1038/nature12835]

27 **Scully KM**, Lahmy R, Signaevskaia L, Sasik R, Medal R, Kim H, French R, James B, Wu Y, Lowy AM, Itkin-Ansari P. E47 Governs the MYC-CDKN1B/p27^{KIP1}-RB Network to Growth Arrest PDA Cells Independent of CDKN2A/p16^{INK4A} and Wild-Type p53. *Cell Mol Gastroenterol Hepatol* 2018; **6**: 181-198 [PMID: 30003124 DOI: 10.1016/j.jcmgh.2018.05.002]

28 **Tatarian T**, Winter JM. Genetics of Pancreatic Cancer and Its Implications on Therapy. *Surg Clin North Am* 2016; **96**: 1207-1221 [PMID: 27865273 DOI: 10.1016/j.suc.2016.07.014]

29 **Das S**, Cardin D. Targeting DNA Damage Repair Pathways in Pancreatic Adenocarcinoma. *Curr Treat Options Oncol* 2020; **21**: 62 [PMID: 32601814 DOI: 10.1007/s11864-020-00763-7]

30 **Hruban RH**, Maitra A, Goggins M. Update on pancreatic intraepithelial neoplasia. *Int J Clin Exp Pathol* 2008; **1**: 306-316 [PMID: 18787611]

31 **Brune K**, Abe T, Canto M, O'Malley L, Klein AP, Maitra A, Volkan Adsay N, Fishman EK, Cameron JL, Yeo CJ, Kern SE, Goggins M, Hruban RH. Multifocal neoplastic precursor lesions associated with lobular atrophy of the pancreas in patients having a strong family history of pancreatic cancer. *Am J Surg Pathol* 2006; **30**: 1067-1076 [PMID: 16931950]

32 **Liu BB**, Wang WH. Survivin and pancreatic cancer. *World J Clin Oncol* 2011; **2**: 164-168 [PMID: 21611091 DOI: 10.5306/wjco.v2.i3.164]

33 **Maitra A**, Ashfaq R, Gunn CR, Rahman A, Yeo CJ, Sohn TA, Cameron JL, Hruban RH, Wilentz RE. Cyclooxygenase 2 expression in pancreatic adenocarcinoma and pancreatic intraepithelial neoplasia: an immunohistochemical analysis with automated cellular imaging. *Am J Clin Pathol* 2002; **118**: 194-201 [PMID: 12162677 DOI: 10.1309/TPG4-CK1C-9V8V-8AWC]

34 **Ohuchida K**, Mizumoto K, Ohhashi S, Yamaguchi H, Konomi H, Nagai E, Yamaguchi K, Tsuneyoshi M, Tanaka M. S100A11, a putative tumor suppressor gene, is overexpressed in pancreatic carcinogenesis. *Clin Cancer Res* 2006; **12**: 5417-5422 [PMID: 17000675 DOI: 10.1158/1078-0432.CCR-06-0222]

35 **Ohuchida K**, Mizumoto K, Egami T, Yamaguchi H, Fujii K, Konomi H, Nagai E, Yamaguchi K, Tsuneyoshi M, Tanaka M. S100P is an early developmental marker of pancreatic carcinogenesis. *Clin Cancer Res* 2006; **12**: 5411-5416 [PMID: 17000674 DOI: 10.1158/1078-0432.CCR-06-0298]

36 **Nagata K**, Horinouchi M, Saitou M, Higashi M, Nomoto M, Goto M, Yonezawa S. Mucin expression profile in pancreatic cancer and the precursor lesions. *J Hepatobiliary Pancreat Surg* 2007; **14**: 243-254 [PMID: 17520199 DOI: 10.1007/s00534-006-1169-2]

37 **Suh H**, Pillai K, Morris DL. Mucins in pancreatic cancer: biological role, implications in carcinogenesis and applications in diagnosis and therapy. *Am J Cancer Res* 2017; **7**: 1372-1383 [PMID: 28670497]

38 **Swartz MJ**, Batra SK, Varshney GC, Hollingsworth MA, Yeo CJ, Cameron JL, Wilentz RE, Hruban RH, Argani P. MUC4 expression increases progressively in pancreatic intraepithelial neoplasia. *Am J Clin Pathol* 2002; **117**: 791-796 [PMID: 12090430 DOI: 10.1309/7Y7N-M1WM-R0YK-M2VA]

39 **Ohuchida K**, Mizumoto K, Yamada D, Fujii K, Ishikawa N, Konomi H, Nagai E, Yamaguchi K, Tsuneyoshi M, Tanaka M. Quantitative analysis of MUC1 and MUC5AC mRNA in pancreatic juice for preoperative diagnosis of pancreatic cancer. *Int J Cancer* 2006; **118**: 405-411 [PMID: 16052519 DOI: 10.1002/ijc.21317]

40 **Singh AP**, Chaturvedi P, Batra SK. Emerging roles of MUC4 in cancer: a novel target for diagnosis and therapy. *Cancer Res* 2007; **67**: 433-436 [PMID: 17234748 DOI: 10.1158/0008-5472.CAN-06-3114]

41 **Collisson EA**, Bailey P, Chang DK, Biankin AV. Molecular subtypes of pancreatic cancer. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 207-220 [PMID: 30718832 DOI: 10.1038/s41575-019-0109-y]

42 **Peng J**, Sun BF, Chen CY, Zhou JY, Chen YS, Chen H, Liu L, Huang D, Jiang J, Cui GS, Yang Y, Wang W, Guo D, Dai M, Guo J, Zhang T, Liao Q, Liu Y, Zhao YL, Han DL, Zhao Y, Yang YG, Wu W. Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma. *Cell Res* 2019; **29**: 725-738 [PMID: 31273297 DOI: 10.1038/s41422-019-0195-y]

43 **Moffitt RA**, Marayati R, Flate EL, Volmar KE, Loeza SG, Hoadley KA, Rashid NU, Williams LA,

Eaton SC, Chung AH, Smyla JK, Anderson JM, Kim HJ, Bentrem DJ, Talamonti MS, Iacobuzio-Donahue CA, Hollingsworth MA, Yeh JJ. Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma. *Nat Genet* 2015; **47**: 1168-1178 [PMID: 26343385 DOI: 10.1038/ng.3398]

44 **Perera RM**, Stoykova S, Nicolay BN, Ross KN, Fitamant J, Boukhali M, Lengrand J, Deshpande V, Selig MK, Ferrone CR, Settleman J, Stephanopoulos G, Dyson NJ, Zoncu R, Ramaswamy S, Haas W, Bardeesy N. Transcriptional control of autophagy-lysosome function drives pancreatic cancer metabolism. *Nature* 2015; **524**: 361-365 [PMID: 26168401 DOI: 10.1038/nature14587]

45 **Bryant KL**, Stalnecker CA, Zeitouni D, Klomp JE, Peng S, Tikunov AP, Gunda V, Pierobon M, Waters AM, George SD, Tomar G, Papke B, Hobbs GA, Yan L, Hayes TK, Diehl JN, Goode GD, Chaika NV, Wang Y, Zhang GF, Witkiewicz AK, Knudsen ES, Petricoin EF 3rd, Singh PK, Macdonald JM, Tran NL, Lyssiotis CA, Ying H, Kimmelman AC, Cox AD, Der CJ. Combination of ERK and autophagy inhibition as a treatment approach for pancreatic cancer. *Nat Med* 2019; **25**: 628-640 [PMID: 30833752 DOI: 10.1038/s41591-019-0368-8]

46 **Kinsey CG**, Camolotto SA, Boespflug AM, Guillen KP, Foth M, Truong A, Schuman SS, Shea JE, Seipp MT, Yap JT, Burrell LD, Lum DH, Whisenant JR, Gilcrease GW 3rd, Cavalieri CC, Rehbein KM, Cutler SL, Affolter KE, Welm AL, Welm BE, Scaife CL, Snyder EL, McMahon M. Protective autophagy elicited by RAF→MEK→ERK inhibition suggests a treatment strategy for RAS-driven cancers. *Nat Med* 2019; **25**: 620-627 [PMID: 30833748 DOI: 10.1038/s41591-019-0367-9]

47 **Shukla SK**, Purohit V, Mehla K, Gunda V, Chaika NV, Vernucci E, King RJ, Abrego J, Goode GD, Dasgupta A, Illies AL, Gebregiworgis T, Dai B, Augustine JJ, Murthy D, Attri KS, Mashadova O, Grandgenett PM, Powers R, Ly QP, Lazenby AJ, Grem JL, Yu F, Matés JM, Asara JM, Kim JW, Hankins JH, Weekes C, Hollingsworth MA, Serkova NJ, Sasson AR, Fleming JB, Oliveto JM, Lyssiotis CA, Cantley LC, Berim L, Singh PK. MUC1 and HIF-1alpha Signaling Crosstalk Induces Anabolic Glucose Metabolism to Impart Gemcitabine Resistance to Pancreatic Cancer. *Cancer Cell* 2017; **32**: 71-87. e7 [PMID: 28697344 DOI: 10.1016/j.ccell.2017.06.004]

48 **Guillaumond F**, Bidaut G, Ouaissi M, Servais S, Gouirand V, Olivares O, Lac S, Borge L, Roques J, Gayet O, Pinault M, Guimaraes C, Nigri J, Loncle C, Lavaut MN, Garcia S, Tailleux A, Staels B, Calvo E, Tomasini R, Iovanna JL, Vasseur S. Cholesterol uptake disruption, in association with chemotherapy, is a promising combined metabolic therapy for pancreatic adenocarcinoma. *Proc Natl Acad Sci USA* 2015; **112**: 2473-2478 [PMID: 25675507 DOI: 10.1073/pnas.1421601112]

49 **Tadros S**, Shukla SK, King RJ, Gunda V, Vernucci E, Abrego J, Chaika NV, Yu F, Lazenby AJ, Berim L, Grem J, Sasson AR, Singh PK. *De Novo* Lipid Synthesis Facilitates Gemcitabine Resistance through Endoplasmic Reticulum Stress in Pancreatic Cancer. *Cancer Res* 2017; **77**: 5503-5517 [PMID: 28811332 DOI: 10.1158/0008-5472.CAN-16-3062]

50 **Kuzmickiene I**, Everatt R, Virviciute D, Tamosiunas A, Radisauskas R, Reklaitiene R, Milinaviciene E. Smoking and other risk factors for pancreatic cancer: a cohort study in men in Lithuania. *Cancer Epidemiol* 2013; **37**: 133-139 [PMID: 23107757 DOI: 10.1016/j.canep.2012.10.001]

51 **Pelucchi C**, Galeone C, Polesel J, Manzari M, Zucchetto A, Talamini R, Franceschi S, Negri E, La Vecchia C. Smoking and body mass index and survival in pancreatic cancer patients. *Pancreas* 2014; **43**: 47-52 [PMID: 24177141 DOI: 10.1097/MPA.0b013e3182a7c74b]

52 **Rahman F**, Cotterchio M, Cleary SP, Gallinger S. Association between alcohol consumption and pancreatic cancer risk: a case-control study. *PLoS One* 2015; **10**: e0124489 [PMID: 25856529 DOI: 10.1371/journal.pone.0124489]

53 **Berrington de Gonzalez A**, Sweetland S, Spencer E. A meta-analysis of obesity and the risk of pancreatic cancer. *Br J Cancer* 2003; **89**: 519-523 [PMID: 12888824 DOI: 10.1038/sj.bjc.6601140]

54 **Lightsey D**; National Council Against Health Fraud and Quackwatch. Comment on 'Red and processed meat consumption and risk of pancreatic cancer: meta-analysis of prospective studies'. *Br J Cancer* 2012; **107**: 754-755 [PMID: 22805322 DOI: 10.1038/bjc.2012.111]

55 **Paluszkiewicz P**, Smolińska K, Dębińska I, Turski WA. Main dietary compounds and pancreatic cancer risk. The quantitative analysis of case-control and cohort studies. *Cancer Epidemiol* 2012; **36**: 60-67 [PMID: 22018953 DOI: 10.1016/j.canep.2011.05.004]

56 **Maisonneuve P**, Lowenfels AB. Risk factors for pancreatic cancer: a summary review of meta-analytical studies. *Int J Epidemiol* 2015; **44**: 186-198 [PMID: 25502106 DOI: 10.1093/ije/dyu240]

57 **Greer JB**, Whitcomb DC, Brand RE. Genetic predisposition to pancreatic cancer: a brief review. *Am J Gastroenterol* 2007; **102**: 2564-2569 [PMID: 17958761 DOI: 10.1111/j.1572-0241.2007.01475.x]

58 **Jacobs EJ**, Chanock SJ, Fuchs CS, Lacroix A, McWilliams RR, Steplowski E, Stolzenberg-Solomon RZ, Arslan AA, Bueno-de-Mesquita HB, Gross M, Helzlsouer K, Petersen G, Zheng W, Agalliu I, Allen NE, Amundadottir L, Boutron-Ruault MC, Buring JE, Canzian F, Clipp S, Dorronsoro M, Gaziano JM, Giovannucci EL, Hankinson SE, Hartge P, Hoover RN, Hunter DJ, Jacobs KB, Jenab M, Kraft P, Kooperberg C, Lynch SM, Sund M, Mendelsohn JB, Mouw T, Newton CC, Overvad K, Palli D, Peeters PH, Rajkovic A, Shu XO, Thomas G, Tobias GS, Trichopoulos D, Virtamo J, Wactawski-Wende J, Wolpin BM, Yu K, Zeleniuch-Jacquotte A. Family history of cancer and risk of pancreatic cancer: a pooled analysis from the Pancreatic Cancer Cohort Consortium (PanScan). *Int J Cancer* 2010; **127**: 1421-1428 [PMID: 20049842 DOI: 10.1002/ijc.25148]

59 **Grover S**, Syngal S. Hereditary pancreatic cancer. *Gastroenterology* 2010; **139**: 1076-1080, 1080.e1-1080. e2 [PMID: 20727885 DOI: 10.1053/j.gastro.2010.08.012]

60 **Raphael KL**, Willingham FF. Hereditary pancreatitis: current perspectives. *Clin Exp Gastroenterol* 2016; **9**: 197-207 [PMID: 27555793 DOI: 10.2147/CEG.S84358]

61 **Huxley R**, Ansary-Moghaddam A, Berrington de González A, Barzi F, Woodward M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. *Br J Cancer* 2005; **92**: 2076-2083 [PMID: 15886696 DOI: 10.1038/sj.bjc.6602619]

62 **Bosetti C**, Rosato V, Li D, Silverman D, Petersen GM, Bracci PM, Neale RE, Muscat J, Anderson K, Gallinger S, Olson SH, Miller AB, Bas Bueno-de-Mesquita H, Scelo G, Janout V, Holcatova I, Lagiou P, Serraino D, Lucenteforte E, Fabianova E, Baghurst PA, Zatonski W, Foretova L, Fontham E, Bamlet WR, Holly EA, Negri E, Hassan M, Prizment A, Cotterchio M, Cleary S, Kurtz RC, Maisonneuve P, Trichopoulos D, Polesel J, Duell EJ, Boffetta P, La Vecchia C, Ghadirian P. Diabetes, antidiabetic medications, and pancreatic cancer risk: an analysis from the International Pancreatic Cancer Case-Control Consortium. *Ann Oncol* 2014; **25**: 2065-2072 [PMID: 25057164 DOI: 10.1093/annonc/mdu276]

63 **Risch HA**, Lu L, Kidd MS, Wang J, Zhang W, Ni Q, Gao YT, Yu H. Helicobacter pylori seropositivities and risk of pancreatic carcinoma. *Cancer Epidemiol Biomarkers Prev* 2014; **23**: 172-178 [PMID: 24234587 DOI: 10.1158/1055-9965.EPI-13-0447]

64 **Ben Q**, Wang K, Yuan Y, Li Z. Pancreatic cancer incidence and outcome in relation to ABO blood groups among Han Chinese patients: a case-control study. *Int J Cancer* 2011; **128**: 1179-1186 [PMID: 20473916 DOI: 10.1002/ijc.25426]

65 **Zhang BL**, He N, Huang YB, Song FJ, Chen KX. ABO blood groups and risk of cancer: a systematic review and meta-analysis. *Asian Pac J Cancer Prev* 2014; **15**: 4643-4650 [PMID: 24969898 DOI: 10.7314/apjcp.2014.15.11.4643]

66 **Shin EJ**, Canto MI. Pancreatic cancer screening. *Gastroenterol Clin North Am* 2012; **41**: 143-157 [PMID: 22341255 DOI: 10.1016/j.gtc.2011.12.001]

67 **Canto MI**. Strategies for screening for pancreatic adenocarcinoma in high-risk patients. *Semin Oncol* 2007; **34**: 295-302 [PMID: 17674957 DOI: 10.1053/j.seminoncol.2007.05.008]

68 **Scarà S**, Bottoni P, Scatena R. CA 19-9: Biochemical and Clinical Aspects. *Adv Exp Med Biol* 2015; **867**: 247-260 [PMID: 26530370 DOI: 10.1007/978-94-017-7215-0_15]

69 **Goggins M**. Identifying molecular markers for the early detection of pancreatic neoplasia. *Semin Oncol* 2007; **34**: 303-310 [PMID: 17674958 DOI: 10.1053/j.seminoncol.2007.05.003]

70 **Bünger S**, Laubert T, Roblick UJ, Habermann JK. Serum biomarkers for improved diagnostic of pancreatic cancer: a current overview. *J Cancer Res Clin Oncol* 2011; **137**: 375-389 [PMID: 21193998 DOI: 10.1007/s00432-010-0965-x]

71 **Zubarik R**, Gordon SR, Lidofsky SD, Anderson SR, Pipas JM, Badger G, Ganguly E, Vecchio J. Screening for pancreatic cancer in a high-risk population with serum CA 19-9 and targeted EUS: a feasibility study. *Gastrointest Endosc* 2011; **74**: 87-95 [PMID: 21704809 DOI: 10.1016/j.gie.2011.03.1235]

72 **Meng Q**, Shi S, Liang C, Liang D, Xu W, Ji S, Zhang B, Ni Q, Xu J, Yu X. Diagnostic and prognostic value of carcinoembryonic antigen in pancreatic cancer: a systematic review and meta-analysis. *Onco Targets Ther* 2017; **10**: 4591-4598 [PMID: 28979147 DOI: 10.2147/OTT.S145708]

73 **Gold DV**, Modrak DE, Ying Z, Cardillo TM, Sharkey RM, Goldenberg DM. New MUC1 serum immunoassay differentiates pancreatic cancer from pancreatitis. *J Clin Oncol* 2006; **24**: 252-258 [PMID: 16344318 DOI: 10.1200/JCO.2005.02.8282]

74 **Gold DV**, Goggins M, Modrak DE, Newsome G, Liu M, Shi C, Hruban RH, Goldenberg DM. Detection of early-stage pancreatic adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 2786-2794 [PMID: 20810605 DOI: 10.1158/1055-9965.EPI-10-0667]

75 **Gold DV**, Karanjawala Z, Modrak DE, Goldenberg DM, Hruban RH. PAM4-reactive MUC1 is a biomarker for early pancreatic adenocarcinoma. *Clin Cancer Res* 2007; **13**: 7380-7387 [PMID: 18094420 DOI: 10.1158/1078-0432.CCR-07-1488]

76 **Sharma C**, Eltawil KM, Renfrew PD, Walsh MJ, Molinari M. Advances in diagnosis, treatment and palliation of pancreatic carcinoma: 1990-2010. *World J Gastroenterol* 2011; **17**: 867-897 [PMID: 21412497 DOI: 10.3748/wjg.v17.i7.867]

77 **Simeone DM**, Ji B, Banerjee M, Arumugam T, Li D, Anderson MA, Bamberger AM, Greenson J, Brand RE, Ramachandran V, Logsdon CD. CEACAM1, a novel serum biomarker for pancreatic cancer. *Pancreas* 2007; **34**: 436-443 [PMID: 17446843 DOI: 10.1097/MPA.0b013e3180333ae3]

78 **Rückert F**, Pilarsky C, Grützmann R. Serum tumor markers in pancreatic cancer-recent discoveries. *Cancers (Basel)* 2010; **2**: 1107-1124 [PMID: 24281109 DOI: 10.3390/cancers2021107]

79 **Bouvet M**, Nardin SR, Burton DW, Lee NC, Yang M, Wang X, Baranov E, Behling C, Moossa AR, Hoffman RM, Deftos LJ. Parathyroid hormone-related protein as a novel tumor marker in pancreatic adenocarcinoma. *Pancreas* 2002; **24**: 284-290 [PMID: 11893937 DOI: 10.1097/00006676-200204000-00012]

80 **Louhimo J**, Alfthan H, Stenman UH, Haglund C. Serum HCG beta and CA 72-4 are stronger prognostic factors than CEA, CA 19-9 and CA 242 in pancreatic cancer. *Oncology* 2004; **66**: 126-131 [PMID: 15138364 DOI: 10.1159/000077438]

81 **Kumar Y**, Gurusamy K, Pamecha V, Davidson BR. Tumor M2-pyruvate kinase as tumor marker in exocrine pancreatic cancer a meta-analysis. *Pancreas* 2007; **35**: 114-119 [PMID: 17632316 DOI: 10.1097/mpa.0b013e3180537237]

82 **Frena A**. SPan-1 and exocrine pancreatic carcinoma. The clinical role of a new tumor marker. *Int J Biol Markers* 2001; **16**: 189-197 [PMID: 11605732]

83 **Wang J**, Raimondo M, Guha S, Chen J, Diao L, Dong X, Wallace MB, Killary AM, Frazier ML, Woodward TA, Wang J, Sen S. Circulating microRNAs in Pancreatic Juice as Candidate Biomarkers of Pancreatic Cancer. *J Cancer* 2014; **5**: 696-705 [PMID: 25258651 DOI: 10.7150/jca.10094]

84 **Tada M**, Komatsu Y, Kawabe T, Sasahira N, Isayama H, Toda N, Shiratori Y, Omata M. Quantitative analysis of K-ras gene mutation in pancreatic tissue obtained by endoscopic ultrasonography-guided fine needle aspiration: clinical utility for diagnosis of pancreatic tumor. *Am J Gastroenterol* 2002; **97**: 2263-2270 [PMID: 12358243 DOI: 10.1111/j.1572-0241.2002.05980.x]

85 **Eshleman JR**, Norris AL, Sadakari Y, Debeljak M, Borges M, Harrington C, Lin E, Brant A, Barkley T, Almario JA, Topazian M, Farrell J, Syngal S, Lee JH, Yu J, Hruban RH, Kanda M, Canto MI, Goggins M. KRAS and guanine nucleotide-binding protein mutations in pancreatic juice collected from the duodenum of patients at high risk for neoplasia undergoing endoscopic ultrasound. *Clin Gastroenterol Hepatol* 2015; **13**: 963-9. e4 [PMID: 25481712 DOI: 10.1016/j.cgh.2014.11.028]

86 **Matsubayashi H**, Canto M, Sato N, Klein A, Abe T, Yamashita K, Yeo CJ, Kalloo A, Hruban R, Goggins M. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. *Cancer Res* 2006; **66**: 1208-1217 [PMID: 16424060 DOI: 10.1158/0008-5472.CAN-05-2664]

87 **Maitra A**, Cohen Y, Gillespie SE, Mambo E, Fukushima N, Hoque MO, Shah N, Goggins M, Califano J, Sidransky D, Chakravarti A. The Human MitoChip: a high-throughput sequencing microarray for mitochondrial mutation detection. *Genome Res* 2004; **14**: 812-819 [PMID: 15123581 DOI: 10.1101/gr.2228504]

88 **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838 [PMID: 15944708 DOI: 10.1038/nature03702]

89 **Zakharova OP**, Karmazanovsky GG, Egorov VI. Pancreatic adenocarcinoma: Outstanding problems. *World J Gastrointest Surg* 2012; **4**: 104-113 [PMID: 22655124 DOI: 10.4240/wjgs.v4.i5.104]

90 **Canto MI**. Screening and surveillance approaches in familial pancreatic cancer. *Gastrointest Endosc Clin N Am* 2008; **18**: 535-553, x [PMID: 18674702 DOI: 10.1016/j.giec.2008.05.012]

91 **Zhao WY**, Luo M, Sun YW, Xu Q, Chen W, Zhao G, Wu ZY. Computed tomography in diagnosing vascular invasion in pancreatic and periampullary cancers: a systematic review and meta-analysis. *Hepatobiliary Pancreat Dis Int* 2009; **8**: 457-464 [PMID: 19822487]

92 **Buchs NC**, Chilcott M, Poletti PA, Buhler LH, Morel P. Vascular invasion in pancreatic cancer: Imaging modalities, preoperative diagnosis and surgical management. *World J Gastroenterol* 2010; **16**: 818-831 [PMID: 20143460 DOI: 10.3748/wjg.v16.i7.818]

93 **Lami G**, Biagini MR, Galli A. Endoscopic ultrasonography for surveillance of individuals at high risk for pancreatic cancer. *World J Gastrointest Endosc* 2014; **6**: 272-285 [PMID: 25031786 DOI: 10.4253/wjge.v6.i7.272]

94 **Steinberg WM**, Barkin JS, Bradley EL 3rd, DiMagno E, Layer P, Canto MI, Levy MJ. Should patients with a strong family history of pancreatic cancer be screened on a periodic basis for cancer of the pancreas? *Pancreas* 2009; **38**: e137-e150 [PMID: 19550273 DOI: 10.1097/MPA.0b013e3181a86b2c]

95 **Vasen HF**, Wasser M, van Mil A, Tollenaar RA, Konstantinovski M, Gruis NA, Bergman W, Hes FJ, Hommes DW, Offerhaus GJ, Morreau H, Bonsing BA, de Vos tot Nederveen Cappel WH. Magnetic resonance imaging surveillance detects early-stage pancreatic cancer in carriers of a p16-Leiden mutation. *Gastroenterology* 2011; **140**: 850-856 [PMID: 21129377 DOI: 10.1053/j.gastro.2010.11.048]

96 **Mitra A**, D'Souza A, Goel M, Shrikhande SV. Surgery for Pancreatic and Periampullary Carcinoma. *Indian J Surg* 2015; **77**: 371-380 [PMID: 26722199 DOI: 10.1007/s12262-015-1358-9]

97 **Ludwig E**, Olson SH, Bayuga S, Simon J, Schattner MA, Gerdes H, Allen PJ, Jarnagin WR, Kurtz RC. Feasibility and yield of screening in relatives from familial pancreatic cancer families. *Am J Gastroenterol* 2011; **106**: 946-954 [PMID: 21468009 DOI: 10.1038/ajg.2011.65]

98 **Tang S**, Huang G, Liu J, Liu T, Treven L, Song S, Zhang C, Pan L, Zhang T. Usefulness of 18F-FDG PET, combined FDG-PET/CT and EUS in diagnosing primary pancreatic carcinoma: a meta-analysis. *Eur J Radiol* 2011; **78**: 142-150 [PMID: 19854016 DOI: 10.1016/j.ejrad.2009.09.026]

99 **Wu LM**, Hu JN, Hua J, Liu MJ, Chen J, Xu JR. Diagnostic value of diffusion-weighted magnetic resonance imaging compared with fluorodeoxyglucose positron emission tomography/computed tomography for pancreatic malignancy: a meta-analysis using a hierarchical regression model. *J Gastroenterol Hepatol* 2012; **27**: 1027-1035 [PMID: 22414092 DOI: 10.1111/j.1440-1746.2012.07112.x]

100 **Wang XY**, Yang F, Jin C, Fu DL. Utility of PET/CT in diagnosis, staging, assessment of resectability and metabolic response of pancreatic cancer. *World J Gastroenterol* 2014; **20**: 15580-15589 [PMID: 25400441 DOI: 10.3748/wjg.v20.i42.15580]

101 **Wakabayashi H**, Nishiyama Y, Otani T, Sano T, Yachida S, Okano K, Izuishi K, Suzuki Y. Role of 18F-fluorodeoxyglucose positron emission tomography imaging in surgery for pancreatic cancer. *World J Gastroenterol* 2008; **14**: 64-69 [PMID: 18176963 DOI: 10.3748/wjg.14.64]

102 **Kauhanen SP**, Komar G, Seppänen MP, Dean KI, Minn HR, Kajander SA, Rinta-Kiikka I, Alanen K, Borra RJ, Puolakkainen PA, Nuutila P, Ovaska JT. A prospective diagnostic accuracy study of 18F-fluorodeoxyglucose positron emission tomography/computed tomography, multidetector row

computed tomography, and magnetic resonance imaging in primary diagnosis and staging of pancreatic cancer. *Ann Surg* 2009; **250**: 957-963 [PMID: 19687736 DOI: 10.1097/SLA.0b013e3181b2fafa]

103 **Strobel K**, Heinrich S, Bhure U, Soyka J, Veit-Haibach P, Pestalozzi BC, Clavien PA, Hany TF. Contrast-enhanced 18F-FDG PET/CT: 1-stop-shop imaging for assessing the resectability of pancreatic cancer. *J Nucl Med* 2008; **49**: 1408-1413 [PMID: 18703604 DOI: 10.2967/jnumed.108.051466]

104 **Wang C**, Xu Y, Lu X. Should preoperative biliary drainage be routinely performed for obstructive jaundice with resectable tumor? *Hepatobiliary Surg Nutr* 2013; **2**: 266-271 [PMID: 24570957 DOI: 10.3978/j.issn.2304-3881.2013.09.01]

105 **Sewnath ME**, Karsten TM, Prins MH, Rauws EJ, Obertop H, Gouma DJ. A meta-analysis on the efficacy of preoperative biliary drainage for tumors causing obstructive jaundice. *Ann Surg* 2002; **236**: 17-27 [PMID: 12131081 DOI: 10.1097/00000658-200207000-00005]

106 **Wang Q**, Gurusamy KS, Lin H, Xie X, Wang C. Preoperative biliary drainage for obstructive jaundice. *Cochrane Database Syst Rev* 2008; CD005444 [PMID: 18677779 DOI: 10.1002/14651858.CD005444.pub2]

107 **Fang Y**, Gurusamy KS, Wang Q, Davidson BR, Lin H, Xie X, Wang C. Meta-analysis of randomized clinical trials on safety and efficacy of biliary drainage before surgery for obstructive jaundice. *Br J Surg* 2013; **100**: 1589-1596 [PMID: 24264780 DOI: 10.1002/bjs.9260]

108 **Eshuis WJ**, van der Gaag NA, Rauws EA, van Eijck CH, Bruno MJ, Kuipers EJ, Coene PP, Kubben FJ, Gerritsen JJ, Greve JW, Gerhards MF, de Hingh IH, Klinkenbijl JH, Nio CY, de Castro SM, Busch OR, van Gulik TM, Bossuyt PM, Gouma DJ. Therapeutic delay and survival after surgery for cancer of the pancreatic head with or without preoperative biliary drainage. *Ann Surg* 2010; **252**: 840-849 [PMID: 21037440 DOI: 10.1097/SLA.0b013e3181fd36a2]

109 **Crippa S**, Cirocchi R, Partelli S, Petrone MC, Muffatti F, Renzi C, Falconi M, Arcidiacono PG. Systematic review and meta-analysis of metal versus plastic stents for preoperative biliary drainage in resectable periampullary or pancreatic head tumors. *Eur J Surg Oncol* 2016; **42**: 1278-1285 [PMID: 27296728 DOI: 10.1016/j.ejso.2016.05.001]

110 **Venkat R**, Edil BH, Schulick RD, Lidor AO, Makary MA, Wolfgang CL. Laparoscopic distal pancreatectomy is associated with significantly less overall morbidity compared to the open technique: a systematic review and meta-analysis. *Ann Surg* 2012; **255**: 1048-1059 [PMID: 22511003 DOI: 10.1097/SLA.0b013e318251ee09]

111 **Jin T**, Altaf K, Xiong JJ, Huang W, Javed MA, Mai G, Liu XB, Hu WM, Xia Q. A systematic review and meta-analysis of studies comparing laparoscopic and open distal pancreatectomy. *HPB (Oxford)* 2012; **14**: 711-724 [PMID: 23043660 DOI: 10.1111/j.1477-2574.2012.00531.x]

112 **Nickel F**, Haney CM, Kowalewski KF, Probst P, Limen EF, Kalkum E, Diener MK, Strobel O, Müller-Stich BP, Hackert T. Laparoscopic Versus Open Pancreaticoduodenectomy: A Systematic Review and Meta-analysis of Randomized Controlled Trials. *Ann Surg* 2020; **271**: 54-66 [PMID: 30973388 DOI: 10.1097/SLA.0000000000003309]

113 **Yoo D**, Song KB, Lee JW, Hwang K, Hong S, Shin D, Hwang DW, Lee JH, Lee W, Kwon J, Park Y, Jun E, Kim SC. A Comparative Study of Laparoscopic versus Open Pancreaticoduodenectomy for Ampulla of Vater Carcinoma. *J Clin Med* 2020; **9** [PMID: 32668683 DOI: 10.3390/jcm9072214]

114 **Chen K**, Zhou Y, Jin W, Zhu Q, Lu C, Niu N, Wang Y, Mou Y, Chen Z. Laparoscopic pancreaticoduodenectomy versus open pancreaticoduodenectomy for pancreatic ductal adenocarcinoma: oncologic outcomes and long-term survival. *Surg Endosc* 2020; **34**: 1948-1958 [PMID: 31317331 DOI: 10.1007/s00464-019-06968-8]

115 **Zhou W**, Jin W, Wang D, Lu C, Xu X, Zhang R, Kuang T, Zhou Y, Wu W, Jin D, Mou Y, Lou W. Laparoscopic versus open pancreaticoduodenectomy for pancreatic ductal adenocarcinoma: a propensity score matching analysis. *Cancer Commun (Lond)* 2019; **39**: 66 [PMID: 31661036 DOI: 10.1186/s40880-019-0410-8]

116 **Chen XM**, Sun DL, Zhang Y. Laparoscopic versus open pancreaticoduodenectomy combined with uncinated process approach: A comparative study evaluating perioperative outcomes (Retrospective cohort study). *Int J Surg* 2018; **51**: 170-173 [PMID: 29408641 DOI: 10.1016/j.ijsu.2018.01.038]

117 **Dang C**, Wang M, Zhu F, Qin T, Qin R. Comparison of laparoscopic and open pancreaticoduodenectomy for the treatment of nonpancreatic periampullary adenocarcinomas: a propensity score matching analysis. *Am J Surg* 2020 [PMID: 33386105 DOI: 10.1016/j.amjsurg.2020.12.023]

118 **Palanivelu C**, Senthilnathan P, Sabnis SC, Babu NS, Srivatsan Gurumurthy S, Anand Vijai N, Nalankilli VP, Praveen Raj P, Parthasarathy R, Rajapandian S. Randomized clinical trial of laparoscopic versus open pancreatoduodenectomy for periampullary tumours. *Br J Surg* 2017; **104**: 1443-1450 [PMID: 28895142 DOI: 10.1002/bjs.10662]

119 **Nassour I**, Wang SC, Porembka MR, Yopp AC, Choti MA, Augustine MM, Polanco PM, Mansour JC, Minter RM. Robotic Versus Laparoscopic Pancreaticoduodenectomy: a NSQIP Analysis. *J Gastrointest Surg* 2017; **21**: 1784-1792 [PMID: 28819886 DOI: 10.1007/s11605-017-3543-6]

120 **Kamarajah SK**, Bundred J, Marc OS, Jiao LR, Manas D, Abu Hilal M, White SA. Robotic versus conventional laparoscopic pancreaticoduodenectomy a systematic review and meta-analysis. *Eur J Surg Oncol* 2020; **46**: 6-14 [PMID: 31409513 DOI: 10.1016/j.ejso.2019.08.007]

121 **Wong JC**, Raman S. Surgical resectability of pancreatic adenocarcinoma: CTA. *Abdom Imaging* 2010; **35**: 471-480 [PMID: 19468791 DOI: 10.1007/s00261-009-9539-2]

122 **Sanjay P**, Takaori K, Govil S, Shrikhande SV, Windsor JA. 'Artery-first' approaches to pancreatoduodenectomy. *Br J Surg* 2012; **99**: 1027-1035 [PMID: 22569924 DOI: 10.1002/bjs.8763]

123 **Yu XZ**, Li J, Fu DL, Di Y, Yang F, Hao SJ, Jin C. Benefit from synchronous portal-superior mesenteric vein resection during pancreaticoduodenectomy for cancer: a meta-analysis. *Eur J Surg Oncol* 2014; **40**: 371-378 [PMID: 24560302 DOI: 10.1016/j.ejso.2014.01.010]

124 **Bell R**, Ao BT, Ironside N, Bartlett A, Windsor JA, Pandanaboyana S. Meta-analysis and cost effective analysis of portal-superior mesenteric vein resection during pancreatoduodenectomy: Impact on margin status and survival. *Surg Oncol* 2017; **26**: 53-62 [PMID: 28317585 DOI: 10.1016/j.suronc.2016.12.007]

125 **Mollberg N**, Rahbari NN, Koch M, Hartwig W, Hoeger Y, Büchler MW, Weitz J. Arterial resection during pancreatectomy for pancreatic cancer: a systematic review and meta-analysis. *Ann Surg* 2011; **254**: 882-893 [PMID: 22064622 DOI: 10.1097/SLA.0b013e31823ac299]

126 **Bassi C**, Marchegiani G, Dervenis C, Sarr M, Abu Hilal M, Adham M, Allen P, Andersson R, Asbun HJ, Besselink MG, Conlon K, Del Chiaro M, Falconi M, Fernandez-Cruz L, Fernandez-Del Castillo C, Fingerhut A, Friess H, Gouma DJ, Hackert T, Izbicki J, Lillemoe KD, Neoptolemos JP, Olah A, Schulick R, Shrikhande SV, Takada T, Takaori K, Traverso W, Vollmer CR, Wolfgang CL, Yeo CJ, Salvia R, Buchler M; International Study Group on Pancreatic Surgery (ISGPS). The 2016 update of the International Study Group (ISGPS) definition and grading of postoperative pancreatic fistula: 11 Years After. *Surgery* 2017; **161**: 584-591 [PMID: 28040257 DOI: 10.1016/j.surg.2016.11.014]

127 **Zhou Y**, Yu J, Wu L, Li B. Meta-analysis of pancreaticogastrostomy versus pancreaticojejunostomy on occurrences of postoperative pancreatic fistula after pancreaticoduodenectomy. *Asian J Surg* 2015; **38**: 155-160 [PMID: 25913732 DOI: 10.1016/j.asjsur.2015.02.002]

128 **Liu FB**, Chen JM, Geng W, Xie SX, Zhao YJ, Yu LQ, Geng XP. Pancreaticogastrostomy is associated with significantly less pancreatic fistula than pancreaticojejunostomy reconstruction after pancreaticoduodenectomy: a meta-analysis of seven randomized controlled trials. *HPB (Oxford)* 2015; **17**: 123-130 [PMID: 24888576 DOI: 10.1111/hpb.12279]

129 **Que W**, Fang H, Yan B, Li J, Guo W, Zhai W, Zhang S. Pancreaticogastrostomy versus pancreaticojejunostomy after pancreaticoduodenectomy: a meta-analysis of randomized controlled trials. *Am J Surg* 2015; **209**: 1074-1082 [PMID: 25743406 DOI: 10.1016/j.amjsurg.2014.07.019]

130 **Kawaida H**, Kono H, Hosomura N, Amemiya H, Itakura J, Fujii H, Ichikawa D. Surgical techniques and postoperative management to prevent postoperative pancreatic fistula after pancreatic surgery. *World J Gastroenterol* 2019; **25**: 3722-3737 [PMID: 31391768 DOI: 10.3748/wjg.v25.i28.3722]

131 **Kleespies A**, Rentsch M, Seeliger H, Albertsmeier M, Jauch KW, Bruns CJ. Blumgart anastomosis for pancreaticojejunostomy minimizes severe complications after pancreatic head resection. *Br J Surg* 2009; **96**: 741-750 [PMID: 19526614 DOI: 10.1002/bjs.6634]

132 **Kawakatsu S**, Inoue Y, Mise Y, Ishizawa T, Ito H, Takahashi Y, Saiura A. Comparison of pancreatojejunostomy techniques in patients with a soft pancreas: Kakita anastomosis and Blumgart anastomosis. *BMC Surg* 2018; **18**: 88 [PMID: 30355352 DOI: 10.1186/s12893-018-0420-5]

133 **Hirono S**, Kawai M, Okada KI, Miyazawa M, Kitahata Y, Hayami S, Ueno M, Yamaue H. Modified Blumgart Mattress Suture Versus Conventional Interrupted Suture in Pancreaticojejunostomy During Pancreaticoduodenectomy: Randomized Controlled Trial. *Ann Surg* 2019; **269**: 243-251 [PMID: 29697455 DOI: 10.1097/SLA.0000000000002802]

134 **Chen Y**, Ke N, Tan C, Zhang H, Wang X, Mai G, Liu X. Continuous versus interrupted suture techniques of pancreaticojejunostomy after pancreaticoduodenectomy. *J Surg Res* 2015; **193**: 590-597 [PMID: 25175768 DOI: 10.1016/j.jss.2014.07.066]

135 **Ji W**, Shao Z, Zheng K, Wang J, Song B, Ma H, Tang L, Shi L, Wang Y, Li X, Zhang Y, Jin G. Pancreaticojejunostomy with double-layer continuous suturing is associated with a lower risk of pancreatic fistula after pancreaticoduodenectomy: a comparative study. *Int J Surg* 2015; **13**: 84-89 [PMID: 25481836 DOI: 10.1016/j.ijsu.2014.11.034]

136 **Peng SY**, Wang JW, Lau WY, Cai XJ, Mou YP, Liu YB, Li JT. Conventional versus binding pancreaticojejunostomy after pancreaticoduodenectomy: a prospective randomized trial. *Ann Surg* 2007; **245**: 692-698 [PMID: 17457161 DOI: 10.1097/01.sla.0000255588.50964.5d]

137 **Berger AC**, Howard TJ, Kennedy EP, Sauter PK, Bower-Cherry M, Dutkevitch S, Hyslop T, Schmidt CM, Rosato EL, Lavu H, Nakeeb A, Pitt HA, Lillemoe KD, Yeo CJ. Does type of pancreaticojejunostomy after pancreaticoduodenectomy decrease rate of pancreatic fistula? *J Am Coll Surg* 2009; **208**: 738-47; discussion 747 [PMID: 19476827 DOI: 10.1016/j.jamcollsurg.2008.12.031]

138 **Bai X**, Zhang Q, Gao S, Lou J, Li G, Zhang Y, Ma T, Xu Y, Liang T. Duct-to-Mucosa vs Invagination for Pancreaticojejunostomy after Pancreaticoduodenectomy: A Prospective, Randomized Controlled Trial from a Single Surgeon. *J Am Coll Surg* 2016; **222**: 10-18 [PMID: 26577499 DOI: 10.1016/j.jamcollsurg.2015.10.003]

139 **Kojima T**, Niguma T, Watanabe N, Sakata T, Mimura T. Modified Blumgart anastomosis with the "complete packing method" reduces the incidence of pancreatic fistula and complications after resection of the head of the pancreas. *Am J Surg* 2018; **216**: 941-948 [PMID: 29606278 DOI: 10.1016/j.amjsurg.2018.03.024]

140 **Bin X**, Lian B, Jianping G, Bin T. Comparison of patient outcomes with and without stenting tube in pancreaticoduodenectomy. *J Int Med Res* 2018; **46**: 403-410 [PMID: 28718685 DOI: 10.1177/0300060517717400]

141 **Suzuki S**, Kaji S, Koike N, Harada N, Tanaka S, Hayashi T, Suzuki M, Hanyu F. Pancreaticojejunostomy of duct to mucosa anastomosis can be performed more safely without than with a stenting tube. *Am J Surg* 2009; **198**: 51-54 [PMID: 19217599 DOI: 10.1016/j.amjsurg.2008.05.008]

142 **Sachs TE**, Pratt WB, Kent TS, Callery MP, Vollmer CM Jr. The pancreaticojejunal anastomotic stent: friend or foe? *Surgery* 2013; **153**: 651-662 [PMID: 23305594 DOI: 10.1016/j.surg.2012.11.007]

143 **Motoi F**, Egawa S, Rikiyama T, Katayose Y, Unno M. Randomized clinical trial of external stent drainage of the pancreatic duct to reduce postoperative pancreatic fistula after pancreaticojejunostomy. *Br J Surg* 2012; **99**: 524-531 [PMID: 22497024 DOI: 10.1002/bjs.8654]

144 **Patel K**, Teta A, Sukharamwala P, Thoens J, Szuchmacher M, DeVito P. External pancreatic duct stent reduces pancreatic fistula: a meta-analysis and systematic review. *Int J Surg* 2014; **12**: 827-832 [PMID: 25003575 DOI: 10.1016/j.ijsu.2014.06.008]

145 **Hong S**, Wang H, Yang S, Yang K. External stent versus no stent for pancreaticojejunostomy: a meta-analysis of randomized controlled trials. *J Gastrointest Surg* 2013; **17**: 1516-1525 [PMID: 23568149 DOI: 10.1007/s11605-013-2187-4]

146 **Zhang GQ**, Li XH, Ye XJ, Chen HB, Fu NT, Wu AT, Li Y. Internal Versus External Drainage With a Pancreatic Duct Stent For Pancreaticojejunostomy During Pancreaticoduodenectomy for Patients at High Risk for Pancreatic Fistula: A Comparative Study. *J Surg Res* 2018; **232**: 247-256 [PMID: 30463725 DOI: 10.1016/j.jss.2018.06.033]

147 **Zhou W**, Lv R, Wang X, Mou Y, Cai X, Herr I. Stapler vs suture closure of pancreatic remnant after distal pancreatectomy: a meta-analysis. *Am J Surg* 2010; **200**: 529-536 [PMID: 20538249 DOI: 10.1016/j.amjsurg.2009.12.022]

148 **Diener MK**, Seiler CM, Rossion I, Kleeff J, Glanemann M, Butturini G, Tomazic A, Bruns CJ, Busch OR, Farkas S, Belyaev O, Neoptolemos JP, Halloran C, Keck T, Niedergethmann M, Gellert K, Witzigmann H, Kollmar O, Langer P, Steger U, Neudecker J, Berrevoet F, Ganzera S, Heiss MM, Luntz SP, Bruckner T, Kieser M, Büchler MW. Efficacy of stapler versus hand-sewn closure after distal pancreatectomy (DISPACT): a randomised, controlled multicentre trial. *Lancet* 2011; **377**: 1514-1522 [PMID: 21529927 DOI: 10.1016/S0140-6736(11)60237-7]

149 **Jensen EH**, Portschy PR, Chowaniec J, Teng M. Meta-analysis of bioabsorbable staple line reinforcement and risk of fistula following pancreatic resection. *J Gastrointest Surg* 2013; **17**: 267-272 [PMID: 22948840 DOI: 10.1007/s11605-012-2016-1]

150 **Klein F**, Glanemann M, Faber W, Gül S, Neuhaus P, Bahra M. Pancreatoenteral anastomosis or direct closure of the pancreatic remnant after a distal pancreatectomy: a single-centre experience. *HPB (Oxford)* 2012; **14**: 798-804 [PMID: 23134180 DOI: 10.1111/j.1477-2574.2012.00538.x]

151 **Meniconi RL**, Caronna R, Borreca D, Schiratti M, Chirletti P. Pancreato-jejunostomy versus hand-sewn closure of the pancreatic stump to prevent pancreatic fistula after distal pancreatectomy: a retrospective analysis. *BMC Surg* 2013; **13**: 23 [PMID: 23819892 DOI: 10.1186/1471-2482-13-23]

152 **Martin I**, Au K. Does fibrin glue sealant decrease the rate of anastomotic leak after a pancreaticoduodenectomy? *HPB (Oxford)* 2013; **15**: 561-566 [PMID: 23458447 DOI: 10.1111/hpb.12018]

153 **Gong J**, He S, Cheng Y, Cheng N, Gong J, Zeng Z. Fibrin sealants for the prevention of postoperative pancreatic fistula following pancreatic surgery. *Cochrane Database Syst Rev* 2018; **6**: CD009621 [PMID: 29934987 DOI: 10.1002/14651858.CD009621.pub3]

154 **Tian Y**, Ma H, Peng Y, Li G, Yang H. Preventive effect of omental flap in pancreaticoduodenectomy against postoperative complications: a meta-analysis. *Hepatogastroenterology* 2015; **62**: 187-189 [PMID: 25911894]

155 **Hassenpflug M**, Hinz U, Strobel O, Volpert J, Knebel P, Diener MK, Doerr-Harim C, Werner J, Hackert T, Büchler MW. Teres Ligament Patch Reduces Relevant Morbidity After Distal Pancreatectomy (the DISCOVER Randomized Controlled Trial). *Ann Surg* 2016; **264**: 723-730 [PMID: 27455155 DOI: 10.1097/SLA.0000000000001913]

156 **Behrman SW**, Zarzaur BL, Parmar A, Riall TS, Hall BL, Pitt HA. Routine drainage of the operative bed following elective distal pancreatectomy does not reduce the occurrence of complications. *J Gastrointest Surg* 2015; **19**: 72-9; discussion 79 [PMID: 25115324 DOI: 10.1007/s11605-014-2608-z]

157 **Van Buren G 2nd**, Bloomston M, Schmidt CR, Behrman SW, Zyromski NJ, Ball CG, Morgan KA, Hughes SJ, Karanicolas PJ, Allendorf JD, Vollmer CM Jr, Ly Q, Brown KM, Velanovich V, Winter JM, McElhany AL, Muscarella P 2nd, Schmidt CM, House MG, Dixon E, Dillhoff ME, Trevino JG, Hallet J, Coburn NSG, Nakeeb A, Behrns KE, Sasson AR, Ceppa EP, Abdel-Misih SRZ, Riall TS, Silberfein EJ, Ellison EC, Adams DB, Hsu C, Tran Cao HS, Mohammed S, Villafañe-Ferriol N, Barakat O, Massarweh NN, Chai C, Mendez-Reyes JE, Fang A, Jo E, Mo Q, Fisher WE. A Prospective Randomized Multicenter Trial of Distal Pancreatectomy With and Without Routine Intraperitoneal Drainage. *Ann Surg* 2017; **266**: 421-431 [PMID: 28692468 DOI: 10.1097/SLA.0000000000002375]

158 **Witzigmann H**, Diener MK, Kienkötter S, Rossion I, Bruckner T, Bärbel Werner, Pridöhl O, Radulova-Mauersberger O, Lauer H, Knebel P, Ulrich A, Strobel O, Hackert T, Büchler MW. No Need for Routine Drainage After Pancreatic Head Resection: The Dual-Center, Randomized, Controlled PANDRA Trial (ISRCTN04937707). *Ann Surg* 2016; **264**: 528-537 [PMID: 27513157 DOI: 10.1097/SLA.0000000000001859]

159 **Van Buren G 2nd**, Bloomston M, Hughes SJ, Winter J, Behrman SW, Zyromski NJ, Vollmer C, Velanovich V, Riall T, Muscarella P, Trevino J, Nakeeb A, Schmidt CM, Behrns K, Ellison EC, Barakat O, Perry KA, Drebin J, House M, Abdel-Misih S, Silberfein EJ, Goldin S, Brown K, Mohammed S, Hodges SE, McElhany A, Issazadeh M, Jo E, Mo Q, Fisher WE. A randomized prospective multicenter trial of pancreaticoduodenectomy with and without routine intraperitoneal drainage. *Ann Surg* 2014; **259**: 605-612 [PMID: 24374513 DOI: 10.1097/SLA.0000000000000460]

160 **Wang YC**, Szatmary P, Zhu JQ, Xiong JJ, Huang W, Gomatos I, Nunes QM, Sutton R, Liu XB. Prophylactic intra-peritoneal drain placement following pancreaticoduodenectomy: a systematic review and meta-analysis. *World J Gastroenterol* 2015; **21**: 2510-2521 [PMID: 25741162 DOI: 10.3748/wjg.v21.i8.2510]

161 **Nagakawa Y**, Matsudo T, Hijikata Y, Kikuchi S, Bunso K, Suzuki Y, Kasuya K, Tsuchida A. Bacterial contamination in ascitic fluid is associated with the development of clinically relevant pancreatic fistula after pancreatoduodenectomy. *Pancreas* 2013; **42**: 701-706 [PMID: 23429497 DOI: 10.1097/MPA.0b013e31826d3a41]

162 **Kurumboor P**, Palaniswami KN, Pramil K, George D, Ponnambathayil S, Varma D, Aikot S. Octreotide Does Not Prevent Pancreatic Fistula Following Pancreatoduodenectomy in Patients with Soft Pancreas and Non-dilated Duct: A Prospective Randomized Controlled Trial. *J Gastrointest Surg* 2015; **19**: 2038-2044 [PMID: 26302879 DOI: 10.1007/s11605-015-2925-x]

163 **Fernández-Cruz L**, Jiménez Chavarría E, Taurà P, Closa D, Boado MA, Ferrer J. Prospective randomized trial of the effect of octreotide on pancreatic juice output after pancreaticoduodenectomy in relation to histological diagnosis, duct size and leakage. *HPB (Oxford)* 2013; **15**: 392-399 [PMID: 23557411 DOI: 10.1111/j.1477-2574.2012.00608.x]

164 **Sarr MG**; Pancreatic Surgery Group. The potent somatostatin analogue vapreotide does not decrease pancreas-specific complications after elective pancreatectomy: a prospective, multicenter, double-blinded, randomized, placebo-controlled trial. *J Am Coll Surg* 2003; **196**: 556-64; discussion 564 [PMID: 12691930 DOI: 10.1159/000345874]

165 **Allen PJ**, Gönen M, Brennan MF, Bucknor AA, Robinson LM, Pappas MM, Carlucci KE, D'Angelica MI, DeMatteo RP, Kingham TP, Fong Y, Jarnagin WR. Pasireotide for postoperative pancreatic fistula. *N Engl J Med* 2014; **370**: 2014-2022 [PMID: 24849084 DOI: 10.1056/NEJMoa1313688]

166 **Young S**, Sung ML, Lee JA, DiFronzo LA, O'Connor VV. Pasireotide is not effective in reducing the development of postoperative pancreatic fistula. *HPB (Oxford)* 2018; **20**: 834-840 [PMID: 30060910 DOI: 10.1016/j.hpb.2018.03.007]

167 **Elliott IA**, Dann AM, Ghukasyan R, Damato L, Girgis MD, King JC, Hines OJ, Reber HA, Donahue TR. Pasireotide does not prevent postoperative pancreatic fistula: a prospective study. *HPB (Oxford)* 2018; **20**: 418-422 [PMID: 29398424 DOI: 10.1016/j.hpb.2017.10.018]

168 **Furuse J**, Shibahara J, Sugiyama M. Development of chemotherapy and significance of conversion surgery after chemotherapy in unresectable pancreatic cancer. *J Hepatobiliary Pancreat Sci* 2018; **25**: 261-268 [PMID: 29651809 DOI: 10.1002/jhbp.547]

169 **Kunzmann V**, Algül H, Goekkurt E, Siegler GM, Martens UM, Waldschmidt D, Network NCC. Pancreatic Adenocarcinoma (Version 1.2021) 2021. [cited 12 December 2020]. Available from: https://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf

170 **Hackert T**, Sachsenmaier M, Hinz U, Schneider L, Michalski CW, Springfeld C, Strobel O, Jäger D, Ulrich A, Büchler MW. Locally Advanced Pancreatic Cancer: Neoadjuvant Therapy With Folfirinox Results in Resectability in 60% of the Patients. *Ann Surg* 2016; **264**: 457-463 [PMID: 27355262 DOI: 10.1097/SLA.0000000000001850]

171 **Macedo FI**, Ryon E, Maithel SK, Lee RM, Kooby DA, Fields RC, Hawkins WG, Williams G, Maduekwe U, Kim HJ, Ahmad SA, Patel SH, Abbott DE, Schwartz P, Weber SM, Scoggins CR, Martin RCG, Dudeja V, Franceschi D, Livingstone AS, Merchant NB. Survival Outcomes Associated With Clinical and Pathological Response Following Neoadjuvant FOLFIRINOX or Gemcitabine/Nab-Paclitaxel Chemotherapy in Resected Pancreatic Cancer. *Ann Surg* 2019; **270**: 400-413 [PMID: 31283563 DOI: 10.1097/SLA.0000000000003468]

172 **Clarke NW**, Ali A, Ingleby FC, Hoyle A, Amos CL, Attard G, Brawley CD, Calvert J, Chowdhury S, Cook A, Cross W, Dearnaley DP, Douis H, Gilbert D, Gillessen S, Jones RJ, Langley RE, MacNair A, Malik Z, Mason MD, Matheson D, Millman R, Parker CC, Ritchie AWS, Rush H, Russell JM, Brown J, Beesley S, Birtle A, Capaldi L, Gale J, Gibbs S, Lydon A, Nikapota A, Omlin A, O'Sullivan JM, Parikh O, Protheroe A, Rudman S, Srihari NN, Simms M, Tanguay JS, Tolan S, Wagstaff J, Wallace J, Wylie J, Zarkar A, Sydes MR, Parmar MKB, James ND; STAMPEDE investigators. Corrigendum to Addition of docetaxel to hormonal therapy in low- and high-burden metastatic hormone sensitive prostate cancer: long-term survival results from the STAMPEDE trial: Ann Oncol 2019; 30: 1992-2003. *Ann Oncol* 2020; **31**: 442 [PMID: 32067690 DOI: 10.1016/j.annonc.2020.01.002]

173 **Michelakos T**, Pergolini I, Castillo CF, Honselmann KC, Cai L, Deshpande V, Wo JY, Ryan DP, Allen JN, Blaszkowsky LS, Clark JW, Murphy JE, Nipp RD, Parikh A, Qadan M, Warshaw AL, Hong TS, Lillemoe KD, Ferrone CR. Predictors of Resectability and Survival in Patients With Borderline and Locally Advanced Pancreatic Cancer who Underwent Neoadjuvant Treatment With FOLFIRINOX. *Ann Surg* 2019; **269**: 733-740 [PMID: 29227344 DOI: 10.1097/SLA.0000000000002600]

174 **Rangelova E**, Wefer A, Persson S, Valente R, Tanaka K, Orsini N, Segersvärd R, Arnelo U, Del

Chiaro M. Surgery Improves Survival After Neoadjuvant Therapy for Borderline and Locally Advanced Pancreatic Cancer: A Single Institution Experience. *Ann Surg* 2021; **273**: 579-586 [PMID: 30946073 DOI: 10.1097/SLA.0000000000003301]

175 **Yoo C**, Shin SH, Kim KP, Jeong JH, Chang HM, Kang JH, Lee SS, Park DH, Song TJ, Seo DW, Lee SK, Kim MH, Park JH, Hwang DW, Song KB, Lee JH, Ryoo BY, Kim SC. Clinical Outcomes of Conversion Surgery after Neoadjuvant Chemotherapy in Patients with Borderline Resectable and Locally Advanced Unresectable Pancreatic Cancer: A Single-Center, Retrospective Analysis. *Cancers (Basel)* 2019; **11** [PMID: 30813624 DOI: 10.3390/cancers11030278]

176 **Satoi S**, Yamamoto T, Yamaki S, Sakaguchi T, Sekimoto M. Surgical indication for and desirable outcomes of conversion surgery in patients with initially unresectable pancreatic ductal adenocarcinoma. *Ann Gastroenterol Surg* 2020; **4**: 6-13 [PMID: 32021953 DOI: 10.1002/ags3.12295]

177 **Tajima H**, Ohta T, Kitagawa H, Okamoto K, Sakai S, Makino I, Kinoshita J, Furukawa H, Nakamura K, Hayashi H, Oyama K, Inokuchi M, Nakagawara H, Fujita H, Takamura H, Ninomiya I, Fushida S, Tani T, Fujimura T, Ikeda H, Kitamura S. Pilot study of neoadjuvant chemotherapy with gemcitabine and oral S-1 for resectable pancreatic cancer. *Exp Ther Med* 2012; **3**: 787-792 [PMID: 22969969 DOI: 10.3892/etm.2012.482]

178 **O'Reilly EM**, Perelshteyn A, Jarnagin WR, Schattner M, Gerdes H, Capanu M, Tang LH, LaValle J, Winston C, DeMatteo RP, D'Angelica M, Kurtz RC, Abou-Alfa GK, Klimstra DS, Lowery MA, Brennan MF, Coit DG, Reidy DL, Kingham TP, Allen PJ. A single-arm, nonrandomized phase II trial of neoadjuvant gemcitabine and oxaliplatin in patients with resectable pancreas adenocarcinoma. *Ann Surg* 2014; **260**: 142-148 [PMID: 24901360 DOI: 10.1097/SLA.0000000000000251]

179 **Motoi F**, Ishida K, Fujishima F, Ottomo S, Oikawa M, Okada T, Shimamura H, Takemura S, Ono F, Akada M, Nakagawa K, Katayose Y, Egawa S, Unno M. Neoadjuvant chemotherapy with gemcitabine and S-1 for resectable and borderline pancreatic ductal adenocarcinoma: results from a prospective multi-institutional phase 2 trial. *Ann Surg Oncol* 2013; **20**: 3794-3801 [PMID: 23838925 DOI: 10.1245/s10434-013-3129-9]

180 **Scott E**, Mamawala M, Epstein JI, Landis P, Wolf S, Trock, Carter HB. Intermediate and longer-term outcomes from a prospective active-surveillance program for favorable-risk prostate cancer. Tosoian JJ, Mamawala M, Epstein JI, Landis P, Wolf S, Trock BJ, Carter HB.J Clin Oncol. 2015 Oct 20;33(30):3379-85. [Epub 2015 Aug 31]. doi: 10.1200/JCO.2015.62.5764. *Urol Oncol* 2017; **35**: 121-122 [PMID: 28159497 DOI: 10.1016/j.urolonc.2016.12.019]

181 **Labori KJ**, Lassen K, Hoem D, Grønbech JE, Søreide JA, Mortensen K, Smaaland R, Sorbye H, Verbeke C, Dueland S. Neoadjuvant chemotherapy versus surgery first for resectable pancreatic cancer (Norwegian Pancreatic Cancer Trial - 1 (NorPACT-1)) - study protocol for a national multicentre randomized controlled trial. *BMC Surg* 2017; **17**: 94 [PMID: 28841916 DOI: 10.1186/s12893-017-0291-1]

182 **Heinrich S**, Pestalozzi B, Lesurtel M, Berrevoet F, Laurent S, Delpero JR, Raoul JL, Bachellier P, Dufour P, Moehler M, Weber A, Lang H, Rogiers X, Clavien PA. Adjuvant gemcitabine versus NEOadjuvant gemcitabine/oxaliplatin plus adjuvant gemcitabine in resectable pancreatic cancer: a randomized multicenter phase III study (NEOPAC study). *BMC Cancer* 2011; **11**: 346 [PMID: 21831266 DOI: 10.1186/1471-2407-11-346]

183 **Sohal D**, McDonough SL, Ahmad SA, Gandhi N, Hochster HS. SWOG S1505: A randomized phase II study of perioperative mFOLFIRINOX vs. gemcitabine/nab-paclitaxel as therapy for resectable pancreatic adenocarcinom. *J Clin Oncol* 2017; **35**: TPS4152-TPS4152 [DOI: 10.1200/JCO.2017.35.15_suppl.TPS4152]

184 **Turrini O**, Viret F, Moureau-Zabotto L, Guiramand J, Moutardier V, Lelong B, de Chaisemartin C, Giovannini M, Delpero JR. Neoadjuvant 5 fluorouracil-cisplatin chemoradiation effect on survival in patients with resectable pancreatic head adenocarcinoma: a ten-year single institution experience. *Oncology* 2009; **76**: 413-419 [PMID: 19407474 DOI: 10.1159/000215928]

185 **Golcher H**, Brunner TB, Witzigmann H, Marti L, Bechstein WO, Bruns C, Jungnickel H, Schreiber S, Grabenbauer GG, Meyer T, Merkel S, Fietkau R, Hohenberger W. Neoadjuvant chemoradiation therapy with gemcitabine/cisplatin and surgery versus immediate surgery in resectable pancreatic cancer: results of the first prospective randomized phase II trial. *Strahlenther Onkol* 2015; **191**: 7-16 [PMID: 25252602 DOI: 10.1007/s00066-014-0737-7]

186 **Okano K**, Suto H, Oshima M, Maeda E, Yamamoto N, Kakinoki K, Kamada H, Masaki T, Takahashi S, Shibata T, Suzuki Y. A Prospective Phase II Trial of Neoadjuvant S-1 with Concurrent Hypofractionated Radiotherapy in Patients with Resectable and Borderline Resectable Pancreatic Ductal Adenocarcinoma. *Ann Surg Oncol* 2017; **24**: 2777-2784 [PMID: 28608121 DOI: 10.1245/s10434-017-5921-4]

187 **Hattangadi JA**, Hong TS, Yeap BY, Mamon HJ. Results and patterns of failure in patients treated with adjuvant combined chemoradiation therapy for resected pancreatic adenocarcinoma. *Cancer* 2009; **115**: 3640-3650 [PMID: 19514088 DOI: 10.1002/cncr.24410]

188 **Smeenk HG**, van Eijck CH, Hop WC, Erdmann J, Tran KC, Debois M, van Cutsem E, van Dekken H, Klinkenbijl JH, Jeekel J. Long-term survival and metastatic pattern of pancreatic and periampullary cancer after adjuvant chemoradiation or observation: long-term results of EORTC trial 40891. *Ann Surg* 2007; **246**: 734-740 [PMID: 17968163 DOI: 10.1097/SLA.0b013e318156eef3]

189 **Neoptolemos JP**, Dunn JA, Stocken DD, Almond J, Link K, Beger H, Bassi C, Falconi M, Pederzoli

P, Dervenis C, Fernandez-Cruz L, Lacaine F, Pap A, Spooner D, Kerr DJ, Friess H, Büchler MW; European Study Group for Pancreatic Cancer. Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. *Lancet* 2001; **358**: 1576-1585 [PMID: 11716884 DOI: 10.1016/s0140-6736(01)06651-x]

190 **Oettle H**, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, Schramm H, Fahlke J, Zuelke C, Burkart C, Gutberlet K, Kettner E, Schmalenberg H, Weigang-Koehler K, Bechstein WO, Niedergethmann M, Schmidt-Wolf I, Roll L, Doerken B, Riess H. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* 2007; **297**: 267-277 [PMID: 17227978 DOI: 10.1001/jama.297.3.267]

191 **Neoptolemos JP**, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, Padbury R, Moore MJ, Gallinger S, Mariette C, Wente MN, Izbicki JR, Friess H, Lerch MM, Dervenis C, Oláh A, Butturini G, Doi R, Lind PA, Smith D, Valle JW, Palmer DH, Buckels JA, Thompson J, McKay CJ, Rawcliffe CL, Büchler MW; European Study Group for Pancreatic Cancer. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. *JAMA* 2010; **304**: 1073-1081 [PMID: 20823433 DOI: 10.1001/jama.2010.1275]

192 **Neoptolemos JP**, Palmer DH, Ghaneh P, Psarelli EE, Valle JW, Halloran CM, Faluyi O, O'Reilly DA, Cunningham D, Wadsley J, Darby S, Meyer T, Gillmore R, Anthoney A, Lind P, Glimelius B, Falk S, Izbicki JR, Middleton GW, Cummins S, Ross PJ, Wasan H, McDonald A, Crosby T, Ma YT, Patel K, Sherriff D, Soomal R, Borg D, Sothi S, Hammel P, Hackert T, Jackson R, Büchler MW; European Study Group for Pancreatic Cancer. Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. *Lancet* 2017; **389**: 1011-1024 [PMID: 28129987 DOI: 10.1016/S0140-6736(16)32409-6]

193 **Conroy T**, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, Choné L, Francois E, Artru P, Biagi JJ, Lecomte T, Assenat E, Faroux R, Ychou M, Volet J, Sauvanet A, Breysacher G, Di Fiore F, Cripps C, Kavan P, Texereau P, Bouhier-Leporrier K, Khemissa-Akouz F, Legoux JL, Juzyna B, Gourgou S, O'Callaghan CJ, Jouffroy-Zeller C, Rat P, Malka D, Castan F, Bachet JB; Canadian Cancer Trials Group and the Unicancer-GI–PRODIGE Group. FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer. *N Engl J Med* 2018; **379**: 2395-2406 [PMID: 30575490 DOI: 10.1056/NEJMoa1809775]

194 **Uesaka K**, Boku N, Fukutomi A, Okamura Y, Konishi M, Matsumoto I, Kaneoka Y, Shimizu Y, Nakamori S, Sakamoto H, Morinaga S, Kainuma O, Imai K, Sata N, Hishinuma S, Ojima H, Yamaguchi R, Hirano S, Sudo T, Ohashi Y; JASPAC 01 Study Group. Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: a phase 3, open-label, randomised, non-inferiority trial (JASPAC 01). *Lancet* 2016; **388**: 248-257 [PMID: 27265347 DOI: 10.1016/S0140-6736(16)30583-9]

195 **Sinn M**, Bahra M, Liersch T, Gellert K, Messmann H, Bechstein W, Waldschmidt D, Jacobasch L, Wilhelm M, Rau BM, Grützmann R, Weinmann A, Maschmeyer G, Pelzer U, Stieler JM, Striefler JK, Ghadimi M, Bischoff S, Dörken B, Oettle H, Riess H. CONKO-005: Adjuvant Chemotherapy With Gemcitabine Plus Erlotinib Versus Gemcitabine Alone in Patients After R0 Resection of Pancreatic Cancer: A Multicenter Randomized Phase III Trial. *J Clin Oncol* 2017; **35**: 3330-3337 [PMID: 28817370 DOI: 10.1200/JCO.2017.72.6463]

196 **Sinn M**, Liersch T, Riess H, Gellert K, Stübs P, Waldschmidt D, Lammert F, Maschmeyer G, Bechstein W, Bitzer M, Denzlinger C, Hofheinz R, Lindig U, Ghadimi M, Hinke A, Striefler JK, Pelzer U, Bischoff S, Bahra M, Oettle H. CONKO-006: A randomised double-blinded phase IIb-study of additive therapy with gemcitabine + sorafenib/placebo in patients with R1 resection of pancreatic cancer - Final results. *Eur J Cancer* 2020; **138**: 172-181 [PMID: 32890813 DOI: 10.1016/j.ejca.2020.06.032]

197 **Conroy T**, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817-1825 [PMID: 21561347 DOI: 10.1056/NEJMoa1011923]

198 **Von Hoff DD**, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* 2013; **369**: 1691-1703 [PMID: 24131140 DOI: 10.1056/NEJMoa1304369]

199 **Wang-Gillam A**, Li CP, Bodoky G, Dean A, Shan YS, Jameson G, Macarulla T, Lee KH, Cunningham D, Blanc JF, Hubner RA, Chiu CF, Schwartsmann G, Siveke JT, Braiteh F, Moyo V, Belanger B, Dhindsa N, Bayever E, Von Hoff DD, Chen LT; NAPOLI-1 Study Group. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global, randomised, open-label, phase 3 trial. *Lancet* 2016; **387**: 545-557 [PMID: 26615328 DOI: 10.1016/S0140-6736(15)00986-1]

200 **Hingorani SR**, Zheng L, Bullock AJ, Seery TE, Harris WP, Sigal DS, Braiteh F, Ritch PS, Zalupski MM, Bahary N, Oberstein PE, Wang-Gillam A, Wu W, Chondros D, Jiang P, Khelifa S, Pu J,

Aldrich C, Hendifar AE. HALO 202: Randomized Phase II Study of PEGPH20 Plus Nab-Paclitaxel/Gemcitabine Versus Nab-Paclitaxel/Gemcitabine in Patients With Untreated, Metastatic Pancreatic Ductal Adenocarcinoma. *J Clin Oncol* 2018; **36**: 359-366 [PMID: 29232172 DOI: 10.1200/JCO.2017.74.9564]

201 **Roth MT**, Cardin DB, Berlin JD. Recent advances in the treatment of pancreatic cancer. *F1000Res* 2020; **9** [PMID: 32148767 DOI: 10.12688/f1000research.21981.1]

202 **Henriksen A**, Dyhl-Polk A, Chen I, Nielsen D. Checkpoint inhibitors in pancreatic cancer. *Cancer Treat Rev* 2019; **78**: 17-30 [PMID: 31325788 DOI: 10.1016/j.ctrv.2019.06.005]

203 **Upadhrasta S**, Zheng L. Strategies in Developing Immunotherapy for Pancreatic Cancer: Recognizing and Correcting Multiple Immune "Defects" in the Tumor Microenvironment. *J Clin Med* 2019; **8** [PMID: 31527414 DOI: 10.3390/jcm8091472]

204 **Marabelle A**, Le DT, Ascierto PA, Di Giacomo AM, De Jesus-Acosta A, Delord JP, Geva R, Gottfried M, Penel N, Hansen AR, Piha-Paul SA, Doi T, Gao B, Chung HC, Lopez-Martin J, Bang YJ, Frommer RS, Shah M, Ghori R, Joe AK, Pruitt SK, Diaz LA Jr. Efficacy of Pembrolizumab in Patients With Noncolorectal High Microsatellite Instability/Mismatch Repair-Deficient Cancer: Results From the Phase II KEYNOTE-158 Study. *J Clin Oncol* 2020; **38**: 1-10 [PMID: 31682550 DOI: 10.1200/JCO.19.02105]

205 **Drilon A**, Laetsch TW, Kummar S, DuBois SG, Lassen UN, Demetri GD, Nathenson M, Doebele RC, Farago AF, Pappo AS, Turpin B, Dowlati A, Brose MS, Mascarenhas L, Federman N, Berlin J, El-Deiry WS, Baik C, Deeken J, Boni V, Nagasubramanian R, Taylor M, Rudzinski ER, Meric-Bernstam F, Sohal DPS, Ma PC, Raez LE, Hechtman JF, Benayed R, Ladanyi M, Tuch BB, Ebata K, Cruickshank S, Ku NC, Cox MC, Hawkins DS, Hong DS, Hyman DM. Efficacy of Larotrectinib in TRK Fusion-Positive Cancers in Adults and Children. *N Engl J Med* 2018; **378**: 731-739 [PMID: 29466156 DOI: 10.1056/NEJMoa1714448]

206 **Drilon A**, Siena S, Ou SI, Patel M, Ahn MJ, Lee J, Bauer TM, Farago AF, Wheler JJ, Liu SV, Doebele R, Giannetta L, Cerea G, Marrapese G, Schirru M, Amatu A, Bencardino K, Palmeri L, Sartore-Bianchi A, Vanzulli A, Cresta S, Damian S, Duca M, Ardini E, Li G, Christiansen J, Kowalski K, Johnson AD, Patel R, Luo D, Chow-Maneval E, Hornby Z, Multani PS, Shaw AT, De Braud FG. Safety and Antitumor Activity of the Multitargeted Pan-TRK, ROS1, and ALK Inhibitor Entrectinib: Combined Results from Two Phase I Trials (ALKA-372-001 and STARTRK-1). *Cancer Discov* 2017; **7**: 400-409 [PMID: 28183697 DOI: 10.1158/2159-8290.CD-16-1237]

207 **Golan T**, Kanji ZS, Epelbaum R, Devaud N, Dagan E, Holter S, Aderka D, Paluch-Shimon S, Kaufman B, Gershoni-Baruch R, Hedley D, Moore MJ, Friedman E, Gallinger S. Overall survival and clinical characteristics of pancreatic cancer in BRCA mutation carriers. *Br J Cancer* 2014; **111**: 1132-1138 [PMID: 25072261 DOI: 10.1038/bjc.2014.418]

208 **Golan T**, Hammel P, Reni M, Van Cutsem E, Macarulla T, Hall MJ, Park JO, Hochhauser D, Arnold D, Oh DY, Reinacher-Schick A, Tortora G, Algül H, O'Reilly EM, McGuinness D, Cui KY, Schlienger K, Locker GY, Kindler HL. Maintenance Olaparib for Germline *BRCA*-Mutated Metastatic Pancreatic Cancer. *N Engl J Med* 2019; **381**: 317-327 [PMID: 31157963 DOI: 10.1056/NEJMoa1903387]

209 **Rouanet M**, Lebrin M, Gross F, Bournet B, Cordelier P, Buscail L. Gene Therapy for Pancreatic Cancer: Specificity, Issues and Hopes. *Int J Mol Sci* 2017; **18** [PMID: 28594388 DOI: 10.3390/ijms18061231]

210 **Chen S**, Xu M, Zhao J, Shen J, Li J, Liu Y, Cao G, Ma J, He W, Chen X, Shan T. MicroRNA-4516 suppresses pancreatic cancer development *via* negatively regulating orthodenticle homeobox 1. *Int J Biol Sci* 2020; **16**: 2159-2169 [PMID: 32549762 DOI: 10.7150/ijbs.45933]

211 **Roth S**, Springfeld C, Diener MK, Tjaden C, Knebel P, Klaiber U, Michalski CW, Mieth M, Jäger D, Büchler MW, Hackert T. Protocol of a prospective, monocentric phase I/II feasibility study investigating the safety of multimodality treatment with a combination of intraoperative chemotherapy and surgical resection in locally confined or borderline resectable pancreatic cancer: the combiCaRe study. *BMJ Open* 2019; **9**: e028696 [PMID: 31434770 DOI: 10.1136/bmjopen-2018-028696]

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REVIEW

Silencing hepatitis B virus covalently closed circular DNA: The potential of an epigenetic therapy approach

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Abstract

Global prophylactic vaccination programmes have helped to curb new hepatitis B virus (HBV) infections. However, it is estimated that nearly 300 million people are chronically infected and have a high risk of developing hepatocellular carcinoma. As such, HBV remains a serious health priority and the development of novel curative therapeutics is urgently needed. Chronic HBV infection has been attributed to the persistence of the covalently closed circular DNA (cccDNA) which establishes itself as a minichromosome in the nucleus of hepatocytes. As the viral transcription intermediate, the cccDNA is responsible for producing new virions and perpetuating infection. HBV is dependent on various host factors for cccDNA formation and the minichromosome is amenable to epigenetic modifications. Two HBV proteins, X (HBx) and core (HBc) promote viral replication by modulating the cccDNA epigenome and regulating host cell responses. This includes viral and host gene expression, chromatin remodeling, DNA methylation, the antiviral immune response, apoptosis, and ubiquitination. Elimination of the cccDNA minichromosome would result in a sterilizing cure; however, this may be difficult to achieve. Epigenetic therapies could permanently silence the cccDNA minichromosome and promote a functional cure. This review explores the cccDNA epigenome, how host and viral factors influence transcription, and the recent epigenetic therapies and epigenome engineering approaches that have been described.

Key Words: Chronic hepatitis B virus; Epigenetic gene silencing; Functional cure; Hepatocellular carcinoma; Hepatitis B surface antigen

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Core Tip: Epigenetic regulation of the hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) minichromosome is important for establishing and maintaining infection. To do this HBV manipulates several cellular pathways, resulting in an intricate and complex interplay between the virus and the host. Epigenetic silencing of the cccDNA could permanently inhibit viral transcription. Therapies such as immune modulators, small molecules, and epigenome engineering tools could silence HBV DNA to promote a functional cure.

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INTRODUCTION

Chronic HBV infection (CHB) remains largely incurable and is a major risk factor for the development of hepatocellular carcinoma (HCC)[1]. Regrettably, only a small percentage of chronically infected individuals are currently afforded therapy[2] and the annual rate of hepatitis-related deaths remains high. This underpins the importance of developing new therapeutics to treat the estimated 257-291 million people afflicted by this life-threatening disease[3]. Licensed direct-acting antivirals such as nucleoside/nucleotide analogues help mitigate infection by preventing viral replication but have little effect on established covalently closed circular DNA (cccDNA)[4]. Immunotherapy approaches using interferon alpha (IFN-α) have demonstrated antiviral efficacy, particularly when using pegylated formulations, and have been paired with various nucleoside/nucleotide analogues for combination therapy[5]. However cost, serious side effects, and mixed results have limited the broad clinical feasibility of IFN-based therapies in their current form. Persistence of the cccDNA as a minichromosome-like structure in the nucleus of long-lived hepatocytes may account for the inability of these antivirals to achieve cure, even after long-term treatment[6]. A sterilizing cure can only be realized following complete elimination of intrahepatic cccDNA, while permanent loss of serum hepatitis B surface antigen (HBsAg) would achieve a functional cure. The cccDNA is thought to endure for years [7] and as the template for viral mRNA synthesis[8], it drives viral replication and remains the major obstacle in the development of curative antiviral therapies.

In recent years, treatments designed to directly silence or eliminate the cccDNA minichromosome have gained interest as they may provide a means for achieving cure [9]. Gene editing tools such as designer nucleases and nickases disrupt the viral DNA *via* site-directed cleavage. This stimulates either targeted mutagenesis or degradation of the cccDNA[10,11]. Zinc finger (ZF) nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided nucleases have all demonstrated antiviral potential in preclinical models of infection. The simplicity of the CRISPR/Cas system has led to a surge in the field of anti-HBV gene editing, particularly as multiple studies have reported degradation of cccDNA following cleavage[12-15]. The CRISPR/Cas constructs developed by Seeger and Sohn[16] were shown to edit HBV DNA 15000 times more efficiently than IFN-α-induced APOBEC deamination. However, concerns regarding off-target cleavage, liver-specific delivery and expression, and Cas9 immunity will need to be addressed to ensure safety without compromising efficacy [17-20]. Viral vectors, including adeno-associated viruses (AAVs) and high-capacity adenoviral vectors (HCAdV) have recently been explored as hepatotropic delivery vehicles[13,21,22]. To overcome the packaging limitations of AAVs, the smaller *Staphylococcus aureus* endonucleases have been combined with HBV-specific guide RNAs to demonstrate cccDNA-targeting and antiviral efficacy in hNTCP-HepG2 cells [22] and transgenic mice[21]. On the other hand, the large packaging capacity of HCAdVs have been exploited to accommodate multiple HBV guide RNAs along with the larger *Streptococcus pyogenes* Cas9[13], which may improve the efficacy of this gene editing approach. Importantly, iterative modifications to the effector domains of traditional gene editing platforms have given rise to several new tools. CRISPR/Cas

base editors comprising an APOBEC1 deaminase, Cas9 nickase, and uracil glycosylase inhibitor introduced point mutations in cccDNA as well as integrated viral DNA[23]. A number of new epigenome engineering platforms have also been developed, which could be used to generate targeted epigenetic changes to control gene expression[24]. Such an approach may be well suited to treating CHB, as epigenetic modifications regulate cccDNA minichromosome organization and can either promote or repress viral transcription[25-27]. Host and/or virus-specific epigenetic therapies could promote silencing of HBV cccDNA and achieve a functional cure. This review will explore the formation and epigenetic regulation of the cccDNA minichromosome, how host and viral factors influence transcription, and whether epigenome editing could be used to silence HBV cccDNA permanently.

CCCDNA FORMATION AND ORGANIZATION OF THE MINICHROMOSOME

cccDNA biogenesis is a multi-step process that relies on a number of host-cell DNA synthesis and repair factors[28,29]. Although the exact process is not fully understood it involves: (1) Removal of the covalently attached viral polymerase from the 5′ end of the negative DNA strand; (2) Removal of the short RNA oligomer attached to the 5′ end of the positive strand which is derived from the priming of positive-strand DNA synthesis; (3) Removal of one copy of the short terminal redundancy from the negative-strand; (4) Positive-strand elongation; and (5) Covalent ligation of the two strands[30-32]. Evidence from animal models has shown that between 1 and 50 copies of cccDNA may accumulate per infected cell[33-36]; however, more recent reports estimate up to 15 copies per cell[37,38]. Multiple rounds of infection are not necessary to establish the cccDNA pool as recycling of newly synthesized relaxed circular DNA (rcDNA) has been shown to occur, and contributes to the maintenance of cccDNA copy numbers[39-41]. Increasing evidence also indicates that the size of the pool is controlled by different host and viral mechanisms[42], but that initial *de novo* cccDNA formation is sufficient to maintain viral replication in the absence of rcDNA recycling [43].

Many different host factors have been implicated in cccDNA biogenesis, including host DNA polymerases[32,44], ligases[45], flap endonuclease I (FEN1)[46], topoisomerase I and II[47], SAMHD1[48], and tyrosyl-DNA phosphodiesterase 2[31]. Five essential components for cccDNA formation have recently been described by Wei and Ploss[49], who developed a cell-free biochemical system to mimic cccDNA biogenesis. The components are proliferating cell nuclear antigen (PCNA), replication factor C complex, DNA polymerase δ, FEN1, and DNA ligase 1. Confirmation of these requirements in hepatocytes will be needed to validate the cell-free biochemical system[50]. Aphidicolin and CD437 were shown to block host DNA polymerases α, which in turn inhibited intracellular amplification of cccDNA[44]. Also disubstituted sulfonamide compounds, CCC-0975 and CCC-0346, influenced conversion of rcDNA to cccDNA, most likely as a result of inhibiting deproteination[51]. Blocking the conversion of rcDNA to cccDNA with small molecule inhibitors may prevent accumulation of new replication-competent viral genomes in the nucleus of infected hepatocytes, but will not prevent *de novo* cccDNA biogenesis.

Once formed, cccDNA is organized into a nucleosome-decorated minichromosome [8,52]. It associates with histone and non-histone proteins, the latter of which are derived from both the host and the virus[29,33]. As a hepatotropic virus, the viral genome contains binding sites for ubiquitous and liver-specific transcription factors [53]. Thus, the recruitment, activity, and dynamic interplay of several host and viral factors are essential for efficient HBV gene expression[25-27]. Several histone proteins, transcription factors, chromatin modifying enzymes, as well as additional host enzymes and coactivators have been reported to bind to cccDNA (Table 1). Recruitment of these factors plays an essential role in regulating viral transcription which is amenable to epigenetic influences. Apart from host factors, viral elements such as HBx (HBV protein X) and HBc (HBV protein core) have been shown to influence gene transcription as well as reduce nucleosomal spacing, indicating a role in promoting viral replication and structural maintenance of the cccDNA minichromosome[8]. Thus, it is conceivable that post-translational modifications (PTMs) such as DNA methylation and histone modifications could be used to regulate cccDNA.

HOST FACTORS AND THE EPIGENETIC REGULATION OF HBV

Epigenetic modifications are reversible heritable phenotypic changes that alter chemical signatures on chromosomes without affecting the DNA sequence. This highly

Table 1 Host cell factors involved in the epigenetic maintenance of the covalently closed circular DNA minichromosome

	Category	Epigenetic effect	Effect on HBV	Ref.
Chromatin remodeling enzymes				
Histone acetyltransferase p300/CBP	Writer	Increases H3K122a	Activation	Ananthanarayanan *et al*[99] and Tropberger *et al*[221]
P300/CBP-associated factor (PCAF)	Writer		Activation	Levrero *et al*[82] and Rivière *et al*[93]
Histone deacetylase 1 (HDAC1)	Eraser	Reduces acetylation of H3K9 and H3K27 with IFN	Inhibition	Pollicino *et al*[57] and Liu *et al*[98]
Sirtuin 1 and 3 (SIRT1/3)	Eraser	Reduces H3K9me3	Inhibition	Belloni *et al*[105] and Ren *et al*[114]
Enhancer of zeste homolog 2 (EZH2)	Writer	Increases H3K27ac and H3K27me3	Inhibition	Zhang *et al*[89]
Histone acetyltransferase 1 (HAT1)	Writer	Overexpression of HAT1 promotes acetylation of H3K27, H4K5 and H4K12	Activation	Yang *et al*[195]
Mixed lineage leukemia protein 3 (MLL3)	Writer	Increases H3K4me3	Activation	Tropberger *et al*[86] and Ananthanarayanan *et al*[99]
Protein arginine methyltransferase (PRMT)	Writer	PRMT5 interacts with HBc to increase H4R3me2s	Inhibition	Zhang *et al*[89]
Demethylases (KDMs)	Eraser	Increases H3K79me and function to transcriptional repression *via* SIRT1-mediated chromatin silencing	Inhibition	Kang *et al*[222]
Histone methyltransferase suppressor of variegation 3-9 homolog 1 (SUV39H1)	Writer	Increases H3K9me3	Inhibition	Peng and Karpen[223,224]
DNA methyltransferase (DNMTs)	Writer		Inhibition	Vivekanandan *et al*[81]
Methyl-CpG binding protein (MBPs)	Reader	Recruit chromatin remodeling and histone-modifying complexes to methylated sites resulting in histone methylation	Inhibition	Lopez-Serra and Esteller[94]
Cellular transcription factors				
Activating transcription factor 2 (ATF 2)		Inhibition viral transcription	Inhibition	Choi *et al*[225]
cAMP response element binding protein (CREB)		Enhances transcription	Activation	Kim *et al*[226] and Song *et al*[227]
Nuclear factor 1 (NF1)	Reader		Activation	Ori *et al*[228] and Shaul *et al*[229]
Transcription factor Yin Yang 1 (YY1)			Inhibition	Belloni *et al*[199] and Nakanishi-Matsui *et al*[230]
Specificity protein 1 (SP1)	Reader		Activation	Raney and McLachlan[231], Raney *et al*[232] and Li and Ou[233]
Nuclear Transcription Factor Y (NF-Y)		Recruit enzymes that both methylate and acetylate histone proteins	Activation	Lu and Yen[234], Maity and de Crombrugghe[235] and Nardini *et al*[236]
Activator protein 1(AP-1)			Activation	Ren *et al*[111] and Choi *et al*[237]
TATA binding protein (TBP)	Reader		Activation	Bogomolski-Yahalom *et al*[238] and Chen *et al*[239]
Prospero-related homeobox protein (Prox1)			Inhibition	Qin *et al*[240]
Nuclear factor kappa-B (NF-κB)			Inhibition	Lin *et al*[241]
Histone-lysine N-methyltransferase SETDB1	Writer	In the absence of HBx, SETDB1 increases H3K9me2 and H3K9me3	Inhibition	Rivière *et al*[93]
Hepatocyte factors				
Retinoid X receptors (RXRα)		Increases acetylation of histones H4 and H3 by recruiting p300 to cccDNA minichromosome	Activation	Zhang *et al*[89] and Nkongolo *et al*[242]
Small heterodimer partner (SHP)			Inhibition	Oropeza *et al*[243]
CAAT enhancer-binding protein		C/EBP in low concentrations; C/EBP in high	Activation;	Raney and McLachlan[231] and

α and ζ (C/EBP)	concentrations	Inhibition	López-Cabrera *et al*[244]
Hepatocyte nuclear factor 1 α and β (HNF1)	HNF1 and Oct 1 are essential co-activators of transcription. High HNF1 levels increase NF-κB expression and resulting in transcription inhibition	Activation and inhibition	Zhou and Yen[245], Zheng *et al*[246] and Lin *et al*[247]
Hepatocyte nuclear factor 3 α, β, and γ (HNF3)	Functions as a chromatin remodeler	Activation	Chen *et al*[248] and Li *et al*[249]
Hepatocyte nuclear factor 4 (HNF4)		Activation	Zheng *et al*[246], Cho *et al*[250] and Long *et al*[251]
Testicular orphan receptor 4 (TR4)		Inhibition	Lin *et al*[252]
Type I interferon (IFN-α)	Reduced acetylation of H3K9and H3K27	Inhibition	Pollicino *et al*[57], Liu *et al*[98] and Yuan *et al*[253]

cccDNA: Covalently closed circular DNA; NF-κB: nuclear factor-kappa B.

regulated process has a critical impact on gene expression, cell function as well as cell behavior[54]. Epigenetic marks can be added directly to DNA or placed on histone tails. Methylation of cytosines within CpG dinucleotides is the most common DNA epigenetic mark, and is critical for controlling transcription, genomic imprinting and cell type identity maintenance[55]. The wrapping of DNA around histones creates highly condensed chromatin structures with protruding N-terminal histone tails amenable to PTMs[56]. Histone acetylation and DNA methylation are required for cccDNA formation and viral replication[57], highlighting the importance of the epigenome in HBV infection.

Numerous proteins are involved in the epigenetic regulation of genes and are generally categorized as writers, readers and erasers (Table 1)[54]. Writers are responsible for establishing epigenetic marks while readers recognize modified residues and recruit other protein complexes to regulate gene expression. Erasers are enzymes that remove epigenetic marks. Epigenetic therapies generally target one or more of these proteins, and studies using this approach for other chronic viral infections[58,59] suggest that this may be a promising treatment strategy for CHB.

Methylation for transcriptional gene silencing

In mammalian cells, methylation is a common cellular defense mechanism known to silence invading foreign DNA[60]. It involves the addition of a methyl group from the methyl donor, S-adenosyl methionine, to the fifth carbon of cytosine in DNA (5mC). DNA methylation is most commonly detected within CpG islands and is associated with transcriptional gene silencing[61]. These inheritable 5mC marks can be 'written' (added) *de novo* in unmethylated regions which are either maintained or actively 'erased' (removed)[62]. In mammals, methyl-CpG binding proteins (MBPs) are responsible for the identification of methylation patterns[63] and a family of DNA methyltransferases (DNMTs) is responsible for establishment and maintenance of these patterns[64]. The DNMT family consists of DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (DNMT3-like). DNMT1 is primarily responsible for the maintenance of the methylation patterns during cell division and shows a preference for hemimethylated DNA[65,66]. *De novo* methylation patterns are established by the two active DNMT3A and DNMT3B enzymes and their activity is enhanced by interaction with DNMT3L which lacks catalytic activity[67,68]. In addition, DNMT2 plays an important role in methylation of structural RNA[69]. More than 20 years ago, it was discovered that integrated HBV DNA could be methylated and this was frequently observed in CHB patients with HCC[70]. Since then methylation of non-integrated HBV DNA as well as cccDNA has been observed in patients' liver samples [71,72]. Several studies have reported that HBV cccDNA can be methylated to various extents, resulting in repression of transcription, decreased viremia and loss of hepatitis B e antigen (HBeAg)[71,73,74]. Thus, the methylation pattern of cccDNA is a crucial component of viral replication and may affect pathogenesis.

HBV cccDNA has three predicted CpG islands which are strategically located in the regulatory elements of the viral genome[75]. The first CpG island (CpG I) overlaps the ATG start site of the sequence encoding the small HBsAg, the second (CpG II) overlaps the enhancer I and II, the core promoter, and HBx promoter sequence, and the third (CpG III) spans the SpI promoter and ATG start codon of the polymerase gene[72,76]. Among the 10 distinct HBV genotypes (A-J), CpG I is present in five genotypes (A, B, D, E, and I) whereas CpG II and III have been shown to exist in all genotypes[76].

Varying degrees of methylation have been reported to occur at the three CpG islands. In patients with CHB, methylation frequencies of 14, 0.6 and 3.7% within CpG I, II and III, respectively, were observed[77]. However, a separate computational study revealed that 50% of HBV sequences lacked CpG I, whereas CpG II and II were conserved across genotypes[76]. This suggests that methylation patterns are likely to differ between the genotypes. Vivekanandan *et al*[72] demonstrated that increased methylation of CpG I and II correlated with reduced viral protein production when analyzing HBV DNA samples isolated from CHB liver biopsies. In addition, the authors showed that patients with occult HBV infection had increased methylation at CpG II when compared to non-occult CHB individuals. A subsequent study compared the methylation status of CpG II in liver tissues from HBeAg+ and HBeAg- individuals [71]. Methylation was higher in HBeAg- samples (48%) compared to HBeAg+ samples (14%), indicating that an increase in methylation may reduce viral protein production. Similarly, a link between methylation of CpG III and lower HBsAg levels has been reported[74]. A cohort of cirrhosis patients failed to show an association between cccDNA methylation and HBsAg expression, but did indicate that higher methylation density was associated with lower viral load and virion productivity[73]. These results show the impact methylation can have on viral gene expression in CHB[78]. A recent comprehensive meta-analysis revealed significant hypermethylation of certain host genes depending on the geographical location of the population[79]. This included six genes in HBV-positive carcinoma tissues (*p16*, *RASSF1A*, *GSTP1*, *APC*, *p15* and *SFRP1)*, two genes in HBV-positive carcinoma sera (*p16* and *APC*) and one gene in HBV-positive adjacent tissues (*GSTP1*). The study also indicated that DNA methylation could lead to the development of HBV-related HCC, an important consideration for epigenetic therapy.

The gene silencing effects of methylation have also been validated in different cell culture models of HBV replication. Transfection of HepG2 cells with methylated HBV DNA led to a reduction in viral mRNA levels, decreased HBV core antigen (HBcAg) and HBsAg expression, and reduced secretion of viral proteins[80]. *In vitro* infection experiments showed an increase in DNMT expression in response to HBV, which led to hypermethylation of the viral DNA, a reduction in viral mRNAs and proteins, and decreased HBV replication[81]. Furthermore, co-transfection of HBV DNA and DNMT3a was associated with decreased production of HBeAg and HBsAg[81].

Histone modifications and the cccDNA epigenome

HBV cccDNA is associated with host histones, whose "bead-on-a-string" organization serves to compact the viral genome and provide a means for regulating gene expression[52]. As such transcription can be controlled by epigenetic modifications to the cccDNA-bound histones, thereby regulating HBV replication[82]. Nucleosome-associated histones undergo numerous types of PTMs that are generally reversible and mainly localized at the amino-terminal histone tails. These direct modifications of the N-terminal tails most commonly include acetylation, methylation, ubiquitination, phosphorylation, SUMOylation, ADP-ribosylation, and deamination[83]. Histone modifications can also indirectly regulate chromatin structure by serving as binding sites for the recruitment of other regulatory proteins[84]. Current studies have shown that PTMs significantly impact HBV replication, maturation and infection[85,86]. Several enzymes such as histone acetyltransferases (HATs) and deacetylases (HDACs), lysine and protein arginine methyltransferases (KMTs and PRMTs), demethylases (HDMTs), kinases, phosphatases, ubiquitin ligase, and deubiquitinases, modify cccDNA-associated histones[83,87]. The epigenome is further influenced by HBx and HBc, adding to the intricate and complex interplay between host and viral proteins on the cccDNA minichromosome (discussed later). Regulation of histone modifications has been proposed as a likely method to reduce cccDNA[8,52,57]. HATs facilitate the acetylation of lysine residues, making the histone-associated DNA more accessible to transcription factors, effectors and RNA polymerase II, to promote gene expression[88, 89]. Conversely, HDACs catalyze the removal of acetyl groups from lysine residues, leading to heterochromatin formation and gene repression[90,91]. Although less characterized, MBPs are reported to recruit HMTs and HDACs to promote histone methylation, which in turn recruits heterochromatin protein 1 factors (HP1) to promote DNA methylation[92-94].

HBV transcription is regulated by the acetylation status of cccDNA bound histones 3 and 4 (H3 and H4) both in cell culture models of viral replication[57,95] and in CHB patients[57]. Recruitment of HDAC1 and hypoacetylation of H3 and H4 is associated with low HBV replication and viremia *in vitro* and *in vivo*[57]. In this study, Pollicino *et al*[57] demonstrated that acetylation of cccDNA-bound H4 correlated with high levels of replication, an effect that was maintained when using HDAC inhibitors. Further-

more, the recruitment of the HATs, CREB-binding protein (CBP) and p300 as well as HDAC1 was associated with low HBV replication in an HBx-dependent manner. NIRF, an E3 ubiquitination ligase, has been shown to reduce acetylation of cccDNA-bound H3 as well as promote proteasomal degradation of HBc[96]. Gong and colleagues previously reported similar results for acetylation of cccDNA-bound H3, and identified roles for histone methylation and phosphorylation in controlling viral replication[85]. Surprisingly, HDAC inhibitors suppressed duck hepatitis B virus (DHBV) cccDNA transcription and reduced viral replication in a dose-dependent manner[97,98], suggesting cell and virus-specific epigenetic factors differ between the avian and human models[98].

Genome wide studies of PTMs on the cccDNA minichromosome revealed high levels of trimethylation at lysine 4 of histone H3 (H3K4me3) and acetylation of lysine residues of histone H3 (H3K27ac and H3K122ac) at specific loci, which lead to gene activation or repression[86]. H3K4me3 modifications are crucial for active transcription of the sodium-taurocholate cotransporting polypeptide (NTCP) gene in HepG2 cells[99]. MLL3, a component of the ASCOM complex, is responsible for this modification and may indirectly facilitate HBV infection, given that NTCP is the hepatotropic receptor[97,99]. Knockdown of another methyltransferase, the enhancer of zeste homolog 2 (EZH2), resulted in upregulation of HBsAg and HBeAg production indicating that EZH2 represses HBV gene expression[89]. A recent study by Zhang *et al*[89] showed that PRMT5 induced symmetric dimethylation of arginine 3 on histone H4 (H4R3me2s) on cccDNA when associated with the hSWI/SNF chromatin remodeling complex and HBc. This epigenetic modification repressed viral transcription by inhibiting binding of RNA polymerase II. Interestingly, encapsidation of pregenomic RNA was also restricted in an epigenetically-independent manner, suggesting multiple antiviral roles for PRMT5[89].

VIRAL FACTORS AS EPIGENETIC REGULATORS

Two viral proteins, HBx and HBc, have been identified as important modulators of HBV transcription which influence the cccDNA epigenome (Figure 1). HBx is a small 17 kDa oncogenic protein that has been shown to interact with multiple host factors to manipulate both host and viral gene expression, mediate host immune responses, and control apoptosis[100]. Although HBx lacks DNA-binding motifs and does not directly bind to the cccDNA minichromosome, it is able to act as a trans-activator of viral replication by recruiting other factors to the minichromosome[101]. HBc is a 21 kDa structural polypeptide that forms the viral nucleocapsid and is essential for reverse transcription of the pgRNA[102]. As such, HBc is important for the packaging and secretion of new virions during HBV replication. Interestingly, it also forms part of the cccDNA minichromosome and is involved in its epigenetic regulation[8,57]. Unlike HBx, the HBc C-terminal domain (CTD) is capable of binding directly to cccDNA[103] as well as host gene promoter regions[104]. As such, HBc binding affects minichromosome organization as it can adjust the number and spacing of cccDNA-associated nucleosomes[57,105,106].

HBx acts as a 'master regulator'

HBx has been shown to recruit a variety of host transcription factors and co-activators to regulate cccDNA transcription (Figure 1). These include ATF/CREB, ATF3, c/EBP, NF-κB, IL-6, Ets, Egr, SMAD4, Oct1, RXR receptor, and p53[82]. HBx-associated protein HBXAP (RSF1) has been shown to bind to HBx *via* its plant homology domain, and acts as a co-activator of viral transcription[107]. Recently, Saeed *et al*[108] demonstrated that HBx can directly bind to parvulin 14 (Par14) and 17 (Par17), which increased the stability of HBx and mediated its translocation to the nucleus and mitochondria. In addition, binding of Par14 and Par17 to the cccDNA at enhancer and promoter regions resulted in upregulation of viral transcription, an effect that was abrogated in the absence of HBx[108]. HBx has also been shown to recruit, block and promote degradation of host antiviral proteins that would normally prevent HBV replication and cccDNA persistence, as well as activate host genes to enhance viral gene expression.

Initiation of viral replication and stabilization of the cccDNA minichromosome is thought to occur through HBx-mediated PTMs of histones associated with hypomethylated CpG islands (mainly GpG I and III)[74,109]. Recruitment of HATs such as CBP, p300, and the p300/CBP-associated factor (PCAF) to the cccDNA minichromosome allows for upregulation of viral transcription[105]. In the presence of

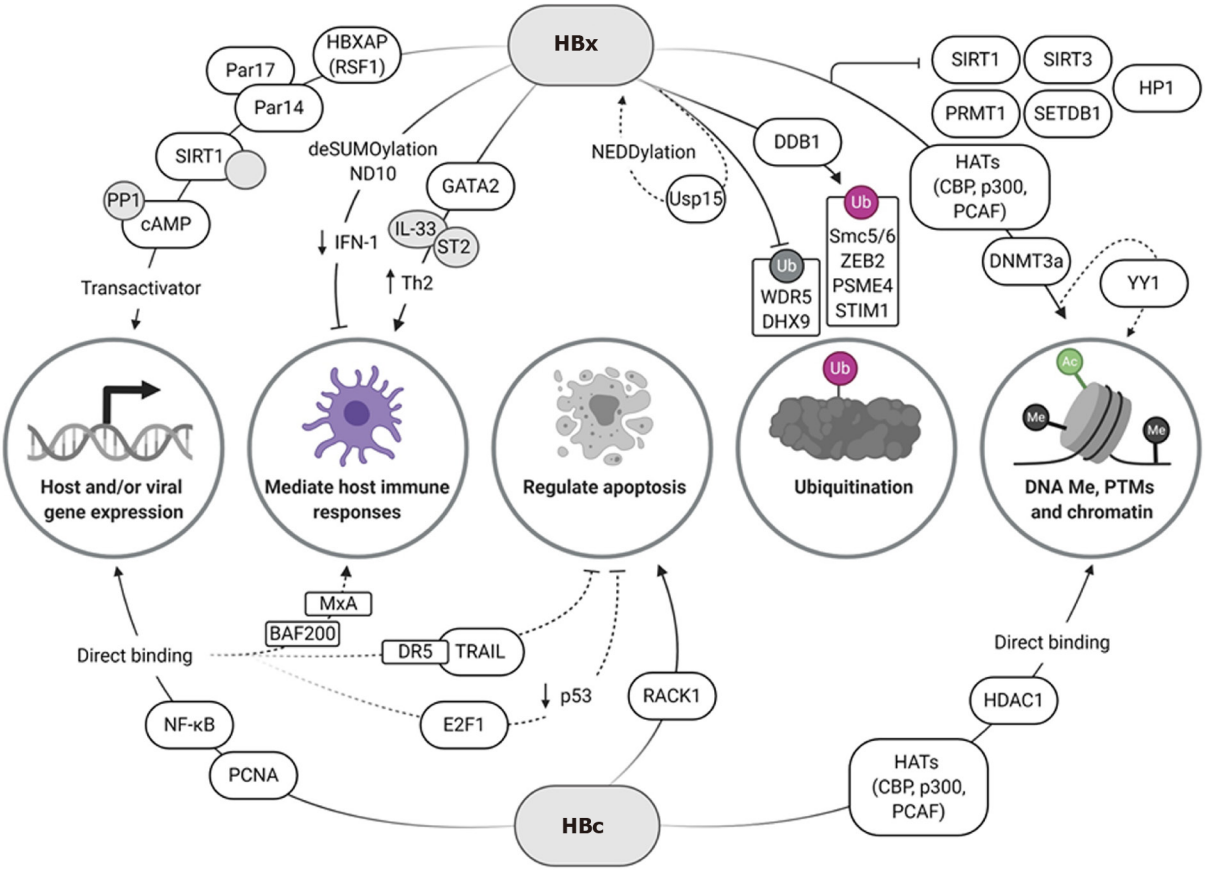


Figure 1 Hepatitis B virus proteins X and core act on multiple host cell pathways to promote viral persistence. An intricate epigenetic regulatory network is established during Hepatitis B virus infection. Hepatitis B virus proteins X and core contribute to this by either directly or indirectly controlling gene expression, modifying chromatin structure or location, mediating host immune responses, regulating apoptosis, and promoting or preventing ubiquitination. Pathways discussed in 'Viral factors as epigenetic regulators' are depicted here. Created with BioRender.com. HBx: Hepatitis B virus X protein; HBc: Hepatitis B virus core protein; IFN: Interferon; IL: Interleukin; NF-κB: nuclear factor-kappa B; PTM: Post-translational modifications; SIRT1: Sirtuin 1; YY1: Yin-Yang 1; HATs: Histone acetyltransferases; HDAC: Histone deacetylase; PCNA: Proliferating cell nuclear antigen; HBXAP: HBx-associated protein; RSF1: Remodeling and spacing factor 1; Par14: Parvulin 14; Par17: Parvulin 17; PP1: Protein phosphatase 1; cAMP: Cyclic adenosine monophosphate; ND10: Nuclear domain 10; GATA2: GATA binding protein 2; ST2: Interleukin 1 receptor-like 1; Th2: T helper cell type 2; Usp15: Ubiquitin-specific peptidase 15; DDB1: Damaged DNA-binding protein 1; Smc5/6: Structural maintenance of chromosomes 5/6; ZEB2: Zinc finger E-box-binding homeobox 2; PSME4: Proteasome activator subunit 4; STIM1: Stromal interaction molecule 1; WDR5: tryptophan-aspartic acid (WD) repeat domain 5 protein; DHX9: DExH-box RNA helicase 9; ub: Ubiquitination; SIRT3: Sirtuin 3; PRMT1: Protein arginine methyltransferase 1; SETDB1: SET domain bifurcated histone lysine methyltransferase 1; HP1: Heterochromatin protein 1 factors; CBP: CREB-binding protein; PCAF: p300/CBP-associated factor; DNMT3a: DNA methyltransferase 3a; MxA: Myxovirus resistance gene A; DR5: Death receptor 5; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; E2F1: E2F transcription factor 1; RACK1: Receptor for activated protein kinase C 1.

an HBx mutant, Belloni *et al*[105] showed increased recruitment of HDAC1 and sirtuin 1 (SIRT1) to the cccDNA, which correlated with a reduction in acetylation of cccDNA-bound H4 and viral transcription. These results suggest a role for HBx in promoting cccDNA histone acetylation to enhance viral transcription. However, the involvement of SIRT1 in the promotion or suppression of HBV replication appears to vary. Other studies found that SIRT1 positively regulated HBV replication by deacetylating PGC-1α and FXRα[110], and promoting binding of activator protein 1 to the core promoter [111]. However, these studies did not investigate the role of SIRT1 in cccDNA-dependent models of viral replication. More recently Deng *et al*[112] showed that in HepG2-NTCP cells, SIRT1 complexes with and stabilizes HBx, and promoted the recruitment of HBx and other co-activating factors to cccDNA. This increased viral transcription in a deacetylase-independent manner. These results suggest a dual role for SIRT1 in the regulation of HBV replication, which may be dependent on the presence or absence of HBx.

Binding of HBx to PRMT1 inhibited the methyltransferase activity of this enzyme, ultimately enhancing viral replication[113]. Recently, HBx was found to inhibit SIRT3 expression by downregulating its expression and possibly inhibiting its recruitment to the cccDNA[114]. SIRT3 promotes cccDNA-histone methylation (H3K9me3) by directly removing H3K9ac marks and recruiting histone methyltransferase SUV39H1 and SETD1A to the cccDNA[114]. However, in the presence of HBx, H3K9me3 marks

were reduced and H3K4me3 and H3K9ac modifications increased to promote HBV transcription. This effect has also been observed for other transcriptional repressors. HBx reduced the chromatin structure alteration-mediated repression of histone-lysine N-methyltransferase SET domain, bifurcate 1 (SETDB1) and HP1[93]; however, the exact molecular mechanisms of this rescue remain to be elucidated. Upregulation of DNMTs by HBx and direct interaction of HBx with DNMT3A has been shown to either upregulate transcription of host genes, or block activity by facilitating host promoter methylation[115]. For example, SOCS-1, SUFU and TIRAP are downregulated, and this could be important in mediating an immune response in CHB, albeit the exact mechanisms still need to be elucidated[81,116]. HBx-mediated downregulation of tumor suppressors may be a factor leading to the development of HCC[115,117-119]. HBx-mediated deSUMOylation removes transcription factors from nuclear domain 10 (ND10), which in turn downregulates IFN-I-response pathway elements and host epigenetic modifications by p300/HDAC1, resulting in viral persistence[120]. Host gene promoters and enhancers can also be stimulated by HBx to regulate HBV replication[121-123]. Upregulation of host *ST2* expression by HBx-GATA2 binding to its promoter and subsequent induction of the IL-33/ST2 axis stimulated a Th2 immune response, instead of the desired antiviral Th1 response[124], which is likely to play a role in the chronicity of infection[125].

As stated before, CREB is recruited by HBx to the cccDNA minichromosome; however, HBx also increases this activator's longevity[126]. By binding to protein phosphatase 1, the activating phosphorylation induced by cAMP has a longer half-life, resulting in increased viral transcription[126]. Interestingly, not only does HBx mediate recruitment of factors directly to the cccDNA, but also promotes spatial localization of the minichromosome. Shen *et al*[127] showed that HBx and the transcription factor Yin-Yang 1 (YY1) aligned the cccDNA minichromosome with a region of the human genome rich in a highly active enhancer element, resulting in increased HBV replication.

Negative regulation by HBx has also been shown through an interaction with peroxiredoxin 1 (Prdx1), a cellular hydrogen peroxide scavenger, which recruits exosome component 5 (Excosc5) to degrade HBV RNA. Although the ability of Prdx1 to bind to HBV RNA was independent of HBx, HBx was required for degradation of the RNA[128].

HBx-mediated ubiquitination

Indirect epigenetic regulation can also occur through HBx-mediated proteasomal degradation of host factors, or by preventing degradation or deactivation of host factors. Damaged DNA-binding protein 1 (DDB1), an adapter protein for the cullin-RING ligase 4 (CRL4) E3 Ligase complex, is responsible for recruitment of DDB1-Cul4-associated factors (DCAFs), which allow for ubiquitination and subsequent degradation of proteins[129]. Interactions between HBx and DDB1 were implicated in early studies[130]. The interaction is mediated by a motif in HBx that mimics the DCAF proteins' binding motif, allowing HBV to hijack the host protein ubiquitination system[131]. Years later, Decorsière *et al*[132] and Murphy *et al*[133] identified the structural maintenance of chromosomes 5/6 (Smc5/6) complex as a target for HBx-mediated proteasomal degradation. The Smc5/6 complex is a host antiviral restriction factor that enables transcriptional repression of the cccDNA minichromosome, by localizing to the ND10 without inducing an innate immune response[134]. However, the epigenetic effect is transient as it does not silence HBx mRNA expression, ultimately leading to Smc5/6 degradation and initiation of viral transcription[135]. Like Smc5/6, other HBV restriction factors are degraded by HBx-mediated polyubiquitination, to increase viral transcription and HBV replication[136]. This includes ZEB2, which inhibits HBV replication by interaction with the core promoter[137], proteasome activator subunit 4 (PSME4), which induces degradation of acetylated histones involved in upregulation of HBV[138,139], and stromal interaction molecule 1 (STIM1), known to regulate cytoplasmic calcium levels[140].

On the other hand, HBx is also able to inhibit proteasomal degradation[141]. This has recently been shown for the WD repeat domain 5 protein (WDR5), a core subunit of H3K4 methyltransferase complexes[142]. HBx stabilized WDR5 by preventing DDB1/cullin-4-induced degradation and in turn, promoted viral gene expression [142]. Shen *et al*[143] demonstrated how HBx could also inhibit MDM2-mediated degradation of DExH-box RNA helicase 9 (DHX9) to enhance viral DNA synthesis.

The epigenetic regulation of viral transcription is further enhanced as a result of HBx-mediated *trans*-activation of its own promoter[144]. Along with this, post-translational modifications of HBx through interactions with the deubiquitinating ubiquitin-specific peptidase 15 (Usp15) enzyme, increase the half-life of the viral protein[145].

This may also be a result of the close interaction of HBx with components of the proteasome machinery, such as the DDB1-CRL4 complex, which may inadvertently result in ubiquitination of HBx itself. HDM2-mediated NEDDylation of HBx was also found to occur, probably because of its close proximity to Cul4, resulting in increased stability and chromatin localization[101,146]. NEDDylation-dependent reduction of ubiquitination and subsequent degradation of HBx by E3 Ligases, such as Siah-1, may account for the increased stability of this viral protein. MLN4924 (pevonedistat), a NEDD8-activating enzyme inhibitor, has been shown to impede HBV replication by reducing cullin[147] and HBx NEDDylation[146]. In addition, MLN4924 promotes upregulation of phosphorylated extracellular signal-regulated kinases (ERKs) resulting in reduced HNF1α, HNF4α and C/EBPα transcription factor levels[147], which are known activators of HBV transcription. Overall, the maintenance of persistent HBV infection by HBx contributes to HCC, by inducing host epigenetic modifications implicated in cancer.

HBc associates with cccDNA and host genes

Direct interaction of the HBc CTD[103] with CpG islands across the cccDNA, especially CpG II, has been shown to induce hypomethylation and subsequent upregulation of viral transcription and regulation[74,148]. This hypomethylation causes increased CBP binding and resulting histone acetylation, further increasing viral gene expression[57,148] albeit at lower levels than with HBx[149,150]. The association of HBc with HDAC1 was also shown[148], as well as P300 and PCAF/CBP [151]. Increased NF-κB binding upstream of *ENII* induced by HBc is thought to increase pre-C promoter activity[152]. Host DNA polymerase coordinator PCNA, which is known to maintain genetic and epigenetic integrity[153], is recruited to cccDNA by HBc which upregulates viral gene expression and plays a role in the development of HCC. However, the exact mechanism of this process is still unclear [154].

HBc can additionally mediate host gene expression through the binding of HBc to endogenous promoter regions. Guo *et al*[104] identified nearly 3100 host promoter regions with potential HBc binding sites. Examples of these include binding to the MxA promoter to evade a host IFN-induced immune response[155] and competitive binding to BAF200, resulting in the downregulation of interferon-induced transmembrane protein 1 (IFITM1) mRNA[156]. Binding of HBc to the death receptor 5 (DR5) promoter reduced DR5 expression, thereby reducing cellular apoptosis through TRAIL which is thought to support CHB infection[157].

Transcription factor binding to mediate host expression has also been shown. The binding of HBc to E2F1 prevented its natural association with the p53 promoter, and hence decreased p53 levels[158]. This could reduce p53-related apoptosis[159] and along with DR5 downregulation further support the development of chronic infection. However, competitive binding of HBc to the receptor for activated protein kinase C 1 (RACK1) prevented phosphorylation of mitogen-activated protein kinase 7 (MKK7) which sensitized cells to apoptosis[160]. This suggests a dual role for HBc in the control of apoptosis and requires further study to determine the impact of these pathways on HBV infection. The subcellular location of HBc is dependent on cell cycle phase, with *in vitro* studies showing that predominantly nuclear localization occurs during G1 phase[161]. Since E2F transcription factors and p53 both have effects on cell cycling[162-164] and are differentially regulated during HBV infection, further investigation of this phenomenon and the possibility of epigenetically controlling the cell cycle is needed. HBc has also been shown to recruit APOBEC3A/B to cccDNA to promote deamination[165]. Since so many HBc binding sites are predicted to occur in endogenous promoter regions, there is the possibility that APOBEC3A/B is recruited to host genes to influence their expression, and may play a role in the development of HCC[166].

REGULATORY NONCODING RNAS

Noncoding RNAs (ncRNAs) consist of small ncRNAs (< 200 nt) and long ncRNA (lncRNA > 200 nt)[167]. Small ncRNAs, including miRNAs, are involved in post-transcriptional gene silencing[167], while lncRNA are implicated in a plethora of host functions including post-transcriptional and chromatin modifications[168]. By altering the host's epigenetic signature, HBV dysregulates the ncRNA landscape to control viral replication and influence hepatocarcinogenesis.

Epigenetic influences of miRNAs

miRNAs have been widely studied in HBV infection, and have been shown to facilitate or inhibit viral replication[169]. An increase in replication can occur by targeting host factors that normally restrict viral replication, for example miR-15b downregulates HNF-1α[170], or by indirectly upregulating factors to promote replication, such as miR-1 upregulation of farnesoid X receptor α (FRXα) expression[171]. Interestingly, miR-1-mediated downregulation of E2F5 and HDAC4 was implicated in G1 cell cycle arrest and upregulation of hepatocyte differentiation factors, to increase HBV replication[171]. This may be related to other viral epigenetic factors such as HBc, which showed similar associations. Indirect FRXα upregulation by miR-449a targeting of CREB5[172], HDAC4 downregulation by miR-548ah[173] and G1 arrest by miR-125b-5p-mediated inhibition of retinoblastoma protein phosphorylation[174] implicate redundant epigenetic pathways in HBV regulation.

A recent study by Moon *et al*[175] has suggested that miR-20a can act in an epigenetic manner and promote methylation of cccDNA. They found that AGO2-miR-20a binding to the cccDNA may recruit DNMT3 to silence gene expression; however, further studies are needed to confirm the exact mechanism. Interestingly miR-146a could play a role in cccDNA formation, through a positive feedback loop with FEN1 [176], and in silencing ZEB2 expression[177].

Induction of autophagy has been associated with many miRNAs including miR-146a-5p[178], the miR-99 family[179] and miR-192-3p, which is downregulated by HBx [180]. miR-155 has also been implicated in autophagy; however, this miRNA is downregulated following HBV infection[181]. HBV upregulates expression of miR-192-5p and miR-215-5p, which results in downregulation of apoptosis[182]. Contrasting effects were observed during HBV-mediated upregulation of miR-194-5p, which resulted in the downregulation of anti-apoptotic proteins SODD and cFLIP and sensitization of liver cells to apoptosis[182]. Once again, the role of HBV in promoting and preventing apoptosis needs further clarification.

Certain cancer-related miRNAs, namely miR-15a/miR-16-1, the miR-17-92 cluster and miR-224, are also associated with decreased viral replication through modification of cccDNA promoters and histones[183]. Interestingly, miR-122, which targets the HBV polymerase ORF and *core* 3'UTR[184], has also been shown to inhibit HBV by targeting cyclin G1 to increase p53-mediated inhibition of replication[185]. However, HBx abrogates these antiviral effects by downregulating miR-122[183]. Host miRNAs can also indirectly inhibit viral replication by regulating various host factors[169] HBx-mediated downregulation of miR-122 alters regulation of its natural host targets, including heme oxygenase-1[186], CCNG1 and NDRG3[187]. Similarly, miR-141 was implicated in targeting both PPARA[188] and SIRT1[189]. Reduced SIRT1 inhibited autophagy, and miR-130a targets liver pyruvate kinase which is thought to reduce energy supply and hence HBV replication[190]. Induction of immune suppression may occur through the increase of miR-199a-5p, miR-221-3p and Let-7a-3p, as shown in immune tolerant HBV-infected patients[191]. HBV-miR-3, a miRNA produced by the virus, reduces replication by directly targeting viral transcripts[192] or upregulating the anti-HBV IFN immune response[193].

Newly discovered lncRNAs implicated in the control of HBV

LncRNAs have been identified as important polyfunctional epigenetic regulators and are associated with disease progression, particularly carcinogenesis[194]. Novel lncRNAs related to development of HBV-associated HCC are continuously being identified; however, a few have also been implicated in epigenetic regulation of viral transcription. LncRNAs highly up-regulated in liver cancer (HULC), DLEU2, and lncRNA 32 have been identified as epigenetic regulators of HBV. HAT1, which is co-activated by HBx-Sp1 binding, is transported to the cccDNA by HULC in an HBc-dependent manner[195]. HULC also mediates upregulation of HBx and the subsequent HBx-STAT upregulation of miR-539, which targets APOBEC3B. This results in reduced cccDNA degradation, with resultant increased transcription of viral genes, viral persistence, and HCC progression[196]. Interestingly, the promotion of HBV transcription by the lncRNA HULC is dependent on both HBx and HBc, which forms a positive feedback loop[195]. In the case of lncRNA DLEU, HBx both upregulates its expression and is co-recruited with it to the cccDNA, where the complex may bind to the histone methyltransferase EZH2[197]. Computational analysis of this interaction suggests that binding alleviates EZH2 transcriptional repression[197]. LncRNAs have also been implicated in the host antiviral response. To increase IFN-stimulated gene (*ISG*) expression, lncRNA 32 binds to activating transcription factor 2 (ATF 2) and promotes gene expression. However, during HBV

infection, the expression of this lncRNA is reduced[198], ultimately dampening the immune response to the virus.

ANTI-HBV EPIGENETIC THERAPY

A number of epigenetic strategies has been identified as having therapeutic potential for CHB[27]. Some promote the host's natural antiviral defense mechanisms while others aim to target specific pathways involved in the epigenetic control of cccDNA.

IFN-α therapy

The antiviral and epigenetic properties of IFN-α therapy are well established[199], and have been adopted as a strategy to treat CHB[5]. Type 1 IFNs interact with the IFN-α/β receptor complex to modulate transcription of ISGs, ultimately to evoke strong innate immune responses against viral infections[200]. Administration of IFN-α to HBV-infected cells and HBV-infected chimeric uPA/SCID mice resulted in the inhibition of viral replication through epigenetic regulation of cccDNA[105]. Hypoacetylation of cccDNA-bound histones was achieved following the active recruitment of histone deacetylases hSIRT1 and HDAC1, methyltransferase EZH2, and the transcriptional repressor YY1[199]. Binding of transcription factors STAT1 and STAT2 to the IFN-α sensitive response element on active cccDNA was shown to be reduced after IFN-α treatment[199]. More recently, the structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1) and promyelocytic leukemia protein have, along with STAT1, been implicated in the stimulation of cccDNA-associated histone PTMs in response to IFN-α[201]. Furthermore, IFN-α-based treatment may regulate the SMC5/6 complex to promote transcriptional repression of cccDNA[202].

When comparing IFN-α treatment to the effects of C646 (a small molecule epigenetic modifying agent that inhibits p300/CBP), Tropberger *et al*[86] demonstrated that C646 reduces HBV transcription in primary human hepatocytes in a dose-dependent manner. Like IFN-α, C646 treatment reduced the levels of active PTMs but without activating the innate immune response. However, neither treatment reduced cccDNA levels[86]. When combined with lymphotoxin-β receptor activation, IFN-α treatment did eliminate cccDNA in an APOBEC-dependent manner[165]. Yet the clinical utility of currently licensed IFN-α-based treatments has proved tricky because some patients respond to therapy, but others show no benefit. High costs and adverse side effects have also limited the use of IFN-α. Alternatives are being investigated and an iterative approach has led to the identification of new IFNs with increased potency, such as the recently described IFN-α 14[203].

Small molecule inhibitors and epigenetic drugs

Epigenetic therapies and immunomodulators have shown promise as anti-HCC agents [204], of which HDAC inhibitors such as 5-Azacytidine (5-Aza) and the EZH2 inhibitor 3-Deazaneplanocin A (DZNep) have recently been explored as combination therapies [205]. Such therapies have also been proposed for the treatment of CHB to silence the cccDNA minichromosome. Two small molecule inhibitors, AGK2 and GS-5801, are currently being investigated as anti-HBV epigenetic drugs[206,207]. AGK2 is an inhibitor of the SIRT2 deacetylase, while GS-5801 prevents erasing of epigenetic marks by lysine demethylase 5 (KDM5). Both have been shown to repress viral replication; however, preliminary results from a single dose of GS-5801 suggest that H3K4me3 may not be permanently associated with cccDNA[207]. Recently Dicoumarol, an inhibitor of NAD(P)H:quinone oxidoreductase 1 (NQO1), has been shown to promote the silencing of cccDNA[208] indirectly. The epigenetic effect of Dicoumarol is thought to arise from the destabilization of the HBx/NQO1 interaction, as the upregulation of NQO1 was shown to increase the half-life of HBx[208]. Although promising, the feasibility of using epigenetic modulators to silence HBV is complex, especially as the virus employs many normal host factors to regulate gene expression. The influence on development of HBV-related HCC and viral integration are additional important considerations for advancing epigenetic therapy. For instance, treating HCC with HDAC inhibitors is likely to promote viral transcription[57], while epigenetic silencing cccDNA gene expression by increasing DNA methylation may promote hepatocarcinogenesis[209].

Targeted epigenetic gene silencing

Epigenome engineering has become a popular targeted gene therapy approach to promote locus-specific epigenetic changes to treat genetic diseases[24]. Effector domains based on a variety of transcriptional activators, repressors, and chromatin remodeling enzymes including the typical writers, readers and erasers (Table 1) have been fused to DNA binding domains (DBD) to promote site-specific modifications. The targeting capabilities of epigenome engineering tools could overcome the non-specific effects epigenetic drugs may have on host gene expression. As such, cccDNA epigenome editors are currently being developed as a novel antiviral gene therapy for CHB (Figure 2).

ZF and transcription activator-like effector (TALE) DBDs have been used to direct different epigenetic effectors, primarily transcriptional repressors, to the HBV genome [210-213]. In 2013, Zhao *et al*[210] described the first ATF which was designed to target an 18 bp region of the HBV X or enhancer I (EnhI) sequence. The ATF was generated by joining six ZF DBDs to the Krüppelassociated box (KRAB) repression domain. Although not tested in a typical HBV replication model, the ATF achieved repression of the HBx promoter in a Hep3B reporter cell line, although accompanying cellular growth arrest was also observed[210]. Building on this study, Luo and colleagues demonstrated the antiviral activity of their EnhI-specific ATF in HepG2.2.15 cells and transgenic mice[211]. Viral replication was inhibited, in this case without affecting cellular growth, despite both models harboring integrated HBV sequences. Interestingly, the EnhI ZF-array alone achieved transcriptional repression, an effect that has previously been reported when using anti-HBV TALENs[214]. The antiviral effect of the ATF was further confirmed in the HBV transgenic mouse model where reductions in viral DNA, HBcAg and HBeAg were observed[211]. By replacing the repressor domain with a methyltransferase, ZF arrays have also been used to achieve targeted methylation of viral DNA. Xirong *et al*[213] fused the C-terminal region of the DNMT3A to a six-finger ZF (XPDnmt3aC) and tested the epigenome editor in HepG2 cells and HBV transgenic mice. The XPDnmt3aC was designed to bind to the HBx promoter region and achieved *de novo* methylation of the HBV DNA in HepG2 cells which was accompanied by a reduction in markers of viral replication. However, in transgenic mice the antiviral effect lasted for about two weeks[213], suggesting that if epigenetic modification was achieved it was not permanent.

Anti-HBV repressor TALEs (rTALEs) are engineered proteins with DBDs derived from the *Xanthomonas* bacteria which have been fused to the KRAB effector domain [212]. Two rTALEs, SPL and SPR, which were designed to bind to the *surface* ORF were tested in both *in vitro* and *in vivo* models of viral replication. Both the SPL and SPR rTALEs inhibited HBV replication, and significant reductions in HBsAg, viral mRNAs, and circulating viral particles were observed[212]. Importantly, a comparison between the liver specific mouse transthyretin (MTTR) or ubiquitously active CMV promoters was performed. The MTTR conferred liver-specific expression which may improve the safety of this epigenome engineering approach. Quantitative DNA methylation analysis using EpiTYPER™ Technology[215] was used to show that increased targeted methylation of intrahepatic HBV DNA was achieved at CpG II [212]. This confirmed the principle of this epigenome engineering approach; however, further validation of these rTALEs on cccDNA is warranted.

Interestingly, despite widespread studies using CRISPR/Cas gene editing technologies to disrupt cccDNA, the alternative epigenome editing platforms have yet to be investigated. CRISPRi and CRISPRa are RNA-guided epigenome modifiers that can repress or promote gene expression, and are generated by fusing single or multiple effector domains to a dead (nuclease deficient) Cas[216]. This means that site-directed epigenetic modifications can be achieved by designing guide RNAs to bind to the cccDNA. Although not yet reported, the field of CRISPR epigenome engineering is constantly expanding and may present a novel way of silencing cccDNA.

CONCLUSION

The epigenetic regulation of the cccDNA minichromosome to either promote or repress viral transcription involves an intricate association between host cell factors and viral proteins that is not yet fully understood. While there is currently debate around whether HBV should be considered a stealth virus[217], it is clear that it can manipulate the host cells replication, transcription and translation machinery for its own benefit. HBx appears to have a key role in viral persistence, not only as a regulator of cccDNA but also as a modulator of RNAs, ubiquitination, apoptosis, and

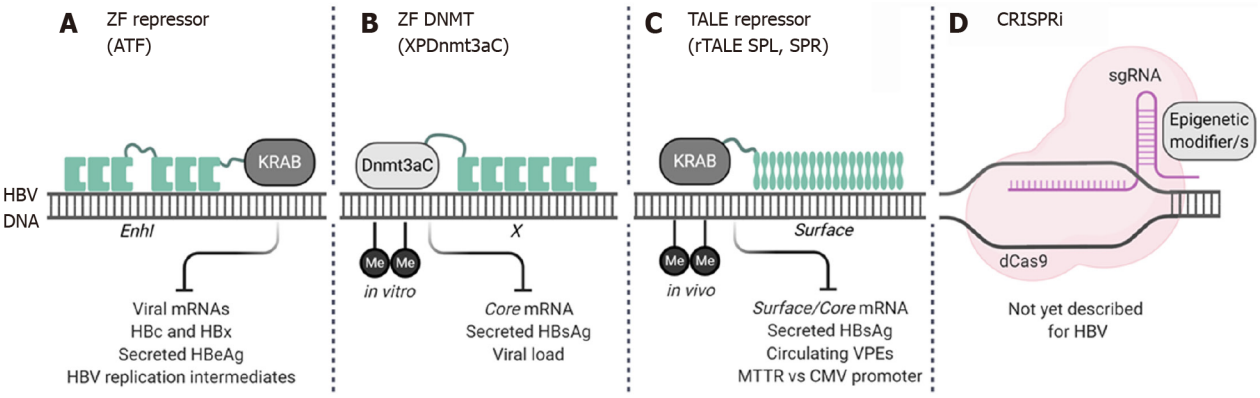


Figure 2 Novel epigenome engineering tools that target hepatitis B virus DNA. Three different types of epigenetic editors have been designed to silence viral gene expression. The Zinc finger (ZF) repressor-based artificial transcription factors (ATF) comprise two three-finger ZF DNA binding domains (DBD) which are fused, with a C-terminal linker peptide, to the Krüppelassociated box (KRAB) repressor domain. ATFs were designed to target the *EnhI* region (A). A ZF-based methyltransferase (DNMT) designed to target the *X* region (XPDnmt3aC). The XPDnmt3aC comprises a six-finger ZF DBD fused to the catalytic domain of Dnmt3a (Dnmt3aC). Methylation of the viral DNA was achieved *in vitro* (B). To generate transcription activator-like effector (TALE) repressors, TALE DBDs targeting two regions of *surface* were fused to the KRAB repressor domain (N-terminal). *In vivo*, methylation of intrahepatic viral DNA was shown (C). Although not yet described for HBV, the CRISPRi epigenome editing tools provide a novel means of silencing covalently closed circular DNA. RNA guides (sgRNA) targeting the viral genome could be used to recruit a dead Cas (dCas9) with one or more epigenetic modifying domains (D). Created with BioRender.com. HBV: Hepatitis B virus; ZF: Zinc finger; ATF: Artificial transcription factor; HBx: Hepatitis B virus X protein; HBc: Hepatitis B virus core protein; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; TALE: Transcription activator-like effector; MTTR: Mouse transthyretin; CMV: Cytomegalovirus; VPE: Viral particle equivalent.

antiviral responses. Although these intricate host-viral pathways require further clarification, the research to date has helped to identify possible feedback loops which could act as new targets for anti-HBV therapy.

There are conflicting views regarding the involvement of certain proteins, for example the epigenetic and deacetylase-independent roles of SIRT1. While some factors may have dual roles, the model systems used to evaluate epigenetic regulation of HBV need to be considered carefully. Although CHB is associated with HCC, epigenetic therapies designed to achieve a functional cure are likely to be given prior to the development of cancer. Using different liver cancer cell lines may skew the HBV epigenetic landscape as regulation of the host cells' epigenome and gene expression is irregular. Establishing an ideal model system is however, easier said than done. Infectious primary human hepatocyte-based models require specialized facilities, as for example is required for the generation of humanized mouse models[218]. Similarly, studies on primary human hepatocytes and three-dimensional liver cultures are costly. Viruses infecting small animals, like the DHBV and woodchuck hepatitis virus, which are commonly used to test novel therapies, may also pose problems for analysis of the cccDNA epigenome. The different effects that HDAC inhibitors had on HBV and DHBV infection suggest differences between the hosts' endogenous epigenetic pathways. Standardizing cccDNA quantification using droplet digital polymerase chain reaction and the liver biopsy-adapted chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction technique (micro-ChIP) may help detect low levels of cccDNA as well as measure epigenetic marks[219].

Epigenetic drugs, improved interferon therapies, and epigenome editing tools are at an interesting stage of development, as new ways to silence cccDNA transcription. Accomplishing targeted epigenetic modifications and long-term viral suppression will be important, and consideration of the HCC epigenetic profile will need careful evaluation. Integrated viral sequences may also be amenable to cccDNA-specific epigenome editing, and undesirable off-target effects on the host genome need to be carefully assessed. Next-generation sequencing platforms like RNA-Seq would help to establish transcriptome profiles and identify potential off-target sites. To date only four targeted epigenome editors have been investigated, and although action on HBV DNA has been shown, a direct effect on the cccDNA minichromome is yet to be established. Furthermore, as with designer nucleases, liver-specific delivery[19] and immune responses to the foreign TALE and Cas proteins will need to be addressed [220]. Delivering epigenome editors as *in vitro* transcribed mRNA transcripts would avoid the packaging constraints of viral vectors[10]. Despite the current challenges associated with epigenetic therapies, there is potential for such an approach to achieve cccDNA gene silencing and perhaps cure. As this relatively new field of research continues to grow, overcoming the hurdles could lead to a promising class of new

antivirals.

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REFERENCES

1 **Levrero M**, Zucman-Rossi J. Mechanisms of HBV-induced hepatocellular carcinoma. *J Hepatol* 2016; **64**: S84-S101 [PMID: 27084040 DOI: 10.1016/j.jhep.2016.02.021]
2 **Hutin Y**, Nasrullah M, Easterbrook P, Nguimfack BD, Burrone E, Averhoff F, Bulterys M. Access to Treatment for Hepatitis B Virus Infection - Worldwide, 2016. *MMWR Morb Mortal Wkly Rep* 2018; **67**: 773-777 [PMID: 30025413 DOI: 10.15585/mmwr.mm6728a2]
3 **Polaris Observatory Collaborators**. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol* 2018; **3**: 383-403 [PMID: 29599078 DOI: 10.1016/S2468-1253(18)30056-6]
4 **Revill PA**, Chisari FV, Block JM, Dandri M, Gehring AJ, Guo H, Hu J, Kramvis A, Lampertico P, Janssen HLA, Levrero M, Li W, Liang TJ, Lim SG, Lu F, Penicaud MC, Tavis JE, Thimme R; Members of the ICE-HBV Working Groups; ICE-HBV Stakeholders Group Chairs; ICE-HBV Senior Advisors, Zoulim F. A global scientific strategy to cure hepatitis B. *Lancet Gastroenterol Hepatol* 2019; **4**: 545-558 [PMID: 30981686 DOI: 10.1016/S2468-1253(19)30119-0]
5 **Tan G**, Song H, Xu F, Cheng G. When Hepatitis B Virus Meets Interferons. *Front Microbiol* 2018; **9**: 1611 [PMID: 30072974 DOI: 10.3389/fmicb.2018.01611]
6 **Lythgoe KA**, Lumley SF, Pellis L, McKeating JA, Matthews PC. Estimating hepatitis B virus cccDNA persistence in chronic infection. *Virus Evol* 2021; **7**: veaa063 [PMID: 33732502 DOI: 10.1093/ve/veaa063]
7 **Nassal M**. Hepatitis B viruses: reverse transcription a different way. *Virus Res* 2008; **134**: 235-249 [PMID: 18339439 DOI: 10.1016/j.virusres.2007.12.024]
8 **Bock CT**, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, Zentgraf H. Structural organization of the hepatitis B virus minichromosome. *J Mol Biol* 2001; **307**: 183-196 [PMID: 11243813 DOI: 10.1006/jmbi.2000.4481]
9 **Bloom K**, Maepa MB, Ely A, Arbuthnot P. Gene Therapy for Chronic HBV-Can We Eliminate cccDNA? *Genes (Basel)* 2018; **9** [PMID: 29649127 DOI: 10.3390/genes9040207]
10 **Ely A**, Singh P, Smith TS, Arbuthnot P. In vitro transcribed mRNA for expression of designer nucleases: Advantages as a novel therapeutic for the management of chronic HBV infection. *Adv Drug Deliv Rev* 2021; **168**: 134-146 [PMID: 32485207 DOI: 10.1016/j.addr.2020.05.010]
11 **Wu X**, Ma W, Mei C, Chen X, Yao Y, Liu Y, Qin X, Yuan Y. Description of CRISPR/Cas9 development and its prospect in hepatocellular carcinoma treatment. *J Exp Clin Cancer Res* 2020; **39**: 97 [PMID: 32487115 DOI: 10.1186/s13046-020-01603-0]
12 **Ramanan V**, Shlomai A, Cox DB, Schwartz RE, Michailidis E, Bhatta A, Scott DA, Zhang F, Rice CM, Bhatia SN. CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Sci Rep* 2015; **5**: 10833 [PMID: 26035283 DOI: 10.1038/srep10833]
13 **Schiwon M**, Ehrke-Schulz E, Oswald A, Bergmann T, Michler T, Protzer U, Ehrhardt A. One-Vector System for Multiplexed CRISPR/Cas9 against Hepatitis B Virus cccDNA Utilizing High-Capacity Adenoviral Vectors. *Mol Ther Nucleic Acids* 2018; **12**: 242-253 [PMID: 30195763 DOI: 10.1016/j.omtn.2018.05.006]
14 **Liu X**, Hao R, Chen S, Guo D, Chen Y. Inhibition of hepatitis B virus by the CRISPR/Cas9 system *via* targeting the conserved regions of the viral genome. *J Gen Virol* 2015; **96**: 2252-2261 [PMID: 25904148 DOI: 10.1099/vir.0.000159]
15 **Kostyushev D**, Brezgin S, Kostyusheva A, Zarifyan D, Goptar I, Chulanov V. Orthologous CRISPR/Cas9 systems for specific and efficient degradation of covalently closed circular DNA of hepatitis B virus. *Cell Mol Life Sci* 2019; **76**: 1779-1794 [PMID: 30673820 DOI: 10.1007/s00018-019-03021-8]
16 **Seeger C**, Sohn JA. Complete Spectrum of CRISPR/Cas9-induced Mutations on HBV cccDNA. *Mol Ther* 2016; **24**: 1258-1266 [PMID: 27203444 DOI: 10.1038/mt.2016.94]
17 **Charlesworth CT**, Deshpande PS, Dever DP, Camarena J, Lemgart VT, Cromer MK, Vakulskas CA, Collingwood MA, Zhang L, Bode NM, Behlke MA, Dejene B, Cieniewicz B, Romano R, Lesch BJ, Gomez-Ospina N, Mantri S, Pavel-Dinu M, Weinberg KI, Porteus MH. Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nat Med* 2019; **25**: 249-254 [PMID: 30692695 DOI: 10.1038/s41591-018-0326-x]
18 **Li A**, Tanner MR, Lee CM, Hurley AE, De Giorgi M, Jarrett KE, Davis TH, Doerfler AM, Bao G, Beeton C, Lagor WR. AAV-CRISPR Gene Editing Is Negated by Pre-existing Immunity to Cas9. *Mol Ther* 2020; **28**: 1432-1441 [PMID: 32348718 DOI: 10.1016/j.ymthe.2020.04.017]
19 **Ates I**, Rathbone T, Stuart C, Bridges PH, Cottle RN. Delivery Approaches for Therapeutic Genome

Editing and Challenges. *Genes (Basel)* 2020; **11** [PMID: 32977396 DOI: 10.3390/genes11101113]
20 **Tong S**, Moyo B, Lee CM, Leong K, Bao G. Engineered materials for *in vivo* delivery of genome-editing machinery. *Nat Rev Mater* 2019; **4**: 726-737 [DOI: 10.1038/s41578-019-0145-9]
21 **Li H**, Sheng C, Liu H, Wang S, Zhao J, Yang L, Jia L, Li P, Wang L, Xie J, Xu D, Sun Y, Qiu S, Song H. Inhibition of HBV Expression in HBV Transgenic Mice Using AAV-Delivered CRISPR-SaCas9. *Front Immunol* 2018; **9**: 2080 [PMID: 30254645 DOI: 10.3389/fimmu.2018.02080]
22 **Scott T**, Moyo B, Nicholson S, Maepa MB, Watashi K, Ely A, Weinberg MS, Arbuthnot P. ssAAVs containing cassettes encoding SaCas9 and guides targeting hepatitis B virus inactivate replication of the virus in cultured cells. *Sci Rep* 2017; **7**: 7401 [PMID: 28785016 DOI: 10.1038/s41598-017-07642-6]
23 **Yang YC**, Chen YH, Kao JH, Ching C, Liu IJ, Wang CC, Tsai CH, Wu FY, Liu CJ, Chen PJ, Chen DS, Yang HC. Permanent Inactivation of HBV Genomes by CRISPR/Cas9-Mediated Non-cleavage Base Editing. *Mol Ther Nucleic Acids* 2020; **20**: 480-490 [PMID: 32278307 DOI: 10.1016/j.omtn.2020.03.005]
24 **Sgro A**, Blancafort P. Epigenome engineering: new technologies for precision medicine. *Nucleic Acids Res* 2020; **48**: 12453-12482 [PMID: 33196851 DOI: 10.1093/nar/gkaa1000]
25 **Dandri M**. Epigenetic modulation in chronic hepatitis B virus infection. *Semin Immunopathol* 2020; **42**: 173-185 [PMID: 32185454 DOI: 10.1007/s00281-020-00780-6]
26 **Hong X**, Kim ES, Guo H. Epigenetic regulation of hepatitis B virus covalently closed circular DNA: Implications for epigenetic therapy against chronic hepatitis B. *Hepatology* 2017; **66**: 2066-2077 [PMID: 28833361 DOI: 10.1002/hep.29479]
27 **Mohd-Ismail NK**, Lim Z, Gunaratne J, Tan YJ. Mapping the Interactions of HBV cccDNA with Host Factors. *Int J Mol Sci* 2019; **20** [PMID: 31480501 DOI: 10.3390/ijms20174276]
28 **Xia Y**, Guo H. Hepatitis B virus cccDNA: Formation, regulation and therapeutic potential. *Antiviral Res* 2020; **180**: 104824 [PMID: 32450266 DOI: 10.1016/j.antiviral.2020.104824]
29 **Schreiner S**, Nassal M. A Role for the Host DNA Damage Response in Hepatitis B Virus cccDNA Formation-and Beyond? *Viruses* 2017; **9** [PMID: 28531167 DOI: 10.3390/v9050125]
30 **Luo J**, Cui X, Gao L, Hu J. Identification of an Intermediate in Hepatitis B Virus Covalently Closed Circular (CCC) DNA Formation and Sensitive and Selective CCC DNA Detection. *J Virol* 2017; **91** [PMID: 28637752 DOI: 10.1128/JVI.00539-17]
31 **Königer C**, Wingert I, Marsmann M, Rösler C, Beck J, Nassal M. Involvement of the host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA persistence reservoir of hepatitis B viruses. *Proc Natl Acad Sci USA* 2014; **111**: E4244-E4253 [PMID: 25201958 DOI: 10.1073/pnas.1409986111]
32 **Qi Y**, Gao Z, Xu G, Peng B, Liu C, Yan H, Yao Q, Sun G, Liu Y, Tang D, Song Z, He W, Sun Y, Guo JT, Li W. DNA Polymerase κ Is a Key Cellular Factor for the Formation of Covalently Closed Circular DNA of Hepatitis B Virus. *PLoS Pathog* 2016; **12**: e1005893 [PMID: 27783675 DOI: 10.1371/journal.ppat.1005893]
33 **Newbold JE**, Xin H, Tencza M, Sherman G, Dean J, Bowden S, Locarnini S. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. *J Virol* 1995; **69**: 3350-3357 [PMID: 7745682 DOI: 10.1128/JVI.69.6.3350-3357.1995]
34 **Moraleda G**, Saputelli J, Aldrich CE, Averett D, Condreay L, Mason WS. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. *J Virol* 1997; **71**: 9392-9399 [PMID: 9371599 DOI: 10.1128/JVI.71.12.9392-9399.1997]
35 **Dandri M**, Burda MR, Will H, Petersen J. Increased hepatocyte turnover and inhibition of woodchuck hepatitis B virus replication by adefovir *in vitro* do not lead to reduction of the closed circular DNA. *Hepatology* 2000; **32**: 139-146 [PMID: 10869302 DOI: 10.1053/jhep.2000.8701]
36 **Wong DK**, Yuen MF, Yuan H, Sum SS, Hui CK, Hall J, Lai CL. Quantitation of covalently closed circular hepatitis B virus DNA in chronic hepatitis B patients. *Hepatology* 2004; **40**: 727-737 [PMID: 15349913 DOI: 10.1002/hep.20353]
37 **Huang JT**, Yang Y, Hu YM, Liu XH, Liao MY, Morgan R, Yuan EF, Li X, Liu SM. A Highly Sensitive and Robust Method for Hepatitis B Virus Covalently Closed Circular DNA Detection in Single Cells and Serum. *J Mol Diagn* 2018; **20**: 334-343 [PMID: 29656833 DOI: 10.1016/j.jmoldx.2018.01.010]
38 **Balagopal A**, Hwang HS, Grudda T, Quinn J, Sterling RK, Sulkowski MS, Thio CL. Single Hepatocyte Hepatitis B Virus Transcriptional Landscape in HIV Coinfection. *J Infect Dis* 2020; **221**: 1462-1469 [PMID: 31740931 DOI: 10.1093/infdis/jiz607]
39 **Wu TT**, Coates L, Aldrich CE, Summers J, Mason WS. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. *Virology* 1990; **175**: 255-261 [PMID: 2155510 DOI: 10.1016/0042-6822(90)90206-7]
40 **Zoulim F**. New insight on hepatitis B virus persistence from the study of intrahepatic viral cccDNA. *J Hepatol* 2005; **42**: 302-308 [PMID: 15710212 DOI: 10.1016/j.jhep.2004.12.015]
41 **Ji M**, Hu K. Recent advances in the study of hepatitis B virus covalently closed circular DNA. *Virol Sin* 2017; **32**: 454-464 [PMID: 29280054 DOI: 10.1007/s12250-017-4009-4]
42 **Köck J**, Rösler C, Zhang JJ, Blum HE, Nassal M, Thoma C. Generation of covalently closed circular DNA of hepatitis B viruses *via* intracellular recycling is regulated in a virus specific manner. *PLoS Pathog* 2010; **6**: e1001082 [PMID: 20824087 DOI: 10.1371/journal.ppat.1001082]
43 **Tu T**, Zehnder B, Qu B, Urban S. *D e novo* synthesis of hepatitis B virus nucleocapsids is

dispensable for the maintenance and transcriptional regulation of cccDNA. *JHEP Rep* 2021; **3**: 100195 [PMID: 33385130 DOI: 10.1016/j.jhepr.2020.100195]

44 **Tang L**, Sheraz M, McGrane M, Chang J, Guo JT. DNA Polymerase alpha is essential for intracellular amplification of hepatitis B virus covalently closed circular DNA. *PLoS Pathog* 2019; **15**: e1007742 [PMID: 31026293 DOI: 10.1371/journal.ppat.1007742]

45 **Long Q**, Yan R, Hu J, Cai D, Mitra B, Kim ES, Marchetti A, Zhang H, Wang S, Liu Y, Huang A, Guo H. The role of host DNA ligases in hepadnavirus covalently closed circular DNA formation. *PLoS Pathog* 2017; **13**: e1006784 [PMID: 29287110 DOI: 10.1371/journal.ppat.1006784]

46 **Kitamura K**, Que L, Shimadu M, Koura M, Ishihara Y, Wakae K, Nakamura T, Watashi K, Wakita T, Muramatsu M. Flap endonuclease 1 is involved in cccDNA formation in the hepatitis B virus. *PLoS Pathog* 2018; **14**: e1007124 [PMID: 29928064 DOI: 10.1371/journal.ppat.1007124]

47 **Sheraz M**, Cheng J, Tang L, Chang J, Guo JT. Cellular DNA Topoisomerases Are Required for the Synthesis of Hepatitis B Virus Covalently Closed Circular DNA. *J Virol* 2019; **93** [PMID: 30867306 DOI: 10.1128/JVI.02230-18]

48 **Wing PA**, Davenne T, Wettengel J, Lai AG, Zhuang X, Chakraborty A, D'Arienzo V, Kramer C, Ko C, Harris JM, Schreiner S, Higgs M, Roessler S, Parish JL, Protzer U, Balfe P, Rehwinkel J, McKeating JA. A dual role for SAMHD1 in regulating HBV cccDNA and RT-dependent particle genesis. *Life Sci Alliance* 2019; **2** [PMID: 30918010 DOI: 10.26508/Lsa.201900355]

49 **Wei L**, Ploss A. Core components of DNA lagging strand synthesis machinery are essential for hepatitis B virus cccDNA formation. *Nat Microbiol* 2020; **5**: 715-726 [PMID: 32152586 DOI: 10.1038/s41564-020-0678-0]

50 **Zhao Q**, Guo JT. Have the Starting Lineup of Five for Hepatitis B Virus Covalently Closed Circular DNA Synthesis Been Identified? *Hepatology* 2020; **72**: 1142-1144 [PMID: 32502295 DOI: 10.1002/hep.31408]

51 **Cai D**, Mills C, Yu W, Yan R, Aldrich CE, Saputelli JR, Mason WS, Xu X, Guo JT, Block TM, Cuconati A, Guo H. Identification of disubstituted sulfonamide compounds as specific inhibitors of hepatitis B virus covalently closed circular DNA formation. *Antimicrob Agents Chemother* 2012; **56**: 4277-4288 [PMID: 22644022 DOI: 10.1128/AAC.00473-12]

52 **Bock CT**, Schranz P, Schröder CH, Zentgraf H. Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. *Virus Genes* 1994; **8**: 215-229 [PMID: 7975268 DOI: 10.1007/BF01703079]

53 **Quasdorff M**, Protzer U. Control of hepatitis B virus at the level of transcription. *J Viral Hepat* 2010; **17**: 527-536 [PMID: 20546497 DOI: 10.1111/j.1365-2893.2010.01315.x]

54 **Allis CD**, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet* 2016; **17**: 487-500 [PMID: 27346641 DOI: 10.1038/nrg.2016.59]

55 **Jeltsch A**, Jurkowska RZ. New concepts in DNA methylation. *Trends Biochem Sci* 2014; **39**: 310-318 [PMID: 24947342 DOI: 10.1016/j.tibs.2014.05.002]

56 **Cutter AR**, Hayes JJ. A brief review of nucleosome structure. *FEBS Lett* 2015; **589**: 2914-2922 [PMID: 25980611 DOI: 10.1016/j.febslet.2015.05.016]

57 **Pollicino T**, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, Levrero M. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. *Gastroenterology* 2006; **130**: 823-837 [PMID: 16530522 DOI: 10.1053/j.gastro.2006.01.001]

58 **Deeks SG**. HIV: Shock and kill. *Nature* 2012; **487**: 439-440 [PMID: 22836995 DOI: 10.1038/487439a]

59 **Ghosh SK**, Perrine SP, Williams RM, Faller DV. Histone deacetylase inhibitors are potent inducers of gene expression in latent EBV and sensitize lymphoma cells to nucleoside antiviral agents. *Blood* 2012; **119**: 1008-1017 [PMID: 22160379 DOI: 10.1182/blood-2011-06-362434]

60 **Barlow DP**. Methylation and imprinting: from host defense to gene regulation? *Science* 1993; **260**: 309-310 [PMID: 8469984 DOI: 10.1126/science.8469984]

61 **Jones PA**. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012; **13**: 484-492 [PMID: 22641018 DOI: 10.1038/nrg3230]

62 **Probst AV**, Dunleavy E, Almouzni G. Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* 2009; **10**: 192-206 [PMID: 19234478 DOI: 10.1038/nrm2640]

63 **Egger G**, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 2004; **429**: 457-463 [PMID: 15164071 DOI: 10.1038/nature02625]

64 **Jones PA**, Liang G. Rethinking how DNA methylation patterns are maintained. *Nat Rev Genet* 2009; **10**: 805-811 [PMID: 19789556 DOI: 10.1038/nrg2651]

65 **Kar S**, Deb M, Sengupta D, Shilpi A, Parbin S, Torrisani J, Pradhan S, Patra S. An insight into the various regulatory mechanisms modulating human DNA methyltransferase 1 stability and function. *Epigenetics* 2012; **7**: 994-1007 [PMID: 22894906 DOI: 10.4161/epi.21568]

66 **Hermann A**, Goyal R, Jeltsch A. The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites. *J Biol Chem* 2004; **279**: 48350-48359 [PMID: 15339928 DOI: 10.1074/jbc.M403427200]

67 **Chedin F**, Lieber MR, Hsieh CL. The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. *Proc Natl Acad Sci USA* 2002; **99**: 16916-16921 [PMID: 12481029 DOI: 10.1073/pnas.262443999]

68 **Gowher H**, Liebert K, Hermann A, Xu G, Jeltsch A. Mechanism of stimulation of catalytic activity of Dnmt3A and Dnmt3B DNA-(cytosine-C5)-methyltransferases by Dnmt3L. *J Biol Chem* 2005;

280: 13341-13348 [PMID: 15671018 DOI: 10.1074/jbc.M413412200]
69 **Goll MG**, Kirpekar F, Maggert KA, Yoder JA, Hsieh CL, Zhang X, Golic KG, Jacobsen SE, Bestor TH. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science* 2006; **311**: 395-398 [PMID: 16424344 DOI: 10.1126/science.1120976]
70 **Chen JY**, Hsu HC, Lee CS, Chen DS, Zuckerman AJ, Harrison TJ. Detection of hepatitis B virus DNA in hepatocellular carcinoma: methylation of integrated viral DNA. *J Virol Methods* 1988; **19**: 257-263 [PMID: 2836461 DOI: 10.1016/0166-0934(88)90020-1]
71 **Guo Y**, Li Y, Mu S, Zhang J, Yan Z. Evidence that methylation of hepatitis B virus covalently closed circular DNA in liver tissues of patients with chronic hepatitis B modulates HBV replication. *J Med Virol* 2009; **81**: 1177-1183 [PMID: 19475606 DOI: 10.1002/jmv.21525]
72 **Vivekanandan P**, Thomas D, Torbenson M. Hepatitis B viral DNA is methylated in liver tissues. *J Viral Hepat* 2008; **15**: 103-107 [PMID: 18184192 DOI: 10.1111/j.1365-2893.2007.00905.x]
73 **Kim JW**, Lee SH, Park YS, Hwang JH, Jeong SH, Kim N, Lee DH. Replicative activity of hepatitis B virus is negatively associated with methylation of covalently closed circular DNA in advanced hepatitis B virus infection. *Intervirology* 2011; **54**: 316-325 [PMID: 21242658 DOI: 10.1159/000321450]
74 **Zhang Y**, Mao R, Yan R, Cai D, Zhang Y, Zhu H, Kang Y, Liu H, Wang J, Qin Y, Huang Y, Guo H, Zhang J. Transcription of hepatitis B virus covalently closed circular DNA is regulated by CpG methylation during chronic infection. *PLoS One* 2014; **9**: e110442 [PMID: 25337821 DOI: 10.1371/journal.pone.0110442]
75 **Jain S**, Chang TT, Chen S, Boldbaatar B, Clemens A, Lin SY, Yan R, Hu CT, Guo H, Block TM, Song W, Su YH. Comprehensive DNA methylation analysis of hepatitis B virus genome in infected liver tissues. *Sci Rep* 2015; **5**: 10478 [PMID: 26000761 DOI: 10.1038/srep10478]
76 **Zhang Y**, Li C, Zhang Y, Zhu H, Kang Y, Liu H, Wang J, Qin Y, Mao R, Xie Y, Huang Y, Zhang J. Comparative analysis of CpG islands among HBV genotypes. *PLoS One* 2013; **8**: e56711 [PMID: 23451072 DOI: 10.1371/journal.pone.0056711]
77 **Kaur P**, Paliwal A, Durantel D, Hainaut P, Scoazec JY, Zoulim F, Chemin I, Herceg Z. DNA methylation of hepatitis B virus (HBV) genome associated with the development of hepatocellular carcinoma and occult HBV infection. *J Infect Dis* 2010; **202**: 700-704 [PMID: 20653444 DOI: 10.1086/655398]
78 **Mogul D**, Torbenson M, Schwarz KB. Epigenetic regulation of hepatitis B virus infection. *Curr Hepatitis Rep* 2011; **10**: 277-284
79 **Zhang C**, Huang C, Sui X, Zhong X, Yang W, Hu X, Li Y. Association between gene methylation and HBV infection in hepatocellular carcinoma: A meta-analysis. *J Cancer* 2019; **10**: 6457-6465 [PMID: 31772678 DOI: 10.7150/jca.33005]
80 **Vivekanandan P**, Thomas D, Torbenson M. Methylation regulates hepatitis B viral protein expression. *J Infect Dis* 2009; **199**: 1286-1291 [PMID: 19301974 DOI: 10.1086/597614]
81 **Vivekanandan P**, Daniel HD, Kannangai R, Martinez-Murillo F, Torbenson M. Hepatitis B virus replication induces methylation of both host and viral DNA. *J Virol* 2010; **84**: 4321-4329 [PMID: 20147412 DOI: 10.1128/JVI.02280-09]
82 **Levrero M**, Pollicino T, Petersen J, Belloni L, Raimondo G, Dandri M. Control of cccDNA function in hepatitis B virus infection. *J Hepatol* 2009; **51**: 581-592 [PMID: 19616338 DOI: 10.1016/j.jhep.2009.05.022]
83 **Kouzarides T**. Chromatin modifications and their function. *Cell* 2007; **128**: 693-705 [PMID: 17320507 DOI: 10.1016/j.cell.2007.02.005]
84 **Strahl BD**, Allis CD. The language of covalent histone modifications. *Nature* 2000; **403**: 41-45 [PMID: 10638745 DOI: 10.1038/47412]
85 **Gong Q**, Chen S, Guo J, Sun H, Zheng G, Liu Q, Ren H, He S. Chromosome remodeling related to hepatitis B virus replication in HepG2 cells. *DNA Cell Biol* 2011; **30**: 347-354 [PMID: 21345131 DOI: 10.1089/dna.2010.1172]
86 **Tropberger P**, Mercier A, Robinson M, Zhong W, Ganem DE, Holdorf M. Mapping of histone modifications in episomal HBV cccDNA uncovers an unusual chromatin organization amenable to epigenetic manipulation. *Proc Natl Acad Sci USA* 2015; **112**: E5715-E5724 [PMID: 26438841 DOI: 10.1073/pnas.1518090112]
87 **Allis CD**, Berger SL, Cote J, Dent S, Jenuwien T, Kouzarides T, Pillus L, Reinberg D, Shi Y, Shiekhattar R, Shilatifard A, Workman J, Zhang Y. New nomenclature for chromatin-modifying enzymes. *Cell* 2007; **131**: 633-636 [PMID: 18022353 DOI: 10.1016/j.cell.2007.10.039]
88 **Berger SL**, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. *Genes Dev* 2009; **23**: 781-783 [PMID: 19339683 DOI: 10.1101/gad.1787609]
89 **Zhang W**, Chen J, Wu M, Zhang X, Zhang M, Yue L, Li Y, Liu J, Li B, Shen F, Wang Y, Bai L, Protzer U, Levrero M, Yuan Z. PRMT5 restricts hepatitis B virus replication through epigenetic repression of covalently closed circular DNA transcription and interference with pregenomic RNA encapsidation. *Hepatology* 2017; **66**: 398-415 [PMID: 28236308 DOI: 10.1002/hep.29133]
90 **Seto E**, Yoshida M. Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harb Perspect Biol* 2014; **6**: a018713 [PMID: 24691964 DOI: 10.1101/cshperspect.a018713]
91 **Kgatle MM**. Recent Advancement in Hepatitis B Virus, Epigenetics Alterations and Related Complications. In: Kgatle MM Advances in Treatment of Hepatitis C and B. IntechOpen, 2017: 149
92 **Wang Z**, Wang W, Wang L. Epigenetic regulation of covalently closed circular DNA minichromosome in hepatitis B virus infection. *Biophys Rep* 2020; 1-12

93 **Rivière L**, Gerossier L, Ducroux A, Dion S, Deng Q, Michel ML, Buendia MA, Hantz O, Neuveut C. HBx relieves chromatin-mediated transcriptional repression of hepatitis B viral cccDNA involving SETDB1 histone methyltransferase. *J Hepatol* 2015; **63**: 1093-1102 [PMID: 26143443 DOI: 10.1016/j.jhep.2015.06.023]

94 **Lopez-Serra L**, Esteller M. Proteins that bind methylated DNA and human cancer: reading the wrong words. *Br J Cancer* 2008; **98**: 1881-1885 [PMID: 18542062 DOI: 10.1038/sj.bjc.6604374]

95 **Lucifora J**, Durantel D, Belloni L, Barraud L, Villet S, Vincent IE, Margeridon-Thermet S, Hantz O, Kay A, Levrero M, Zoulim F. Initiation of hepatitis B virus genome replication and production of infectious virus following delivery in HepG2 cells by novel recombinant baculovirus vector. *J Gen Virol* 2008; **89**: 1819-1828 [PMID: 18632952 DOI: 10.1099/vir.0.83659-0]

96 **Qian G**, Hu B, Zhou D, Xuan Y, Bai L, Duan C. NIRF, a Novel Ubiquitin Ligase, Inhibits Hepatitis B Virus Replication Through Effect on HBV Core Protein and H3 Histones. *DNA Cell Biol* 2015; **34**: 327-332 [PMID: 25664994 DOI: 10.1089/dna.2014.2714]

97 **Yan H**, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 2012; **1**: e00049 [PMID: 23150796 DOI: 10.7554/eLife.00049]

98 **Liu F**, Campagna M, Qi Y, Zhao X, Guo F, Xu C, Li S, Li W, Block TM, Chang J, Guo JT. Alpha-interferon suppresses hepadnavirus transcription by altering epigenetic modification of cccDNA minichromosomes. *PLoS Pathog* 2013; **9**: e1003613 [PMID: 24068929 DOI: 10.1371/journal.ppat.1003613]

99 **Ananthanarayanan M**, Li Y, Surapureddi S, Balasubramaniyan N, Ahn J, Goldstein JA, Suchy FJ. Histone H3K4 trimethylation by MLL3 as part of ASCOM complex is critical for NR activation of bile acid transporter genes and is downregulated in cholestasis. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G771-G781 [PMID: 21330447 DOI: 10.1152/ajpgi.00499.2010]

100 **Murakami S**. Hepatitis B virus X protein: a multifunctional viral regulator. *J Gastroenterol* 2001; **36**: 651-660 [PMID: 11686474 DOI: 10.1007/s005350170027]

101 **Prescott NA**, Bram Y, Schwartz RE, David Y. Targeting Hepatitis B Virus Covalently Closed Circular DNA and Hepatitis B Virus X Protein: Recent Advances and New Approaches. *ACS Infect Dis* 2019; **5**: 1657-1667 [PMID: 31525994 DOI: 10.1021/acsinfecdis.9b00249]

102 **Zlotnick A**, Venkatakrishnan B, Tan Z, Lewellyn E, Turner W, Francis S. Core protein: A pleiotropic keystone in the HBV lifecycle. *Antiviral Res* 2015; **121**: 82-93 [PMID: 26129969 DOI: 10.1016/j.antiviral.2015.06.020]

103 **Koumbi L**, Pollicino T, Raimondo G, Stampoulis D, Khakoo S, Karayiannis P. Hepatitis B virus basal core promoter mutations show lower replication fitness associated with cccDNA acetylation status. *Virus Res* 2016; **220**: 150-160 [PMID: 27132039 DOI: 10.1016/j.virusres.2016.04.022]

104 **Guo Y**, Kang W, Lei X, Li Y, Xiang A, Liu Y, Zhao J, Zhang J, Yan Z. Hepatitis B viral core protein disrupts human host gene expression by binding to promoter regions. *BMC Genomics* 2012; **13**: 563 [PMID: 23088787 DOI: 10.1186/1471-2164-13-563]

105 **Belloni L**, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M, Raimondo G, Levrero M. Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. *Proc Natl Acad Sci USA* 2009; **106**: 19975-19979 [PMID: 19906987 DOI: 10.1073/pnas.0908365106]

106 **Shi L**, Li S, Shen F, Li H, Qian S, Lee DH, Wu JZ, Yang W. Characterization of nucleosome positioning in hepadnaviral covalently closed circular DNA minichromosomes. *J Virol* 2012; **86**: 10059-10069 [PMID: 22787202 DOI: 10.1128/JVI.00535-12]

107 **Shamay M**, Barak O, Doitsh G, Ben-Dor I, Shaul Y. Hepatitis B virus pX interacts with HBXAP, a PHD finger protein to coactivate transcription. *J Biol Chem* 2002; **277**: 9982-9988 [PMID: 11788598 DOI: 10.1074/jbc.M111354200]

108 **Saeed U**, Kim J, Piracha ZZ, Kwon H, Jung J, Chwae YJ, Park S, Shin HJ, Kim K. Parvulin 14 and Parvulin 17 Bind to HBx and cccDNA and Upregulate Hepatitis B Virus Replication from cccDNA to Virion in an HBx-Dependent Manner. *J Virol* 2019; **93** [PMID: 30567987 DOI: 10.1128/JVI.01840-18]

109 **Nassal M**. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. *Gut* 2015; **64**: 1972-1984 [PMID: 26048673 DOI: 10.1136/gutjnl-2015-309809]

110 **Curtil C**, Enache LS, Radreau P, Dron AG, Scholtès C, Deloire A, Roche D, Lotteau V, André P, Ramière C. The metabolic sensors FXRα, PGC-1α, and SIRT1 cooperatively regulate hepatitis B virus transcription. *FASEB J* 2014; **28**: 1454-1463 [PMID: 24297698 DOI: 10.1096/fj.13-236372]

111 **Ren JH**, Tao Y, Zhang ZZ, Chen WX, Cai XF, Chen K, Ko BC, Song CL, Ran LK, Li WY, Huang AL, Chen J. Sirtuin 1 regulates hepatitis B virus transcription and replication by targeting transcription factor AP-1. *J Virol* 2014; **88**: 2442-2451 [PMID: 24335313 DOI: 10.1128/JVI.02861-13]

112 **Deng JJ**, Kong KE, Gao WW, Tang HV, Chaudhary V, Cheng Y, Zhou J, Chan CP, Wong DK, Yuen MF, Jin DY. Interplay between SIRT1 and hepatitis B virus X protein in the activation of viral transcription. *Biochim Biophys Acta Gene Regul Mech* 2017; **1860**: 491-501 [PMID: 28242208 DOI: 10.1016/j.bbagrm.2017.02.007]

113 **Benhenda S**, Ducroux A, Rivière L, Sobhian B, Ward MD, Dion S, Hantz O, Protzer U, Michel ML, Benkirane M, Semmes OJ, Buendia MA, Neuveut C. Methyltransferase PRMT1 is a binding partner of HBx and a negative regulator of hepatitis B virus transcription. *J Virol* 2013; **87**: 4360-4371

[PMID: 23388725 DOI: 10.1128/JVI.02574-12]

114 **Ren JH**, Hu JL, Cheng ST, Yu HB, Wong VKW, Law BYK, Yang YF, Huang Y, Liu Y, Chen WX, Cai XF, Tang H, Hu Y, Zhang WL, Liu X, Long QX, Zhou L, Tao NN, Zhou HZ, Yang QX, Ren F, He L, Gong R, Huang AL, Chen J. SIRT3 restricts hepatitis B virus transcription and replication through epigenetic regulation of covalently closed circular DNA involving suppressor of variegation 3-9 homolog 1 and SET domain containing 1A histone methyltransferases. *Hepatology* 2018; **68**: 1260-1276 [PMID: 29624717 DOI: 10.1002/hep.29912]

115 **Zheng DL**, Zhang L, Cheng N, Xu X, Deng Q, Teng XM, Wang KS, Zhang X, Huang J, Han ZG. Epigenetic modification induced by hepatitis B virus X protein *via* interaction with de novo DNA methyltransferase DNMT3A. *J Hepatol* 2009; **50**: 377-387 [PMID: 19070387 DOI: 10.1016/j.jhep.2008.10.019]

116 **Fu X**, Song X, Li Y, Tan D, Liu G. Hepatitis B virus X protein upregulates DNA methyltransferase 3A/3B and enhances SOCS-1CpG island methylation. *Mol Med Rep* 2016; **13**: 301-308 [PMID: 26573490 DOI: 10.3892/mmr.2015.4545]

117 **Park IY**, Sohn BH, Yu E, Suh DJ, Chung YH, Lee JH, Surzycki SJ, Lee YI. Aberrant epigenetic modifications in hepatocarcinogenesis induced by hepatitis B virus X protein. *Gastroenterology* 2007; **132**: 1476-1494 [PMID: 17408664 DOI: 10.1053/j.gastro.2007.01.034]

118 **Park ES**, Park YK, Shin CY, Park SH, Ahn SH, Kim DH, Lim KH, Kwon SY, Kim KP, Yang SI, Seong BL, Kim KH. Hepatitis B virus inhibits liver regeneration *via* epigenetic regulation of urokinase-type plasminogen activator. *Hepatology* 2013; **58**: 762-776 [PMID: 23483589 DOI: 10.1002/hep.26379]

119 **Koumbi L**, Karayiannis P. The Epigenetic Control of Hepatitis B Virus Modulates the Outcome of Infection. *Front Microbiol* 2015; **6**: 1491 [PMID: 26779147 DOI: 10.3389/fmicb.2015.01491]

120 **Sengupta I**, Das D, Singh SP, Chakravarty R, Das C. Host transcription factor Speckled 110 kDa (Sp110), a nuclear body protein, is hijacked by hepatitis B virus protein X for viral persistence. *J Biol Chem* 2017; **292**: 20379-20393 [PMID: 29046350 DOI: 10.1074/jbc.M117.796839]

121 **Colgrove R**, Simon G, Ganem D. Transcriptional activation of homologous and heterologous genes by the hepatitis B virus X gene product in cells permissive for viral replication. *J Virol* 1989; **63**: 4019-4026 [PMID: 2788226 DOI: 10.1128/JVI.63.9.4019-4026.1989]

122 **Cougot D**, Wu Y, Cairo S, Caramel J, Renard CA, Lévy L, Buendia MA, Neuveut C. Correction: The hepatitis B virus X protein functionally interacts with CREB-binding protein/p300 in the regulation of CREB-mediated transcription. *J Biol Chem* 2020; **295**: 2888 [PMID: 32111723 DOI: 10.1074/jbc.AAC120.012898]

123 **Cougot D**, Wu Y, Cairo S, Caramel J, Renard CA, Lévy L, Buendia MA, Neuveut C. The hepatitis B virus X protein functionally interacts with CREB-binding protein/p300 in the regulation of CREB-mediated transcription. *J Biol Chem* 2007; **282**: 4277-4287 [PMID: 17158882 DOI: 10.1074/jbc.M606774200]

124 **Chen S**, Wu L, Peng L, Wang X, Tang N. Hepatitis B virus X protein (HBx) promotes ST2 expression by GATA2 in liver cells. *Mol Immunol* 2020; **123**: 32-39 [PMID: 32413787 DOI: 10.1016/j.molimm.2020.04.024]

125 **Volarevic V**, Mitrovic M, Milovanovic M, Zelen I, Nikolic I, Mitrovic S, Pejnovic N, Arsenijevic N, Lukic ML. Protective role of IL-33/ST2 axis in Con A-induced hepatitis. *J Hepatol* 2012; **56**: 26-33 [PMID: 21703183 DOI: 10.1016/j.jhep.2011.03.022]

126 **Cougot D**, Allemand E, Rivière L, Benhenda S, Duroure K, Levillayer F, Muchardt C, Buendia MA, Neuveut C. Inhibition of PP1 phosphatase activity by HBx: a mechanism for the activation of hepatitis B virus transcription. *Sci Signal* 2012; **5**: ra1 [PMID: 22215732 DOI: 10.1126/scisignal.2001906]

127 **Shen C**, Feng X, Mao T, Yang D, Zou J, Zao X, Deng Q, Chen X, Lu F. Yin-Yang 1 and HBx protein activate HBV transcription by mediating the spatial interaction of cccDNA minichromosome with cellular chromosome 19p13.11. *Emerg Microbes Infect* 2020; **9**: 2455-2464 [PMID: 33084547 DOI: 10.1080/22221751.2020.1840311]

128 **Deng L**, Gan X, Ito M, Chen M, Aly HH, Matsui C, Abe T, Watashi K, Wakita T, Suzuki T, Okamoto T, Matsuura Y, Mizokami M, Shoji I, Hotta H. Peroxiredoxin 1, a Novel HBx-Interacting Protein, Interacts with Exosome Component 5 and Negatively Regulates Hepatitis B Virus (HBV) Propagation through Degradation of HBV RNA. *J Virol* 2019; **93** [PMID: 30567989 DOI: 10.1128/JVI.02203-18]

129 **Jackson S**, Xiong Y. CRL4s: the CUL4-RING E3 ubiquitin ligases. *Trends Biochem Sci* 2009; **34**: 562-570 [PMID: 19818632 DOI: 10.1016/j.tibs.2009.07.002]

130 **Leupin O**, Bontron S, Schaeffer C, Strubin M. Hepatitis B virus X protein stimulates viral genome replication *via* a DDB1-dependent pathway distinct from that leading to cell death. *J Virol* 2005; **79**: 4238-4245 [PMID: 15767425 DOI: 10.1128/JVI.79.7.4238-4245.2005]

131 **Li T**, Robert EI, van Breugel PC, Strubin M, Zheng N. A promiscuous alpha-helical motif anchors viral hijackers and substrate receptors to the CUL4-DDB1 ubiquitin ligase machinery. *Nat Struct Mol Biol* 2010; **17**: 105-111 [PMID: 19966799 DOI: 10.1038/nsmb.1719]

132 **Decorsière A**, Mueller H, van Breugel PC, Abdul F, Gerossier L, Beran RK, Livingston CM, Niu C, Fletcher SP, Hantz O, Strubin M. Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. *Nature* 2016; **531**: 386-389 [PMID: 26983541 DOI: 10.1038/nature17170]

133 **Murphy CM**, Xu Y, Li F, Nio K, Reszka-Blanco N, Li X, Wu Y, Yu Y, Xiong Y, Su L. Hepatitis B Virus X Protein Promotes Degradation of SMC5/6 to Enhance HBV Replication. *Cell Rep* 2016; **16**:

2846-2854 [PMID: 27626656 DOI: 10.1016/j.celrep.2016.08.026]
134 **Niu C**, Livingston CM, Li L, Beran RK, Daffis S, Ramakrishnan D, Burdette D, Peiser L, Salas E, Ramos H, Yu M, Cheng G, Strubin M, Delaney WE IV, Fletcher SP. The Smc5/6 Complex Restricts HBV when Localized to ND10 without Inducing an Innate Immune Response and Is Counteracted by the HBV X Protein Shortly after Infection. *PLoS One* 2017; **12**: e0169648 [PMID: 28095508 DOI: 10.1371/journal.pone.0169648]
135 **Mitra B**, Guo H. Hepatitis B virus X protein crosses out Smc5/6 complex to maintain covalently closed circular DNA transcription. *Hepatology* 2016; **64**: 2246-2249 [PMID: 27639252 DOI: 10.1002/hep.28834]
136 **Minor MM**, Hollinger FB, McNees AL, Jung SY, Jain A, Hyser JM, Bissig KD, Slagle BL. Hepatitis B Virus HBx Protein Mediates the Degradation of Host Restriction Factors through the Cullin 4 DDB1 E3 Ubiquitin Ligase Complex. *Cells* 2020; **9** [PMID: 32235678 DOI: 10.3390/cells9040834]
137 **He Q**, Li W, Ren J, Huang Y, Hu Q, Chen J, Chen W. ZEB2 inhibits HBV transcription and replication by targeting its core promoter. *Oncotarget* 2016; **7**: 16003-16011 [PMID: 26895378 DOI: 10.18632/oncotarget.7435]
138 **Qian MX**, Pang Y, Liu CH, Haratake K, Du BY, Ji DY, Wang GF, Zhu QQ, Song W, Yu Y, Zhang XX, Huang HT, Miao S, Chen LB, Zhang ZH, Liang YN, Liu S, Cha H, Yang D, Zhai Y, Komatsu T, Tsuruta F, Li H, Cao C, Li W, Li GH, Cheng Y, Chiba T, Wang L, Goldberg AL, Shen Y, Qiu XB. Acetylation-mediated proteasomal degradation of core histones during DNA repair and spermatogenesis. *Cell* 2013; **153**: 1012-1024 [PMID: 23706739 DOI: 10.1016/j.cell.2013.04.032]
139 **Mandemaker IK**, Geijer ME, Kik I, Bezstarosti K, Rijkers E, Raams A, Janssens RC, Lans H, Hoeijmakers JH, Demmers JA, Vermeulen W, Marteijn JA. DNA damage-induced replication stress results in PA200-proteasome-mediated degradation of acetylated histones. *EMBO Rep* 2018; **19** [PMID: 30104204 DOI: 10.15252/embr.201745566]
140 **Liou J**, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE Jr, Meyer T. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol* 2005; **15**: 1235-1241 [PMID: 16005298 DOI: 10.1016/j.cub.2005.05.055]
141 **Hu Z**, Zhang Z, Doo E, Coux O, Goldberg AL, Liang TJ. Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome complex. *J Virol* 1999; **73**: 7231-7240 [PMID: 10438810 DOI: 10.1128/JVI.73.9.7231-7240.1999]
142 **Gao W**, Jia Z, Tian Y, Yang P, Sun H, Wang C, Ding Y, Zhang M, Zhang Y, Yang D, Tian Z, Zhou J, Ruan Z, Wu Y, Ni B. HBx Protein Contributes to Liver Carcinogenesis by H3K4me3 Modification Through Stabilizing WD Repeat Domain 5 Protein. *Hepatology* 2020; **71**: 1678-1695 [PMID: 31544250 DOI: 10.1002/hep.30947]
143 **Shen B**, Chen Y, Hu J, Qiao M, Ren J, Chen J, Tang N, Huang A, Hu Y. Hepatitis B virus X protein modulates upregulation of DHX9 to promote viral DNA replication. *Cell Microbiol* 2020; **22**: e13148 [PMID: 31829498 DOI: 10.1111/cmi.13148]
144 **Tang H**, Oishi N, Kaneko S, Murakami S. Molecular functions and biological roles of hepatitis B virus x protein. *Cancer Sci* 2006; **97**: 977-983 [PMID: 16984372 DOI: 10.1111/j.1349-7006.2006.00299.x]
145 **Su ZJ**, Cao JS, Wu YF, Chen WN, Lin X, Wu YL. Deubiquitylation of hepatitis B virus X protein (HBx) by ubiquitin-specific peptidase 15 (USP15) increases HBx stability and its transactivation activity. *Sci Rep* 2017; **7**: 40246 [PMID: 28074857 DOI: 10.1038/srep40246]
146 **Liu N**, Zhang J, Yang X, Jiao T, Zhao X, Li W, Zhu J, Yang P, Jin J, Peng J, Li Z, Ye X. HDM2 Promotes NEDDylation of Hepatitis B Virus HBx To Enhance Its Stability and Function. *J Virol* 2017; **91** [PMID: 28592528 DOI: 10.1128/JVI.00340-17]
147 **Xie M**, Guo H, Lou G, Yao J, Liu Y, Sun Y, Yang Z, Zheng M. Neddylation inhibitor MLN4924 has anti-HBV activity *via* modulating the ERK-HNF1α-C/EBPα-HNF4α axis. *J Cell Mol Med* 2021; **25**: 840-854 [PMID: 33263949 DOI: 10.1111/jcmm.16137]
148 **Guo YH**, Li YN, Zhao JR, Zhang J, Yan Z. HBc binds to the CpG islands of HBV cccDNA and promotes an epigenetic permissive state. *Epigenetics* 2011; **6**: 720-726 [PMID: 21546797 DOI: 10.4161/epi.6.6.15815]
149 **Williams JS**, Andrisani OM. The hepatitis B virus X protein targets the basic region-leucine zipper domain of CREB. *Proc Natl Acad Sci USA* 1995; **92**: 3819-3823 [PMID: 7731990 DOI: 10.1073/pnas.92.9.3819]
150 **Andrisani OM**. CREB-mediated transcriptional control. *Crit Rev Eukaryot Gene Expr* 1999; **9**: 19-32 [PMID: 10200909]
151 **Chong CK**, Cheng CYS, Tsoi SYJ, Huang FY, Liu F, Seto WK, Lai CL, Yuen MF, Wong DK. Role of hepatitis B core protein in HBV transcription and recruitment of histone acetyltransferases to cccDNA minichromosome. *Antiviral Res* 2017; **144**: 1-7 [PMID: 28499864 DOI: 10.1016/j.antiviral.2017.05.003]
152 **Kwon JA**, Rho HM. Hepatitis B viral core protein activates the hepatitis B viral enhancer II/pregenomic promoter through the nuclear factor kappaB binding site. *Biochem Cell Biol* 2002; **80**: 445-455 [PMID: 12234098 DOI: 10.1139/o02-133]
153 **Park SY**, Jeong MS, Han CW, Yu HS, Jang SB. Structural and Functional Insight into Proliferating Cell Nuclear Antigen. *J Microbiol Biotechnol* 2016; **26**: 637-647 [PMID: 26699741 DOI: 10.4014/jmb.1509.09051]
154 **Feng J**, Yang G, Liu Y, Gao Y, Zhao M, Bu Y, Yuan H, Yuan Y, Yun H, Sun M, Gao H, Zhang S,

Liu Z, Yin M, Song X, Miao Z, Lin Z, Zhang X. LncRNA PCNAP1 modulates hepatitis B virus replication and enhances tumor growth of liver cancer. *Theranostics* 2019; **9**: 5227-5245 [PMID: 31410212 DOI: 10.7150/thno.34273]

155 **Fernández M**, Quiroga JA, Carreño V. Hepatitis B virus downregulates the human interferon-inducible MxA promoter through direct interaction of precore/core proteins. *J Gen Virol* 2003; **84**: 2073-2082 [PMID: 12867637 DOI: 10.1099/vir.0.18966-0]

156 **Li T**, Ke Z, Liu W, Xiong Y, Zhu Y, Liu Y. Human Hepatitis B Virus Core Protein Inhibits IFNα-Induced IFITM1 Expression by Interacting with BAF200. *Viruses* 2019; **11** [PMID: 31075894 DOI: 10.3390/v11050427]

157 **Du J**, Liang X, Liu Y, Qu Z, Gao L, Han L, Liu S, Cui M, Shi Y, Zhang Z, Yu L, Cao L, Ma C, Zhang L, Chen Y, Sun W. Hepatitis B virus core protein inhibits TRAIL-induced apoptosis of hepatocytes by blocking DR5 expression. *Cell Death Differ* 2009; **16**: 219-229 [PMID: 18927587 DOI: 10.1038/cdd.2008.144]

158 **Kwon JA**, Rho HM. Transcriptional repression of the human p53 gene by hepatitis B viral core protein (HBc) in human liver cells. *Biol Chem* 2003; **384**: 203-212 [PMID: 12675512 DOI: 10.1515/BC.2003.022]

159 **Fridman JS**, Lowe SW. Control of apoptosis by p53. *Oncogene* 2003; **22**: 9030-9040 [PMID: 14663481 DOI: 10.1038/sj.onc.1207116]

160 **Jia B**, Guo M, Li G, Yu D, Zhang X, Lan K, Deng Q. Hepatitis B virus core protein sensitizes hepatocytes to tumor necrosis factor-induced apoptosis by suppression of the phosphorylation of mitogen-activated protein kinase kinase 7. *J Virol* 2015; **89**: 2041-2051 [PMID: 25428880 DOI: 10.1128/JVI.03106-14]

161 **Yeh CT**, Wong SW, Fung YK, Ou JH. Cell cycle regulation of nuclear localization of hepatitis B virus core protein. *Proc Natl Acad Sci USA* 1993; **90**: 6459-6463 [PMID: 8341655 DOI: 10.1073/pnas.90.14.6459]

162 **Chen J**. The Cell-Cycle Arrest and Apoptotic Functions of p53 in Tumor Initiation and Progression. *Cold Spring Harb Perspect Med* 2016; **6**: a026104 [PMID: 26931810 DOI: 10.1101/cshperspect.a026104]

163 **Giacinti C**, Giordano A. RB and cell cycle progression. *Oncogene* 2006; **25**: 5220-5227 [PMID: 16936740 DOI: 10.1038/sj.onc.1209615]

164 **Henley SA**, Dick FA. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell Div* 2012; **7**: 10 [PMID: 22417103 DOI: 10.1186/1747-1028-7-10]

165 **Lucifora J**, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, Sprinzl MF, Koppensteiner H, Makowska Z, Volz T, Remouchamps C, Chou WM, Thasler WE, Hüser N, Durantel D, Liang TJ, Münk C, Heim MH, Browning JL, Dejardin E, Dandri M, Schindler M, Heikenwalder M, Protzer U. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science* 2014; **343**: 1221-1228 [PMID: 24557838 DOI: 10.1126/science.1243462]

166 **Diab A**, Foca A, Zoulim F, Durantel D, Andrisani O. The diverse functions of the hepatitis B core/capsid protein (HBc) in the viral life cycle: Implications for the development of HBc-targeting antivirals. *Antiviral Res* 2018; **149**: 211-220 [PMID: 29183719 DOI: 10.1016/j.antiviral.2017.11.015]

167 **Gomes AQ**, Nolasco S, Soares H. Non-coding RNAs: multi-tasking molecules in the cell. *Int J Mol Sci* 2013; **14**: 16010-16039 [PMID: 23912238 DOI: 10.3390/ijms140816010]

168 **Quinn JJ**, Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet* 2016; **17**: 47-62 [PMID: 26666209 DOI: 10.1038/nrg.2015.10]

169 **Bandopadhyay M**, Bharadwaj M. Exosomal miRNAs in hepatitis B virus related liver disease: a new hope for biomarker. *Gut Pathog* 2020; **12**: 23 [PMID: 32346400 DOI: 10.1186/s13099-020-00353-w]

170 **Dai X**, Zhang W, Zhang H, Sun S, Yu H, Guo Y, Kou Z, Zhao G, Du L, Jiang S, Zhang J, Li J, Zhou Y. Modulation of HBV replication by microRNA-15b through targeting hepatocyte nuclear factor 1α. *Nucleic Acids Res* 2014; **42**: 6578-6590 [PMID: 24705650 DOI: 10.1093/nar/gku260]

171 **Zhang X**, Zhang E, Ma Z, Pei R, Jiang M, Schlaak JF, Roggendorf M, Lu M. Modulation of hepatitis B virus replication and hepatocyte differentiation by MicroRNA-1. *Hepatology* 2011; **53**: 1476-1485 [PMID: 21520166 DOI: 10.1002/hep.24195]

172 **Zhang X**, Liu H, Xie Z, Deng W, Wu C, Qin B, Hou J, Lu M. Epigenetically regulated miR-449a enhances hepatitis B virus replication by targeting cAMP-responsive element binding protein 5 and modulating hepatocytes phenotype. *Sci Rep* 2016; **6**: 25389 [PMID: 27138288 DOI: 10.1038/srep25389]

173 **Xing T**, Zhu J, Xian J, Li A, Wang X, Wang W, Zhang Q. miRNA-548ah promotes the replication and expression of hepatitis B virus by targeting histone deacetylase 4. *Life Sci* 2019; **219**: 199-208 [PMID: 30615846 DOI: 10.1016/j.lfs.2018.12.057]

174 **Deng W**, Zhang X, Ma Z, Lin Y, Lu M. MicroRNA-125b-5p mediates post-transcriptional regulation of hepatitis B virus replication *via* the LIN28B/Let-7 axis. *RNA Biol* 2017; **14**: 1389-1398 [PMID: 28267418 DOI: 10.1080/15476286.2017.1293770]

175 **Moon IY**, Choi JH, Chung JW, Jang ES, Jeong SH, Kim JW. MicroRNA20 induces methylation of hepatitis B virus covalently closed circular DNA in human hepatoma cells. *Mol Med Rep* 2019; **20**: 2285-2293 [PMID: 31257511 DOI: 10.3892/mmr.2019.10435]

176 **Ji M**, Mei X, Jing X, Xu X, Chen X, Pan W. The cooperative complex of Argonaute-2 and

microRNA-146a regulates hepatitis B virus replication through flap endonuclease 1. *Life Sci* 2020; **257**: 118089 [PMID: 32659369 DOI: 10.1016/j.lfs.2020.118089]

177 **Wang Y**, Li Y. miR-146 promotes HBV replication and expression by targeting ZEB2. *Biomed Pharmacother* 2018; **99**: 576-582 [PMID: 29902868 DOI: 10.1016/j.biopha.2018.01.097]

178 **Fu L**, Fu X, Mo J, Li X, Li R, Peng S. miR-146a-5p enhances hepatitis B virus replication through autophagy to promote aggravation of chronic hepatitis B. *IUBMB Life* 2019; **71**: 1336-1346 [PMID: 31018043 DOI: 10.1002/iub.2044]

179 **Lin Y**, Deng W, Pang J, Kemper T, Hu J, Yin J, Zhang J, Lu M. The microRNA-99 family modulates hepatitis B virus replication by promoting IGF-1R/PI3K/Akt/mTOR/ULK1 signaling-induced autophagy. *Cell Microbiol* 2017; **19** [PMID: 27886437 DOI: 10.1111/cmi.12709]

180 **Wang J**, Chen J, Liu Y, Zeng X, Wei M, Wu S, Xiong Q, Song F, Yuan X, Xiao Y, Cao Y, Li C, Chen L, Guo M, Shi YB, Sun G, Guo D. Hepatitis B Virus Induces Autophagy to Promote its Replication by the Axis of miR-192-3p-XIAP Through NF kappa B Signaling. *Hepatology* 2019; **69**: 974-992 [PMID: 30180281 DOI: 10.1002/hep.30248]

181 **Chen L**, Ming X, Li W, Bi M, Yan B, Wang X, Yang P, Yang B. The microRNA-155 mediates hepatitis B virus replication by reinforcing SOCS1 signalling-induced autophagy. *Cell Biochem Funct* 2020; **38**: 436-442 [PMID: 31930529 DOI: 10.1002/cbf.3488]

182 **Nielsen KO**, Jacobsen KS, Mirza AH, Winther TN, Størling J, Glebe D, Pociot F, Hogh B. Hepatitis B virus upregulates host microRNAs that target apoptosis-regulatory genes in an *in vitro* cell model. *Exp Cell Res* 2018; **371**: 92-103 [PMID: 30059664 DOI: 10.1016/j.yexcr.2018.07.044]

183 **Zhang X**, Hou J, Lu M. Regulation of hepatitis B virus replication by epigenetic mechanisms and microRNAs. *Front Genet* 2013; **4**: 202 [PMID: 24133502 DOI: 10.3389/fgene.2013.00202]

184 **Chen Y**, Shen A, Rider PJ, Yu Y, Wu K, Mu Y, Hao Q, Liu Y, Gong H, Zhu Y, Liu F, Wu J. A liver-specific microRNA binds to a highly conserved RNA sequence of hepatitis B virus and negatively regulates viral gene expression and replication. *FASEB J* 2011; **25**: 4511-4521 [PMID: 21903935 DOI: 10.1096/fj.11-187781]

185 **Wang S**, Qiu L, Yan X, Jin W, Wang Y, Chen L, Wu E, Ye X, Gao GF, Wang F, Chen Y, Duan Z, Meng S. Loss of microRNA 122 expression in patients with hepatitis B enhances hepatitis B virus replication through cyclin G(1) -modulated P53 activity. *Hepatology* 2012; **55**: 730-741 [PMID: 22105316 DOI: 10.1002/hep.24809]

186 **Qiu L**, Fan H, Jin W, Zhao B, Wang Y, Ju Y, Chen L, Chen Y, Duan Z, Meng S. miR-122-induced down-regulation of HO-1 negatively affects miR-122-mediated suppression of HBV. *Biochem Biophys Res Commun* 2010; **398**: 771-777 [PMID: 20633528 DOI: 10.1016/j.bbrc.2010.07.021]

187 **Fan CG**, Wang CM, Tian C, Wang Y, Li L, Sun WS, Li RF, Liu YG. miR-122 inhibits viral replication and cell proliferation in hepatitis B virus-related hepatocellular carcinoma and targets NDRG3. *Oncol Rep* 2011; **26**: 1281-1286 [PMID: 21725618 DOI: 10.3892/or.2011.1375]

188 **Hu W**, Wang X, Ding X, Li Y, Zhang X, Xie P, Yang J, Wang S. MicroRNA-141 represses HBV replication by targeting PPARA. *PLoS One* 2012; **7**: e34165 [PMID: 22479552 DOI: 10.1371/journal.pone.0034165]

189 **Yang Y**, Liu Y, Xue J, Yang Z, Shi Y, Lou G, Wu S, Qi J, Liu W, Wang J, Chen Z. MicroRNA-141 Targets Sirt1 and Inhibits Autophagy to Reduce HBV Replication. *Cell Physiol Biochem* 2017; **41**: 310-322 [PMID: 28135713 DOI: 10.1159/000456162]

190 **Duan X**, Li S, Holmes JA, Tu Z, Li Y, Cai D, Liu X, Li W, Yang C, Jiao B, Schaefer EA, Fusco DN, Salloum S, Chen L, Lin W, Chung RT. MicroRNA 130a Regulates both Hepatitis C Virus and Hepatitis B Virus Replication through a Central Metabolic Pathway. *J Virol* 2018; **92** [PMID: 29321333 DOI: 10.1128/JVI.02009-17]

191 **Singh AK**, Rooge SB, Varshney A, Vasudevan M, Bhardwaj A, Venugopal SK, Trehanpati N, Kumar M, Geffers R, Kumar V, Sarin SK. Global microRNA expression profiling in the liver biopsies of hepatitis B virus-infected patients suggests specific microRNA signatures for viral persistence and hepatocellular injury. *Hepatology* 2018; **67**: 1695-1709 [PMID: 29194684 DOI: 10.1002/hep.29690]

192 **Yang X**, Li H, Sun H, Fan H, Hu Y, Liu M, Li X, Tang H. Hepatitis B Virus-Encoded MicroRNA Controls Viral Replication. *J Virol* 2017; **91** [PMID: 28148795 DOI: 10.1128/JVI.01919-16]

193 **Zhao X**, Sun L, Mu T, Yi J, Ma C, Xie H, Liu M, Tang H. An HBV-encoded miRNA activates innate immunity to restrict HBV replication. *J Mol Cell Biol* 2020; **12**: 263-276 [PMID: 31865380 DOI: 10.1093/jmcb/mjz104]

194 **Morlando M**, Fatica A. Alteration of Epigenetic Regulation by Long Noncoding RNAs in Cancer. *Int J Mol Sci* 2018; **19** [PMID: 29443889 DOI: 10.3390/ijms19020570]

195 **Yang G**, Feng J, Liu Y, Zhao M, Yuan Y, Yuan H, Yun H, Sun M, Bu Y, Liu L, Liu Z, Niu JQ, Yin M, Song X, Miao Z, Lin Z, Zhang X. HAT1 signaling confers to assembly and epigenetic regulation of HBV cccDNA minichromosome. *Theranostics* 2019; **9**: 7345-7358 [PMID: 31695772 DOI: 10.7150/thno.37173]

196 **Liu Y**, Feng J, Sun M, Yang G, Yuan H, Wang Y, Bu Y, Zhao M, Zhang S, Zhang X. Long non-coding RNA HULC activates HBV by modulating HBx/STAT3/miR-539/APOBEC3B signaling in HBV-related hepatocellular carcinoma. *Cancer Lett* 2019; **454**: 158-170 [PMID: 30981758 DOI: 10.1016/j.canlet.2019.04.008]

197 **Salerno D**, Chiodo L, Alfano V, Floriot O, Cottone G, Paturel A, Pallocca M, Plissonnier ML, Jeddari S, Belloni L, Zeisel M, Levrero M, Guerrieri F. Hepatitis B protein HBx binds the DLEU2 LncRNA to sustain cccDNA and host cancer-related gene transcription. *Gut* 2020; **69**: 2016-2024

[PMID: 32114505 DOI: 10.1136/gutjnl-2019-319637]

198 **Nishitsuji H**, Ujino S, Yoshio S, Sugiyama M, Mizokami M, Kanto T, Shimotohno K. Long noncoding RNA #32 contributes to antiviral responses by controlling interferon-stimulated gene expression. *Proc Natl Acad Sci USA* 2016; **113**: 10388-10393 [PMID: 27582466 DOI: 10.1073/pnas.1525022113]

199 **Belloni L**, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, Petersen J, Raimondo G, Dandri M, Levrero M. IFN-α inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. *J Clin Invest* 2012; **122**: 529-537 [PMID: 22251702 DOI: 10.1172/JCI58847]

200 **Schoggins JW**, Rice CM. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol* 2011; **1**: 519-525 [PMID: 22328912 DOI: 10.1016/j.coviro.2011.10.008]

201 **Cheng J**, Zhao Q, Zhou Y, Tang L, Sheraz M, Chang J, Guo JT. Interferon Alpha Induces Multiple Cellular Proteins That Coordinately Suppress Hepadnaviral Covalently Closed Circular DNA Transcription. *J Virol* 2020; **94** [PMID: 32581092 DOI: 10.1128/JVI.00442-20]

202 **Allweiss L**, Giersch K, Volz T, Lohse AW, Petersen J, Urban S, Luetgehmann M, Dandri M. PS-155-HBV entry inhibition after interferon alpha treatment hinders HBV rebound in hepatocytes that became negative for all HBV markers during interferon treatment. *J Hepatol* 2019; **70**: e98 [DOI: 10.1016/S0618-8278(19)30173-2]

203 **Chen J**, Li Y, Lai F, Wang Y, Sutter K, Dittmer U, Ye J, Zai W, Liu M, Shen F, Wu M, Hu K, Li B, Lu M, Zhang X, Zhang J, Li J, Chen Q, Yuan Z. Functional Comparison of Interferon-α Subtypes Reveals Potent Hepatitis B Virus Suppression by a Concerted Action of Interferon-α and Interferon-γ Signaling. *Hepatology* 2021; **73**: 486-502 [PMID: 32333814 DOI: 10.1002/hep.31282]

204 **Neureiter D**, Stintzing S, Kiesslich T, Ocker M. Hepatocellular carcinoma: Therapeutic advances in signaling, epigenetic and immune targets. *World J Gastroenterol* 2019; **25**: 3136-3150 [PMID: 31333307 DOI: 10.3748/wjg.v25.i25.3136]

205 **Hong YK**, Li Y, Pandit H, Li S, Pulliam Z, Zheng Q, Yu Y, Martin RCG. Epigenetic modulation enhances immunotherapy for hepatocellular carcinoma. *Cell Immunol* 2019; **336**: 66-74 [PMID: 30626493 DOI: 10.1016/j.cellimm.2018.12.010]

206 **Yu HB**, Jiang H, Cheng ST, Hu ZW, Ren JH, Chen J. AGK2, A SIRT2 Inhibitor, Inhibits Hepatitis B Virus Replication *In Vitro* And *In Vivo*. *Int J Med Sci* 2018; **15**: 1356-1364 [PMID: 30275764 DOI: 10.7150/ijms.26125]

207 **Gilmore S**, Tam D, Dick R, Appleby T, Birkus G, Willkom M, Delaney WE, Notte GT, Feierbach B. SAT-160 - Antiviral activity of GS-5801, a liver-targeted prodrug of a lysine demethylase 5 inhibitor, in a hepatitis B virus primary human hepatocyte infection model. *J Hepatol* 2017; **66**: S690-S691 [DOI: 10.1016/S0168-8278(17)31855-X]

208 **Cheng ST**, Hu JL, Ren JH, Yu HB, Zhong S, Wai Wong VK, Kwan Law BY, Chen WX, Xu HM, Zhang ZZ, Cai XF, Hu Y, Zhang WL, Long QX, Ren F, Zhou HZ, Huang AL, Chen J. Dicoumarol, an NQO1 inhibitor, blocks cccDNA transcription by promoting degradation of HBx. *J Hepatol* 2021; **74**: 522-534 [PMID: 32987030 DOI: 10.1016/j.jhep.2020.09.019]

209 **Shim YH**, Yoon GS, Choi HJ, Chung YH, Yu E. p16 Hypermethylation in the early stage of hepatitis B virus-associated hepatocarcinogenesis. *Cancer Lett* 2003; **190**: 213-219 [PMID: 12565176 DOI: 10.1016/s0304-3835(02)00613-4]

210 **Zhao X**, Zhao Z, Guo J, Huang P, Zhu X, Zhou X, Yang Z, Zhao L, Xu L, Xu J, Fu L, Zhang J, Zhang X, Dong Y, Huang G, Wang Q, Li B, Song X, Yang X, Liu S, Yi S, Yu T, Yu C, Hou L, Li J, Chen W. Creation of a six-fingered artificial transcription factor that represses the hepatitis B virus HBx gene integrated into a human hepatocellular carcinoma cell line. *J Biomol Screen* 2013; **18**: 378-387 [PMID: 23042077 DOI: 10.1177/1087057112463066]

211 **Luo W**, Wang J, Xu D, Bai H, Zhang Y, Li X. Engineered zinc-finger transcription factors inhibit the replication and transcription of HBV in vitro and in vivo. *Int J Mol Med* 2018; **41**: 2169-2176 [PMID: 29344646 DOI: 10.3892/ijmm.2018.3396]

212 **Bloom K**, Kaldine H, Cathomen T, Mussolino C, Ely A, Arbuthnot P. Inhibition of replication of hepatitis B virus using transcriptional repressors that target the viral DNA. *BMC Infect Dis* 2019; **19**: 802 [PMID: 31510934 DOI: 10.1186/s12879-019-4436-y]

213 **Xirong L**, Rui L, Xiaoli Y, Qiuyan H, Bikui T, Sibo Z, Naishuo Z. Hepatitis B virus can be inhibited by DNA methyltransferase 3a *via* specific zinc-finger-induced methylation of the X promoter. *Biochemistry (Mosc)* 2014; **79**: 111-123 [PMID: 24794726 DOI: 10.1134/S0006297914020047]

214 **Bloom K**, Ely A, Mussolino C, Cathomen T, Arbuthnot P. Inactivation of hepatitis B virus replication in cultured cells and *in vivo* with engineered transcription activator-like effector nucleases. *Mol Ther* 2013; **21**: 1889-1897 [PMID: 23883864 DOI: 10.1038/mt.2013.170]

215 **Kunze S**. Quantitative Region-Specific DNA Methylation Analysis by the EpiTYPER™ Technology. *Methods Mol Biol* 2018; **1708**: 515-535 [PMID: 29224161 DOI: 10.1007/978-1-4939-7481-8_26]

216 **Nakamura M**, Gao Y, Dominguez AA, Qi LS. CRISPR technologies for precise epigenome editing. *Nat Cell Biol* 2021; **23**: 11-22 [PMID: 33420494 DOI: 10.1038/s41556-020-00620-7]

217 **Broering R**, Luo X, Liu J, Lu M. Controversial: Early Innate Responses to Hepatitis B Virus Infection, an Explanation for Viral Persistence? *Virol Sin* 2021; **36**: 163-166 [PMID: 32632817 DOI: 10.1007/s12250-020-00235-0]

218 **Grompe M**, Strom S. Mice with human livers. *Gastroenterology* 2013; **145**: 1209-1214 [PMID: 24042096 DOI: 10.1053/j.gastro.2013.09.009]

219 **Lebossé F**, Inchauspé A, Locatelli M, Miaglia C, Diederichs A, Fresquet J, Chapus F, Hamed K, Testoni B, Zoulim F. Quantification and epigenetic evaluation of the residual pool of hepatitis B covalently closed circular DNA in long-term nucleoside analogue-treated patients. *Sci Rep* 2020; **10**: 21097 [PMID: 33273565 DOI: 10.1038/s41598-020-78001-1]

220 **Crudele JM**, Chamberlain JS. Cas9 immunity creates challenges for CRISPR gene editing therapies. *Nat Commun* 2018; **9**: 3497 [PMID: 30158648 DOI: 10.1038/s41467-018-05843-9]

221 **Tropberger P**, Pott S, Keller C, Kamieniarz-Gdula K, Caron M, Richter F, Li G, Mittler G, Liu ET, Bühler M, Margueron R, Schneider R. Regulation of transcription through acetylation of H3K122 on the lateral surface of the histone octamer. *Cell* 2013; **152**: 859-872 [PMID: 23415232 DOI: 10.1016/j.cell.2013.01.032]

222 **Kang JY**, Kim JY, Kim KB, Park JW, Cho H, Hahm JY, Chae YC, Kim D, Kook H, Rhee S, Ha NC, Seo SB. KDM2B is a histone H3K79 demethylase and induces transcriptional repression *via* sirtuin-1-mediated chromatin silencing. *FASEB J* 2018; **32**: 5737-5750 [PMID: 29763382 DOI: 10.1096/fj.201800242R]

223 **Peng JC**, Karpen GH. H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. *Nat Cell Biol* 2007; **9**: 25-35 [PMID: 17159999 DOI: 10.1038/ncb1514]

224 **Peng JC**, Karpen GH. Heterochromatic genome stability requires regulators of histone H3 K9 methylation. *PLoS Genet* 2009; **5**: e1000435 [PMID: 19325889 DOI: 10.1371/journal.pgen.1000435]

225 **Choi CY**, Choi BH, Park GT, Rho HM. Activating transcription factor 2 (ATF2) down-regulates hepatitis B virus X promoter activity by the competition for the activating protein 1 binding site and the formation of the ATF2-Jun heterodimer. *J Biol Chem* 1997; **272**: 16934-16939 [PMID: 9202004 DOI: 10.1074/jbc.272.27.16934]

226 **Kim BK**, Lim SO, Park YG. Requirement of the cyclic adenosine monophosphate response element-binding protein for hepatitis B virus replication. *Hepatology* 2008; **48**: 361-373 [PMID: 18615500 DOI: 10.1002/hep.22359]

227 **Song CL**, Ren JH, Ran LK, Li YG, Li XS, Chen X, Li WY, Huang AL, Chen J. Cyclin D2 plays a regulatory role in HBV replication. *Virology* 2014; **462-463**: 149-157 [PMID: 24992041 DOI: 10.1016/j.virol.2014.05.027]

228 **Ori A**, Atzmony D, Haviv I, Shaul Y. An NF1 motif plays a central role in hepatitis B virus enhancer. *Virology* 1994; **204**: 600-608 [PMID: 7941327 DOI: 10.1006/viro.1994.1574]

229 **Shaul Y**, Ben-Levy R, De-Medina T. High affinity binding site for nuclear factor I next to the hepatitis B virus S gene promoter. *EMBO J* 1986; **5**: 1967-1971 [PMID: 3463507]

230 **Nakanishi-Matsui M**, Hayashi Y, Kitamura Y, Koike K. Integrated hepatitis B virus DNA preserves the binding sequence of transcription factor Yin and Yang 1 at the virus-cell junction. *J Virol* 2000; **74**: 5562-5568 [PMID: 10823863 DOI: 10.1128/jvi.74.12.5562-5568.2000]

231 **Raney AK**, McLachlan A. Characterization of the hepatitis B virus large surface antigen promoter Sp1 binding site. *Virology* 1995; **208**: 399-404 [PMID: 11831727 DOI: 10.1006/viro.1995.1167]

232 **Raney AK**, Le HB, McLachlan A. Regulation of transcription from the hepatitis B virus major surface antigen promoter by the Sp1 transcription factor. *J Virol* 1992; **66**: 6912-6921 [PMID: 1331502 DOI: 10.1128/JVI.66.12.6912-6921.1992]

233 **Li J**, Ou JH. Differential regulation of hepatitis B virus gene expression by the Sp1 transcription factor. *J Virol* 2001; **75**: 8400-8406 [PMID: 11507185 DOI: 10.1128/jvi.75.18.8400-8406.2001]

234 **Lu CC**, Yen TS. Activation of the hepatitis B virus S promoter by transcription factor NF-Y *via* a CCAAT element. *Virology* 1996; **225**: 387-394 [PMID: 8918925 DOI: 10.1006/viro.1996.0613]

235 **Maity SN**, de Crombrugghe B. Role of the CCAAT-binding protein CBF/NF-Y in transcription. *Trends Biochem Sci* 1998; **23**: 174-178 [PMID: 9612081 DOI: 10.1016/s0968-0004(98)01201-8]

236 **Nardini M**, Gnesutta N, Donati G, Gatta R, Forni C, Fossati A, Vonrhein C, Moras D, Romier C, Bolognesi M, Mantovani R. Sequence-specific transcription factor NF-Y displays histone-like DNA binding and H2B-like ubiquitination. *Cell* 2013; **152**: 132-143 [PMID: 23332751 DOI: 10.1016/j.cell.2012.11.047]

237 **Choi BH**, Park CJ, Rho HM. Insulin activates the hepatitis B virus X gene through the activating protein-1 binding site in HepG2 cells. *DNA Cell Biol* 1998; **17**: 951-956 [PMID: 9839804 DOI: 10.1089/dna.1998.17.951]

238 **Bogomolski-Yahalom V**, Klein A, Greenblat I, Haviv Y, Tur-Kaspa R. The TATA-less promoter of hepatitis B virus S gene contains a TBP binding site and an active initiator. *Virus Res* 1997; **49**: 1-7 [PMID: 9178491 DOI: 10.1016/s0168-1702(96)01429-3]

239 **Chen IH**, Huang CJ, Ting LP. Overlapping initiator and TATA box functions in the basal core promoter of hepatitis B virus. *J Virol* 1995; **69**: 3647-3657 [PMID: 7745713 DOI: 10.1128/JVI.69.6.3647-3657.1995]

240 **Qin J**, Zhai J, Hong R, Shan S, Kong Y, Wen Y, Wang Y, Liu J, Xie Y. Prospero-related homeobox protein (Prox1) inhibits hepatitis B virus replication through repressing multiple cis regulatory elements. *J Gen Virol* 2009; **90**: 1246-1255 [PMID: 19264593 DOI: 10.1099/vir.0.006007-0]

241 **Lin YC**, Hsu EC, Ting LP. Repression of hepatitis B viral gene expression by transcription factor nuclear factor-kappaB. *Cell Microbiol* 2009; **11**: 645-660 [PMID: 19141126 DOI: 10.1111/j.1462-5822.2008.01280.x]

242 **Nkongolo S**, Nußbaum L, Lempp FA, Wodrich H, Urban S, Ni Y. The retinoic acid receptor (RAR) α-specific agonist Am80 (tamibarotene) and other RAR agonists potently inhibit hepatitis B virus transcription from cccDNA. *Antiviral Res* 2019; **168**: 146-155 [PMID: 31018112 DOI:

10.1016/j.antiviral.2019.04.009]

243 **Oropeza CE**, Li L, McLachlan A. Differential inhibition of nuclear hormone receptor-dependent hepatitis B virus replication by the small heterodimer partner. *J Virol* 2008; **82**: 3814-3821 [PMID: 18234786 DOI: 10.1128/JVI.02507-07]

244 **López-Cabrera M**, Letovsky J, Hu KQ, Siddiqui A. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: trans-activation and repression by CCAAT/enhancer binding protein. *Proc Natl Acad Sci USA* 1990; **87**: 5069-5073 [PMID: 2367525 DOI: 10.1073/pnas.87.13.5069]

245 **Zhou DX**, Yen TS. The ubiquitous transcription factor Oct-1 and the liver-specific factor HNF-1 are both required to activate transcription of a hepatitis B virus promoter. *Mol Cell Biol* 1991; **11**: 1353-1359 [PMID: 1996097 DOI: 10.1128/mcb.11.3.1353]

246 **Zheng Y**, Li J, Ou JH. Regulation of hepatitis B virus core promoter by transcription factors HNF1 and HNF4 and the viral X protein. *J Virol* 2004; **78**: 6908-6914 [PMID: 15194767 DOI: 10.1128/JVI.78.13.6908-6914.2004]

247 **Lin J**, Gu C, Shen Z, Liu Y, Wang W, Tao S, Cui X, Liu J, Xie Y. Hepatocyte nuclear factor 1α downregulates HBV gene expression and replication by activating the NF-κB signaling pathway. *PLoS One* 2017; **12**: e0174017 [PMID: 28319127 DOI: 10.1371/journal.pone.0174017]

248 **Chen M**, Hieng S, Qian X, Costa R, Ou JH. Regulation of hepatitis B virus ENI enhancer activity by hepatocyte-enriched transcription factor HNF3. *Virology* 1994; **205**: 127-132 [PMID: 7975208 DOI: 10.1006/viro.1994.1627]

249 **Li M**, Xie Y, Wu X, Kong Y, Wang Y. HNF3 binds and activates the second enhancer, ENII, of hepatitis B virus. *Virology* 1995; **214**: 371-378 [PMID: 8553537 DOI: 10.1006/viro.1995.0046]

250 **Cho EY**, Kim HJ, Park C, So HS, Park RK, Kim HC. Impact of Nucleotide Mutations at the HNF3- and HNF4-Binding Sites in Enhancer 1 on Viral Replication in Patients with Chronic Hepatitis B Virus Infection. *Gut Liver* 2013; **7**: 569-575 [PMID: 24073315 DOI: 10.5009/gnl.2013.7.5.569]

251 **Long Y**, Chen E, Liu C, Huang F, Zhou T, He F, Liu L, Liu F, Tang H. The correlation of hepatocyte nuclear factor 4 alpha and 3 beta with hepatitis B virus replication in the liver of chronic hepatitis B patients. *J Viral Hepat* 2009; **16**: 537-546 [PMID: 19302415 DOI: 10.1111/j.1365-2893.2009.01089.x]

252 **Lin TJ**, Yang RY, Lee HJ. Collective repression of the hepatitis B virus enhancer II by human TR4 and TR2 orphan receptors. *Hepatol Res* 2008; **38**: 79-84 [PMID: 17645519 DOI: 10.1111/j.1872-034X.2007.00208.x]

253 **Yuan Y**, Yuan H, Yang G, Yun H, Zhao M, Liu Z, Zhao L, Geng Y, Liu L, Wang J, Zhang H, Wang Y, Zhang XD. IFN-α confers epigenetic regulation of HBV cccDNA minichromosome by modulating GCN5-mediated succinylation of histone H3K79 to clear HBV cccDNA. *Clin Epigenetics* 2020; **12**: 135 [PMID: 32894195 DOI: 10.1186/s13148-020-00928-z]

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REVIEW

COVID-19-associated diarrhea

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) recently emerged as a highly virulent respiratory pathogen that is known as the causative agent of coronavirus disease 2019 (COVID-19). Diarrhea is a common early symptom in a significant proportion of patients with SARS-CoV-2 infection. SARS-CoV-2 can infect and replicate in esophageal cells and enterocytes, leading to direct damage to the intestinal epithelium. The infection decreases the level of angiotensin-converting enzyme 2 receptors, thereby altering the composition of the gut microbiota. SARS-CoV-2 elicits a cytokine storm, which contributes to gastrointestinal inflammation. The direct cytopathic effects of SARS-CoV-2, gut dysbiosis, and aberrant immune response result in increased intestinal permeability, which may exacerbate existing symptoms and worsen the prognosis. By exploring the elements of pathogenesis, several therapeutic options have emerged for the treatment of COVID-19 patients, such as biologics and biotherapeutic agents. However, the presence of SARS-CoV-2 in the feces may facilitate the spread of COVID-19 through fecal-oral transmission and contaminate the environment. Thus gastrointestinal SARS-CoV-2 infection has important epidemiological significance. The development of new therapeutic and preventive options is necessary to treat and restrict the spread of this severe and widespread infection more effectively. Therefore, we summarize the key elements involved in the pathogenesis and the epidemiology of COVID-19-associated diarrhea.

Key Words: COVID-19; Diarrhea; Ionic imbalance; Viroporin; Angiotensin-converting enzyme type 2; Leaky gut

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Core Tip: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) replicates in enterocytes, triggers ionic imbalances, activates the NLRP3 inflammasome pathway, induces apoptosis, and exerts a dual effect on the autophagic process. These effects of SARS-CoV-2 lead to the development of leaky gut. Increased permeability triggers the absorption of lipopolysaccharide into the circulation, further exacerbating inflammation induced by viral infection. In addition to drugs that affect the inflammatory response and viral replication, agents targeting autophagy and apoptosis appear to be potentially suitable for the treatment of coronavirus disease 2019 (COVID-19). The fecal-oral route of SARS-CoV-2 transmission calls for strict and more consistent adherence to hygiene rules to prevent the spread of COVID-19.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) recently emerged as a highly virulent respiratory pathogen that is known as the causative agent of coronavirus disease 2019 (COVID-19)[1]. SARS-CoV-2 enters the human body through the airways and multiplies in the lungs. This novel coronavirus causes mild, severe, and critical respiratory disease in 81%, 14%, and 5% of cases, respectively[2]. It may also enter the bloodstream, which results in viremia and systemic spread throughout the body.

In addition to the airways, the virus can multiply in the gastrointestinal (GI) tract (GIT), urinary tract, and central nervous system. The infection elicits an intemperate immune response characterized by a life-threatening cytokine storm and a corrupted interferon (IFN) system, which is unable to eliminate the pathogen effectively. As a result, a systemic inflammatory response syndrome occurs[3,4]. In the severe and critical clinical manifestations of COVID-19, atypical pneumonia leading to progressive respiratory failure develops[2].

As of the 13th of January 2021, about 90 million people have been infected, and nearly 2 million people have died during the COVID-19 pandemic[5]. Although the leading COVID-19 symptoms are due to involvement of the respiratory system, it often causes GI symptoms as well. Thus, we examined the current state of knowledge on the pathogenesis, occurrence rate, clinical significance, and epidemiological consequences of COVID-19-associated diarrhea.

TAXONOMIC CLASSIFICATION, STRUCTURE, AND REPLICATION OF SARS-COV-2

SARS-CoV-2 belongs to the genus *Betacoronavirus* of the family *Coronaviridae*, which comprises enveloped viruses with positive-sense single-stranded RNA genomes[1,6,7]. The spherical or elliptical virions are pleomorphic with diameters of 80-160 nm[8,9]. The capsid has helical symmetry, which is built up by the nucleocapsid (N) protein. The spike (S), membrane (M), and envelope (E) proteins are located in the virion envelope[10]. The S protein forms protrusions of 20 nm in length that provide a characteristic crown-like appearance, which is reflected in the name of the viral family. The S-protein is responsible for binding to the cell surface receptor[10].

Besides the S, E, M, and N structural protein genes, the genome of SARS-CoV-2 contains open reading frames (Orfs) that encode nine accessory proteins (3a, 3b, 6, 7a, 7b, 8, 9b, 9c, and 10) and two polyproteins (pp1a and pp1ab)[10-15]. Polyproteins pp1a and pp1ab are cleaved by viral proteases to form unique non-structural proteins (Nsp), which play an important role in viral replication[10]. Although the accessory proteins of SARS-CoV-2 are not essential for viral multiplication, they are implicated in the pathogenesis[10].

As the first step in infection, the S protein of SARS-CoV-2 binds to its corresponding cell-surface receptor, angiotensin-converting enzyme type 2 (ACE2)[16,17]. The S protein has two subunits: S1 and S2. The S1 subunit has a receptor-binding domain and is responsible for receptor engagement, whereas the S2 subunit is involved in the fusion process[17-19]. Following ACE2 binding, cellular proteases such as transmembrane protease/serine subfamily member 2 (TMPRSS2), TMPRSS4, and cathepsin L cleave S protein into S1 and S2 subunits, and the virus enters the host cell by receptor-mediated endocytosis[17,19-23]. Other proteases have also been shown to be able to cleave S protein, including furin, trypsin-like proteases, elastase, plasmin, and factor Xa. This suggests that these enzymes may also facilitate entry or expand the tissue tropism of SARS-CoV-2[23-27].

Within the endosome, cathepsin-mediated activation of the S protein continues, eventually causing the S2 subunit to gain a fusogenic effect that triggers the fusion of the viral envelope and the endosomal membrane[25]. The nucleocapsid is then released into the cytoplasm, where the translation of Orf1a and Orf1b results in the formation of pp1a and pp1ab, from which Nsp1-16 are generated by proteolysis. Nsp12 functions as an RNA-dependent RNA polymerase and associates with Nsp7 and Nsp8 to form the core of the replication and transcription complex (RTC) of SARS-CoV-2[28,29]. The cofactors Nsp7 and Nsp8 form a hexadecameric ring structure that has a primase function and generates RNA primers for the synthesis of the negative-sense RNA[28,29].

RTC synthesizes the genomic RNA and a set of SARS-CoV-2 mRNAs through full-length and subgenomic negative-sense RNA intermediates[28,29]. The replication of the viral genome and transcription of viral genes takes place in double-membraned vesicles[30,31]. The SARS-CoV-2 replication compartment provides a protected environment which inhibits the antiviral effects of IFN and other cellular antiviral defense mechanisms by hiding the viral genome, transcripts, and replicative intermediates from cellular nucleic acid sensors.

The viral mRNAs are translated in the rough endoplasmic reticulum, leading to the formation of accessory proteins and structural proteins (N, M, E, and S). The M, E, and S proteins then become embedded in the endoplasmic reticulum, whereas the N proteins assemble with the newly synthesized full-length positive-sense RNA to form the nucleocapsid[30,31]. After being transported to the ERGIC (endoplasmic reticulum-Golgi intermediate compartment), the nucleocapsids bud through the ERGIC membrane into its lumen[30,31]. The mature virions reach the cytoplasm membrane *via* vesicular transport and are released from the cell[30,31].

THE MAIN CELLULAR EFFECTS OF SARS-COV-2

During multiplication, SARS-CoV-2 modulates several cellular aspects, including signaling, transcription, translation, cell division, the IFN system, autophagy, and apoptosis, as well as the biogenesis, function, and morphology of mitochondria and intracellular vesicles. Phosphoproteomic profiling has revealed that SARS-CoV-2 infection affects the activity of 97 kinases. The activities of several members of the p38 pathway and the guanosine monophosphate-dependent protein kinases are upregulated, while cell cycle kinases (CDK1/2/5), cell growth-related signaling pathway kinases (AKT1/2), and regulators of the cytoskeleton are down-regulated [32]. The functional changes in the signal transduction pathways have been shown to play an important role in SARS-CoV-2-induced cytoskeletal damage, cytokine production, and slow-down in cell proliferation at the S/G2 transition phase[32].

Transcriptomic profiles of SARS-CoV-2-infected primary human bronchial epithelial cells, lung biopsy, and bronchoalveolar lavage fluid samples of COVID-19 patients have demonstrated upregulated expression of genes implicated in metabolism, immunity, and the stress responses of the endoplasmic reticulum and mitochondria [33-35]. It has been shown that the M protein, Nsp7, and ORF9c stimulate lipogenesis, while Nsp7, Nsp12, and ORF8 trigger endoplasmic stress response, and Nsp7 induces mitochondrial dysfunction[34]. Moreover, the M and E proteins, along with Nsp3a, Nsp6, Nsp8, Nsp10, and Nsp13, were shown to be able to modify the structure and function of the endomembrane system and vesicle trafficking, thereby facilitating several steps of viral multiplication[36].

Interestingly, the expression of genes involved in the humoral immune response and innate immune response-activating signal transduction are increased, whereas genes implicated in cytokine-mediated signaling pathways are down-regulated[33]. A multiplex gene expression analysis showed that the genes involved in type I IFN

signaling were highly up-regulated, whereas the expression of IFN-stimulated genes (*ISGs*) was decreased in severe COVID-19 patients[37]. The levels of pro-inflammatory cytokines measured in sera of COVID-19 patients were highly increased in a pattern corresponding to a cytokine storm[38-40].

Consistent with this observation, transcriptional activation of pro-inflammatory cytokine genes was also detected in peripheral blood mononuclear cells and bronchoalveolar lavage fluid[41]. The sera and lung tissue samples of patients have shown interleukin (IL)-1β, IL-6, IL-10, IL-18, IL-33, transforming growth factor-β, IFN-γ, CSF2/GM-CSF (colony-stimulating factor 2/granulocyte-macrophage colony-stimulating factor), CSF3/G-CSF, CC chemokines [CCL2/MCP-1, CCL3/MIP-1A, CCL4/MIP-1B, CCL5/RANTES, CCL8, CCL3L1] and CXC chemokines [CXCL1, CXCL2 and CXCL10/IP10][38,39,41,42]. However, during SARS-CoV-2 infection, the production of type I and III IFNs is decreased[37,43]. Thus, these data clearly demonstrate that SARS-CoV-2 infection alters both the transcriptional and translational patterns in cells profoundly[32-43].

Other observations indicate that SARS-CoV-2 could trigger several cell-death processes, including apoptosis, necrosis, pyroptosis, and anoikis, depending on the type of cell[44-47]. The death of infected cells may contribute to tissue damage and induce an inflammatory reaction[44-47]. It has also been revealed that SARS-CoV-2 Orf 3a stimulates the formation of the autophagic Beclin-1-Vps34-Atg14 complex while simultaneously inhibiting the Beclin-1 complex containing the UVRAG adaptor protein[48]. Orf 3a thereby exerts a dual effect on the autophagic process manifesting in the induction of the initial steps and a block in the fusion of the autophagosomes with lysosomes[48].

DIARRHEA IN COVID-19

GIT involvement is frequent in COVID-19 patients and includes anorexia, nausea, vomiting, diarrhea, and abdominal pain[49-62]. Among the specific GI symptoms, diarrhea is the most common. Based on different studies, the prevalence of diarrhea might range from 2% to 49.5%[50,61,63]. COVID-19-associated diarrhea is characterized by loose or watery stools and is usually mild, self-limiting, and can even be the only symptom of the infection[49,52,58,59,63]. The average frequency of bowel movements is in the range of 3.3-4.3 times per day[53,58], and the average duration of diarrhea is 3-5.4 d[52,53,58,59,63]. In some cases, however, diarrhea is more severe, with patients experiencing more frequent bowel movements of up to 18-30 times per day[58,63].

Rare cases with more severe GI symptoms have also been reported, such as acute hemorrhagic colitis and GI bleeding[53,54,64]. Furthermore, the relationship between GI symptoms and the severity of the disease has been investigated. Statistically significant differences were not observed between COVID-19 patients with and without GI symptoms in clinical severity, length of hospital stay, and mortality rate[49,59,60]. SARS-CoV-2 mRNA could be detected in the stool of COVID-19 patients in 22%-54.5% of cases, and occasionally, the virus is detectable in the stool even after the airway samples become negative[54,57,58,65-69]. Positive results have been obtained from real-time reverse transcriptase-polymerase chain reaction (Rrt-PCR) tests of stool even in patients without GI symptoms[58]. In patients with GI symptoms, the total time between the onset of symptoms and viral clearance is significantly longer than in those with only respiratory manifestations[58,70].

The reason why GI symptoms occur in only a subset of COVID-19 patients is currently unknown. There are no significant differences between the two patient groups in terms of demographics and certain coexisting conditions, such as pregnancy, cancer, chronic renal disease, chronic obstructive pulmonary disease, or immunosuppression. A study conducted by Jin *et al*[52] revealed that the rate of chronic liver disease in COVID-19 patients with GI symptoms is much higher than among those without GI symptoms. Moreover, the incidence of COVID-19 with GI symptoms displays familial clustering[52]. Based on these interesting observations, it is reasonable to infer that genetic, immunological, and epidemiological factors are involved in the development of COVID-19-associated diarrhea.

PATHOGENESIS OF COVID-19-ASSOCIATED DIARRHEA

ACE2, the cellular receptor of SARS-CoV-2, is widely expressed in many types of cells and tissues of the GIT, including the esophagus, stomach, small intestine, colon, rectum, pancreatic exocrine glands and islets, and gallbladder[71]. The expression level of ACE2 in the GIT is highest in the ileum epithelial cells, especially in the absorptive enterocytes[72]. It has also been demonstrated that ACE2 is co-expressed with TMPRSS2/4 proteases in the GIT, with the highest level in the ileum[73]. These observations indicate that several cell types in the GIT are potentially susceptible to SARS-CoV-2 infection[71-73].

Studies demonstrating that viral RNA can be detected in the stool samples of COVID-19 patients indicate that SARS-CoV-2 can indeed infect the GIT[54,57-59,66-68]. It is estimated that feces and GI tissues contain 10^4-10^8 and 10^0-10^4 RNAs per gram, respectively[74,75]. Further studies revealed that SARS-CoV-2 establishes a productive infection in intestinal epithelial cells and human small intestinal organoids, leading to the production of new infectious progeny virions[20,76]. Viral particles within intracytoplasmic vesicles and aggregates of SARS-CoV-2 virions attached to the surface of enterocytes have been detected in intestinal organoids and post-mortem GIT samples from COVID-19 patients by electron microscopy[76,77]. These observations indicate that the GIT can be an entry site and an extra-pulmonary target organ of SARS-CoV-2[20,71-73,76,77].

Further analyses were performed to determine whether infectious viruses are present in the GIT or feces. In most cases, efforts to cultivate infectious SARS-CoV-2 from feces have failed, although Xiao *et al*[78] recently reported the successful isolation of the virus from stool samples by using the Vero E6 cell line. Simulated large intestinal fluid was shown to reduce the infectivity of the virus significantly[20]. Thus, it is possible that most of the virus that multiplies in the enterocytes may be inactivated in the lumen of intestines within a short time after release.

In vitro cultivation of SARS-CoV-2 has demonstrated that this virus elicits a cytopathic effect (CPE) on some cell lines, whereas in other cell types, no cytomorphological abnormalities could be observed despite efficient viral replication[79]. In human airway epithelial cells, SARS-CoV-2 causes CPE characterized by the formation of multinucleated syncytia and cilium shrinking, and cell death largely occurs by way of apoptosis[45]. In contrast, the colorectal adenocarcinoma Caco-2 cell line proved to be susceptible to infection, but the multiplication of SARS-CoV-2 was not accompanied by a visible CPE[79]. Likewise, intense tissue damage was not observed in the GIT of COVID-19 patients[80].

SARS-CoV-2 can establish a persistent infection in human C2BBe1 intestinal cells expressing a brush border[81]. Moreover, SARS-CoV-2 was shown to be more effective in inducing the production of IFN-α, IFN-β, IFN-λ1, IFN-λ2, and IFN-λ3 in human intestinal tissues *ex vivo* than in lung tissue[80]. Therefore, it is also conceivable that a specific immuno-inflammatory environment develops in the lungs and GIT as a result of infection, which affects the rate of viral replication and cell demise in different ways.

Although SARS-CoV-2 causes no extensive tissue damage in the intestines, the infection seems to harm the enterocytes in a much more sophisticated way. E protein was shown to bind to the tight junction-associated PALS1 (Proteins Associated with Lin Seven 1)[82]. PALS1 interacts with PATJ (PALS1-Associated Tight Junction protein) and CRB3 (Crumbs 3), and the PALS1/PATJ/CRB3 complex that forms is essential for the maintenance of tight junctions connecting epithelial cells[83]. E protein causes functional impairment of PALS1 and interferes with the formation of tight junctions, leading to the disruption of intestinal barrier integrity[82]. By using a biomimetic gut-on-chip system, Guo et. al. elegantly demonstrated that SARS-CoV-2 infection destroys tight junctions and adherent junctions in both the endothelium and intestinal epithelium, which in turn may lead to leaky gut syndrome, local and systemic invasion of normal microbiota members, and immune activation[84] (Figure 1).

The E protein of SARS-CoV-2 is a single-spanning membrane protein that forms a homopentameric ion channel, which displays selective permeability for monovalent ions (Na^+, K^+, and Cl^-) and Ca^{2+}[85]. E protein accumulates in the endoplasmic reticulum and ERGIC/Golgi membranes and transports Ca^{2+} from these compartments to the cytoplasm. Elevated cytoplasmic Ca^{2+} concentration can increase the rate of apical Cl^- exit across the Ca^{2+}-activated Cl^- channels and cyclic-nucleotide-activated cystic fibrosis transmembrane conductance regulator[86].

SARS-CoV-2 also has another ion-channel protein, Orf3a, which is a K^+ ion channel viroporin that exhibits plasma membrane and endomembrane localization[46,87]. Orf3a in the cytoplasmic membrane may cause leakage of K^+ ions from enterocytes.

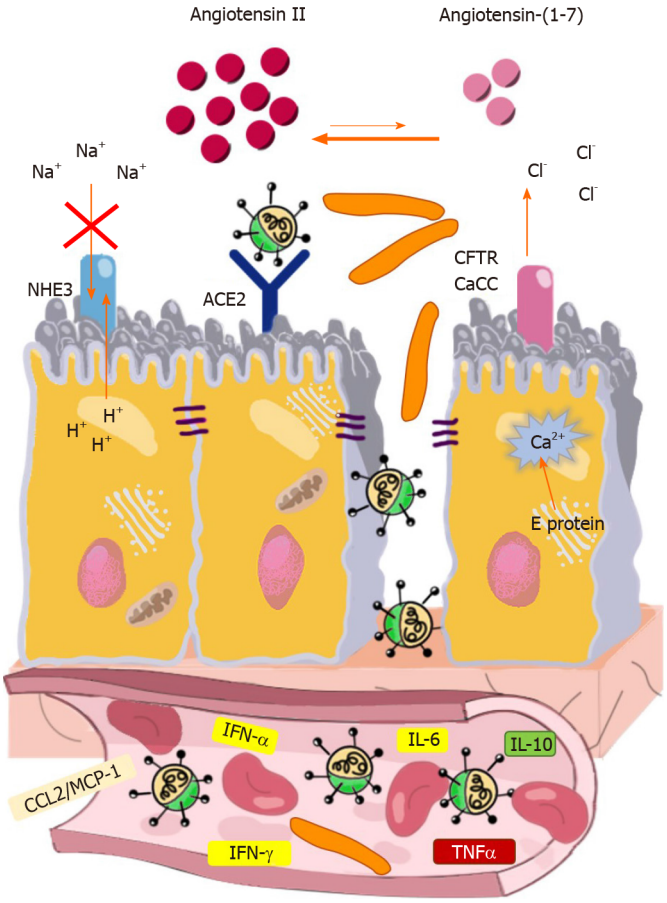


Figure 1 Mechanism involved in coronavirus disease 2019-associated diarrhea. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binds to its corresponding cell-surface receptor, angiotensin-converting enzyme type 2. In the intestines, SARS-CoV-2 viroporins, E protein, dysregulation of the renin-angiotensin-aldosterone system triggering ionic imbalance, disruption of barrier integrity and inflammation play important roles in the development of coronavirus disease 2019-associated secretory diarrhea and leaky gut. ACE: Angiotensin-converting enzyme; IFN: Interferon; IL: Interleukin; TNFα: tumor necrosis factor alpha; CFTR: CF transmembrane conductance regulator; CaCC: Ca^{2+}-activated Cl^- channel; NHE3: Na^+-H^+ exchanger 3.

Moreover, intracellular ionic imbalances triggered by SARS-CoV-2 viroporins (E protein and Orf3a) can lead to the activation of the NLRP3 inflammasome (NOD-, LRR-, and Pyrin domain-containing 3). This results in the secretion of IL-1β and cell death in a process called pyroptosis[44,46]. By activating innate immune cells, IL-1β contributes to the development of a local inflammatory environment and a systemic cytokine storm. The direct action of viroporins and the indirect effects of cytokines together can trigger an ionic imbalance of enterocytes, which may contribute to the development of diarrhea (Figure 1).

During its multiplication, SARS-CoV-2 disturbs the function of the renin-angiotensin-aldosterone system (RAAS). The main components of this system are ACE, angiotensin II, and AT1R. Angiotensin II is known to elicit vasoconstriction, oxidative stress, and inflammation following binding to AT1R[88]. The ACE2/angiotensin (1-7)/Mas pathway is an important physiological negative regulator of the ACE/angiotensin II/AT1R axis and exerts anti-inflammatory effects[88]. SARS-CoV-2 uses ACE2 for entry as a receptor, which becomes degraded in the endolysosomal compartment after being internalized along with the virion particles[17,19,20].

The viral infection has been shown to increase the expression of ADAM (A Disintegrin and Metalloprotease) metallopeptidase domain 17 enzyme, which is endowed with sheddase activity[89]. ADAM17 functions in the ectodomain shedding of tumor necrosis factor alpha (TNF-α), EGFR ligands, and ACE2[90,91]. ADAM17-mediated cleavage decreases ACE2 Levels on the cytoplasm membrane and thereby shifts the delicate balance towards the ACE/angiotensin II/AT1R pathway. In turn, this can lead to pro-inflammatory predominance. It has also been demonstrated recently that ACE2 forms dimer-of-heterodimer complexes with the neutral amino acid transporter B^0AT1 (Broad neutral Amino acid Transporter 1)[91]. B^0AT1 is involved in the Na^+-coupled transportation of tryptophan, phenylalanine, glutamine, and leucine[91].

The ADAM17-mediated cleavage of ACE2 ectodomain and attachment of SARS-CoV-2 to the ACE2:B^0AT1 complex may potentially compromise the transport of Na^+ and neutral amino acids[88]. Impairment of the ACE2:B^0AT1 complex and the consequential amino acid starvation can decrease Na^+ uptake and affect the activation state of the mechanistic target of rapamycin (mTOR) complex, which is an important regulator of autophagy, xenophagy, metabolism, and various immune processes[88,92-95]. Dysregulated RAAS may aggravate ionic imbalance and inflammation, which may affect the metabolic state of cells, the composition of the microbiota, and cell viability, leading to increasingly severe intestinal dysfunction[88,90,91,94,95] (Figure 1).

VIRUS-INDEPENDENT CAUSES OF COVID-19-ASSOCIATED DIARRHEA

If diarrhea is not included in the presenting symptoms and develops after admission, it becomes challenging to ascertain the cause of diarrhea. Several confounding variables, such as the hyperinflammatory response, altered gut flora, secondary bacterial infections, antiviral agents, antibiotics, enteral feeding, and the use of proton pump inhibitors can potentially cause diarrhea in hospitalized COVID-19 patients.

In approximately 20% of COVID-19 patients, the infection progresses to severe and critical phases in which an extrapulmonary hyperinflammatory state develops due to cytokine release syndrome[96]. Several cytokines may affect the course and clinical manifestations of SARS-CoV-2 infection by increasing the intestinal and vascular permeability as well as triggering the formation of thrombi in the small blood vessels and the alteration of intestinal microbiota, leading to bacterial translocation towards the bloodstream and the mesenteric lymph node[3,4]. The cytokine-mediated GI damage may thereby further intensify the systemic immunological response and contribute to the deterioration of the patient's condition. A study conducted by Zhang *et al*[97] revealed that the pro-inflammatory cytokine patterns are different in COVID-19 patients with and without diarrhea. Moreover, diarrhea patients were more likely to develop cytokine release syndrome and multi-organ damage[97]. Interestingly, the levels of TNF-α, IL-6, and IL-10 were significantly higher in the sera of diarrhea patients than in the non-diarrhea group[97]. TNF-α is known to increase the expression of adhesion molecules on the surface of endothelial cells, platelets, and leukocytes, thereby facilitating the adhesion of thrombocytes to the vessels and initiating the formation of thrombi in the microcirculation of the GIT and other organs. These effects increase vascular permeability and can lead to inflammation and disseminated intravascular coagulation. Furthermore, TNF-α has the ability to disrupt the intestinal tight junction barrier, which in turn contributes to the development of leaky gut[98]. IL-6 exerts dual effects on the intestinal epithelium. It increases gut permeability to small molecules with a radius < 4Å (< 0.4 nm) *via* activating claudin-2 gene expression[99]. However, by stimulating epithelial proliferation and regeneration, IL-6 plays a beneficial role in the maintenance of intestinal epithelial integrity during acute injury[100]. IL-10 is an anti-inflammatory cytokine that restricts uncontrolled immune responses to the intestinal microbiota and defends gut barrier integrity[101,102]. In light of these data, it is reasonable to infer that TNF-α may be an important factor in COVID-19-associated diarrhea, whereas without IL-10, the cytokine storm and intestinal injury would be even more devastating. However, further studies are needed to identify the precise role of each cytokine in the development of COVID-19-associated diarrhea. Such studies could also contribute to a better understanding of the potential adverse effects of anti-cytokine therapies on the GIT.

Interesting observations revealed that the composition of intestinal microbiota is profoundly altered in COVID-19 patients: The diversity is highly reduced, the proportion of beneficial commensal bacteria is decreased and the opportunistic pathogens are enriched compared with that found in healthy controls[103,104]. It has also been demonstrated that some *Bacteroides spp.*, capable of decreasing ACE2 expression in mice, displayed inverse correlation with the fecal SARS-CoV-2 load [104]. The immune system and the intestinal microbiota are in a continuous dialog. The presence of commensal microorganisms shapes host immunity, and alterations in microbiota composition may lead to increased susceptibility to various pathological conditions, including infections, inflammation, and metabolic and autoimmune disorders. Thus, the altered microbiota observed in COVID-19 patients may be an additional factor contributing to the development of diarrhea by weakening colonization resistance, decreasing the production of beneficial bacterial metabolites, and triggering a local immune recalibration.

For clinical improvement and treatment of secondary bacterial infections, COVID-19 patients are treated with antiviral agents, antibiotics, and corticosteroids. Antiviral agents such as the RNA polymerase inhibitors favipiravir and remdesivir may cause diarrhea[105]. Diarrhea is also a common adverse drug reaction to antibiotics such as cephalosporins, macrolides and fluoroquinolones, largely due to destruction of the normal intestinal microbiota. Moreover, treatment of COVID-19 patients with broad-spectrum antibiotics has the potential to increase the risk of *Clostridioides difficile* (*C. difficile*) infection, including in survivors even long after recovery. In co-infections with SARS-CoV-2 and *C. difficile*, intestinal damage is more extensive and diarrhea symptoms are more severe[106]. To counteract the detrimental effects of various proinflammatory cytokines, biological therapy is used in selected patient groups. IL-6 and IL-6 receptor inhibitors, such as tocilizumab, sarilumab and siltuximab, represent another class of drugs that often cause diarrhea[107]. Although enteral feeding has well- established, clear advantages over parenteral nutrition[108], adverse events, like diarrhea, may develop. Tube feeding-related diarrhea can occur for several reasons, mostly related to the circumstances of feeding (adaptation time, perfusion speed, temperature) or the composition of the used enteral formula (osmolarity, fat content, nutrient intolerance), and can be managed easily with careful observation of the patients[109]. Not only is the use of PPIs during the course of COVID-19 infection controversial[110], such drugs may induce diarrhea in general through the alteration of GI microbiota by different mechanisms, including the direct consequences of increased gastric pH itself. This safety issue was evaluated by a number of meta-analyses based on retrospective observational or case-control studies, which suggested an increased risk for enteral infections, especially *C. difficile* infections, in PPI-treated patients[111-113]. In contrast, a recent long-term prospective study (COMPASS) failed to show an increased risk of *C. difficile* infection in PPI users and only a slight increase of enteral infections in general[114].

POTENTIAL CONSEQUENCES OF COVID-19-ASSOCIATED DIARRHEA

A great body of experimental and clinical evidence demonstrates that SARS-CoV-2 infects and replicates in the GIT, and the stool contains high copies of viral RNA, although the amount of infectious virus in the stool appears to be low. The presence of SARS-CoV-2 in the feces may potentially facilitate the spread of COVID-19 through fecal-oral transmission among humans and contaminate the environment[115-117]. Thus, SARS-CoV-2 infection of the GIT has important epidemiological significance.

The feces of COVID-19 patients pose a serious epidemiological risk, which justifies the use of all available methods of prevention, including protective equipment, disinfection procedures, and vaccination. However, further studies are needed to establish the efficiency of the fecal-oral spread of SARS-CoV-2 precisely. It would be very useful if the concentration of infectious virion particles in the stool were determined in asymptomatic individuals and different patient groups under standardized parameters when discharge frequencies and the grade on the Bristol stool scale are precisely recorded. It is possible that the rate of virus inactivation in the intestinal lumen may significantly differ in COVID-19 patients.

SARS-CoV-2 can extensively contaminate the environment, and viral RNA can be detected in sewage and solid waste[118,119]. Measurement of SARS-CoV-2 RNA in wastewater is used for local monitoring of the epidemic situation, which facilitates the implementation of preventive measures. Wastewater epidemiology involves using Rrt-PCR to determine SARS-CoV-2 RNA in sewage, but how long the virus survives in this environment has not been measured[118,119]. It would be essential to determine the concentration of infectious virion particles to elucidate the risk of SARS-CoV-2 transmission *via* wastewater contamination.

CONCLUSION

Among the specific GI symptoms, diarrhea is the most common in COVID-19 patients. The ACE2 receptor and other elements required for the attachment of this virus to the various cell types are extensively expressed throughout the GIT. SARS-CoV-2 can establish a productive infection in the enterocytes, leading to mild cellular damage. The infection evokes an inflammatory response in the intestines, which is characterized by the production of various pro-inflammatory cytokines and chemokines, many of which are known to increase intestinal permeability. Direct effects of SARS-

CoV-2 viroporins and dysregulation of the intestinal RAAS triggering ionic imbalance and inflammation in the intestines seem to play important roles in the development of COVID-19-associated secretory diarrhea and leaky gut.

Infection in the lungs and GIT also seems to display some different tissue-specific features. The production of type I and III IFNs is more efficient in the GIT than in the lungs. The antiviral IFNs may restrict viral replication in the GIT to some extent, which may allow the development of a less cytopathogenic or persistent form of infection in this anatomical region. SARS-CoV-2-mediated dysregulation of the ACE2:B^0AT1 complex may modify the biological response of cells to the infection, and in enterocytes, it may contribute to the development of diarrhea by inducing amino acid starvation, which can decrease Na^+ uptake. These effects are not seen in the lungs, however, as ACE2 does not form a complex with B^0AT1 in this organ. SARS-CoV-2 infection of the GIT is of pivotal epidemiological significance, but further studies are needed to assess the extent of this risk.

REFERENCES

1 **Coronaviridae Study Group of the International Committee on Taxonomy of Viruses**. The species Severe acute respiratory syndrome-related coronavirus: lassifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* 2020; **5**: 536-544 [PMID: 32123347 DOI: 10.1038/s41564-020-0695-z]

2 **Hu B**, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. *Nat Rev Microbiol* 2021; **19**: 141-154 [PMID: 33024307 DOI: 10.1038/s41579-020-00459-7]

3 **Tay MZ**, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: immunity, inflammation and intervention. *Nat Rev Immunol* 2020; **20**: 363-374 [PMID: 32346093 DOI: 10.1038/s41577-020-0311-8]

4 **Vabret N**, Britton GJ, Gruber C, Hegde S, Kim J, Kuksin M, Levantovsky R, Malle L, Moreira A, Park MD, Pia L, Risson E, Saffern M, Salomé B, Esai Selvan M, Spindler MP, Tan J, van der Heide V, Gregory JK, Alexandropoulos K, Bhardwaj N, Brown BD, Greenbaum B, Gümüş ZH, Homann D, Horowitz A, Kamphorst AO, Curotto de Lafaille MA, Mehandru S, Merad M, Samstein RM; Sinai Immunology Review Project. Immunology of COVID-19: Current State of the Science. *Immunity* 2020; **52**: 910-941 [PMID: 32505227 DOI: 10.1016/j.immuni.2020.05.002]

5 **World Health Organization**. WHO Coronavirus Disease (COVID-19) Dashboard. [cited 13 January 2021]. In: World Health Organization [Internet]. Available from: https://covid19.who.int

6 **Helmy YA**, Fawzy M, Elaswad A, Sobieh A, Kenney SP, Shehata AA. The COVID-19 Pandemic: A Comprehensive Review of Taxonomy, Genetics, Epidemiology, Diagnosis, Treatment, and Control. *J Clin Med* 2020; **9** [PMID: 32344679 DOI: 10.3390/jcm9041225]

7 **Lu R**, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 2020; **395**: 565-574 [PMID: 32007145 DOI: 10.1016/S0140-6736(20)30251-8]

8 **Miller SE**, Goldsmith CS. Caution in Identifying Coronaviruses by Electron Microscopy. *J Am Soc Nephrol* 2020; **31**: 2223-2224 [PMID: 32651224 DOI: 10.1681/ASN.2020050755]

9 **Caldas LA**, Carneiro FA, Higa LM, Monteiro FL, da Silva GP, da Costa LJ, Durigon EL, Tanuri A, de Souza W. Ultrastructural analysis of SARS-CoV-2 interactions with the host cell *via* high resolution scanning electron microscopy. *Sci Rep* 2020; **10**: 16099 [PMID: 32999356 DOI: 10.1038/s41598-020-73162-5]

10 **Naqvi AAT**, Fatima K, Mohammad T, Fatima U, Singh IK, Singh A, Atif SM, Hariprasad G, Hasan GM, Hassan MI. Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. *Biochim Biophys Acta Mol Basis Dis* 2020; **1866**: 165878 [PMID: 32544429 DOI: 10.1016/j.bbadis.2020.165878]

11 **Khailany RA**, Safdar M, Ozaslan M. Genomic characterization of a novel SARS-CoV-2. *Gene Rep* 2020; **19**: 100682 [PMID: 32300673 DOI: 10.1016/j.genrep.2020.100682]

12 **Michel CJ**, Mayer C, Poch O, Thompson JD. Characterization of accessory genes in coronavirus genomes. *Virol J* 2020; **17**: 131 [PMID: 32854725 DOI: 10.1186/s12985-020-01402-1]

13 **Finkel Y**, Mizrahi O, Nachshon A, Weingarten-Gabbay S, Morgenstern D, Yahalom-Ronen Y, Tamir H, Achdout H, Stein D, Israeli O, Beth-Din A, Melamed S, Weiss S, Israely T, Paran N, Schwartz M, Stern-Ginossar N. The coding capacity of SARS-CoV-2. *Nature* 2021; **589**: 125-130 [PMID: 32906143 DOI: 10.1038/s41586-020-2739-1]

14 **Wu F**, Zhao S, Yu B, Chen YM, Wang W, Song ZG, Hu Y, Tao ZW, Tian JH, Pei YY, Yuan ML, Zhang YL, Dai FH, Liu Y, Wang QM, Zheng JJ, Xu L, Holmes EC, Zhang YZ. A new coronavirus associated with human respiratory disease in China. *Nature* 2020; **579**: 265-269 [PMID: 32015508 DOI: 10.1038/s41586-020-2008-3]

15 **Srinivasan S**, Cui H, Gao Z, Liu M, Lu S, Mkandawire W, Narykov O, Sun M, Korkin D. Structural Genomics of SARS-CoV-2 Indicates Evolutionary Conserved Functional Regions of Viral Proteins.

Viruses 2020; **12** [PMID: 32218151 DOI: 10.3390/v12040360]

16 **Zhou P**, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, Guo H, Jiang RD, Liu MQ, Chen Y, Shen XR, Wang X, Zheng XS, Zhao K, Chen QJ, Deng F, Liu LL, Yan B, Zhan FX, Wang YY, Xiao GF, Shi ZL. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020; **579**: 270-273 [PMID: 32015507 DOI: 10.1038/s41586-020-2012-7]

17 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280. e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]

18 **Bosch BJ**, van der Zee R, de Haan CA, Rottier PJ. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J Virol* 2003; **77**: 8801-8811 [PMID: 12885899 DOI: 10.1128/JVI.77.16.8801-8811.2003]

19 **Letko M**, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol* 2020; **5**: 562-569 [PMID: 32094589 DOI: 10.1038/s41564-020-0688-y]

20 **Zang R**, Gomez Castro MF, McCune BT, Zeng Q, Rothlauf PW, Sonnek NM, Liu Z, Brulois KF, Wang X, Greenberg HB, Diamond MS, Ciorba MA, Whelan SPJ, Ding S. TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. *Sci Immunol* 2020; **5** [PMID: 32404436 DOI: 10.1126/sciimmunol.abc3582]

21 **Wruck W**, Adjaye J. SARS-CoV-2 receptor ACE2 is co-expressed with genes related to transmembrane serine proteases, viral entry, immunity and cellular stress. *Sci Rep* 2020; **10**: 21415 [PMID: 33293627 DOI: 10.1038/s41598-020-78402-2]

22 **Hussain M**, Jabeen N, Amanullah A, Baig AA, Aziz B, Shabbir S, Raza F, Uddin N. Molecular docking between human TMPRSS2 and SARS-CoV-2 spike protein: conformation and intermolecular interactions. *AIMS Microbiol* 2020; **6**: 350-360 [PMID: 33029570 DOI: 10.3934/microbiol.2020021]

23 **Walls AC**, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell* 2020; **181**: 281-292. e6 [PMID: 32155444 DOI: 10.1016/j.cell.2020.02.058]

24 **Belouzard S**, Madu I, Whittaker GR. Elastase-mediated activation of the severe acute respiratory syndrome coronavirus spike protein at discrete sites within the S2 domain. *J Biol Chem* 2010; **285**: 22758-22763 [PMID: 20507992 DOI: 10.1074/jbc.M110.103275]

25 **Ou X**, Liu Y, Lei X, Li P, Mi D, Ren L, Guo L, Guo R, Chen T, Hu J, Xiang Z, Mu Z, Chen X, Chen J, Hu K, Jin Q, Wang J, Qian Z. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun* 2020; **11**: 1620 [PMID: 32221306 DOI: 10.1038/s41467-020-15562-9]

26 **Du L**, Kao RY, Zhou Y, He Y, Zhao G, Wong C, Jiang S, Yuen KY, Jin DY, Zheng BJ. Cleavage of spike protein of SARS coronavirus by protease factor Xa is associated with viral infectivity. *Biochem Biophys Res Commun* 2007; **359**: 174-179 [PMID: 17533109 DOI: 10.1016/j.bbrc.2007.05.092]

27 **Ji HL**, Zhao R, Matalon S, Matthay MA. Elevated Plasmin(ogen) as a Common Risk Factor for COVID-19 Susceptibility. *Physiol Rev* 2020; **100**: 1065-1075 [PMID: 32216698 DOI: 10.1152/physrev.00013.2020]

28 **Gao Y**, Yan L, Huang Y, Liu F, Zhao Y, Cao L, Wang T, Sun Q, Ming Z, Zhang L, Ge J, Zheng L, Zhang Y, Wang H, Zhu Y, Zhu C, Hu T, Hua T, Zhang B, Yang X, Li J, Yang H, Liu Z, Xu W, Guddat LW, Wang Q, Lou Z, Rao Z. Structure of the RNA-dependent RNA polymerase from COVID-19 virus. *Science* 2020; **368**: 779-782 [PMID: 32277040 DOI: 10.1126/science.abb7498]

29 **Wang Q**, Wu J, Wang H, Gao Y, Liu Q, Mu A, Ji W, Yan L, Zhu Y, Zhu C, Fang X, Yang X, Huang Y, Gao H, Liu F, Ge J, Sun Q, Xu W, Liu Z, Yang H, Lou Z, Jiang B, Guddat LW, Gong P, Rao Z. Structural Basis for RNA Replication by the SARS-CoV-2 Polymerase. *Cell* 2020; **182**: 417-428. e13 [PMID: 32526208 DOI: 10.1016/j.cell.2020.05.034]

30 **Mendonça L**, Howe A, Gilchrist JB, Sun D, Knight ML, Zanetti-Domingues LC, Bateman B, Krebs AS, Chen L, Radecke J, Sheng Y, Li VD, Ni T, Kounatidis I, Koronfel MA, Szynkiewicz M, Harkiolaki M, Martin-Fernandez ML, James W, Zhang P. SARS-CoV-2 Assembly and Egress Pathway Revealed by Correlative Multi-modal Multi-scale Cryo-imaging. *bioRxiv* 2020 [PMID: 33173874 DOI: 10.1101/2020.11.05.370239]

31 **Klein S**, Cortese M, Winter SL, Wachsmuth-Melm M, Neufeldt CJ, Cerikan B, Stanifer ML, Boulant S, Bartenschlager R, Chlanda P. SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. *Nat Commun* 2020; **11**: 5885 [PMID: 33208793 DOI: 10.1038/s41467-020-19619-7]

32 **Bouhaddou M**, Memon D, Meyer B, White KM, Rezelj VV, Correa Marrero M, Polacco BJ, Melnyk JE, Ulferts S, Kaake RM, Batra J, Richards AL, Stevenson E, Gordon DE, Rojc A, Obernier K, Fabius JM, Soucheray M, Miorin L, Moreno E, Koh C, Tran QD, Hardy A, Robinot R, Vallet T, Nilsson-Payant BE, Hernandez-Armenta C, Dunham A, Weigang S, Knerr J, Modak M, Quintero D, Zhou Y, Dugourd A, Valdeolivas A, Patil T, Li Q, Hüttenhain R, Cakir M, Muralidharan M, Kim M, Jang G, Tutuncuoglu B, Hiatt J, Guo JZ, Xu J, Bouhaddou S, Mathy CJP, Gaulton A, Manners EJ, Félix E, Shi Y, Goff M, Lim JK, McBride T, O'Neal MC, Cai Y, Chang JCJ, Broadhurst DJ, Klippsten S, De Wit E, Leach AR, Kortemme T, Shoichet B, Ott M, Saez-Rodriguez J, tenOever

BR, Mullins RD, Fischer ER, Kochs G, Grosse R, García-Sastre A, Vignuzzi M, Johnson JR, Shokat KM, Swaney DL, Beltrao P, Krogan NJ. The Global Phosphorylation Landscape of SARS-CoV-2 Infection. *Cell* 2020; **182**: 685-712. e19 [PMID: 32645325 DOI: 10.1016/j.cell.2020.06.034]

33 **Cavalli E**, Petralia MC, Basile MS, Bramanti A, Bramanti P, Nicoletti F, Spandidos DA, Shoenfeld Y, Fagone P. Transcriptomic analysis of COVID19 Lungs and bronchoalveolar lavage fluid samples reveals predominant B cell activation responses to infection. *Int J Mol Med* 2020; **46**: 1266-1273 [PMID: 32945352 DOI: 10.3892/ijmm.2020.4702]

34 **Ehrlich A**, Uhl S, Ioannidis K, Hofree M. The SARS-CoV-2 transcriptional metabolic signature in lung epithelium. *SSRN* 2020 [DOI: 10.2139/ssrn.3650499]

35 **Fagone P**, Ciurleo R, Lombardo SD, Iacobello C, Palermo CI, Shoenfeld Y, Bendtzen K, Bramanti P, Nicoletti F. Transcriptional landscape of SARS-CoV-2 infection dismantles pathogenic pathways activated by the virus, proposes unique sex-specific differences and predicts tailored therapeutic strategies. *Autoimmun Rev* 2020; **19**: 102571 [PMID: 32376402 DOI: 10.1016/j.autrev.2020.102571]

36 **Gordon DE**, Jang GM, Bouhaddou M, Xu J, Obernier K, White KM, O'Meara MJ, Rezelj VV, Guo JZ, Swaney DL, Tummino TA, Hüttenhain R, Kaake RM, Richards AL, Tutuncuoglu B, Foussard H, Batra J, Haas K, Modak M, Kim M, Haas P, Polacco BJ, Braberg H, Fabius JM, Eckhardt M, Soucheray M, Bennett MJ, Cakir M, McGregor MJ, Li Q, Meyer B, Roesch F, Vallet T, Mac Kain A, Miorin L, Moreno E, Naing ZZC, Zhou Y, Peng S, Shi Y, Zhang Z, Shen W, Kirby IT, Melnyk JE, Chorba JS, Lou K, Dai SA, Barrio-Hernandez I, Memon D, Hernandez-Armenta C, Lyu J, Mathy CJP, Perica T, Pilla KB, Ganesan SJ, Saltzberg DJ, Rakesh R, Liu X, Rosenthal SB, Calviello L, Venkataramanan S, Liboy-Lugo J, Lin Y, Huang XP, Liu Y, Wankowicz SA, Bohn M, Safari M, Ugur FS, Koh C, Savar NS, Tran QD, Shengjuler D, Fletcher SJ, O'Neal MC, Cai Y, Chang JCJ, Broadhurst DJ, Klippsten S, Sharp PP, Wenzell NA, Kuzuoglu-Ozturk D, Wang HY, Trenker R, Young JM, Cavero DA, Hiatt J, Roth TL, Rathore U, Subramanian A, Noack J, Hubert M, Stroud RM, Frankel AD, Rosenberg OS, Verba KA, Agard DA, Ott M, Emerman M, Jura N, von Zastrow M, Verdin E, Ashworth A, Schwartz O, d'Enfert C, Mukherjee S, Jacobson M, Malik HS, Fujimori DG, Ideker T, Craik CS, Floor SN, Fraser JS, Gross JD, Sali A, Roth BL, Ruggero D, Taunton J, Kortemme T, Beltrao P, Vignuzzi M, García-Sastre A, Shokat KM, Shoichet BK, Krogan NJ. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 2020; **583**: 459-468 [PMID: 32353859 DOI: 10.1038/s41586-020-2286-9]

37 **Hadjadj J**, Yatim N, Barnabei L, Corneau A, Boussier J, Smith N, Péré H, Charbit B, Bondet V, Chenevier-Gobeaux C, Breillat P, Carlier N, Gauzit R, Morbieu C, Pène F, Marin N, Roche N, Szwebel TA, Merkling SH, Treluyer JM, Veyer D, Mouthon L, Blanc C, Tharaux PL, Rozenberg F, Fischer A, Duffy D, Rieux-Laucat F, Kernéis S, Terrier B. Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. *Science* 2020; **369**: 718-724 [PMID: 32661059 DOI: 10.1126/science.abc6027]

38 **Wang J**, Jiang M, Chen X, Montaner LJ. Cytokine storm and leukocyte changes in mild *vs* severe SARS-CoV-2 infection: Review of 3939 COVID-19 patients in China and emerging pathogenesis and therapy concepts. *J Leukoc Biol* 2020; **108**: 17-41 [PMID: 32534467 DOI: 10.1002/JLB.3COVR0520-272R]

39 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

40 **Chen G**, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang M, Wu S, Song J, Chen T, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest* 2020; **130**: 2620-2629 [PMID: 32217835 DOI: 10.1172/JCI137244]

41 **Xiong Y**, Liu Y, Cao L, Wang D, Guo M, Jiang A, Guo D, Hu W, Yang J, Tang Z, Wu H, Lin Y, Zhang M, Zhang Q, Shi M, Zhou Y, Lan K, Chen Y. Transcriptomic characteristics of bronchoalveolar lavage fluid and peripheral blood mononuclear cells in COVID-19 patients. *Emerg Microbes Infect* 2020; **9**: 761-770 [PMID: 32228226 DOI: 10.1080/22221751.2020.1747363]

42 **Blanco-Melo D**, Nilsson-Payant BE, Liu WC, Uhl S, Hoagland D, Møller R, Jordan TX, Oishi K, Panis M, Sachs D, Wang TT, Schwartz RE, Lim JK, Albrecht RA, tenOever BR. Imbalanced Host Response to SARS-CoV-2 Drives Development of COVID-19. *Cell* 2020; **181**: 1036-1045. e9 [PMID: 32416070 DOI: 10.1016/j.cell.2020.04.026]

43 **Galani IE**, Rovina N, Lampropoulou V, Triantafyllia V, Manioudaki M, Pavlos E, Koukaki E, Fragkou PC, Panou V, Rapti V, Koltsida O, Mentis A, Koulouris N, Tsiodras S, Koutsoukou A, Andreakos E. Untuned antiviral immunity in COVID-19 revealed by temporal type I/III interferon patterns and flu comparison. *Nat Immunol* 2021; **22**: 32-40 [PMID: 33277638 DOI: 10.1038/s41590-020-00840-x]

44 **Theobald SJ**, Simonis A, Kreer C, Zehner M, Fischer J, Albert M-C, Malin JJ, Gräb J, Winter S, Silva US de, Böll B, Köhler P, Gruell H, Suàrez I, Hallek M, Fätkenheuer G, Jung N, Cornely O, Lehmann C, Kashkar H, Klein F, Rybniker J. The SARS-CoV-2 spike protein primes inflammasome-mediated interleukin-1-beta secretion in COVID-19 patient-derived macrophages. *Res Sq* 2020 [DOI: 10.21203/rs.3.rs-30407/v1]

45 **Zhu N**, Wang W, Liu Z, Liang C, Ye F, Huang B, Zhao L, Wang H, Zhou W, Deng Y, Mao L, Su C, Qiang G, Jiang T, Zhao J, Wu G, Song J, Tan W. Morphogenesis and cytopathic effect of SARS-CoV-2 infection in human airway epithelial cells. *Nat Commun* 2020; **11**: 3910 [PMID: 32764693

DOI: 10.1038/s41467-020-17796-z]
46 **Xu H**, Chitre SA, Akinyemi IA, Loeb JC, Lednicky JA, McIntosh MT, Bhaduri-McIntosh S. SARS-CoV-2 viroporin triggers the NLRP3 inflammatory pathway. 2020 Preprint. Available from: bioRxiv:2020.10.27.357731 [DOI: 10.1101/2020.10.27.357731]
47 **Li S**, Zhang Y, Guan Z, Li H, Ye M, Chen X, Shen J, Zhou Y, Shi ZL, Zhou P, Peng K. SARS-CoV-2 triggers inflammatory responses and cell death through caspase-8 activation. *Signal Transduct Target Ther* 2020; **5**: 235 [PMID: 33037188 DOI: 10.1038/s41392-020-00334-0]
48 **Qu Y**, Wang X, Zhu Y, Wang Y, Yang X, Hu G, Liu C, Li J, Ren S, Xiao Z, Liu Z, Wang W, Li P, Zhang R, Liang Q. ORF3a mediated-incomplete autophagy facilitates SARS-CoV-2 replication. 2020 Preprint. Available from: bioRxiv:2020.11.12.380709 [DOI: 10.1101/2020.11.12.380709]
49 **Ramachandran P**, Onukogu I, Ghanta S, Gajendran M, Perisetti A, Goyal H, Aggarwal A. Gastrointestinal Symptoms and Outcomes in Hospitalized Coronavirus Disease 2019 Patients. *Dig Dis* 2020; **38**: 373-379 [PMID: 32599601 DOI: 10.1159/000509774]
50 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]
51 **Li LQ**, Huang T, Wang YQ, Wang ZP, Liang Y, Huang TB, Zhang HY, Sun W, Wang Y. COVID-19 patients' clinical characteristics, discharge rate, and fatality rate of meta-analysis. *J Med Virol* 2020; **92**: 577-583 [PMID: 32162702 DOI: 10.1002/jmv.25757]
52 **Jin X**, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, Hao SR, Jia HY, Cai H, Zhang XL, Yu GD, Xu KJ, Wang XY, Gu JQ, Zhang SY, Ye CY, Jin CL, Lu YF, Yu X, Yu XP, Huang JR, Xu KL, Ni Q, Yu CB, Zhu B, Li YT, Liu J, Zhao H, Zhang X, Yu L, Guo YZ, Su JW, Tao JJ, Lang GJ, Wu XX, Wu WR, Qv TT, Xiang DR, Yi P, Shi D, Chen Y, Ren Y, Qiu YQ, Li LJ, Sheng J, Yang Y. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. *Gut* 2020; **69**: 1002-1009 [PMID: 32213556 DOI: 10.1136/gutjnl-2020-320926]
53 **Fang D**, Ma J, Guan J, Wang M, Song Y, Tian D, Li P. Manifestations of Digestive system in hospitalized patients with novel coronavirus pneumonia in Wuhan, China: a single-center, descriptive study. *Zhonghua Xiaohua Zazhi* 2020; **40**: E005-E005 [DOI: 10.3760/cma.j.issn.0254-1432.2020.0005]
54 **Xiao F**, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for Gastrointestinal Infection of SARS-CoV-2. *Gastroenterology* 2020; **158**: 1831-1833. e3 [PMID: 32142773 DOI: 10.1053/j.gastro.2020.02.055]
55 **Parasa S**, Desai M, Thoguluva Chandrasekar V, Patel HK, Kennedy KF, Roesch T, Spadaccini M, Colombo M, Gabbiadini R, Artifon ELA, Repici A, Sharma P. Prevalence of Gastrointestinal Symptoms and Fecal Viral Shedding in Patients With Coronavirus Disease 2019: A Systematic Review and Meta-analysis. *JAMA Netw Open* 2020; **3**: e2011335 [PMID: 32525549 DOI: 10.1001/jamanetworkopen.2020.11335]
56 **Galanopoulos M**, Gkeros F, Doukatas A, Karianakis G, Pontas C, Tsoukalas N, Viazis N, Liatsos C, Mantzaris GJ. COVID-19 pandemic: Pathophysiology and manifestations from the gastrointestinal tract. *World J Gastroenterol* 2020; **26**: 4579-4588 [PMID: 32884218 DOI: 10.3748/wjg.v26.i31.4579]
57 **Wang D**, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 2020; **323**: 1061-1069 [PMID: 32031570 DOI: 10.1001/jama.2020.1585]
58 **Han C**, Duan C, Zhang S, Spiegel B, Shi H, Wang W, Zhang L, Lin R, Liu J, Ding Z, Hou X. Digestive Symptoms in COVID-19 Patients With Mild Disease Severity: Clinical Presentation, Stool Viral RNA Testing, and Outcomes. *Am J Gastroenterol* 2020; **115**: 916-923 [PMID: 32301761 DOI: 10.14309/ajg.0000000000000664]
59 **Pan L**, Mu M, Yang P, Sun Y, Wang R, Yan J, Li P, Hu B, Wang J, Hu C, Jin Y, Niu X, Ping R, Du Y, Li T, Xu G, Hu Q, Tu L. Clinical Characteristics of COVID-19 Patients With Digestive Symptoms in Hubei, China: A Descriptive, Cross-Sectional, Multicenter Study. *Am J Gastroenterol* 2020; **115**: 766-773 [PMID: 32287140 DOI: 10.14309/ajg.0000000000000620]
60 **Redd WD**, Zhou JC, Hathorn KE, McCarty TR, Bazarbashi AN, Thompson CC, Shen L, Chan WW. Prevalence and Characteristics of Gastrointestinal Symptoms in Patients With Severe Acute Respiratory Syndrome Coronavirus 2 Infection in the United States: A Multicenter Cohort Study. *Gastroenterology* 2020; **159**: 765-767. e2 [PMID: 32333911 DOI: 10.1053/j.gastro.2020.04.045]
61 **Chen N**, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020; **395**: 507-513 [PMID: 32007143 DOI: 10.1016/S0140-6736(20)30211-7]
62 **Mo P**, Xing Y, Xiao Y, Deng L, Zhao Q, Wang H, Xiong Y, Cheng Z, Gao S, Liang K, Luo M, Chen T, Song S, Ma Z, Chen X, Zheng R, Cao Q, Wang F, Zhang Y. Clinical characteristics of refractory COVID-19 pneumonia in Wuhan, China. *Clin Infect Dis* 2020 [PMID: 32173725 DOI: 10.1093/cid/ciaa270]

63 **Leung WK**, To KF, Chan PK, Chan HL, Wu AK, Lee N, Yuen KY, Sung JJ. Enteric involvement of severe acute respiratory syndrome-associated coronavirus infection. *Gastroenterology* 2003; **125**: 1011-1017 [PMID: 14517783 DOI: 10.1016/j.gastro.2003.08.001]

64 **Carvalho A**, Alqusairi R, Adams A, Paul M, Kothari N, Peters S, DeBenedet AT. SARS-CoV-2 Gastrointestinal Infection Causing Hemorrhagic Colitis: Implications for Detection and Transmission of COVID-19 Disease. *Am J Gastroenterol* 2020; **115**: 942-946 [PMID: 32496741 DOI: 10.14309/ajg.0000000000000667]

65 **Pan Y**, Zhang D, Yang P, Poon LLM, Wang Q. Viral load of SARS-CoV-2 in clinical samples. *Lancet Infect Dis* 2020; **20**: 411-412 [PMID: 32105638 DOI: 10.1016/S1473-3099(20)30113-4]

66 **Young BE**, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, Tan TY, Ng TY, Cui L, Said Z, Kurupatham L, Chen MI, Chan M, Vasoo S, Wang LF, Tan BH, Lin RTP, Lee VJM, Leo YS, Lye DC; Singapore 2019 Novel Coronavirus Outbreak Research Team. Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore. *JAMA* 2020; **323**: 1488-1494 [PMID: 32125362 DOI: 10.1001/jama.2020.3204]

67 **Peng L**, Liu J, Xu W, Luo Q, Chen D, Lei Z, Huang Z, Li X, Deng K, Lin B, Gao Z. SARS-CoV-2 can be detected in urine, blood, anal swabs, and oropharyngeal swabs specimens. *J Med Virol* 2020; **92**: 1676-1680 [PMID: 32330305 DOI: 10.1002/jmv.25936]

68 **Wu Y**, Guo C, Tang L, Hong Z, Zhou J, Dong X, Yin H, Xiao Q, Tang Y, Qu X, Kuang L, Fang X, Mishra N, Lu J, Shan H, Jiang G, Huang X. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *Lancet Gastroenterol Hepatol* 2020; **5**: 434-435 [PMID: 32199469 DOI: 10.1016/S2468-1253(20)30083-2]

69 **Chen Y**, Chen L, Deng Q, Zhang G, Wu K, Ni L, Yang Y, Liu B, Wang W, Wei C, Yang J, Ye G, Cheng Z. The presence of SARS-CoV-2 RNA in the feces of COVID-19 patients. *J Med Virol* 2020; **92**: 833-840 [PMID: 32243607 DOI: 10.1002/jmv.25825]

70 **Ling Y**, Xu SB, Lin YX, Tian D, Zhu ZQ, Dai FH, Wu F, Song ZG, Huang W, Chen J, Hu BJ, Wang S, Mao EQ, Zhu L, Zhang WH, Lu HZ. Persistence and clearance of viral RNA in 2019 novel coronavirus disease rehabilitation patients. *Chin Med J (Engl)* 2020; **133**: 1039-1043 [PMID: 32118639 DOI: 10.1097/CM9.0000000000000774]

71 **Hikmet F**, Méar L, Edvinsson Å, Micke P, Uhlén M, Lindskog C. The protein expression profile of ACE2 in human tissues. *Mol Syst Biol* 2020; **16**: e9610 [PMID: 32715618 DOI: 10.15252/msb.20209610]

72 **Zou X**, Chen K, Zou J, Han P, Hao J, Han Z. Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Front Med* 2020; **14**: 185-192 [PMID: 32170560 DOI: 10.1007/s11684-020-0754-0]

73 **Zhang H**, Kang Z, Gong H, Xu D, Wang J, Li Z, Cui X, Xiao J, Zhan J, Meng T, Zhou W, Liu J, Xu H. Digestive system is a potential route of COVID-19: an analysis of single-cell coexpression pattern of key proteins in viral entry process. *Gut* 2020; **69**: 1010-1018 [DOI: 10.1136/gutjnl-2020-320953]

74 **Bar-On YM**, Flamholz A, Phillips R, Milo R. SARS-CoV-2 (COVID-19) by the numbers. *Elife* 2020; **9** [PMID: 32228860 DOI: 10.7554/eLife.57309]

75 **Sender R**, Bar-On YM, Flamholz A, Gleizer S, Bernsthein B, Phillips R, Milo R. The total number and mass of SARS-CoV-2 virions in an infected person. *medRxiv* 2020 [PMID: 33236021 DOI: 10.1101/2020.11.16.20232009]

76 **Lamers MM**, Beumer J, van der Vaart J, Knoops K, Puschhof J, Breugem TI, Ravelli RBG, Paul van Schayck J, Mykytyn AZ, Duimel HQ, van Donselaar E, Riesebosch S, Kuijpers HJH, Schipper D, van de Wetering WJ, de Graaf M, Koopmans M, Cuppen E, Peters PJ, Haagmans BL, Clevers H. SARS-CoV-2 productively infects human gut enterocytes. *Science* 2020; **369**: 50-54 [PMID: 32358202 DOI: 10.1126/science.abc1669]

77 **Bradley BT**, Maioli H, Johnston R, Chaudhry I, Fink SL, Xu H, Najafian B, Deutsch G, Lacy JM, Williams T, Yarid N, Marshall DA. Histopathology and ultrastructural findings of fatal COVID-19 infections in Washington State: a case series. *Lancet* 2020; **396**: 320-332 [PMID: 32682491 DOI: 10.1016/S0140-6736(20)31305-2]

78 **Xiao F**, Sun J, Xu Y, Li F, Huang X, Li H, Zhao J, Huang J. Infectious SARS-CoV-2 in Feces of Patient with Severe COVID-19. *Emerg Infect Dis* 2020; **26**: 1920-1922 [PMID: 32421494 DOI: 10.3201/eid2608.200681]

79 **Wurtz N**, Penant G, Jardot P, Duclos N, La Scola B. Culture of SARS-CoV-2 in a panel of laboratory cell lines, permissivity, and differences in growth profile. *Eur J Clin Microbiol Infect Dis* 2021; **40**: 477-484 [PMID: 33389257 DOI: 10.1007/s10096-020-04106-0]

80 **Chu H**, Chan JF, Wang Y, Yuen TT, Chai Y, Shuai H, Yang D, Hu B, Huang X, Zhang X, Hou Y, Cai JP, Zhang AJ, Zhou J, Yuan S, To KK, Hung IF, Cheung TT, Ng AT, Hau-Yee Chan I, Wong IY, Law SY, Foo DC, Leung WK, Yuen KY. SARS-CoV-2 Induces a More Robust Innate Immune Response and Replicates Less Efficiently Than SARS-CoV in the Human Intestines: An Ex Vivo Study With Implications on Pathogenesis of COVID-19. *Cell Mol Gastroenterol Hepatol* 2021; **11**: 771-781 [PMID: 33010495 DOI: 10.1016/j.jcmgh.2020.09.017]

81 **Lee S**, Yoon GY, Myoung J, Kim SJ, Ahn DG. Robust and persistent SARS-CoV-2 infection in the human intestinal brush border expressing cells. *Emerg Microbes Infect* 2020; **9**: 2169-2179 [PMID: 32969768 DOI: 10.1080/22221751.2020.1827985]

82 **De Maio F**, Lo Cascio E, Babini G, Sali M, Della Longa S, Tilocca B, Roncada P, Arcovito A, Sanguinetti M, Scambia G, Urbani A. Improved binding of SARS-CoV-2 Envelope protein to tight

junction-associated PALS1 could play a key role in COVID-19 pathogenesis. *Microbes Infect* 2020; **22**: 592-597 [PMID: 32891874 DOI: 10.1016/j.micinf.2020.08.006]

83 **Straight SW**, Shin K, Fogg VC, Fan S, Liu CJ, Roh M, Margolis B. Loss of PALS1 expression leads to tight junction and polarity defects. *Mol Biol Cell* 2004; **15**: 1981-1990 [PMID: 14718565 DOI: 10.1091/mbc.E03-08-0620]

84 **Guo Y**, Luo R, Wang Y, Deng P, Song T, Zhang M, Wang P, Zhang X, Cui K, Tao T, Li Z, Chen W, Zheng Y, Qin J. SARS-CoV-2 induced intestinal responses with a biomimetic human gut-on-chip. *Sci Bull (Beijing)* 2021; **66**: 783-793 [PMID: 33282445 DOI: 10.1016/j.scib.2020.11.015]

85 **Cao Y**, Yang R, Wang W, Lee I, Zhang R, Zhang W, Sun J, Xu B, Meng X. Computational Study of the Ion and Water Permeation and Transport Mechanisms of the SARS-CoV-2 Pentameric E Protein Channel. *Front Mol Biosci* 2020; **7**: 565797 [PMID: 33173781 DOI: 10.3389/fmolb.2020.565797]

86 **Barrett KE**, Keely SJ. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. *Annu Rev Physiol* 2000; **62**: 535-572 [PMID: 10845102 DOI: 10.1146/annurev.physiol.62.1.535]

87 **Ren Y**, Shu T, Wu D, Mu J, Wang C, Huang M, Han Y, Zhang XY, Zhou W, Qiu Y, Zhou X. The ORF3a protein of SARS-CoV-2 induces apoptosis in cells. *Cell Mol Immunol* 2020; **17**: 881-883 [PMID: 32555321 DOI: 10.1038/s41423-020-0485-9]

88 **Obukhov AG**, Stevens BR, Prasad R, Li Calzi S, Boulton ME, Raizada MK, Oudit GY, Grant MB. SARS-CoV-2 Infections and ACE2: Clinical Outcomes Linked With Increased Morbidity and Mortality in Individuals With Diabetes. *Diabetes* 2020; **69**: 1875-1886 [PMID: 32669391 DOI: 10.2337/dbi20-0019]

89 **Xu J**, Xu X, Jiang L, Dua K, Hansbro PM, Liu G. SARS-CoV-2 induces transcriptional signatures in human lung epithelial cells that promote lung fibrosis. *Respir Res* 2020; **21**: 182 [PMID: 32664949 DOI: 10.1186/s12931-020-01445-6]

90 **Jia HP**, Look DC, Tan P, Shi L, Hickey M, Gakhar L, Chappell MC, Wohlford-Lenane C, McCray PB Jr. Ectodomain shedding of angiotensin converting enzyme 2 in human airway epithelia. *Am J Physiol Lung Cell Mol Physiol* 2009; **297**: L84-L96 [PMID: 19411314 DOI: 10.1152/ajplung.00071.2009]

91 **Andring JT**, McKenna R, Stevens BR. Amino acid transporter B0AT1 influence on ADAM17 interactions with SARS-CoV-2 receptor ACE2 putatively expressed in intestine, kidney, and cardiomyocytes. 2020 Preprint. Available from: bioRxiv: 2020.10.30.361873 [DOI: 10.1101/2020.10.30.361873]

92 **Deretic V**, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. *Nat Rev Immunol* 2013; **13**: 722-737 [PMID: 24064518 DOI: 10.1038/nri3532]

93 **Sarkar S**. Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. *Biochem Soc Trans* 2013; **41**: 1103-1130 [PMID: 24059496 DOI: 10.1042/BST20130134]

94 **Delorme-Axford E**, Klionsky DJ. Highlights in the fight against COVID-19: does autophagy play a role in SARS-CoV-2 infection? *Autophagy* 2020; **16**: 2123-2127 [PMID: 33153403 DOI: 10.1080/15548627.2020.1844940]

95 **Shojaei S**, Suresh M, Klionsky DJ, Labouta HI, Ghavami S. Autophagy and SARS-CoV-2 infection: Apossible smart targeting of the autophagy pathway. *Virulence* 2020; **11**: 805-810 [PMID: 32567972 DOI: 10.1080/21505594.2020.1780088]

96 **Siddiqi HK**, Mehra MR. COVID-19 illness in native and immunosuppressed states: A clinical-therapeutic staging proposal. *J Heart Lung Transplant* 2020; **39**: 405-407 [PMID: 32362390 DOI: 10.1016/j.healun.2020.03.012]

97 **Zhang L**, Han C, Zhang S, Duan C, Shang H, Bai T, Hou X. Diarrhea and altered inflammatory cytokine pattern in severe coronavirus disease 2019: Impact on disease course and in-hospital mortality. *J Gastroenterol Hepatol* 2021; **36**: 421-429 [PMID: 32602128 DOI: 10.1111/jgh.15166]

98 **Ma TY**, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, Said HM. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G367-G376 [PMID: 14766535 DOI: 10.1152/ajpgi.00173.2003]

99 **Al-Sadi R**, Ye D, Boivin M, Guo S, Hashimi M, Ereifej L, Ma TY. Interleukin-6 modulation of intestinal epithelial tight junction permeability is mediated by JNK pathway activation of claudin-2 gene. *PLoS One* 2014; **9**: e85345 [PMID: 24662742 DOI: 10.1371/journal.pone.0085345]

100 **Kuhn KA**, Manieri NA, Liu TC, Stappenbeck TS. IL-6 stimulates intestinal epithelial proliferation and repair after injury. *PLoS One* 2014; **9**: e114195 [PMID: 25478789 DOI: 10.1371/journal.pone.0114195]

101 **Kühn R**, Löhler J, Rennick D, Rajewsky K, Müller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993; **75**: 263-274 [PMID: 8402911 DOI: 10.1016/0092-8674(93)80068-p]

102 **Lorén V**, Cabré E, Ojanguren I, Domènech E, Pedrosa E, García-Jaraquemada A, Mañosa M, Manyé J. Interleukin-10 Enhances the Intestinal Epithelial Barrier in the Presence of Corticosteroids through p38 MAPK Activity in Caco-2 Monolayers: A Possible Mechanism for Steroid Responsiveness in Ulcerative Colitis. *PLoS One* 2015; **10**: e0130921 [PMID: 26090671 DOI: 10.1371/journal.pone.0130921]

103 **Gu S**, Chen Y, Wu Z, Gao H, Lv L, Guo F, Zhang X, Luo R, Huang C, Lu H, Zheng B, Zhang J,

Yan R, Zhang H, Jiang H, Xu Q, Guo J, Gong Y, Tang L, Li L. Alterations of the Gut Microbiota in Patients With Coronavirus Disease 2019 or H1N1 Influenza. *Clin Infect Dis* 2020; **71**: 2669-2678 [PMID: 32497191 DOI: 10.1093/cid/ciaa709]

104 **Zuo T**, Zhang F, Lui GCY, Yeoh YK, Li AYL, Zhan H, Wan Y, Chung ACK, Cheung CP, Chen N, Lai CKC, Chen Z, Tso EYK, Fung KSC, Chan V, Ling L, Joynt G, Hui DSC, Chan FKL, Chan PKS, Ng SC. Alterations in Gut Microbiota of Patients With COVID-19 During Time of Hospitalization. *Gastroenterology* 2020; **159**: 944-955. e8 [PMID: 32442562 DOI: 10.1053/j.gastro.2020.05.048]

105 **Mifsud EJ**, Hayden FG, Hurt AC. Antivirals targeting the polymerase complex of influenza viruses. *Antiviral Res* 2019; **169**: 104545 [PMID: 31247246 DOI: 10.1016/j.antiviral.2019.104545]

106 **Granata G**, Bartoloni A, Codeluppi M, Contadini I, Cristini F, Fantoni M, Ferraresi A, Fornabaio C, Grasselli S, Lagi F, Masucci L, Puoti M, Raimondi A, Taddei E, Trapani FF, Viale P, Johnson S, Petrosillo N; On Behalf Of The CloVid Study Group. The Burden of Clostridioides Difficile Infection during the COVID-19 Pandemic: A Retrospective Case-Control Study in Italian Hospitals (CloVid). *J Clin Med* 2020; **9** [PMID: 33260943 DOI: 10.3390/jcm9123855]

107 **Hanna R**, Dalvi S, Sălăgean T, Pop ID, Bordea IR, Benedicenti S. Understanding COVID-19 Pandemic: Molecular Mechanisms and Potential Therapeutic Strategies. An Evidence-Based Review. *J Inflamm Res* 2021; **14**: 13-56 [PMID: 33447071 DOI: 10.2147/JIR.S282213]

108 **Gramlich L**, Kichian K, Pinilla J, Rodych NJ, Dhaliwal R, Heyland DK. Does enteral nutrition compared to parenteral nutrition result in better outcomes in critically ill adult patients? *Nutrition* 2004; **20**: 843-848 [PMID: 15474870 DOI: 10.1016/j.nut.2004.06.003]

109 **Blumenstein I**, Shastri YM, Stein J. Gastroenteric tube feeding: techniques, problems and solutions. *World J Gastroenterol* 2014; **20**: 8505-8524 [PMID: 25024606 DOI: 10.3748/wjg.v20.i26.8505]

110 **Li GF**, An XX, Yu Y, Jiao LR, Canarutto D, Yu G, Wang G, Wu DN, Xiao Y. Do proton pump inhibitors influence SARS-CoV-2 related outcomes? *Gut* 2020 [PMID: 33172925 DOI: 10.1136/gutjnl-2020-323366]

111 **Leonard J**, Marshall JK, Moayyedi P. Systematic review of the risk of enteric infection in patients taking acid suppression. *Am J Gastroenterol* 2007; **102**: 2047-56; quiz 2057 [PMID: 17509031 DOI: 10.1111/j.1572-0241.2007.01275.x]

112 **Tariq R**, Singh S, Gupta A, Pardi DS, Khanna S. Association of Gastric Acid Suppression With Recurrent Clostridium difficile Infection: A Systematic Review and Meta-analysis. *JAMA Intern Med* 2017; **177**: 784-791 [PMID: 28346595 DOI: 10.1001/jamainternmed.2017.0212]

113 **Hafiz RA**, Wong C, Paynter S, David M, Peeters G. The Risk of Community-Acquired Enteric Infection in Proton Pump Inhibitor Therapy: Systematic Review and Meta-analysis. *Ann Pharmacother* 2018; **52**: 613-622 [PMID: 29457492 DOI: 10.1177/1060028018760569]

114 **Moayyedi P**, Eikelboom JW, Bosch J, Connolly SJ, Dyal L, Shestakovska O, Leong D, Anand SS, Störk S, Branch KRH, Bhatt DL, Verhamme PB, O'Donnell M, Maggioni AP, Lonn EM, Piegas LS, Ertl G, Keltai M, Bruns NC, Muehlhofer E, Dagenais GR, Kim JH, Hori M, Steg PG, Hart RG, Diaz R, Alings M, Widimsky P, Avezum A, Probstfield J, Zhu J, Liang Y, Lopez-Jaramillo P, Kakkar AK, Parkhomenko AN, Ryden L, Pogosova N, Dans AL, Lanas F, Commerford PJ, Torp-Pedersen C, Guzik TJ, Vinereanu D, Tonkin AM, Lewis BS, Felix C, Yusoff K, Metsarinne KP, Fox KAA, Yusuf S; COMPASS Investigators. Safety of Proton Pump Inhibitors Based on a Large, Multi-Year, Randomized Trial of Patients Receiving Rivaroxaban or Aspirin. *Gastroenterology* 2019; **157**: 682-691. e2 [PMID: 31152740 DOI: 10.1053/j.gastro.2019.05.056]

115 **Amirian ES**. Potential fecal transmission of SARS-CoV-2: Current evidence and implications for public health. *Int J Infect Dis* 2020; **95**: 363-370 [PMID: 32335340 DOI: 10.1016/j.ijid.2020.04.057]

116 **Arslan M**, Xu B, Gamal El-Din M. Transmission of SARS-CoV-2 *via* fecal-oral and aerosols-borne routes: Environmental dynamics and implications for wastewater management in underprivileged societies. *Sci Total Environ* 2020; **743**: 140709 [PMID: 32652357 DOI: 10.1016/j.scitotenv.2020.140709]

117 **Hindson J**. COVID-19: faecal-oral transmission? *Nat Rev Gastroenterol Hepatol* 2020; **17**: 259 [PMID: 32214231 DOI: 10.1038/s41575-020-0295-7]

118 **Ahmed W**, Angel N, Edson J, Bibby K, Bivins A, O'Brien JW, Choi PM, Kitajima M, Simpson SL, Li J, Tscharke B, Verhagen R, Smith WJM, Zaugg J, Dierens L, Hugenholtz P, Thomas KV, Mueller JF. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community. *Sci Total Environ* 2020; **728**: 138764 [PMID: 32387778 DOI: 10.1016/j.scitotenv.2020.138764]

119 **Medema G**, Heijnen L, Elsinga G, Italiaander R, Brouwer A. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. *Environ Sci Technol Lett* 2020; **7**: 511-516 [DOI: 10.1021/acs.estlett.0c00357]

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REVIEW

Assessment of liver disease in patients with chronic hepatitis C and unhealthy alcohol use

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Abstract

Hepatitis C virus (HCV) infection and unhealthy alcohol use are major drivers of the burden of liver disease worldwide and commonly co-occur. Assessment of underlying liver damage is a cornerstone of the clinical care of patients with chronic HCV infection and/or unhealthy alcohol use because many of them are diagnosed at advanced stages of disease. Early diagnosis of liver disease before decompensated liver cirrhosis becomes established is essential for treatment with direct acting antivirals and/or abstinence from alcohol consumption, which are the main therapeutic approaches for clinical management. In this review, we discuss current knowledge around the use of non-invasive methods to assess liver disease, such as abdominal ultrasound, controlled attenuation parameter, transient elastography, magnetic resonance imaging, and indices based on serum markers of liver injury.

Key Words: Hepatitis C virus; Alcohol; Liver fibrosis; Non-invasive methods; Ultrasound; Transient elastography

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Core Tip: In this review, we discuss current knowledge around the use of non-invasive methods to assess underlying liver disease in patients with hepatitis C virus infection and/or unhealthy alcohol use. A timely diagnosis of liver disease is of the outmost

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importance to avoid progression to decompensated liver disease and liver cancer. Antiviral treatment and abstinence from alcohol use are cornerstones of clinical care for these patients.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection and unhealthy alcohol intake are common co-occurring conditions, in part because people with HCV often consume more alcohol than the general population[1]. Conversely, alcohol use is associated with a higher prevalence of HCV infection exposure and greater HCV infection persistence; patients with alcohol use have a lower likelihood of spontaneously clearing acute HCV infection[2]. For these reasons, the prevalence of HCV infection among patients with alcohol use is higher than in the general population[3], which is usually around 1% in Western countries[4]. We have found a prevalence > 20% in our cohort of patients with alcohol use disorder (AUD) and admitted for hospital detoxification[5], which is much higher than many values reported in a meta-analysis published in 2013[3]. This contrast underscores the relevance of a history of prior injection drug use and other risk factors for the acquisition of HCV in an urban population of patients with AUD[6].

In addition to higher infection exposure and persistence, alcohol use may affect HCV viral replication in certain subsets of patients[7] and is associated with progressive liver fibrosis and more extensive liver injury than in patients without alcohol use [8]. HCV is also associated with increased mortality and other poor health outcomes in different subsets of patients with alcohol-use problems[9,10].

Currently, alcohol use is a major contributor to decompensated cirrhosis in patients with HCV infection[11]. Moreover, with the current availability of efficacious treatments for viral hepatitis, alcohol-related liver disease is becoming a major driver of liver disease burden worldwide[12].

HCV infection needs to be detected as early as possible among patients with unhealthy alcohol intake, and accurate assessment of alcohol intake is necessary in patients with HCV infection[12]. Assessment of underlying liver disease is crucial, given that many patients are diagnosed only after their liver disease has reached an advanced stage[13].

ASSESSMENT OF ALCOHOL USE IN PATIENTS WITH LIVER DISEASE

The American Association for the Study of Liver Diseases and European Association for the Study of the Liver guidelines for the treatment of alcohol-related liver disease recommend using the alcohol use disorders identification test (AUDIT) to identify patients with AUD[14,15]. AUDIT includes 10 questions and explores consumption, dependence and alcohol-related problems according to the number of positive answers[16]. For patients with an AUDIT > 7, Diagnostic and Statistical Manual of Mental Disorders 5th edition is an appropriate tool used for the assessment of clinical, personal and social alcohol-related problems[17]. The severity of AUD, that encompasses both alcohol dependence and alcohol abuse, is based on the number of criteria that the patient meets (mild AUD: 2-3 criteria, moderate AUD: 4-5 criteria and severe AUD > 6). AUD represents the more extreme form of unhealthy alcohol use, a term that will be used throughout the manuscript[17].

In terms of amount, unhealthy alcohol use means drinking more than the recommended amount of alcohol by the National Institute of Alcohol abuse and Alcoholism [2 United States standard drinks (12oz of beer or 5oz of wine) *per* day or 14 drinks *per* week for men and 1 drink *per* day or 7 United States standard drinks *per* week for women or men aged > 65], what includes the spectrum from risky alcohol use to AUD[18].

It is important to note that for those who already have HCV infection there is no safe threshold of alcohol consumption and abstinence from alcohol should be the main treatment goal[12].

PATHOLOGIC FEATURES OF HCV-RELATED LIVER DISEASE AND ALCOHOL-RELATED LIVER DISEASE

The natural history of HCV-related liver disease is characterized by progressive liver fibrosis that eventually leads to liver cirrhosis and hepatocellular carcinoma[19]. Many cofactors are associated with a faster progression of liver fibrosis in patients with HCV infection, including age, duration of HCV infection, male sex, obesity, the presence of hepatitis B virus (HBV) and/or human immunodeficiency virus (HIV) co-infection, alcohol use, and immune suppression[20].

Alcohol-related liver disease includes several histological abnormalities ranging from simple steatosis to cirrhosis and liver cancer[21,22]. These various histological abnormalities can be present at the same time in a liver biopsy[21,22]. Alcoholic hepatitis is a complication associated with acute liver failure, an increased risk of infection, and high mortality that can be seen at any point in the natural history of alcohol-related liver disease[12].

Liver fibrosis is the best predictor of progression to decompensated cirrhosis in both chronic HCV infection and alcohol-related liver damage[23]. Liver biopsy is the preferred method to evaluate liver fibrosis, but it is costly, invasive[24] and rarely performed in patients with current alcohol or other substance use disorders[25]. In the present review, we will not discuss the particularities of liver biopsy[26] and focus instead on currently available non-invasive methods to assess the extent of liver damage in patients with HCV and/or unhealthy alcohol use.

THE NEED FOR ASSESSMENT OF LIVER DAMAGE

A timely diagnosis of liver disease in asymptomatic patients with HCV infection and/or AUD contributes to a better prognosis and facilitates treatment[27,28]. As noted, in patients with AUD, liver involvement is the main culprit of alcohol-related disease burden, and liver disease is usually detected in the advanced stages[28]. Almost 75% of patients with alcohol-related liver disease present with a non-elective hospital admission because of decompensated cirrhosis[29], so efforts should prioritize early identification of patients at risk for end-stage liver disease.

Despite the availability of efficacious treatments for viral hepatitis, the liver disease burden is expected to increase because of the growing prevalence of overweight and obesity and other unhealthy lifestyles in developed countries, which is predicted to drive an increase in the prevalence of non-alcoholic liver disease. In addition, many patients with metabolic syndrome also drink alcohol, and many patients with AUD have overweight or obesity and/or a sedentary lifestyle[30].

NON-INVASIVE METHODS FOR ASSESSMENT OF LIVER DAMAGE

Liver ultrasound

Liver ultrasound is both inexpensive and easily available, but it is rarely performed in patients with AUD and no apparent liver disease. The latest American Association for the Study of Liver Diseases guidelines for the management of chronic HCV infection are focused on the test-and-treat paradigm but recommend abdominal ultrasound for hepatocellular carcinoma surveillance only if the patient has already developed cirrhosis[31]. Although the latest European guidelines recommend liver ultrasound to early detect non-alcoholic steatohepatitis[32], there are no guidelines for the early detection of liver abnormalities among patients with high alcohol intake. Screening approaches for patients at risk for alcohol-related liver disease thus are a matter of debate[33,34]. It is also important to note that, in general, liver cancer surveillance among individuals with alcohol-related liver disease is far from optimal, and hepatocellular carcinoma is usually diagnosed at an advanced stage[22].

On abdominal ultrasound, liver steatosis has a hyperechogenic appearance because of the increased parenchymal reflectivity of intracellular fat accumulation[35]. Fat

content affects the sensitivity of abdominal ultrasound for detecting liver steatosis, and sensitivity severely decreases at fat contents lower than 10%–20%[28].

The precision of abdominal ultrasound in differentiating between steatosis and fibrosis is higher when liver fibrosis is more advanced because of an increase in coarse echoes without posterior beam attenuation[35-37]. Steatosis is usually classified as "mild," "moderate," or "severe"[37,38].

Despite operator dependency for the assessment of steatosis, there is agreement that liver ultrasound is inexpensive and reliable for the detection of moderate or severe liver steatosis[39,40]. The accuracy of abdominal ultrasound is lower for mild steatosis, however, it can be increased with a computer-aided method[40]. Ultrasound findings contribute to the detection of alcohol-related liver disease features in addition to liver steatosis, including (among others): Liver size, edge bluntness, parenchyma coarseness, surface nodularity, inferior vena cava abnormalities, the presence of portal hypertension, and spleen size[41].

The authors of a Cochrane review published in 2016 commented on the need for studies of large sample size to assess the efficacy of abdominal ultrasound in patients with alcohol-related liver disease[42], in part because their review could only include two studies[42]. Of these two studies, the first was performed in France in 1985 and included 126 patients with alcoholism[43]. In addition to abdominal ultrasound performed in the entire cohort, 100 patients also underwent liver biopsy. In that group of 100 patients, cirrhosis of the liver was confirmed in 72, and abdominal ultrasound had a sensitivity of 81% and a specificity of 79% to detect cirrhosis[43].

The second study was published in 2013 and performed in Korea and included 230 patients (81% male) who underwent liver biopsy, elastography and abdominal ultrasound[44]. The mean age of participants was 50.4 years. Ultrasound suggested heterogeneous liver in 199 patients (86.5%) and liver cirrhosis in 170 (74%). Cirrhosis of the liver was found in 111 participants, for a sensitivity of 94% and specificity of 49% for ultrasound detection of cirrhosis of the liver[44]. Because of the small number of patients included and the differences in the selection criteria in each of these two studies, the authors of the review could not reach any conclusion about the use of abdominal ultrasound for detecting underlying advanced liver disease among patients with unhealthy alcohol use[42].

In 2018, we published a study describing ultrasound findings among 301 consecutive AUD patients without clinically relevant liver disease who were admitted for hospital treatment of the disorder at Hospital Universitari Germans Trias i Pujol [34].

We obtained clinical and laboratory parameters at admission. An ultrasound was performed so as to identify the presence of liver steatosis, hepatomegaly, heterogeneous liver, and portal hypertension.

For this work, we studied unadjusted associations of ultrasound abnormalities with three prevalent conditions: Alcohol-related liver injury (ALI), advanced liver fibrosis (ALF) and HCV infection. ALI was defined as the presence of at least two of the following: Aspartate aminotransferase (AST) levels ≥ 74 < 300 U/L, AST/alanine aminotransferase (ALT) ratio > 2, and total bilirubin> 1.2 mg/dL. ALF was measured with fibrosis-4 (FIB-4)[45] and was defined as a FIB-4 score ≥ 3.25. We performed logistic regressions to detect if ALI, ALF and/or HCV were associated with having two or more abnormalities in the liver ultrasound.

In summary, 80% of the patients were male, had a median age of 46 years, drank a median of 180 grams of alcohol *per* day upon admission and 21.2% had HCV infection. Median serum AST was 42 U/L, median serum ALT was 35 U/L, the prevalence of ALI and ALF was 16% and 24%, respectively. A total of 57.2% patients had steatosis, 49.5% had hepatomegaly, 17% had heterogeneous liver, while 16% had portal hypertension. Of interest, 77% of patients had one abnormality, and 45% had ≥ 2.

In the multivariate models, both ALI and ALF were significantly associated with the presence of ≥ 2 abnormalities, with odds ratios (ORs) of 5.2 for ALI and 4.7 for ALF. HCV infection did not predict the presence of more than two abnormalities[34].

Most patients included in the study who had only one ultrasound abnormality had mild to moderate steatosis or hepatomegaly, which are both potentially reversible with alcohol cessation. In light of those findings, we believe that abdominal ultrasound could be implemented in the everyday clinical care of patients seeking treatment for AUD, as it could help in clinical decision-making and treatment selection.

Liver abnormalities were quite common in the study, even in patients without HCV, ALI, or ALF, and only 31% of patients without any of those conditions had a completely normal ultrasound. Accordingly, we think that sharing information about ultrasound abnormalities may promote alcohol cessation in patients with AUD who are unaware of their underlying liver disease[34]. Other researchers have previously

reported that sharing findings suggestive of poor health outcomes with patients leads to a decrease in unhealthy alcohol use[46].

Liver cancer has an incidence of 2.9% *per* year in individuals who have cirrhosis of the liver[22]. Hepatocellular carcinoma entails a dire prognosis if not detected early [47], which is why patients with alcohol-related end stage liver disease should undergo an abdominal ultrasound every 6 months[22]. However, periodic surveillance is challenging because many patients miss appointments[22], and less than 30% of liver cancers are detected by surveillance in Europe and the United States[22,48]. In our series, liver cancer was found in two participants[34].

Besides the early identification of steatosis or cirrhosis of the liver, abdominal ultrasound could be used for detecting other forms of liver damage. In fact, alcohol use is common among individuals with other forms of liver disease, and there is no safe level of alcohol consumption in these situations[8,30]. As previously mentioned, alcohol use is a cofactor in liver disease progression in individuals with HCV, HBV, non-alcoholic fatty liver disease, and hemochromatosis, among others. A threshold of a consumption of more than 30 grams of alcohol *per* day for men and 20 g for women is arbitrarily used to support the diagnosis of alcohol-related liver disease[12]. Nevertheless, as overweight and obesity are highly prevalent in the developed world, alcohol-related and non-alcoholic steatosis can co-occur[49]. In our case series of AUD patients, the median body mass index was 24.7, indicating that a sizable proportion of participants were overweight or obese[34].

In addition, liver ultrasound can also detect other forms of liver disease that are less prevalent, like parenchymatous or vascular diseases (non-cirrhotic portal hypertension, hepatic vein congestion, and Budd Chiari).

Transient elastography

Transient elastography has been used for more than a decade to accurately assess liver fibrosis, measuring liver stiffness in patients with chronic HCV infection or HBV infection, with or without HIV co-infection, and in non-alcoholic liver disease[50-52]. In transient elastography, a piston vibrator that is placed in the intercostal space generates a shear wave, and velocity is measured below the skin surface. It measures liver stiffness in kilopascals (kPa), and elastography readings usually range from 2.5 to 75 kPa[53].

The method has also been used to a lesser extent for analyzing liver injury in patients with AUD. A study that included 199 consecutive patients at risk for alcohol-related liver disease found that transient elastography provided an assessment of fibrosis (either significant fibrosis, Ishak score ≥ 3) and cirrhosis (Ishak score ≥ 5) that was comparable to liver biopsy with high accuracy (area under the curve ≥ 0.92)[33]. Some authors have pointed out that steatohepatitis may lead to overestimation of liver fibrosis[54]. The risk of overestimation especially applies in patients who are abstinent, as liver stiffness decreases with cessation of alcohol use, especially with baseline values higher than 7 kPa[55].

In 2016, a systematic review and meta-analysis found that transient elastography could reliably exclude ALF or cirrhosis of the liver. The authors suggested that the same cut-offs described for viral hepatitis-related liver disease should be applied with caution to other forms of liver damage, particularly alcohol-related liver disease[56]. Mueller and colleagues have suggested adapting elastography cut-offs in patients who present with elevated AST or bilirubin[54]. The studies in that meta-analysis, which included only patients with alcohol-related liver disease, had a wide range of participant numbers and an ALF prevalence ranging from 53% to 80%[57-61].

Results of an individual-level meta-analysis published in 2018 that included 1026 patients suggested that cut-offs for transient elastography should be higher[58]. In that study, higher concentrations of AST and bilirubin were significantly associated with higher liver stiffness values ($P < 0.01$)[58]. In addition, the presence of non-severe alcoholic hepatitis was associated with liver stiffness ($P < 0.0001$). Also, higher AST ($P < 0.01$) and higher bilirubin ($P = 0.01$) levels and increased prothrombin activity ($P = 0.01$) were significantly associated with non-severe alcoholic hepatitis. That meta-analysis described specific liver stiffness cut-offs based on concentrations of AST and bilirubin[58].

French researchers used transient elastography to assess ALF in a cohort study of patients with HCV/HIV co-infection. They found that ALF was more prevalent among patients with an alcohol-related diagnosis (OR 3.06; 95%CI: 1.42–6.60) compared to those with non-hazardous alcohol intake[62].

A recently published study describes the use of transient elastography for the measurement of liver and spleen stiffness (a surrogate marker of portal hypertension), as well as spleen length in a retrospective cohort of 499 patients with HCV or alcohol-

related liver disease[63]. Participants with HCV had higher mean spleen stiffness and spleen length but lower liver stiffness when compared to participants with alcohol-related liver disease. The authors concluded that the use of those measurements, as well as the spleen stiffness/liver stiffness and spleen length/spleen stiffness ratios, could be helpful to measure burden of disease and risk for disease-specific complications[63].

Other researchers have confirmed that elastography is a good predictor of clinical events[64]. Transient elastography has also been proven to be cost-effective in primary care[65], and other authors have used it to detect chronic alcohol-related liver disease after alcohol cessation[66] or to detect improvements in liver fibrosis after successful HCV antiviral therapy[67].

Transient elastography thus represents a promising tool for assessing underlying liver damage in patients with unhealthy alcohol use and/or HCV infection[28]. It is important to note that transient elastography results may be affected by the thickness of subcutaneous fat, width of intercostal spaces, by the presence of ascites, by the patients' breathing or by an uneven distribution of liver fibrosis. In addition, the presence of hepatic congestion or steatosis may also distort transient elastography results[28,56].

Table 1 includes a summary of the most relevant studies that have used transient elastography in patients with alcohol-related liver disease.

Controlled attenuation parameter

The controlled attenuation parameter (CAP) is a non-invasive tool for detecting steatosis. It measures ultrasound attenuation during transmission through fatty liver tissue[68]. CAP software can be incorporated into transient elastography devices, thus facilitating the non-invasive measurement of steatosis and fibrosis[28].

Cut-offs for moderate and severe liver steatosis were defined in a recently published patient-level meta-analysis that included 2735 participants with liver biopsy and CAP. Their diagnostic accuracy ranged from 0.65 to 0.90[69]. The etiology of liver disease was HBV in 37% of patients, HCV in 36% and or non-alcoholic fatty liver disease in 20%, and participants with unhealthy alcohol use were under-represented[69]. As all patients included had liver diseases that are strongly associated with liver fibrosis, these cut-offs require further validation in other and healthier populations[70].

A study by Thiele and colleagues included 562 patients with alcohol-related liver steatosis and found that CAP above 290 dB/m ruled in any steatosis with a 88% specificity and a 92% positive predictive value, whereas CAP below 220 dB/m ruled out steatosis with a 90% sensitivity but with a 62% negative predictive value[71]. The authors concluded that it was useful for the diagnosis of severe alcohol-related steatosis and could also rule in any liver steatosis. In patients admitted for alcohol detoxification who did not have obesity, CAP rapidly declined[71]. This finding underscores the synergic effect of obesity and alcohol use in patients seen in clinical practice[72].

A study by Unalp-Arida and colleagues measured liver stiffness and CAP in 4870 participants in the National Health and Nutrition Examination Survey cohort, and found that liver stiffness in the highest quartile was associated, among others, with HCV infection, increased age and body mass index and CAP[73].

Virtual touch quantification

Virtual touch quantification is a point shear wave elastography technique, using acoustic radiation force impulse technology also offers good diagnostic accuracy for liver fibrosis assessment[74]. It has been used in patients with chronic liver disease of different origins, mainly due to viral hepatitis[75,76].

Magnetic resonance imaging

Magnetic resonance imaging and proton magnetic resonance spectroscopy are promising methods for quantifying hepatic fat content[35]. Magnetic resonance measurement of liver steatosis does not appear to be affected by the type of liver disease or the presence of liver inflammation or iron overload, features that may distort results obtained with other non-invasive methods. Despite these potential advantages, magnetic resonance imaging is costly and time-consuming[28]. Moreover, information is scarce about its applicability in alcohol-related liver disease.

Laboratory-driven indices

There are several non-invasive indices that are derived from laboratory parameters aimed for the estimation of liver fibrosis. Some of those parameters are used in

Table 1 Studies that have used transient elastography in patients with alcohol-related liver disease

Ref.	Number of patients	Advanced liver fibrosis (≥ 3)	Sensitivity[1]	Specificity[1]
Nguyen-Khac *et al*[58], 2018	103	51%	87%	80%
Nahon *et al*[59], 2008	147	75%	87%	89%
Kim *et al*[107], 2009	45	80%	97%	78%
Mueller *et al*[57], 2010	101	45%	91%	75%
Janssens *et al*[60], 2010	49	65%	72%	76%
Fernandez *et al*[61], 2015	135	53%	91%	68%

[1]Sensitivity and specificity are calculated using liver biopsy as the gold standard.

everyday clinical practice, including AST, ALT, and platelets. As far back as 1991, Poynard and colleagues described the poly-gamma-l-glutamic acid index, which included prothrombin time, gamma-glutamyl transferase (GGT), and apolipoprotein A1[77]. In a group of 333 individuals with high alcohol consumption, the index correctly classified a heavy drinker as harboring liver cirrhosis most of the time (89%) at a value ≥ 9[77]. The accuracy increased with the addition of alpha-2 macroglobulin (known as the PGAA index)[78].

Other tests, such as the enhanced liver fibrosis (ELF) test, FibroTest (FT), and HepaSCORE, are commercially available and combine different serum markers that are not used in everyday clinical practice. ELF includes type III procollagen peptide, hyaluronic acid, and tissue inhibitor of metalloproteinase-1[79]. FT, also known as FibroSure in the United States, combines age and sex, alpha-2 macroglobulin, haptoglobulin, apolipoprotein A1, GGT, total bilirubin, and ALT[80]. HepaSCORE includes bilirubin, GGT, hyaluronic acid, alpha-2 macroglobulin, age, and sex[81]. The patented indices seem to perform better than non-patented versions against the gold standard of liver biopsy[82], but because of their cost, they are less frequently used in health systems with budget constraints.

Among the indices that include routinely measured parameters, the most commonly used are FIB-4[45] and the AST/platelet ratio index (APRI)[83]. Both indices have been validated against liver biopsy in patients with HCV and in patients with HCV and HIV co-infection[84-86]. Both FIB-4 and APRI are better suited for the detection of the absence of liver fibrosis or the presence of ALF[45,83]. Experience with their use in patients with unhealthy alcohol use is far more limited[87], and some authors have expressed concerns around the potential overestimation of liver fibrosis in patients with alcohol-related liver disease[87,88].

Lieber and colleagues, publishing in 2006, reported assessment results for 1308 patients from two United States veterans' administration cooperative studies of alcoholic liver disease for the accuracy of APRI in non-invasive detection of liver fibrosis[87]. APRI had low sensitivity (13.2%) and specificity (77.6%) for the non-invasive estimation of significant fibrosis in individuals with alcohol-related liver disease, including those who also had HCV.

In different series of patients with HCV with or without co-infection, results have been mixed regarding if non-invasive indices are able to detect the association between alcohol use and liver fibrosis, probably because of how alcohol consumption was assessed and because of unmeasured confounding in the different cohorts studied. A cross-sectional study in a cohort of patients with HIV/AIDS in Baltimore found that heavy alcohol use was associated with ALF measured with the APRI score[89]. However, when patients were stratified according to the presence of HCV infection, the association between APRI score and unhealthy alcohol use was only found among those with no HCV infection[89].

In a cohort that only included women, Blackard and colleagues did not find an association between alcohol use and FIB-4 values as a continuous variable among patients with HCV/HIV co-infection, whereas immune suppression was associated with higher FIB-4 values[90]. In a more recent study in the same cohort, Kelly and colleagues found that light or moderate alcohol consumption (1-7 drinks *per* week), which was reported in 35.7% of the 686 patients included, was not associated with fibrosis progression measured by several FIB-4 determinations over time[91]. Conversely, drinking 8–14 drinks *per* week was associated with minimal acceleration of fibrosis progression, while drinking more than 14 drinks *per* week was clearly

associated with increased fibrosis progression[91].

In a study performed in our cohort of patients with AUD who were admitted for hospital treatment in 2012, FIB-4 used as a continuous variable was significantly higher among those with HCV/HIV co-infection compared to those with HCV infection only[92]. When we stratified results by the presence of HIV infection, we found that alcohol use affected FIB-4 values only in those with HCV infection, whereas immune depression exerted a more negative role than alcohol consumption in those with co-infection[92].

In the HIV-LIVE cohort in Boston, involving a cohort of patients with HIV infection and alcohol problems, lifetime alcohol consumption measured with the Lifetime Drinking History questionnaire was not associated with FIB-4 values < 1.45 consistent with the absence of fibrosis. The adjusted (A)ORs were 1.12 (95%CI: 0.25–2.52) for a lifetime consumption of 150–600 kg of alcohol *vs* < 150 kg (reference category) and 1.11 [(95%CI: 0.52–2.36) for > 600 kg *vs* < 150 kg; global $P = 0.95$][93]. Similar results were found for the presence of ALF (FIB-4 > 3.25) and with the use of APRI instead of FIB-4. Results did not differ among patients with HCV co-infection[93].

An analysis from the veterans aging cohort study (VACS) included a large number of patients (701 with HIV/HCV coinfection, 1410 with HIV infection, with 296 HCV infection, and 1158 with neither HIV nor HCV infection) and a different measure of alcohol consumption (AUDIT-C questionnaire and/or presence of alcohol-related diagnoses). The authors reported greater risks for ALF (measured with FIB-4) among patients with co-infection who had nonhazardous drinking (OR = 14.2; 95%CI: 5.91–34.0) or hazardous/binge drinking (OR = 18.9; 95%CI: 7.98–44.8), or who exhibited alcohol-related diagnoses (OR = 25.2; 95%CI: 10.6–59.7) in comparison to uninfected Veterans who were nonhazardous drinkers[94].

In a more recent study by our group that included 1313 patients with AUD who were admitted for hospital detoxification, 30.6% had ALF, estimated with a FIB-4 value > 3.25. Patients with HCV infection, who represented 18% of the total study population, were two times more likely than those without HCV infection to present with ALF (OR = 2.1; 95%CI: 1.5–3.1, $P < 0.01$)[25].

Despite these different results in terms of accuracy or the ability to detect associations between the presence either of AUD or HCV and ALF, non-invasive indices are performed in patients with alcohol or other substance use disorders that will not undergo a biopsy[93,95]. In addition, non-invasive indices can predict mid-term mortality in epidemiological studies[95,96], which is why FIB-4 has been included in the VACS cohort index[97]. This index, initially intended to predict mortality, also predicts the occurrence of other health outcomes[98], such as incident heart failure in patients living with HIV[99].

The Forns index, which was described in 2002, has been less frequently used in patients with unhealthy alcohol use[100]. Originally developed for detection of the absence of liver fibrosis in a cohort of patients with HCV, it includes platelets, cholesterol, and GGT levels, as well as age[100]. In a study by Naveau and colleagues published in 2009, the accuracy of the Forns index for detecting biopsy-proven ALF was lower than that of other non-patented biomarkers (APRI and FIB-4)[82]. Furthermore, other researchers have expressed concerns regarding the use of the Forns index in alcohol-related liver disease, given that GGT levels differ between patients with HCV or alcohol-related liver disease[101]. Despite those concerns, in our group, we have used the Forns index to estimate liver fibrosis in drug users with HCV infection with or without HIV infection[96] and in patients with AUD[25]. The Forns index accurately predicted mid-term mortality and detected ALF in a fashion that was comparable to APRI and FIB-4[25].

Table 2 includes a summary of the more relevant papers describing assessment of alcohol use with non-patented indices to estimate liver fibrosis in patients with HCV infection.

Combination of non-invasive methods

Because non-invasive indices are better suited for the detection of either the absence of liver fibrosis or the presence of ALF, several approaches of the sequential use of different methods have been proposed. A study published in 2017 by Spanish researchers showed that use of the ELF test with or without a confirmation elastography was cost-effective compared with a single liver biopsy for the detection of liver fibrosis in patients with HCV and alcohol-related liver disease[102].

Another new approach is the use of both FIB-4 and transient elastography to detect ALF in patients with FIB-4 values within the intermediate range (1.45–3.25)[103]. This approach has been used in a Russian cohort of individuals with high alcohol intake, HIV infection, and a high prevalence of chronic HCV infection[103].

Future directions for the non-invasive assessment of liver damage

The measurement of small non-coding microRNAs (miRNAs) is a promising field [104]. In fact, miR-155 and miR-122 have been proposed as potential markers of different forms of liver diseases, including those related to HCV and alcohol[105].

Other markers that could be used in the future are extracellular vesicles, the small membrane vesicles released by cells that transport mRNA, miRNA, proteins, and lipids, and circulating nucleic acids, including cell-free DNA and cell-free non-coding RNA[106].

CONCLUSION

Underlying liver disease can be accurately estimated with the use of several non-invasive methods that spare the necessity of performing a liver biopsy. The widespread use of these methods can help to accurately identify patients at risk for the development of end-stage liver disease. In addition to treatment with direct-acting antivirals for those with HCV infection, abstinence from alcohol consumption should be strongly recommended, given the overlap between alcohol-related and metabolic liver diseases.

Table 2 Studies that used non-patented indices to estimate liver fibrosis in patients with hepatitis C virus infection and unhealthy alcohol use

Ref.	Setting	Non-invasive method	Method for detecting alcohol consumption	Finding
Lieber *et al*[87], 2006	Veterans' affairs studies (2) of alcoholic liver disease	APRI[1]	Average alcohol intake	Low sensitivity and specificity of APRI in comparison to liver biopsy, especially in patients with HCV
Chaudhry *et al*[89], 2009	HIV Hopkins clinical cohort	APRI[1]	Past 6 mo of hazardous drinking	No effect of alcohol on APRI values in HCV/HIV co-infection
Blackard *et al*[90], 2011	WIHS cohort	FIB-4[2]	Recent drinking	No association between alcohol intake and FIB-4 values in HCV/HIV co-infection
Muga *et al*[92], 2012	Patients with AUD admitted for detoxification	FIB-4[2]	Past 6 mo of unhealthy drinking	No association between FIB-4 and alcohol use in HCV/HIV co-infection
Fuster *et al*[93], 2013	HIV-LIVE cohort	FIB-4[2] and APRI[1]	LDH	No association between LDH and liver fibrosis measured with FIB-4 or APRI
Lim *et al*[94], 2014	VACS cohort	FIB-4[2]	AUDIT-C[3]	Advanced liver fibrosis correlated with alcohol use
Kelly *et al*[91], 2017	WIHS cohort	FIB-4[2]	Average number of drinks *per* week, past 6 mo	Light/moderate drinking was not associated with accelerated fibrosis progression
Sanvisens *et al*[25], 2018	Patients with AUD admitted for detoxification	FIB-4[2]; APRI[1]; Forns	Past 6 mo, daily alcohol intake	Patients with HCV were two times as likely to present with advanced liver fibrosis

[1]Aspartate aminotransferase (AST)/platelet ratio index: AST to platelet ratio index = {[Patient AST/AST upper limit of normal (IU/L)]/platelet count (10^9/L)} × 100.
[2]FIB-4 = Age × AST (IU/L)/platelet count (10^9/L) × alanine aminotransferase (IU/L)$^{1/2}$.
[3]AUDIT-C: Alcohol use disorders identification test. HCV: Hepatitis C virus; AST: Aspartate aminotransferase; APRI: AST/platelet ratio index; FIB-4: Fibrosis-4; AUD: Alcohol use disorder; HIV: Human immunodeficiency virus; LDH: Lifetime drinking History; WIHS: Women's interagency HIV study; VACS: Veterans aging cohort study.

REFERENCES

1 **Stoller EP**, Hund AJ, Webster NJ, Blixen CE, Perzynski AT, McCormick RA, Kanuch SW, Dawson NV. Alcohol consumption within the context of hepatitis C: a qualitative study of non-problematic drinkers. *Alcohol Alcohol* 2006; **41**: 546-552 [PMID: 16855001 DOI: 10.1093/alcalc/agl055]
2 **Fuster D**, Sanvisens A, Bolao F, Rivas I, Tor J, Muga R. Alcohol use disorder and its impact on chronic hepatitis C virus and human immunodeficiency virus infections. *World J Hepatol* 2016; **8**: 1295-1308 [PMID: 27872681 DOI: 10.4254/wjh.v8.i31.1295]
3 **Novo-Veleiro I**, Calle Cde L, Domínguez-Quibén S, Pastor I, Marcos M, Laso FJ. Prevalence of hepatitis C virus infection in alcoholic patients: cohort study and systematic review. *Alcohol Alcohol* 2013; **48**: 564-569 [PMID: 23690232 DOI: 10.1093/alcalc/agt044]
4 **Armstrong GL**, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. *Ann Intern Med* 2006; **144**: 705-714 [PMID: 16702586 DOI: 10.7326/0003-4819-144-10-200605160-00004]
5 **Fuster D**, Sanvisens A, Bolao F, Serra I, Rivas I, Tor J, Muga R. Impact of hepatitis C virus infection on the risk of death of alcohol-dependent patients. *J Viral Hepat* 2015; **22**: 18-24 [PMID: 25131721 DOI: 10.1111/jvh.12290]
6 **Campbell JV**, Hagan H, Latka MH, Garfein RS, Golub ET, Coady MH, Thomas DL, Strathdee SA; STRIVE Project. High prevalence of alcohol use among hepatitis C virus antibody positive injection drug users in three US cities. *Drug Alcohol Depend* 2006; **81**: 259-265 [PMID: 16129567 DOI: 10.1016/j.drugalcdep.2005.07.005]
7 **Anand BS**, Thornby J. Alcohol has no effect on hepatitis C virus replication: a meta-analysis. *Gut* 2005; **54**: 1468-1472 [PMID: 16162952 DOI: 10.1136/gut.2004.056697]
8 **Hutchinson SJ**, Bird SM, Goldberg DJ. Influence of alcohol on the progression of hepatitis C virus infection: a meta-analysis. *Clin Gastroenterol Hepatol* 2005; **3**: 1150-1159 [PMID: 16271348 DOI: 10.1016/s1542-3565(05)00407-6]
9 **Tsui JI**, Pletcher MJ, Vittinghoff E, Seal K, Gonzales R. Hepatitis C and hospital outcomes in patients admitted with alcohol-related problems. *J Hepatol* 2006; **44**: 262-266 [PMID: 16226823 DOI: 10.1016/j.jhep.2005.07.027]
10 **Muga R**, Sanvisens A, Jarrin I, Fuster D, Bolao F, Tor J, Muñoz A. Hepatitis C infection substantially reduces survival of alcohol-dependent patients. *Clin Epidemiol* 2018; **10**: 897-905 [PMID: 30123002 DOI: 10.2147/CLEP.S162308]
11 **Alavi M**, Janjua NZ, Chong M, Grebely J, Aspinall EJ, Innes H, Valerio HM, Hajarizadeh B, Hayes PC, Krajden M, Amin J, Law MG, George J, Goldberg DJ, Hutchinson SJ, Dore GJ. The

contribution of alcohol use disorder to decompensated cirrhosis among people with hepatitis C: An international study. *J Hepatol* 2018; **68**: 393-401 [PMID: 29107152 DOI: 10.1016/j.jhep.2017.10.019]

12 **Fuster D**, Samet JH. Alcohol Use in Patients with Chronic Liver Disease. *N Engl J Med* 2018; **379**: 1251-1261 [PMID: 30257164 DOI: 10.1056/NEJMra1715733]

13 **Bataller R**, Arteel GE, Moreno C, Shah V. Alcohol-related liver disease: Time for action. *J Hepatol* 2019; **70**: 221-222 [PMID: 30658723 DOI: 10.1016/j.jhep.2018.12.007]

14 **O'Shea RS**, Dasarathy S, McCullough AJ; Practice Guideline Committee of the American Association for the Study of Liver Diseases; Practice Parameters Committee of the American College of Gastroenterology. Alcoholic liver disease. *Hepatology* 2010; **51**: 307-328 [PMID: 20034030 DOI: 10.1002/hep.23258]

15 **European Association for the Study of the Liver**. European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of alcohol-related liver disease. *J Hepatol* 2018; **69**: 154-181 [PMID: 29628280 DOI: 10.1016/j.jhep.2018.03.018]

16 **Saunders JB**, Aasland OG, Babor TF, de la Fuente JR, Grant M. Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption--II. *Addiction* 1993; **88**: 791-804 [PMID: 8329970 DOI: 10.1111/j.1360-0443.1993.tb02093.x]

17 **American Psychiatric Association**. Diagnostic and Statistical Manual of Mental Disorders, DSM-V. 1994: 4-8

18 **Smith PC**, Schmidt SM, Allensworth-Davies D, Saitz R. Primary care validation of a single-question alcohol screening test. *J Gen Intern Med* 2009; **24**: 783-788 [PMID: 19247718 DOI: 10.1007/s11606-009-0928-6]

19 **Lauer GM**, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41-52 [PMID: 11439948 DOI: 10.1056/NEJM200107053450107]

20 **Thomas DL**, Seeff LB. Natural history of hepatitis C. *Clin Liver Dis* 2005; **9**: 383-398, vi [PMID: 16023972 DOI: 10.1016/j.cld.2005.05.003]

21 **Seitz HK**, Bataller R, Cortez-Pinto H, Gao B, Gual A, Lackner C, Mathurin P, Mueller S, Szabo G, Tsukamoto H. Alcoholic liver disease. *Nat Rev Dis Primers* 2018; **4**: 16 [PMID: 30115921 DOI: 10.1038/s41572-018-0014-7]

22 **Ganne-Carrié N**, Nahon P. Hepatocellular carcinoma in the setting of alcohol-related liver disease. *J Hepatol* 2019; **70**: 284-293 [PMID: 30658729 DOI: 10.1016/j.jhep.2018.10.008]

23 **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218 [PMID: 15690074 DOI: 10.1172/JCI24282]

24 **Gebo KA**, Herlong HF, Torbenson MS, Jenckes MW, Chander G, Ghanem KG, El-Kamary SS, Sulkowski M, Bass EB. Role of liver biopsy in management of chronic hepatitis C: a systematic review. *Hepatology* 2002; **36**: S161-S172 [PMID: 12407590 DOI: 10.1053/jhep.2002.36989]

25 **Sanvisens A**, Muñoz A, Bolao F, Zuluaga P, Farré M, Jarrin I, Tor J, Muga R. Do serum markers of liver fibrosis vary by HCV infection in patients with alcohol use disorder? *Drug Alcohol Depend* 2018; **188**: 180-186 [PMID: 29778771 DOI: 10.1016/j.drugalcdep.2018.04.008]

26 **Pasha T**, Gabriel S, Therneau T, Dickson ER, Lindor KD. Cost-effectiveness of ultrasound-guided liver biopsy. *Hepatology* 1998; **27**: 1220-1226 [PMID: 9581674 DOI: 10.1002/hep.510270506]

27 **Ratib S**, Fleming KM, Crooks CJ, Walker AJ, West J. Causes of death in people with liver cirrhosis in England compared with the general population: a population-based cohort study. *Am J Gastroenterol* 2015; **110**: 1149-1158 [PMID: 26169512 DOI: 10.1038/ajg.2015.191]

28 **Moreno C**, Mueller S, Szabo G. Non-invasive diagnosis and biomarkers in alcohol-related liver disease. *J Hepatol* 2019; **70**: 273-283 [PMID: 30658728 DOI: 10.1016/j.jhep.2018.11.025]

29 **Williams R**, Aspinall R, Bellis M, Camps-Walsh G, Cramp M, Dhawan A, Ferguson J, Forton D, Foster G, Gilmore I, Hickman M, Hudson M, Kelly D, Langford A, Lombard M, Longworth L, Martin N, Moriarty K, Newsome P, O'Grady J, Pryke R, Rutter H, Ryder S, Sheron N, Smith T. Addressing liver disease in the UK: a blueprint for attaining excellence in health care and reducing premature mortality from lifestyle issues of excess consumption of alcohol, obesity, and viral hepatitis. *Lancet* 2014; **384**: 1953-1997 [PMID: 25433429 DOI: 10.1016/S0140-6736(14)61838-9]

30 **Hagström H**. Alcohol Consumption in Concomitant Liver Disease: How Much is Too Much? *Curr Hepatol Rep* 2017; **16**: 152-157 [PMID: 28706775 DOI: 10.1007/s11901-017-0343-0]

31 **Ghany MG**, Morgan TR; AASLD-IDSA Hepatitis C Guidance Panel. Hepatitis C Guidance 2019 Update: American Association for the Study of Liver Diseases-Infectious Diseases Society of America Recommendations for Testing, Managing, and Treating Hepatitis C Virus Infection. *Hepatology* 2020; **71**: 686-721 [PMID: 31816111 DOI: 10.1002/hep.31060]

32 **Ratziu V**, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J Hepatol* 2010; **53**: 372-384 [PMID: 20494470 DOI: 10.1016/j.jhep.2010.04.008]

33 **Thiele M**, Detlefsen S, Sevelsted Møller L, Madsen BS, Fuglsang Hansen J, Fialla AD, Trebicka J, Krag A. Transient and 2-Dimensional Shear-Wave Elastography Provide Comparable Assessment of Alcoholic Liver Fibrosis and Cirrhosis. *Gastroenterology* 2016; **150**: 123-133 [PMID: 26435270 DOI: 10.1053/j.gastro.2015.09.040]

34 **Fuster D**, Garcia-Calvo X, Zuluaga P, Rivas I, Sanvisens A, Tor J, Muga R. Ultrasound findings of liver damage in a series of patients consecutively admitted for treatment of alcohol use disorder. *Drug Alcohol Depend* 2018; **190**: 195-199 [PMID: 30048873 DOI:

10.1016/j.drugalcdep.2018.06.012]
35 **Schwenzer NF**, Springer F, Schraml C, Stefan N, Machann J, Schick F. Non-invasive assessment and quantification of liver steatosis by ultrasound, computed tomography and magnetic resonance. *J Hepatol* 2009; **51**: 433-445 [PMID: 19604596 DOI: 10.1016/j.jhep.2009.05.023]
36 **Palmentieri B**, de Sio I, La Mura V, Masarone M, Vecchione R, Bruno S, Torella R, Persico M. The role of bright liver echo pattern on ultrasound B-mode examination in the diagnosis of liver steatosis. *Dig Liver Dis* 2006; **38**: 485-489 [PMID: 16716779 DOI: 10.1016/j.dld.2006.03.021]
37 **Hamaguchi M**, Kojima T, Itoh Y, Harano Y, Fujii K, Nakajima T, Kato T, Takeda N, Okuda J, Ida K, Kawahito Y, Yoshikawa T, Okanoue T. The severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation. *Am J Gastroenterol* 2007; **102**: 2708-2715 [PMID: 17894848 DOI: 10.1111/j.1572-0241.2007.01526.x]
38 **Saverymuttu SH**, Joseph AE, Maxwell JD. Ultrasound scanning in the detection of hepatic fibrosis and steatosis. *Br Med J (Clin Res Ed)* 1986; **292**: 13-15 [PMID: 3080046 DOI: 10.1136/bmj.292.6512.13]
39 **Strauss S**, Gavish E, Gottlieb P, Katsnelson L. Interobserver and intraobserver variability in the sonographic assessment of fatty liver. *AJR Am J Roentgenol* 2007; **189**: W320-W323 [PMID: 18029843 DOI: 10.2214/AJR.07.2123]
40 **Mancini M**, Prinster A, Annuzzi G, Liuzzi R, Giacco R, Medagli C, Cremone M, Clemente G, Maurea S, Riccardi G, Rivellese AA, Salvatore M. Sonographic hepatic-renal ratio as indicator of hepatic steatosis: comparison with (1)H magnetic resonance spectroscopy. *Metabolism* 2009; **58**: 1724-1730 [PMID: 19716568 DOI: 10.1016/j.metabol.2009.05.032]
41 **American College of Radiology**. American College of Radiology, 2012. ACR–AIUM–SPR–SRU Practice Parameter for the Performance of an Ultrasound Examination of the Abdomen and/or Retroperitoneum. [cited 14 January 2021]. Available from: https://www.acr.org/-/media/ACR/Files/Practice-Parameters/us-abd-retro.pdf?la=en
42 **Pavlov CS**, Casazza G, Semenistaia M, Nikolova D, Tsochatzis E, Liusina E, Ivashkin VT, Gluud C. Ultrasonography for diagnosis of alcoholic cirrhosis in people with alcoholic liver disease. *Cochrane Database Syst Rev* 2016; **3**: CD011602 [PMID: 26934429 DOI: 10.1002/14651858.CD011602.pub2]
43 **Richard P**, Bonniaud P, Barthélémy C, Etaix JP, Veyret C, Audigier JC, Fraisse H. [Value of ultrasonography in the diagnosis of cirrhoses. Prospective study of 128 patients]. *J Radiol* 1985; **66**: 503-506 [PMID: 3912496]
44 **Moon KM**, Kim G, Baik SK, Choi E, Kim MY, Kim HA, Cho MY, Shin SY, Kim JM, Park HJ, Kwon SO, Eom YW. Ultrasonographic scoring system score versus liver stiffness measurement in prediction of cirrhosis. *Clin Mol Hepatol* 2013; **19**: 389-398 [PMID: 24459644 DOI: 10.3350/cmh.2013.19.4.389]
45 **Sterling RK**, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, S Sulkowski M, Torriani FJ, Dieterich DT, Thomas DL, Messinger D, Nelson M; APRICOT Clinical Investigators. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006; **43**: 1317-1325 [PMID: 16729309 DOI: 10.1002/hep.21178]
46 **Tsui JI**, Saitz R, Cheng DM, Nunes D, Libman H, Alperen JK, Samet JH. Awareness of hepatitis C diagnosis is associated with less alcohol use among persons co-infected with HIV. *J Gen Intern Med* 2007; **22**: 822-825 [PMID: 17503108 DOI: 10.1007/s11606-007-0147-y]
47 **Schütte K**, Bornschein J, Kahl S, Seidensticker R, Arend J, Ricke J, Malfertheiner P. Delayed Diagnosis of HCC with Chronic Alcoholic Liver Disease. *Liver Cancer* 2012; **1**: 257-266 [PMID: 24159590 DOI: 10.1159/000343840]
48 **Davila JA**, Morgan RO, Richardson PA, Du XL, McGlynn KA, El-Serag HB. Use of surveillance for hepatocellular carcinoma among patients with cirrhosis in the United States. *Hepatology* 2010; **52**: 132-141 [PMID: 20578139 DOI: 10.1002/hep.23615]
49 **Ajmera VH**, Terrault NA, Harrison SA. Is moderate alcohol use in nonalcoholic fatty liver disease good or bad? *Hepatology* 2017; **65**: 2090-2099 [PMID: 28100008 DOI: 10.1002/hep.29055]
50 **de Lédinghen V**, Douvin C, Kettaneh A, Ziol M, Roulot D, Marcellin P, Dhumeaux D, Beaugrand M. Diagnosis of hepatic fibrosis and cirrhosis by transient elastography in HIV/hepatitis C virus-coinfected patients. *J Acquir Immune Defic Syndr* 2006; **41**: 175-179 [PMID: 16394849 DOI: 10.1097/01.qai.0000194238.15831.c7]
51 **Fernández-Montero JV**, Barreiro P, Vispo E, Labarga P, Sánchez-Parra C, de Mendoza C, Treviño A, Soriano V. Liver fibrosis progression in HIV-HCV-coinfected patients treated with distinct antiretroviral drugs and impact of pegylated interferon/ribavirin therapy. *Antivir Ther* 2014; **19**: 287-292 [PMID: 24192598 DOI: 10.3851/IMP2703]
52 **Wong VW**, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, Choi PC, Kowo M, Chan AW, Merrouche W, Sung JJ, de Lédinghen V. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51**: 454-462 [PMID: 20101745 DOI: 10.1002/hep.23312]
53 **Singh S**, Muir AJ, Dieterich DT, Falck-Ytter YT. American Gastroenterological Association Institute Technical Review on the Role of Elastography in Chronic Liver Diseases. *Gastroenterology* 2017; **152**: 1544-1577 [PMID: 28442120 DOI: 10.1053/j.gastro.2017.03.016]
54 **Mueller S**, Englert S, Seitz HK, Badea RI, Erhardt A, Bozaari B, Beaugrand M, Lupșor-Platon M. Inflammation-adapted liver stiffness values for improved fibrosis staging in patients with hepatitis C virus and alcoholic liver disease. *Liver Int* 2015; **35**: 2514-2521 [PMID: 26121926 DOI:

10.1111/liv.12904]

55 **Gianni E**, Forte P, Galli V, Razzolini G, Bardazzi G, Annese V. Prospective Evaluation of Liver Stiffness Using Transient Elastography in Alcoholic Patients Following Abstinence. *Alcohol Alcohol* 2017; **52**: 42-47 [PMID: 27542989 DOI: 10.1093/alcalc/agw053]

56 **Pavlov CS**, Casazza G, Nikolova D, Tsochatzis E, Gluud C. Systematic review with meta-analysis: diagnostic accuracy of transient elastography for staging of fibrosis in people with alcoholic liver disease. *Aliment Pharmacol Ther* 2016; **43**: 575-585 [PMID: 26791825 DOI: 10.1111/apt.13524]

57 **Mueller S**, Millonig G, Sarovska L, Friedrich S, Reimann FM, Pritsch M, Eisele S, Stickel F, Longerich T, Schirmacher P, Seitz HK. Increased liver stiffness in alcoholic liver disease: differentiating fibrosis from steatohepatitis. *World J Gastroenterol* 2010; **16**: 966-972 [PMID: 20180235 DOI: 10.3748/wjg.v16.i8.966]

58 **Nguyen-Khac E**, Thiele M, Voican C, Nahon P, Moreno C, Boursier J, Mueller S, de Ledinghen V, Stärkel P, Gyune Kim S, Fernandez M, Madsen B, Naveau S, Krag A, Perlemuter G, Ziol M, Chatelain D, Diouf M. Non-invasive diagnosis of liver fibrosis in patients with alcohol-related liver disease by transient elastography: an individual patient data meta-analysis. *Lancet Gastroenterol Hepatol* 2018; **3**: 614-625 [PMID: 29983372 DOI: 10.1016/S2468-1253(18)30124-9]

59 **Nahon P**, Kettaneh A, Tengher-Barna I, Ziol M, de Lédinghen V, Douvin C, Marcellin P, Ganne-Carrié N, Trinchet JC, Beaugrand M. Assessment of liver fibrosis using transient elastography in patients with alcoholic liver disease. *J Hepatol* 2008; **49**: 1062-1068 [PMID: 18930329 DOI: 10.1016/j.jhep.2008.08.011]

60 **Janssens F**, de Suray N, Piessevaux H, Horsmans Y, de Timary P, Stärkel P. Can transient elastography replace liver histology for determination of advanced fibrosis in alcoholic patients: a real-life study. *J Clin Gastroenterol* 2010; **44**: 575-582 [PMID: 20104185 DOI: 10.1097/MCG.0b013e3181cb4216]

61 **Fernandez M**, Trépo E, Degré D, Gustot T, Verset L, Demetter P, Devière J, Adler M, Moreno C. Transient elastography using Fibroscan is the most reliable noninvasive method for the diagnosis of advanced fibrosis and cirrhosis in alcoholic liver disease. *Eur J Gastroenterol Hepatol* 2015; **27**: 1074-1079 [PMID: 26011235 DOI: 10.1097/MEG.0000000000000392]

62 **Marcellin F**, Roux P, Loko MA, Lions C, Caumont-Prim A, Dabis F, Salmon-Ceron D, Spire B, Carrieri MP; HEPAVIH (ANRS CO13) Study Group. High levels of alcohol consumption increase the risk of advanced hepatic fibrosis in HIV/hepatitis C virus-coinfected patients: a sex-based analysis using transient elastography at enrollment in the HEPAVIH ANRS CO13 cohort. *Clin Infect Dis* 2014; **59**: 1190-1192 [PMID: 25015913 DOI: 10.1093/cid/ciu525]

63 **Elshaarawy O**, Mueller J, Guha IN, Chalmers J, Harris R, Krag A, Madsen BS, Stefanescu H, Farcau O, Ardelean A, Procopet B, Thiele M, Mueller S. Spleen stiffness to liver stiffness ratio significantly differs between ALD and HCV and predicts disease-specific complications. *JHEP Rep* 2019; **1**: 99-106 [PMID: 32039357 DOI: 10.1016/j.jhepr.2019.05.003]

64 **Jayaswal ANA**, Levick C, Selvaraj EA, Dennis A, Booth JC, Collier J, Cobbold J, Tunnicliffe EM, Kelly M, Barnes E, Neubauer S, Banerjee R, Pavlides M. Prognostic value of multiparametric magnetic resonance imaging, transient elastography and blood-based fibrosis markers in patients with chronic liver disease. *Liver Int* 2020; **40**: 3071-3082 [PMID: 32730664 DOI: 10.1111/liv.14625]

65 **Serra-Burriel M**, Graupera I, Torán P, Thiele M, Roulot D, Wai-Sun Wong V, Neil Guha I, Fabrellas N, Arslanow A, Expósito C, Hernández R, Lai-Hung Wong G, Harman D, Darwish Murad S, Krag A, Pera G, Angeli P, Galle P, Aithal GP, Caballeria L, Castera L, Ginès P, Lammert F; investigators of the LiverScreen Consortium. Transient elastography for screening of liver fibrosis: Cost-effectiveness analysis from six prospective cohorts in Europe and Asia. *J Hepatol* 2019; **71**: 1141-1151 [PMID: 31470067 DOI: 10.1016/j.jhep.2019.08.019]

66 **Legros L**, Bardou-Jacquet E, Turlin B, Michalak S, Hamonic S, Le Gruyer A, Aziz K, Lemoine C, Bouvard N, Chavagnat JJ, Silvain C, Kerjean J, Le Dréau G, Lacave-Oberti N, Oberti F, Le Lan C, Guyader D, Moirand R. Transient Elastography Accurately Screens for Compensated Advanced Chronic Liver Disease in Patients With Ongoing or Recent Alcohol Withdrawal. *Clin Gastroenterol Hepatol* 2021 [PMID: 33588101 DOI: 10.1016/j.cgh.2021.02.021]

67 **Doyle MA**, Lee T, Singer J, Crawley A, Klein M, Cooper C. Evaluation of Safety and Effectiveness of Elvitegravir/Cobicistat/Emtricitabine/Tenofovir Alafenamide Switch Followed by Ledipasvir/Sofosbuvir HCV Therapy in HIV-HCV Coinfection. *Open Forum Infect Dis* 2019; **6** [PMID: 31363776 DOI: 10.1093/ofid/ofz318]

68 **Sasso M**, Beaugrand M, de Ledinghen V, Douvin C, Marcellin P, Poupon R, Sandrin L, Miette V. Controlled attenuation parameter (CAP): a novel VCTE™ guided ultrasonic attenuation measurement for the evaluation of hepatic steatosis: preliminary study and validation in a cohort of patients with chronic liver disease from various causes. *Ultrasound Med Biol* 2010; **36**: 1825-1835 [PMID: 20870345 DOI: 10.1016/j.ultrasmedbio.2010.07.005]

69 **Karlas T**, Petroff D, Sasso M, Fan JG, Mi YQ, de Lédinghen V, Kumar M, Lupsor-Platon M, Han KH, Cardoso AC, Ferraioli G, Chan WK, Wong VW, Myers RP, Chayama K, Friedrich-Rust M, Beaugrand M, Shen F, Hiriart JB, Sarin SK, Badea R, Jung KS, Marcellin P, Filice C, Mahadeva S, Wong GL, Crotty P, Masaki K, Bojunga J, Bedossa P, Keim V, Wiegand J. Individual patient data meta-analysis of controlled attenuation parameter (CAP) technology for assessing steatosis. *J Hepatol* 2017; **66**: 1022-1030 [PMID: 28039099 DOI: 10.1016/j.jhep.2016.12.022]

70 **Romero-Gómez M**, Cortez-Pinto H. Detecting liver fat from viscoelasticity: How good is CAP in

clinical practice? *J Hepatol* 2017; **66**: 886-887 [PMID: 28189753 DOI: 10.1016/j.jhep.2017.01.029]
71 **Thiele M**, Rausch V, Fluhr G, Kjærgaard M, Piecha F, Mueller J, Straub BK, Lupșor-Platon M, De-Ledinghen V, Seitz HK, Detlefsen S, Madsen B, Krag A, Mueller S. Controlled attenuation parameter and alcoholic hepatic steatosis: Diagnostic accuracy and role of alcohol detoxification. *J Hepatol* 2018; **68**: 1025-1032 [PMID: 29343427 DOI: 10.1016/j.jhep.2017.12.029]
72 **Bellentani S**, Saccoccio G, Masutti F, Crocè LS, Brandi G, Sasso F, Cristanini G, Tiribelli C. Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med* 2000; **132**: 112-117 [PMID: 10644271 DOI: 10.7326/0003-4819-132-2-200001180-00004]
73 **Unalp-Arida A**, Ruhl CE. Transient Elastography-Assessed Hepatic Steatosis and Fibrosis Are Associated With Body Composition in the United States. *Clin Gastroenterol Hepatol* 2021 [PMID: 33549867 DOI: 10.1016/j.cgh.2021.02.009]
74 **Summers JA**, Radhakrishnan M, Morris E, Chalkidou A, Rua T, Patel A, McMillan V, Douiri A, Wang Y, Ayis S, Higgins J, Keevil S, Lewis C, Peacock J. Virtual Touch™ Quantification to Diagnose and Monitor Liver Fibrosis in Hepatitis B and Hepatitis C: A NICE Medical Technology Guidance. *Appl Health Econ Health Policy* 2017; **15**: 139-154 [PMID: 27601240 DOI: 10.1007/s40258-016-0277-7]
75 **Bâldea V**, Sporea I, Tudor A, Popescu A, Bende F, Șirli R. Virtual Touch Quantification using Acoustic Radiation Force Impulse Imaging Technology versus Transient Elastography for the Noninvasive Assessment of Liver Fibrosis in Patients with Chronic Hepatitis B or C using Liver Biopsy as the Gold Standard. *J Gastrointestin Liver Dis* 2020; **29**: 181-190 [PMID: 32530985 DOI: 10.15403/jgld-2256]
76 **Sporea I**, Mare R, Lupusoru R, Popescu A, Danila M, Bende F, Sirli R. Comparative study between four ultrasound Shear Waves Elastographic methods for liver fibrosis assessment. *Med Ultrason* 2018; **20**: 265-271 [PMID: 30167577 DOI: 10.11152/mu-1487]
77 **Poynard T**, Aubert A, Bedossa P, Abella A, Naveau S, Paraf F, Chaput JC. A simple biological index for detection of alcoholic liver disease in drinkers. *Gastroenterology* 1991; **100**: 1397-1402 [PMID: 1672859]
78 **Naveau S**, Essoh BM, Ghinoiu M, Marthey L, Njiké-Nakseu M, Balian A, Lachgar M, Prévot S, Perlemuter G. Comparison of Fibrotest and PGAA for the diagnosis of fibrosis stage in patients with alcoholic liver disease. *Eur J Gastroenterol Hepatol* 2014; **26**: 404-411 [PMID: 24561990 DOI: 10.1097/MEG.0000000000000041]
79 **Vali Y**, Lee J, Boursier J, Spijker R, Löffler J, Verheij J, Brosnan MJ, Böcskei Z, Anstee QM, Bossuyt PM, Zafarmand MH; LITMUS systematic review team(†). Enhanced liver fibrosis test for the non-invasive diagnosis of fibrosis in patients with NAFLD: A systematic review and meta-analysis. *J Hepatol* 2020; **73**: 252-262 [PMID: 32275982 DOI: 10.1016/j.jhep.2020.03.036]
80 **Poynard T**, de Ledinghen V, Zarski JP, Stanciu C, Munteanu M, Vergniol J, France J, Trifan A, Le Naour G, Vaillant JC, Ratziu V, Charlotte F; Fibrosis-TAGS group. Relative performances of FibroTest, Fibroscan, and biopsy for the assessment of the stage of liver fibrosis in patients with chronic hepatitis C: a step toward the truth in the absence of a gold standard. *J Hepatol* 2012; **56**: 541-548 [PMID: 21889468 DOI: 10.1016/j.jhep.2011.08.007]
81 **Adams LA**, Bulsara M, Rossi E, DeBoer B, Speers D, George J, Kench J, Farrell G, McCaughan GW, Jeffrey GP. Hepascore: an accurate validated predictor of liver fibrosis in chronic hepatitis C infection. *Clin Chem* 2005; **51**: 1867-1873 [PMID: 16055434 DOI: 10.1373/clinchem.2005.048389]
82 **Naveau S**, Gaudé G, Asnacios A, Agostini H, Abella A, Barri-Ova N, Dauvois B, Prévot S, Ngo Y, Munteanu M, Balian A, Njiké-Nakseu M, Perlemuter G, Poynard T. Diagnostic and prognostic values of noninvasive biomarkers of fibrosis in patients with alcoholic liver disease. *Hepatology* 2009; **49**: 97-105 [PMID: 19053048 DOI: 10.1002/hep.22576]
83 **Wai CT**, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 518-526 [PMID: 12883497 DOI: 10.1053/jhep.2003.50346]
84 **Nunes D**, Fleming C, Offner G, O'Brien M, Tumilty S, Fix O, Heeren T, Koziel M, Graham C, Craven DE, Stuver S, Horsburgh CR Jr. HIV infection does not affect the performance of noninvasive markers of fibrosis for the diagnosis of hepatitis C virus-related liver disease. *J Acquir Immune Defic Syndr* 2005; **40**: 538-544 [PMID: 16284529 DOI: 10.1097/01.qai.0000184856.31695.bf]
85 **Loko MA**, Castera L, Dabis F, Le Bail B, Winnock M, Coureau G, Bioulac-Sage P, de Ledinghen V, Neau D; Groupe d'Epidémiologie Clinique du SIDA en Aquitaine (GECSA). Validation and comparison of simple noninvasive indexes for predicting liver fibrosis in HIV-HCV-coinfected patients: ANRS CO3 Aquitaine cohort. *Am J Gastroenterol* 2008; **103**: 1973-1980 [PMID: 18796094 DOI: 10.1111/j.1572-0241.2008.01954.x]
86 **Vallet-Pichard A**, Mallet V, Nalpas B, Verkarre V, Nalpas A, Dhalluin-Venier V, Fontaine H, Pol S. FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. *Hepatology* 2007; **46**: 32-36 [PMID: 17567829 DOI: 10.1002/hep.21669]
87 **Lieber CS**, Weiss DG, Morgan TR, Paronetto F. Aspartate aminotransferase to platelet ratio index in patients with alcoholic liver fibrosis. *Am J Gastroenterol* 2006; **101**: 1500-1508 [PMID: 16863553 DOI: 10.1111/j.1572-0241.2006.00610.x]
88 **Trabut JB**, Thépot V, Terris B, Sogni P, Nalpas B, Pol S. [Prognosis assessment of alcoholic liver disease: how and why? *Presse Med* 2014; **43**: 124-134 [PMID: 24140195 DOI: 10.1016/j.lpm.2013.04.016]

89 **Chaudhry AA**, Sulkowski MS, Chander G, Moore RD. Hazardous drinking is associated with an elevated aspartate aminotransferase to platelet ratio index in an urban HIV-infected clinical cohort. *HIV Med* 2009; **10**: 133-142 [PMID: 19207596 DOI: 10.1111/j.1468-1293.2008.00662.x]

90 **Blackard JT**, Welge JA, Taylor LE, Mayer KH, Klein RS, Celentano DD, Jamieson DJ, Gardner L, Sherman KE. HIV mono-infection is associated with FIB-4 - A noninvasive index of liver fibrosis - in women. *Clin Infect Dis* 2011; **52**: 674-680 [PMID: 21248367 DOI: 10.1093/cid/ciq199]

91 **Kelly EM**, Dodge JL, Bacchetti P, Sarkar M, French AL, Tien PC, Glesby MJ, Golub ET, Augenbraun M, Plankey M, Peters MG. Moderate Alcohol Use Is Not Associated With Fibrosis Progression in Human Immunodeficiency Virus/Hepatitis C Virus-Coinfected Women: A Prospective Cohort Study. *Clin Infect Dis* 2017; **65**: 2050-2056 [PMID: 29020382 DOI: 10.1093/cid/cix716]

92 **Muga R**, Sanvisens A, Fuster D, Tor J, Martínez E, Pérez-Hoyos S, Muñoz A. Unhealthy alcohol use, HIV infection and risk of liver fibrosis in drug users with hepatitis C. *PLoS One* 2012; **7**: e46810 [PMID: 23056462 DOI: 10.1371/journal.pone.0046810]

93 **Fuster D**, Tsui JI, Cheng DM, Quinn EK, Bridden C, Nunes D, Libman H, Saitz R, Samet JH. Impact of lifetime alcohol use on liver fibrosis in a population of HIV-infected patients with and without hepatitis C coinfection. *Alcohol Clin Exp Res* 2013; **37**: 1527-1535 [PMID: 23647488 DOI: 10.1111/acer.12129]

94 **Lim JK**, Tate JP, Fultz SL, Goulet JL, Conigliaro J, Bryant KJ, Gordon AJ, Gibert C, Rimland D, Goetz MB, Klein MB, Fiellin DA, Justice AC, Lo Re V 3rd. Relationship between alcohol use categories and noninvasive markers of advanced hepatic fibrosis in HIV-infected, chronic hepatitis C virus-infected, and uninfected patients. *Clin Infect Dis* 2014; **58**: 1449-1458 [PMID: 24569533 DOI: 10.1093/cid/ciu097]

95 **Nunes D**, Fleming C, Offner G, Craven D, Fix O, Heeren T, Koziel MJ, Graham C, Tumilty S, Skolnik P, Stuver S, Horsburgh CR Jr, Cotton D. Noninvasive markers of liver fibrosis are highly predictive of liver-related death in a cohort of HCV-infected individuals with and without HIV infection. *Am J Gastroenterol* 2010; **105**: 1346-1353 [PMID: 20179698 DOI: 10.1038/ajg.2009.746]

96 **Sanvisens A**, Fuster D, Serra I, Tor J, Tural C, Rey-Joly C, Muga R. Estimated liver fibrosis and its impact on all-cause mortality of HCV-monoinfected and HCV/HIV-coinfected drug users. *Curr HIV Res* 2011; **9**: 256-262 [PMID: 21675942 DOI: 10.2174/157016211796320298]

97 **Justice AC**, Freiberg MS, Tracy R, Kuller L, Tate JP, Goetz MB, Fiellin DA, Vanasse GJ, Butt AA, Rodriguez-Barradas MC, Gibert C, Oursler KA, Deeks SG, Bryant K; VACS Project Team. Does an index composed of clinical data reflect effects of inflammation, coagulation, and monocyte activation on mortality among those aging with HIV? *Clin Infect Dis* 2012; **54**: 984-994 [PMID: 22337823 DOI: 10.1093/cid/cir989]

98 **Bambha K**, Pierce C, Cox C, French AL, Tien PC, Sharp GB, Augenbraun M, Glesby MJ, Villacres MC, Plankey M, Strickler HD, Gange SJ, Peters MG. Assessing mortality in women with hepatitis C virus and HIV using indirect markers of fibrosis. *AIDS* 2012; **26**: 599-607 [PMID: 22156972 DOI: 10.1097/QAD.0b013e32834fa121]

99 **So-Armah KA**, Lim JK, Lo Re V, Tate JP, Chang CH, Butt AA, Gibert CL, Rimland D, Marconi VC, Goetz MB, Rodriguez-Barradas MC, Budoff MJ, Tindle HA, Samet JH, Justice AC, Freiberg MS; Veterans Aging Cohort Study Project Team. FIB-4 stage of liver fibrosis predicts incident heart failure among HIV-infected and uninfected patients. *Hepatology* 2017; **66**: 1286-1295 [PMID: 28543215 DOI: 10.1002/hep.29285]

100 **Forns X**, Ampurdanès S, Llovet JM, Aponte J, Quintó L, Martínez-Bauer E, Bruguera M, Sánchez-Tapias JM, Rodés J. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. *Hepatology* 2002; **36**: 986-992 [PMID: 12297848 DOI: 10.1053/jhep.2002.36128]

101 **Altamirano J**, Qi Q, Choudhry S, Abdallah M, Singal AK, Humar A, Bataller R, Borhani AA, Duarte-Rojo A. Non-invasive diagnosis: non-alcoholic fatty liver disease and alcoholic liver disease. *Transl Gastroenterol Hepatol* 2020; **5**: 31 [PMID: 32258535 DOI: 10.21037/tgh.2019.11.14]

102 **Soto M**, Sampietro-Colom L, Lasalvia L, Mira A, Jiménez W, Navasa M. Cost-effectiveness of enhanced liver fibrosis test to assess liver fibrosis in chronic hepatitis C virus and alcoholic liver disease patients. *World J Gastroenterol* 2017; **23**: 3163-3173 [PMID: 28533673 DOI: 10.3748/wjg.v23.i17.3163]

103 **Gnatienko N**, Freiberg MS, Blokhina E, Yaroslavtseva T, Bridden C, Cheng DM, Chaisson CE, Lioznov D, Bendiks S, Koerbel G, Coleman SM, Krupitsky E, Samet JH. Design of a randomized controlled trial of zinc supplementation to improve markers of mortality and HIV disease progression in HIV-positive drinkers in St. Petersburg, Russia. *HIV Clin Trials* 2018; **19**: 101-111 [PMID: 29663871 DOI: 10.1080/15284336.2018.1459344]

104 **Gao B**, Ahmad MF, Nagy LE, Tsukamoto H. Inflammatory pathways in alcoholic steatohepatitis. *J Hepatol* 2019; **70**: 249-259 [PMID: 30658726 DOI: 10.1016/j.jhep.2018.10.023]

105 **Torres JL**, Novo-Veleiro I, Manzanedo L, Alvela-Suárez L, Macías R, Laso FJ, Marcos M. Role of microRNAs in alcohol-induced liver disorders and non-alcoholic fatty liver disease. *World J Gastroenterol* 2018; **24**: 4104-4118 [PMID: 30271077 DOI: 10.3748/wjg.v24.i36.4104]

106 **Mann J**, Reeves HL, Feldstein AE. Liquid biopsy for liver diseases. *Gut* 2018; **67**: 2204-2212 [PMID: 30177542 DOI: 10.1136/gutjnl-2017-315846]

107 **Kim SG**, Kim YS, Jung SW, Kim HK, Jang JY, Moon JH, Kim HS, Lee JS, Lee MS, Shim CS, Kim BS. [The usefulness of transient elastography to diagnose cirrhosis in patients with alcoholic liver disease]. *Korean J Hepatol* 2009; **15**: 42-51 [PMID: 19346784 DOI: 10.3350/kjhep.2009.15.1.42]

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MINIREVIEWS

Clinical indicators for progression of nonalcoholic steatohepatitis to cirrhosis

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Abstract

Non-alcoholic fatty liver disease (NAFLD), is a disease spectrum characterized by fat accumulation in hepatocytes presenting as hepatic steatosis to advance disease with active hepatic inflammation, known as nonalcoholic steatohepatitis. Chronic steatohepatitis will lead to progressive hepatic fibrosis causing cirrhosis and increased risk for developing hepatocellular carcinoma (HCC). Fatty liver disease prevalence has increased at alarming rates alongside obesity, diabetes and metabolic syndrome to become the second most common cause of cirrhosis after alcohol related liver disease worldwide. Given this rise in prevalence, it is becoming increasingly more important to find non-invasive methods to diagnose disease early and stage hepatic fibrosis. Providing clinicians with the tools to diagnose and treat the full spectrum of NAFLD will help prevent known complications such as cirrhosis and HCC and improve quality of life for the patients suffering from this disease. This article discusses the utility of current non-invasive liver function testing in the clinical progression of fatty liver disease along with the imaging modalities that are available. Additionally, we summarize available treatment options including targeted medical therapy through four different pathways, surgical or endoscopic intervention.

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Core Tip: Fatty liver disease rates along with obesity, diabetes and metabolic syndrome continue to increase and now is the second leading cause of cirrhosis secondary to alcohol related liver disease. The need for consistent and readily available methods to accurately diagnose and stage hepatic fibrosis becomes increasingly necessary. With an up to date armamentarium to diagnose and treat the full spectrum of non-alcoholic fatty liver disease will decrease complications such as cirrhosis and hepatocellular carcinoma and will improve the likelihood for patients to have a higher quality of life.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) was first introduced by Schaffner and Thaler [1] in 1986. They assembled a group of non-alcoholic patients with liver diseases and biopsy specimens of liver pathology similar to that of alcoholic liver disease. They defined these subsets of patients as NAFLD. Over the last 20 years, the "non-alcoholic" portion of the diagnosis has been heavily criticized, as it carries an unfavorable connotation for patients that may negatively impact their overall care. In 2019, a group of international experts suggested the term metabolic (dysfunction) associated fatty liver disease "MAFLD" as a more appropriate diagnosis for NAFLD. As the underlying pathology is more related to metabolic dysfunction rather than the exclusion of alcohol[2]. Over the decades, NAFLD has grown to become the second most common cause of liver cirrhosis after alcohol related liver disease. The prevalence of NAFLD has grown every year in the United States secondary to a rise in diabetes, obesity and metabolic syndrome, with an incidence of 31% in 2012 as opposed to 18% in 1988–1991[3,4]. NAFLD refers to a spectrum of liver injury due to accumulation of triglycerides in hepatocytes presenting as a spectrum of conditions, ranging from a simple hepatic steatosis characterized by fat accumulation in the absence of hepatic inflammation to a more severe disease form characterized by active hepatic inflammation, also known as nonalcoholic steatohepatitis (NASH). Progressive hepatic inflammation will lead to cirrhosis and increase the risk of developing hepatocellular carcinoma (HCC) as shown in (Figure 1). Up to 1/3 of NAFLD patients will have NASH which is a risk factor for fibrosis progression, and approximately 40% of NASH patients will experience fibrosis progression[5]. The recent estimated annual progression of fibrosis from NAFLD is up to 0.09% with an incidence of advanced fibrosis as 70 per 1000 patients[6]. The high prevalence of NASH among the biopsied patients could be explained secondary to the indication for biopsy in these patients with elevated liver function tests (LFTs), and the data cannot be extrapolated to the subset of NAFLD patients with normal LFTs where biopsy is not performed often. In the same study, the prevalence of NASH in patients without indication for biopsy was 6.7%[5]. The annual incidence of HCC in NAFLD patients is 44% per 1000 person-years. NAFLD-related HCC amounts to about 2% to 4% of annual cases[7].

Liver biopsy is not always performed and the diagnosis is often made with available non-invasive tests including blood tests and elastography [magnetic resonance elastography (MRE), Fibroscan]. Advantages of fibroscan, other than being a non-invasive modality that helps in sequential assessment of progression or regression of steatosis/fibrosis, include elimination of sampling error experienced by liver biopsy. Liver biopsy is the gold standard in confirming the diagnosis of NAFLD and allowing accurate hepatitis fibrosis staging. The major histologic features include steatosis, lobular inflammation, and cytological ballooning; these findings help in grading and

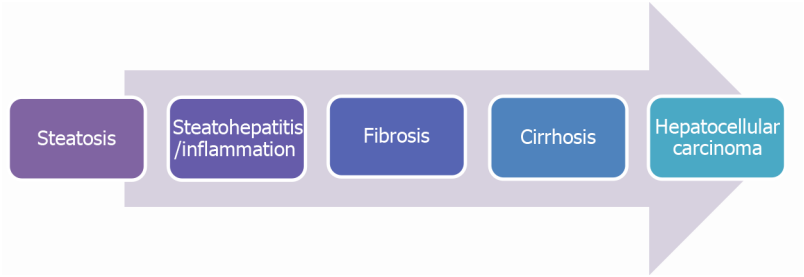


Figure 1 Histologic progression of nonalcoholic fatty liver disease from simple steatosis to cirrhosis.

staging the disease[8,9]. Moreover, the diagnostic tests especially non-invasive fibrosis assessment testing helps to monitor NAFLD stages to prevent disease progression and diagnose cancer early. Our review article discusses the indicators that help in understanding the progression of the disease including symptomatic worsening, liver function testing, imaging, and histopathological changes.

REVIEW

NAFLD encompasses a spectrum of conditions which ranges from bland hepatic steatosis to steatohepatitis causing hepatic fibrosis which will lead to cirrhosis, liver failure and increase the risk of HCC. The risk factors of fatty liver disease are similar to those of metabolic syndrome which leads to insulin resistance[5]. This includes diabetes mellitus, dyslipidemia and elevated body mass index (BMI). It is important to distinguish simple hepatic steatosis, which carries very low risk of developing chronic disease and cirrhosis *vs* NASH which carries a risk of progressive fibrosis, cirrhosis, liver failure and HCC. Overall, one fifth of NASH patients can progress to advanced hepatic fibrosis[10-12]. Hence, the assessment of the degree of hepatic fibrosis with noninvasive diagnostic panels and imaging is important in monitoring disease progression. Several scoring systems and specialized biomarkers have been developed by combining various serologic and clinical parameters for the prediction of fibrosis in NAFLD[13-18]. Despite the advancement of many diagnostic noninvasive fibrosis assessment modalities, one fourth of advanced fibrosis NASH patients can be misclassified as mild hepatic fibrosis[14].

The mortality of NAFLD is not merely targeting the liver. The majority of NAFLD patients are at risk of developing atherosclerotic coronary artery disease carrying higher mortality rate approaching[19]. Understanding patients' risk factors and stage of hepatic fibrosis can help predict patients' clinical outcomes[10]. Multiple clinical indicators and serological markers of disease progression remains an area of intensive clinical and basic science research till this day (Tables 1 and 2).

ROLE OF NONINVASIVE LIVER FUNCTION TESTING IN CLINICAL PROGRESSION OF NASH

Clinical assessment in progression of nash

NASH is a histological diagnosis characterized by hepatocytic inflammation that may progress to fibrosis. Hepatic fibrosis divided into four stages. Stage I describes as mild hepatic fibrosis, stage II moderate hepatic fibrosis, stage III moderate to severe fibrosis, and stage IV severe or advanced fibrosis. It is crucial to identify advanced fibrosis stage as these patients are at-risk to develop decompensated cirrhosis and end-stage liver disease. A number of clinical factors help clinicians to predict the likelihood of the patient progressing into devastating categories of this disease.

Role of liver chemistry in the clinical progression of NAFLD

Liver chemistry test identify active hepatic inflammation. This includes alanine aminotransferases (ALT) and aspartate aminotransferases (AST), alkaline phosphatase (ALP) and direct and indirect bilirubin. Other laboratory data should be monitored in NASH patients are platelet count and coagulation panel, fasting blood glucose and glycosylated proteins and lipid panel. Serum hyaluronic acid tissue metalloproteinase,

Table 1 Role of noninvasive liver function testing in clinical progression of nonalcoholic steatohepatitis

Sr. No.	Indicator	Ref.	Journal	Year	Results
1	Bilirubin	Demir *et al*[24]	*PLoS One*	2013	Total bilirubin was identified as a significant predictor of advanced fibrosis and used to construct the NIKEI score which can reliably exclude advanced fibrosis in subjects with NAFLD
		Ratziu *et al*[25]	*BMC Gastroenterol*	2006	FibroTest which includes total bilirubin in its panel is a simple and noninvasive quantitative estimate of liver fibrosis which reliably predicts advanced fibrosis
		Adams *et al*[11]	*J Hepatol*	2005	Hepascore, a model of 4 serum markers plus age and sex provides clinically useful information regarding different fibrosis stages among hepatitis C patients
2	Serum AST/ALT	Martin-Rodriguez *et al* [20]	*Medicine (Baltimore)*	2017	Serum ALT level is the most predictive laboratory investigation for the NAFLD. The AST-ALT Ratio (AAR) is higher in increasing liver fat content, fibrosis and other metabolic derangements like diabetes and dyslipidemia
		Enomoto *et al* [21]	*World J Gastroenterol*	2015	AAR > 1 is consistent with NASH
		Arora *et al*[22]	*J Clin Exp Hepatol*	2012	AAR > 1 may indicate the progression of NAFLD and aid in diagnosing liver fibrosis
		Shah *et al*[17]	*Clin Gastroenterol Hepatol*	2009	The FIB-4 score composed of age, AST and ALT and platelet counts is an invasive and inexpensive method which has shown superiority to BAAT (BMI, Age, ALT, Triglycerides) and BARD (BMI, AST: ALT, Diabetes) scores in monitoring the progress of NASH
		McPherson *et al* [18]	*Eur J Gastroenterol Hepatol*	2013	The FIB-4 score was reliable in ruling out advanced fibrosis in patients with histological evidence of NAFLD who had normal or increased levels of ALT, thus decreasing the need for invasive liver biopsy
3	Platelet Count	Enomoto *et al* [21]	*World J Gastroenterol*	2015	A reducing level of platelet count has been well documented in advancing liver diseases
		Kawamura *et al* [26]	*Hepatol Int*	2015	FSN score of 17 variables including platelet count could accurately predict fibrotic stage and discriminates patients with advanced fibrosis of NASH
		Kessoku *et al* [27]	*World J Gastroenterol*	2014	PLALA Score is a very unique scoring system as it has shown usefulness in distinguishing cirrhosis in NAFLD when compared with most fibrosis scoring systems
		Abdel-Razik A *et al*[47]	*Eur J Gastroenterol*	2016	MPV is a noninvasive novel marker to predict advanced disease as it was increased in NASH patients and advance liver fibrosis
		Cengiz *et al*[48]	*Eur J Gastroenterol*	2015	Red cell volume distribution width-to-platelet ratio was both correlated and able to predict liver fibrosis. It may reduce liver biopsy in NAFLD
4	Fasting blood glucose and glycosylated protein	Pelusi *et al*[49]	*PLoS One*	2016	Nonalcoholic steatohepatitis with greater degree of fibrosis was discovered in patients with insulin resistance. Type 2 diabetes in patients with NAFLD tends to drive the rate of fibrosis
5	Hyaluronic acid (hyluroante) tissue metaloproteinase	Arora *et al*[22]	*J Clin Exp Hepatol*	2012	European Liver Fibrosis score ELF scoring system has indicators for cellular matrix activities including Hyaluronic acid (hyluroante) tissue metalloproteinase which has been indicative of fibrosis
6	Type IV collagen	Nakamura *et al* [30]	*J Diabetes Investig*	2013	NAFIC Score including type IV collagen 7S and Modified NAFIC score were proven to be clinically useful in screening for NASH in NAFLD patients
7	Glycosylated Albumin to Glycosylated Hemoglobin Ratio	Hu *et al*[28]	*World J Gastroenterol*	2014	HOMA-IR score indicates NAFLD progression using a formula that involves insulin levels and fasting glucose to calculate insulin resistance. The score has a high sensitivity for NASH
		Stål[29]	*World J Gastroenterol*	2015	NAFLD fibrosis score, a non- invasive score which includes the presence of diabetes or impaired fasting glucose is the most predictive of mortality in NASH as compared to NAFL patients
8	Prothrombin time	Assy *et al*[31]	*World J Gastroenterol*	2005	Increase prothrombin time is usually associated with cirrhotic changes
9	Albumin	Bazick *et al*[5]	*Diabetes Care*	2015	Serum albumin gets reduced in patients progressing to NASH and fibrosis from NAFLD

ALT: Alanine aminotransferases; BMI: Body mass index; NAFIC: Nonalcoholic steatohepatitis, ferritin, insulin, and type IV collagen 7S; NASH:

Nonalcoholic steatohepatitis; AAR: Aspartate aminotransferases-alanine aminotransferases ratio; RPR: Red cell volume distribution width-to-platelet ratio; NFS: Non-alcoholic fatty liver disease fibrosis score; NIKEI: Non-invasive koeln-essen-index; NAFLD: Non-alcoholic fatty liver disease.

Table 2 Role of imaging techniques in clinical progression of nonalcoholic steatohepatitis

Sr. No.	Imaging modality	Ref.	Journal	Year	Results
1	Ultrasound	Sanyal [32]	*Gastroenterology*	2002	US is currently the preferred method in United States for screening asymptomatic patients with elevated liver enzymes and suspected NAFLD with sensitivity in detecting steatosis varying between 60%–94%
2	Magnetic Resonance Elastography	Iijima *et al* [35]	*Hepatol Res*	2007	Magnetic resonance elastography has excellent diagnostic accuracy with sensitivity and specificity of 98% and 99%, respectively, for detecting all grades of fibrosis
		Huwart *et al*[36]	*Gastroenterology*	2008	Magnetic resonance elastography was associated with a higher technical success rate than US elastography
3	Fibroscan	Wong *et al*[39]	*Gut*	2012	Transient elastography had shown good results in patients with NAFLD. It is a non-invasive method of assessing liver fibrosis which can be performed at the bedside or in the outpatient clinic
		Wong *et al*[40]	*Hepatology*	2010	Transient elastography had shown good results in patients with NAFLD. It is a non-invasive method of assessing liver fibrosis which can be performed at the bedside or in the outpatient clinic
		Ratziu *et al*[43]	*Gastroenterology*	2005	Fibroscan has now been validated in NAFLD, and represents a useful tool for rapid, non-invasive assessment of liver fibrosis and determining the need for biopsy. Nonetheless, fibroscan values should be interpreted in consonance with clinical, biological, and morphological data

US: Ultrasonography; NAFLD: Non-alcoholic fatty liver disease.

and type 4 collagen are serological markers help in assessing fibrosis stage.

AST and ALT: In a cross-sectional study, Martin-Rodriguez *et al*[20] reported that serum ALT level is the most predictive laboratory investigation for NAFLD. The AST-ALT Ratio (AAR) is higher in increased liver fat content, fibrosis, and other metabolic derangements like diabetes and dyslipidemia. Steatosis or steatohepatitis can be observed, but nevertheless patients have normal serum ALT levels[8]. An AAR > 1 is consistent with a diagnosis of NASH. This forms the basis of several other laboratory combinations that may indicate the progression of NAFLD and diagnosing liver fibrosis including BAAT (which uses BMI, age, ALT, and triglycerides), BARD (which uses BMI, AST: ALT, and diabetes), and FIB-4 scores[21,22]. The FIB-4 score is a simple, noninvasive and inexpensive test superior to BAAT and BARD scores in monitoring the progress of NASH[17]. The FIB-4 score is reliable in ruling out advanced fibrosis in patients with histological evidence of NAFLD who had normal or increased levels of ALT, thus decreasing the need for invasive liver biopsy with sensitivity 84%-94%[18].

ALP: Few subsets of NASH patients present with an isolated ALP elevation[23]. Cholestasis also has been noted on histology in NASH[23]. Elevated ALP should be accompanied by an increase in γ-glutamyltransferase (GGT) enzyme suggesting hepatic inflammation. Otherwise elevated ALP without GGT elevation are seen in pregnancy, muscular disease and bone disease such as Paget's disease.

Bilirubin: Bilirubin is synthetic marker for liver function alongside with PT/INR. Also, it is a part of various scoring system used to estimate the degree of fibrosis. Demir *et al*[24] introduced the non-invasive koeln-essen-index (NIKEI) score which uses age, AST, AST/ALT ratio, and total bilirubin. In a prospective study by Ratziu *et al*[25], the diagnostic utility of FibroTest, a noninvasive marker of fibrosis, was determined in a sample of 170 patients with NAFLD. The FibroTest includes α-2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin, and γ-glutamyl-transpeptidase. Ratziu concluded this simple and noninvasive quantitative estimate of liver fibrosis reliably predicts advanced fibrosis[25]. Hepascore, a combination of bilirubin, γ-glutamyl-transpeptidase, hyaluronic acid, and 2-macroglobulin together with age and sex, is an accurate and reliable panel in predicting different stages of fibrosis. However, the limitation of this study included validation of this score among

only patients with hepatitis C[21]. NIKEI had superior negative predictive value for advanced fibrosis compared to the FIB-4 score (which uses age, AST, ALT, and platelet counts)[24].

Platelet count: Platelet count has great value in assessing degree of fibrosis. Thrombocytopenia occurs in cirrhosis secondary to thrombopoietin deficiency and splenic sequestration from underlying splenomegaly occurring from portal hypertension. Platelet level has been used in combination with other biochemical parameters such as in AST/platelet ratio index and FIB-4 score to monitor liver disease progression[21]. Kawamura established the fibrosis score for NASH (FSN), a new scoring system specific to the fibrotic stage of NASH[26]. FSN can accurately predict the fibrotic stage and distinguishes patients with advanced fibrosis of NASH. The platelet albumin AAR (PLALA) score is unique in that it distinguishes cirrhosis in NAFLD compared to most other fibrosis scoring systems. Each factor (platelet count < 15.3×10^4/μL; albumin < 4 g/dL; AAR > 0.9) is awarded 1 point, and a PLALA score of 2 or 3 may be predictive of cirrhosis in patients with NAFLD[27]. The PLALA score may be an ideal scoring system for detecting cirrhosis in NAFLD patients with sufficient accuracy and simplicity for clinical use. Mean platelet volume (MPV) was elevated in NASH and advanced liver fibrosis (stages 3–4) patients, making MPV a noninvasive, novel marker to predict advanced disease. Another study looked into the performance of red cell volume distribution width-to-platelet ratio in predicting liver fibrosis in patients with NAFLD[27]. This ratio was both correlated and able to predict liver fibrosis.

Fasting blood glucose and glycosylated protein: In their observational cohort of 118 patients, assessing the clinical determinants of fibrosis progression rate in NAFLD patients with baseline and follow-up histological evaluation. Advanced fibrosis is more likely to be found in patients with underlying type 2 diabetes[28]. These patients had histological evidence of more inflammation in the fibrous portal areas in those already developing cirrhosis than those at an earlier stage of the disease. Furthermore, this study also observed that type 2 diabetes can drive fibrosis in the absence of hepatic inflammation.

Glycosylated albumin to glycosylated hemoglobin ratio: Glycosylated albumin (GA) and glycosylated hemoglobin (HbA1c), which are indicators of glycemic control, show a strong relationship with advanced liver fibrosis. The GA/HbA1c ratio, which is typically 3 in a healthy individual, is higher in liver fibrosis patients. Patients with chronic liver diseases have reduced albumin turnover resulting in an elevated level of GA. Also, they have a reduced erythrocyte lifespan which accounts for changes in the increased ratio. The GA/HbA1c ratio's accuracy in detecting liver fibrosis might be limited by other concurrent diseases that can affect plasma and hemoglobin levels[25]. The HOMA-insulin resistance score is a somewhat rigorous and reliable scoring system that indicates NAFLD progression using a formula that involves insulin levels and fasting glucose to calculate insulin resistance[28]. The HOMA-insulin resistance score has a high sensitivity for NASH. The NAFLD fibrosis score (NFS) is a noninvasive score (using age, albumin, AST/ALT ratio, BMI, the presence of diabetes or impaired fasting glucose, and platelet count) most predictive of mortality in NASH compared to NAFLD[29]. As of 2015, the NFS score was endorsed by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association as a screening guideline in clinical practice. These screening tools may be more important in detecting NASH in people with diabetes.

Hyaluronic acid tissue metalloproteinase: A high level of hyaluronic acid (hyaluronate) tissue metalloproteinase 1 has been indicative of fibrosis. The European liver fibrosis scoring system has indicators for cellular matrix activities including age, the amino-terminal peptide of procollagen III, tissue metalloproteinase 1 inhibitor, and hyaluronic acid[22].

Type IV collagen: The FSN score, which includes type IV collagen 7S, platelet count, AST, and ALT, has been more efficient in distinguishing the advanced fibrosis stages 3–4 of NASH compared to other scoring systems including APRI (AST to platelet ratio index), NAFLD Score, FIB-4 Index, BARD, and NIKEI[26]. The nonalcoholic steatohepatitis, ferritin, insulin, and type IV collagen 7S (NAFIC) score, and modified NAFIC score were proven to be clinically useful in screening for fatty liver patients[30].

Albumin: Bazick *et al*[5] demonstrated that serum albumin gets reduced drastically in patients presenting with NASH. Their clinical variable could be used to guide clinical

decision making about referring patients with diabetes and NAFLD to hepatolo-gists [5].

Prothrombin time: In a study by Assy *et al*[31], up to 46% of patients with NAFLD showed thrombotic risk factors. The presence of thrombotic risk factors correlated with the extent of hepatic fibrosis. This is consistent with known coagulopathy in those with altered synthetic function due to hepatic fibrosis.

ROLE OF IMAGING TECHNIQUES IN THE CLINICAL PROGRESSION OF NASH

Noninvasive techniques such as ultrasonography (US), computed tomography, magnetic resonance imaging, and proton magnetic resonance spectroscopy can detect hepatic steatosis but cannot reliably distinguish simple steatosis from NASH[32].

Liver US

US is the preferred cost effective method in the United States for screening patients with suspected NAFLD. The findings on US include: diffuse increase in echogenicity of the liver parenchyma, hepatomegaly and vascular blunting[33]. The sensitivity of US in detecting hepatic steatosis up to 94%. The sensitivity decreases as the degree of steatosis dropped below 30%[33-35]. US cannot differentiate between simple hepatic steatosis *vs* NASH. Thus, laboratory serological and histological data is helpful in pointing towards NASH[22].

MRE

MRE stands for magnetic resonance elastography which is an imaging technique that combines MRI imaging with low-frequency vibrations to measure hepatic stiffness. MRE equivalent of transient elastography has recently demonstrated excellent diagnostic accuracy. It has shown a sensitivity and specificity of 98% and 99%, respectively, for detecting all grades of fibrosis[35]. Huwart *et al*[36] conducted a prospective blind comparison of MRE, US elastography, and APRI (AST to platelet ratio index) in a study of 141 patients who underwent liver biopsy for chronic liver disease. They found MRE was associated with a higher technical success rate than US elastography[36].

Fibroscan

Transient Elastography (Fibroscan, Echosens, Paris, France) is a noninvasive method of assessing liver fibrosis. It can be performed at the bedside or in the outpatient clinic. It employs US-based technology to measure liver stiffness and has been validated for use in patients with chronic hepatitis C and B[37,38]. However, studies have shown good results in patients with NAFLD[39,40]. In only 5% of the cases, it has failed to show any readings. This is mostly seen in obese patients. This limits the TE's utility in the NAFLD cohort. However, a recently introduced XL probe may reduce this problem[41]. In a meta-analysis for NASH with advanced fibrosis, pooled area under the receiver operating characteristic curve, sensitivity and specificity of NFS, and fibroscan are 0.85 (0.80-0.93), 0.90 (0.82-0.99), 0.97 (0.94-0.99), and 0.94 (0.90-0.99), 0.94 (0.88-0.99) and 0.95 (0.89-0.99), respectively[16]. Fibroscan is validated in NAFLD and represents a useful tool for rapid, noninvasive assessment of liver fibrosis and determining the need for biopsy. As this modality evaluates liver stiffness (related to fibrosis, inflammation, and portal hypertension), Fibroscan values should be interpreted in context of the morphological, biological, and clinical data.

ROLE OF LIVER BIOPSY IN THE CLINICAL PROGRESSION OF NASH

A percutaneous liver biopsy is currently the gold standard to assess hepatic fibrosis and inflammation in chronic liver disease[42]. However, liver biopsy is an invasive procedure with associated costs, complications, and inherent inaccuracy due to sampling error and inter-observer and intra-observer variability in histopathological interpretation[42,43]. Despite the criticism of liver biopsy's associated risks, there are 3 basic histological systems that can be used to monitor the progression of NASH (Figure 2). These systems are the steatosis activity and fibrosis score, the NASH

Table 3 Nonalcoholic steatohepatitis activity score; steatosis, activity, and fibrosis score; and brunt grading and staging systems

NASH activity score	Steatosis, activity and fibrosis score	Brunt grading and staging
Steatosis grade 0-3	Steatosis S0-S3	Grade 1 (Mild)
Lobular inflammation 0-3	Activity A1-A3	Grade 2 (Moderate)
Ballooning 0-2	Lobular inflammation 0-2	Grade 3 (Severe)
Fibrosis 0-4 (grade 1 has subgrade A, B, C)	Ballooning 0-2	Stages fibrosis
	Fibrosis F0-F4	Stage 1-4

NASH: Nonalcoholic steatohepatitis.

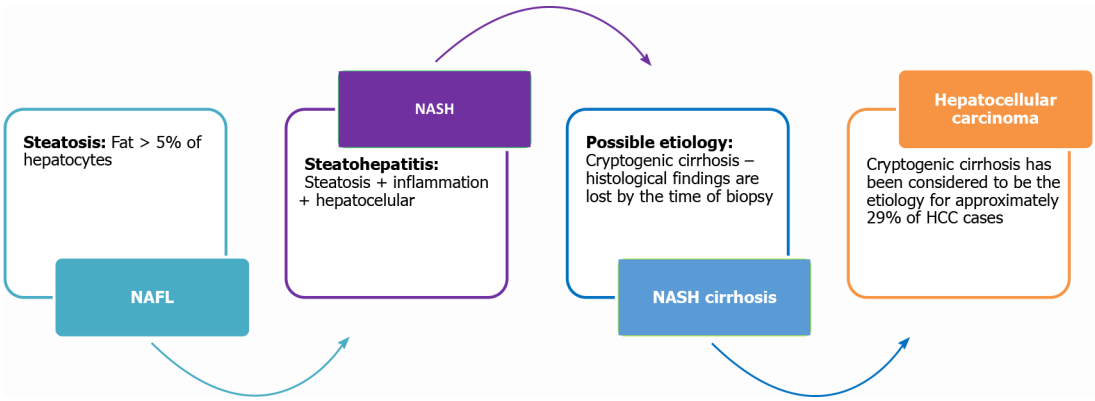


Figure 2 The spectrum of nonalcoholic fatty liver disease. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

activity score, and the Brunt system that grades and stages NASH (Table 3)[28,44]. Due to the risks and limitations associated with liver biopsy, it is controversial to perform liver biopsy on every patient suspected of having NAFLD. Therefore, it cannot be considered a "screening" tool[45]. However, there are studies that support the importance of liver biopsy. An older study, by Skelly *et al*[46] showed that biopsy on 354 patients with abnormal liver tests-66% had fatty liver, 50% of those had steatohepatitis, and approximately 19% of the remaining biopsies had other treatable causes diagnosed by the pathology evaluation. This included autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis and alcoholic liver disease. An adequate liver biopsy, with appropriate clinical history, interpreted by a trained liver pathologist, is not only pivotal for an accurate and complete diagnosis (or exclusion) of NAFLD (or NASH), but also is optimal for obtaining detailed information regarding disease pattern, severity and fibrosis. It not only provides important information with respect to subtypes, potential future risks, possible etiology, and natural history of disease, but also sets the groundwork for future molecular studies and clinical trials, assisting clinical colleagues and patients with treatments and follow-up[47-49].

CONCLUSION

NASH-related cirrhosis is the most common cause of chronic liver disease and indication for liver transplant. The increasing number of affected people imposes a strain on available organs. There are many comorbidities and risk factors implicated in NASH severity and progression to chronic liver disease.

Due to the increasing prevalence of NAFLD in the population, there is an increasing need to find non- invasive methods to diagnose and stage NAFLD. The ideal test should be reproducible, cheap, and able to diagnose full spectrum of NAFLD, predict fibrosis, and reflect changes that occur with treatment. Preliminary evaluation includes clinical presentation with consideration of comorbidities and liver function test in the blood. Noninvasive imaging such as MRE and fibroscan can provide objective measures of liver steatosis and stiffness in patients without advanced fibrosis or

cirrhosis. Due to the limitations, risks and cost of liver biopsy-it cannot be used as a screening test, although is typically relied upon to confirm the diagnosis. Several different methodologies including imaging modalities, serum markers and combined tests are currently being investigated.

REFERENCES

1 **Schaffner F**, Thaler H. Nonalcoholic fatty liver disease. *Prog Liver Dis* 1986; **8**: 283-298 [PMID: 3086934]
2 **Fouad Y**, Waked I, Bollipo S, Gomaa A, Ajlouni Y, Attia D. What's in a name? *Liver Int* 2020; **40**: 1254-1261 [PMID: 32301554 DOI: 10.1111/Liv.14478]
3 **Ruhl CE**, Everhart JE. Fatty liver indices in the multiethnic United States National Health and Nutrition Examination Survey. *Aliment Pharmacol Ther* 2015; **41**: 65-76 [PMID: 25376360 DOI: 10.1111/apt.13012]
4 **Bellentani S**, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. *Dig Dis* 2010; **28**: 155-161 [PMID: 20460905 DOI: 10.1159/000282080]
5 **Bazick J**, Donithan M, Neuschwander-Tetri BA, Kleiner D, Brunt EM, Wilson L, Doo E, Lavine J, Tonascia J, Loomba R. Clinical Model for NASH and Advanced Fibrosis in Adult Patients With Diabetes and NAFLD: Guidelines for Referral in NAFLD. *Diabetes Care* 2015; **38**: 1347-1355 [PMID: 25887357 DOI: 10.2337/dc14-1239]
6 **Tevar AD**, Clarke C, Wang J, Rudich SM, Woodle ES, Lentsch AB, Edwards ML. Clinical review of nonalcoholic steatohepatitis in liver surgery and transplantation. *J Am Coll Surg* 2010; **210**: 515-526 [PMID: 20347746 DOI: 10.1016/j.jamcollsurg.2010.01.020]
7 **Takahashi Y**, Fukusato T. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol* 2014; **20**: 15539-15548 [PMID: 25400438 DOI: 10.3748/wjg.v20.i42.15539]
8 **Satapathy SK**, Sanyal AJ. Epidemiology and Natural History of Nonalcoholic Fatty Liver Disease. *Semin Liver Dis* 2015; **35**: 221-235 [PMID: 26378640 DOI: 10.1055/s-0035-1562943]
9 **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]
10 **Harrison SA**, Torgerson S, Hayashi PH. The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. *Am J Gastroenterol* 2003; **98**: 2042-2047 [PMID: 14499785 DOI: 10.1111/j.1572-0241.2003.07659.x]
11 **Adams LA**, Sanderson S, Lindor KD, Angulo P. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005; **42**: 132-138 [PMID: 15629518 DOI: 10.1016/j.jhep.2004.09.012]
12 **Machado MV**, Cortez-Pinto H. Non-alcoholic fatty liver disease: what the clinician needs to know. *World J Gastroenterol* 2014; **20**: 12956-12980 [PMID: 25278691 DOI: 10.3748/wjg.v20.i36.12956]
13 **Rosenberg WM**, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, Hubscher S, Roskams T, Pinzani M, Arthur MJ; European Liver Fibrosis Group. Serum markers detect the presence of liver fibrosis: a cohort study. *Gastroenterology* 2004; **127**: 1704-1713 [PMID: 15578508 DOI: 10.1053/j.gastro.2004.08.052]
14 **Guha IN**, Parkes J, Roderick P, Chattopadhyay D, Cross R, Harris S, Kaye P, Burt AD, Ryder SD, Aithal GP, Day CP, Rosenberg WM. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. *Hepatology* 2008; **47**: 455-460 [PMID: 18038452 DOI: 10.1002/hep.21984]
15 **Angulo P**, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, Saksena S, Burt AD, Bida JP, Lindor K, Sanderson SO, Lenzi M, Adams LA, Kench J, Therneau TM, Day CP. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007; **45**: 846-854 [PMID: 17393509 DOI: 10.1002/hep.21496]
16 **Musso G**, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011; **43**: 617-649 [PMID: 21039302 DOI: 10.3109/07853890.2010.518623]
17 **Shah AG**, Lydecker A, Murray K, Tetri BN, Contos MJ, Sanyal AJ; Nash Clinical Research Network. Comparison of noninvasive markers of fibrosis in patients with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2009; **7**: 1104-1112 [PMID: 19523535 DOI: 10.1016/j.cgh.2009.05.033]
18 **McPherson S**, Anstee QM, Henderson E, Day CP, Burt AD. Are simple noninvasive scoring systems for fibrosis reliable in patients with NAFLD and normal ALT levels? *Eur J Gastroenterol Hepatol* 2013; **25**: 652-658 [PMID: 23325287 DOI: 10.1097/MEG.0b013e32835d72cf]
19 **Perumpail BJ**, Khan MA, Yoo ER, Cholankeril G, Kim D, Ahmed A. Clinical epidemiology and disease burden of nonalcoholic fatty liver disease. *World J Gastroenterol* 2017; **23**: 8263-8276 [PMID: 29307986 DOI: 10.3748/wjg.v23.i47.8263]
20 **Martin-Rodriguez JL**, Gonzalez-Cantero J, Gonzalez-Cantero A, Arrebola JP, Gonzalez-Calvin JL. Diagnostic accuracy of serum alanine aminotransferase as biomarker for nonalcoholic fatty liver disease and insulin resistance in healthy subjects, using 3T MR spectroscopy. *Medicine (Baltimore)*

2017; **96**: e6770 [PMID: 28445310 DOI: 10.1097/MD.0000000000006770]
21 **Enomoto H**, Bando Y, Nakamura H, Nishiguchi S, Koga M. Liver fibrosis markers of nonalcoholic steatohepatitis. *World J Gastroenterol* 2015; **21**: 7427-7435 [PMID: 26139988 DOI: 10.3748/wjg.v21.i24.7427]
22 **Arora A**, Sharma P. Non-invasive Diagnosis of Fibrosis in Non-alcoholic Fatty Liver Disease. *J Clin Exp Hepatol* 2012; **2**: 145-155 [PMID: 25755423 DOI: 10.1016/S0973-6883(12)60103-0]
23 **Pantsari MW**, Harrison SA. Nonalcoholic fatty liver disease presenting with an isolated elevated alkaline phosphatase. *J Clin Gastroenterol* 2006; **40**: 633-635 [PMID: 16917408 DOI: 10.1097/00004836-200608000-00015]
24 **Demir M**, Lang S, Schlattjan M, Drebber U, Wedemeyer I, Nierhoff D, Kaul I, Sowa J, Canbay A, Töx U, Steffen HM. NIKEI: a new inexpensive and non-invasive scoring system to exclude advanced fibrosis in patients with NAFLD. *PLoS One* 2013; **8**: e58360 [PMID: 23555578 DOI: 10.1371/journal.pone.0058360]
25 **Ratziu V**, Massard J, Charlotte F, Messous D, Imbert-Bismut F, Bonyhay L, Tahiri M, Munteanu M, Thabut D, Cadranel JF, Le Bail B, de Ledinghen V, Poynard T; LIDO Study Group; CYTOL study group. Diagnostic value of biochemical markers (FibroTest-FibroSURE) for the prediction of liver fibrosis in patients with non-alcoholic fatty liver disease. *BMC Gastroenterol* 2006; **6**: 6 [PMID: 16503961 DOI: 10.1186/1471-230x-6-6]
26 **Kawamura Y**, Ikeda K, Arase Y, Sorin Y, Fukushima T, Kunimoto H, Hosaka T, Kobayashi M, Saitoh S, Sezaki H, Akuta N, Suzuki F, Suzuki Y, Kumada H. New discriminant score to predict the fibrotic stage of non-alcoholic steatohepatitis in Japan. *Hepatol Int* 2015; **9**: 269-277 [PMID: 25788193 DOI: 10.1007/s12072-014-9605-x]
27 **Kessoku T**, Ogawa Y, Yoneda M, Imajo K, Sumida Y, Eguchi Y, Fujii H, Hyogo H, Ono M, Suzuki Y, Kawaguchi T, Chayama K, Tanaka S, Fujimoto K, Anzai K, Saibara T, Sata M, Itoh Y, Nakajima A, Okanoue T; Japan Study Group of NAFLD (JSG-NAFLD). Simple scoring system for predicting cirrhosis in nonalcoholic fatty liver disease. *World J Gastroenterol* 2014; **20**: 10108-10114 [PMID: 25110437 DOI: 10.3748/wjg.v20.i29.10108]
28 **Hu KC**, Wang HY, Liu SC, Liu CC, Hung CL, Bair MJ, Liu CJ, Wu MS, Shih SC. Nonalcoholic fatty liver disease: updates in noninvasive diagnosis and correlation with cardiovascular disease. *World J Gastroenterol* 2014; **20**: 7718-7729 [PMID: 24976709 DOI: 10.3748/wjg.v20.i24.7718]
29 **Stål P**. Liver fibrosis in non-alcoholic fatty liver disease - diagnostic challenge with prognostic significance. *World J Gastroenterol* 2015; **21**: 11077-11087 [PMID: 26494963 DOI: 10.3748/wjg.v21.i39.11077]
30 **Nakamura A**, Yoneda M, Sumida Y, Eguchi Y, Fujii H, Hyogo H, Ono M, Suzuki Y, Kawaguchi T, Aoki N, Okanoue T, Nakajima A, Maeda S, Terauchi Y. Modification of a simple clinical scoring system as a diagnostic screening tool for non-alcoholic steatohepatitis in Japanese patients with non-alcoholic fatty liver disease. *J Diabetes Investig* 2013; **4**: 651-658 [PMID: 24843721 DOI: 10.1111/jdi.12101]
31 **Assy N**, Bekirov I, Mejritsky Y, Solomon L, Szvalb S, Hussein O. Association between thrombotic risk factors and extent of fibrosis in patients with non-alcoholic fatty liver diseases. *World J Gastroenterol* 2005; **11**: 5834-5839 [PMID: 16270394 DOI: 10.3748/wjg.v11.i37.5834]
32 **Sanyal AJ**; American Gastroenterological Association. AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology* 2002; **123**: 1705-1725 [PMID: 12404245 DOI: 10.1053/gast.2002.36572]
33 **Mishra P**, Younossi ZM. Abdominal ultrasound for diagnosis of nonalcoholic fatty liver disease (NAFLD). *Am J Gastroenterol* 2007; **102**: 2716-2717 [PMID: 18042105 DOI: 10.1111/j.1572-0241.2007.01520.x]
34 **Strauss S**, Gavish E, Gottlieb P, Katsnelson L. Interobserver and intraobserver variability in the sonographic assessment of fatty liver. *AJR Am J Roentgenol* 2007; **189**: W320-W323 [PMID: 18029843 DOI: 10.2214/ajr.07.2123]
35 **Iijima H**, Moriyasu F, Tsuchiya K, Suzuki S, Yoshida M, Shimizu M, Sasaki S, Nishiguchi S, Maeyama S. Decrease in accumulation of ultrasound contrast microbubbles in non-alcoholic steatohepatitis. *Hepatol Res* 2007; **37**: 722-730 [PMID: 17559420 DOI: 10.1111/j.1872-034X.2007.00130.x]
36 **Huwart L**, Sempoux C, Vicaut E, Salameh N, Annet L, Danse E, Peeters F, ter Beek LC, Rahier J, Sinkus R, Horsmans Y, Van Beers BE. Magnetic resonance elastography for the noninvasive staging of liver fibrosis. *Gastroenterology* 2008; **135**: 32-40 [PMID: 18471441 DOI: 10.1053/j.gastro.2008.03.076]
37 **Boursier J**, de Ledinghen V, Zarski JP, Rousselet MC, Sturm N, Foucher J, Leroy V, Fouchard-Hubert I, Bertrais S, Gallois Y, Oberti F, Dib N, Calès P. A new combination of blood test and fibroscan for accurate non-invasive diagnosis of liver fibrosis stages in chronic hepatitis C. *Am J Gastroenterol* 2011; **106**: 1255-1263 [PMID: 21468012 DOI: 10.1038/ajg.2011.100]
38 **Osakabe K**, Ichino N, Nishikawa T, Sugiyama H, Kato M, Kitahara S, Hashimoto S, Kawabe N, Harata M, Nitta Y, Murao M, Nakano T, Shimazaki H, Arima Y, Suzuki K, Yoshioka K. Reduction of liver stiffness by antiviral therapy in chronic hepatitis B. *J Gastroenterol* 2011; **46**: 1324-1334 [PMID: 21822591 DOI: 10.1007/s00535-011-0444-4]
39 **Wong VW**, Chu WC, Wong GL, Chan RS, Chim AM, Ong A, Yeung DK, Yiu KK, Chu SH, Woo J, Chan FK, Chan HL. Prevalence of non-alcoholic fatty liver disease and advanced fibrosis in Hong Kong Chinese: a population study using proton-magnetic resonance spectroscopy and transient

elastography. *Gut* 2012; **61**: 409-415 [PMID: 21846782 DOI: 10.1136/gutjnl-2011-300342]

40 **Wong VW**, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, Choi PC, Kowo M, Chan AW, Merrouche W, Sung JJ, de Lédinghen V. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51**: 454-462 [PMID: 20101745 DOI: 10.1002/hep.23312]

41 **de Lédinghen V**, Vergniol J, Foucher J, El-Hajbi F, Merrouche W, Rigalleau V. Feasibility of liver transient elastography with FibroScan using a new probe for obese patients. *Liver Int* 2010; **30**: 1043-1048 [PMID: 20492500 DOI: 10.1111/j.1478-3231.2010.02258.x]

42 **Myers RP**, Fong A, Shaheen AA. Utilization rates, complications and costs of percutaneous liver biopsy: a population-based study including 4275 biopsies. *Liver Int* 2008; **28**: 705-712 [PMID: 18433397 DOI: 10.1111/j.1478-3231.2008.01691.x]

43 **Ratziu V**, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, Grimaldi A, Capron F, Poynard T; LIDO Study Group. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005; **128**: 1898-1906 [PMID: 15940625 DOI: 10.1053/j.gastro.2005.03.084]

44 **Benedict M**, Zhang X. Non-alcoholic fatty liver disease: An expanded review. *World J Hepatol* 2017; **9**: 715-732 [PMID: 28652891 DOI: 10.4254/wjh.v9.i16.715]

45 **Nalbantoglu IL**, Brunt EM. Role of liver biopsy in nonalcoholic fatty liver disease. *World J Gastroenterol* 2014; **20**: 9026-9037 [PMID: 25083076 DOI: 10.3748/wjg.v20.i27.9026]

46 **Skelly MM**, James PD, Ryder SD. Findings on liver biopsy to investigate abnormal liver function tests in the absence of diagnostic serology. *J Hepatol* 2001; **35**: 195-199 [PMID: 11580141 DOI: 10.1016/s0168-8278(01)00094-0]

47 **Abdel-Razik A**, Mousa N, Shabana W, Refaey M, ElMahdy Y, Elhelaly R, Elzehery R, Zalata K, Arafa M, Elbaz S, Hafez M, Awad M. A novel model using mean platelet volume and neutrophil to lymphocyte ratio as a marker of nonalcoholic steatohepatitis in NAFLD patients: multicentric study. *Eur J Gastroenterol Hepatol* 2016; **28**: e1-9 [PMID: 26469357 DOI: 10.1097/meg.0000000000000486]

48 **Cengiz M**, Ozenirler S. Comparative diagnostic accuracy of red cell distribution width-to-platelet ratio versus noninvasive fibrosis scores for the diagnosis of liver fibrosis in biopsy-proven nonalcoholic fatty liver disease. *Eur J Gastroenterol Hepatol* 2015; **27**: 1293-1299 [PMID: 26302023 DOI: 10.1097/meg.0000000000000445]

49 **Pelusi S**, Petta S, Rosso C, Borroni V, Fracanzani AL, Dongiovanni P, Craxi A, Bugianesi E, Fargion S, Valenti L. Renin-Angiotensin System Inhibitors, Type 2 Diabetes and Fibrosis Progression: An Observational Study in Patients with Nonalcoholic Fatty Liver Disease. *PLoS One* 2016; **11**: e0163069 [PMID: 27649410 DOI: 10.1371/journal.pone.0163069]

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MINIREVIEWS

Update on the management and treatment of viral hepatitis

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Abstract

This review aims to summarize the current evidence on the treatment of viral hepatitis, focusing on its clinical management. Also, future treatment options and areas of potential research interest are detailed. PubMed and Scopus databases were searched for primary studies published within the last ten years. Keywords included hepatitis A virus, hepatitis B virus (HBV), hepatitis C virus, hepatitis D virus (HDV), hepatitis E virus, and treatment. Outcomes reported in the studies were summarized, tabulated, and synthesized. Significant advances in viral hepatitis treatment were accomplished, such as the advent of curative therapies for hepatitis C and the development and improvement of hepatitis A, hepatitis B, and hepatitis E vaccination. Drugs that cure hepatitis B, going beyond viral suppression, are so far unavailable; however, targeted antiviral drugs against HBV (immunomodulatory therapies and gene silencing technologies) are promising approaches to eradicating the virus. Ultimately, high vaccination coverage and large-scale test-and-treat programmes with high screening rates may eliminate viral hepatitis and mitigate their burden on health systems. The development of curative hepatitis C treatment renewed the enthusiasm for curing hepatitis B, albeit further investigation is required. Novel therapeutic options targeting HDV life cycle are currently under clinical investigation.

Key Words: Viral hepatitis; Hepatitis A virus; Hepatitis B virus; Hepatitis C virus; Hepatitis D virus; Hepatitis E virus

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Core Tip: Viral hepatitis is a major global public health problem due to the risk of progression to chronic hepatitis, cirrhosis, and hepatocellular carcinoma development. The clinical management and treatment of these infections have evolved over the last decade. Even though remarkable achievements have been accomplished, such as the development of curative hepatitis C treatment, drugs that cure hepatitis B are still missing. In addition, programmes to enhance viral hepatitis testing and treatment together with broad vaccination coverage are required. In this review, we summarize the current evidence on the treatment of viral hepatitis and detail future treatment options, and potential areas of research.

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INTRODUCTION

Viral hepatitis is a major public health problem given that it can become chronic, and eventually lead to end-stage liver disease and/or hepatocellular carcinoma (HCC) development[1,2]. Consequently, viral hepatitis is one of the leading indications for liver transplantation and, thus, contributes to the discrepancy between donor organ supply and demand[3].

Whereas hepatitis A and E usually present with a self-limited course followed by complete recovery, hepatitis B and C often result in chronic infection and they are responsible for the most adverse consequences of this disease[4]. Worldwide, approximately 100 million people have the antibody against the hepatitis C virus (HCV) and 71 million present HCV viremia, according to the World Health Organization (WHO)[5]. In a multicentre international study, with the participation of 161 countries, the prevalence of the hepatitis B virus (HBV) surface antigen (HBsAg) was 3.61%[6]. Due to the high prevalence, WHO has set targets for eliminating hepatitis B and C by 2030. These targets include optimizing measures to prevent disease transmission and improving antiviral treatment offering[7]. In the last decade, rapid and significant advances in diagnosing and managing viral hepatitis have been made and have changed its treatment. Despite these advances, issues with screening, diagnosis, referral, and treatment of viral hepatitis still persist.

Therefore, due to the high prevalence of viral hepatitis and its serious consequences, research activity in this field has always been intense, and new and increasingly effective treatments have gradually emerged. This review aims to summarize the current evidence on the treatment of viral hepatitis, focusing on its clinical management. Also, future treatment options and areas of potential research interest are detailed.

HEPATITIS A

Hepatitis A is caused by the hepatitis A virus (HAV), a ribonucleic acid (RNA) picornavirus. The virus is transmitted by the faecal–oral route and this is a major cause of acute viral hepatitis. Clinical manifestations range from asymptomatic infection to acute liver failure (ALF), occurring in less than 1% of cases, and there is no progression to chronic hepatitis[8]. Globally, an estimated 1.4 million cases of hepatitis A occur each year and 27731 deaths were registered in 2010[8]. This disease can occur sporadically or in an epidemic form and risk factors for transmission are mainly person-to-person contact related or *via* contaminated food or water[9,10].

Hepatic injury results from the host immune response to the HAV. Viral replication occurs in the hepatocyte cytoplasm and hepatocellular damage is caused by the destruction of infected cells mediated by human leukocyte antigen-restricted HAV-specific CD8+ T lymphocytes and natural killer cells[8]. Exaggerated host response and marked reduction of circulation HAV RNA during acute infection are associated

with severe hepatitis. The development of symptomatic hepatitis is usually related to patient age as more than 70% of infected adults develop symptoms[8]. Full clinical and biochemical recovery is observed within two to three months in 85% of patients and complete recovery is observed by six months in nearly all patients[11]. The diagnosis is established by detection of serum immunoglobulin M antibody to HAV, which remains detectable for approximately three to six months. Serum immunoglobulin G antibodies appear early in the convalescent phase of the disease, remain detectable for decades, and are associated with lifelong protective immunity[8,11].

To date, there are no specific drugs against HAV infection available; thus, treatment consists mostly of supportive care[8,11]. Prevention of HAV infection includes vaccination, immune globulin, and attention to hygienic practices-handwashing, avoiding consumption of tap water and raw foods in areas with poor sanitation, and heating foods appropriately[12]. In summary, indications for vaccination include children aged 2-18 years who have not previously received hepatitis A vaccine, all persons aged more than one year infected with human immunodeficiency virus, and specific risk groups (individuals with chronic liver disease, travellers, men who have sex with men, *etc*.). Also, vaccination strategies may vary according to local public health policies in each country[8,12].

HEPATITIS B

Although an effective preventive hepatitis B vaccine has existed for over 30 years, HBV infection is still a major cause of chronic liver disease worldwide[13]. HBV is a small deoxyribonucleic acid (DNA) virus of the Hepadnaviridae family. HBV infects hepatocytes and establishes its replication cycle *via* an RNA intermediate (through reverse transcription) and can integrate into the host genome, thus being able to persist in the nucleus of hepatocytes[13,14]. The viral envelope involves a nucleocapsid that contains a partially double-stranded and relaxed circular DNA genome (rcDNA)[15]. In the cytoplasm of infected hepatocytes, the nucleocapsid is transported to the nucleus and then the rcDNA is released and converted into a covalently closed circular DNA (cccDNA) by host factors, forming a stable minichromosome[15,16].

Chronic hepatitis B is a dynamic infectious disease with a pattern of progression strongly dependent on the interaction between the host immune response and the virus. Over two-thirds of patients with chronic hepatitis B are inactive carriers. They present a low viral replication rate and minimal or no liver necroinflammation, secondary to weak activation of the innate immunity and HBV-specific immunological response[17].

The definition of goals for HBV treatment is essential. A virological response during nucleos(t)ide analogue (NA) therapy is defined as a decrease in serum HBV DNA to undetectable levels by tests with a lower limit of detection of 10–20 IU/mL. If interferon (IFN) alpha is used for treatment, the virological response is defined as a serum level of HBV DNA below 2000 IU/mL, assessed at 6 mo after the start of treatment and at the end of the therapy[14]. The biochemical response is defined as the normalization of serum alanine aminotransferase. Biochemical response allied to a reduction in HBV viral load is an important goal to be achieved because they are both associated with a decreased risk of progression to cirrhosis and HCC[14,18].

Current key targets of HBV treatment are a functional cure and a complete or "sterilizing" cure[19,20]. A functional or partial cure is defined as a sustained loss of HBsAg with or without anti-HBs seroconversion, based on assays with a lower limit of HBsAg detection of 0.05 IU/mL. Complete cure is defined as the elimination of cccDNA together with sustained loss of HBsAg and undetectable serum HBV DNA[19,20]. Whilst liver biopsy is currently necessary to measure the intrahepatic activity of cccDNA, serum biomarkers that reflect this indicator have been examined for this purpose[21].

The persistence of cccDNA in the hepatocyte nucleus is the greatest therapeutic challenge in hepatitis B patient care. Even among patients who recover from acute infection, presenting HBsAg loss with HBsAg seroconversion, HBV may persist in a latent state. These patients are potentially at risk of reactivation if exposed to either cancer chemotherapy or immunosuppressive therapies (after transplantation, for example)[22,23].

Although lamivudine was used for many decades to treat chronic hepatitis B-due to its safety and low cost, the low genetic barrier and the risk of developing drug resistance resulted in this being a less effective therapy compared to other treatment agents. Currently, lamivudine therapy is reserved for specific situations, for example,

the unavailability of entecavir or tenofovir[24]. In addition, this treatment may still play a role in HIV-coinfected patients when used as part of an antiretroviral regimen [24].

The two formulations of IFN (conventional and pegylated) and five NAs [telbivudine, entecavir, tenofovir disoproxil fumarate (TDF), tenofovir alafenamide fumarate (TAF), and besifovir dipivoxil] are antiviral agents used for chronic hepatitis B treatment. Albeit these drugs strongly suppress HBV replication, reduce the risk of cirrhosis, and prevent further disease progression, they are not curative and have no proved positive impact on the existing viral hepatocyte reservoir[24]. According to major hepatology societies, entecavir, TDF, TAF, and pegylated (Peg) IFN alpha are currently the first-line anti-HBV agents recommended for chronic hepatitis B treatment [25-27].

Over the last few years, TAF was developed as a safer alternative to TDF because the latter is associated with both proximal renal tubular dysfunction and low bone mineral density. Due to the pharmacological properties of TAF, far more active drug is delivered to target cells while much less is measurable in the bloodstream, reducing systemic toxicity[28,29]. These properties are especially beneficial for elderly patients, patients with renal dysfunction, or osteoporosis[26,28-30].

NAs and IFN have different modes of action as well as particular advantages and disadvantages. On the one hand, compared to NA, IFN has the advantages of being a treatment with a finite duration, absence of resistance, and a higher chance of off-treatment sustained virological response (SVR); as well as potentially offering a greater opportunity for sustained loss of HBsAg/anti-HBs seroconversion. Yet, IFN has the disadvantage of moderate antiviral effects, low tolerability, and an increased risk of adverse events[24,31]. On the other hand, compared to IFN, NA therapy has higher rates of undetectable serum HBV DNA and transaminase normalization after treatment, whilst requiring long-term therapy-hardly envisioning withdrawal-due to the high rate of disease recurrence after discontinuing the medication[19,20].

Importantly, proper patient selection for better clinical efficacy in HBV treatment with Peg-IFN alpha is essential. Female gender, young age, high level of transaminases, lower level of HBV DNA, high rate of liver inflammation on biopsy samples (at least METAVIR A2), HBV genotype A or B, and low viral load increase the chance of a more favourable response to treatment[19,20,24,31]. For patients treated with NA for a longer time without serum hepatitis B e antigen (HBeAg) seroconversion or loss of HBsAg, add-on or switch to Peg-IFN therapy is an option to enhance patient response, although a protocol for this has not been determined. Large randomized controlled trials are waited to provide definitive evidence of these strategies[32-34].

Elimination or inactivation of HBV cccDNA is the central focus of HBV research nowadays. Figure 1 illustrates treatment options that target cccDNA to attack HBV persistence, and these include interventions aiming to prevent cccDNA formation, affect its stability or even its activity. Although mechanistically these therapies would potentially offer a cure for the infection, further basic research and more detailed molecular studies are needed to evaluate the translational potential of novel antiviral strategies. New drugs that target HBV are required and immunomodulatory therapies and gene silencing technologies are the most promising approaches to eradicate HBV without killing the infected hepatocytes[17,21,35]. The advent of curative therapies for hepatitis C has renewed enthusiasm for also curing hepatitis B, going beyond viral suppression. Currently, there are numerous drugs under investigation to enable the cure of HBV infection[36]. In addition, efforts to increase hepatitis B vaccination coverage must be a priority.

HEPATITIS C

The Nobel Prize in Physiology or Medicine in 2020 was awarded to three scientists, Harvey Alter, Michael Houghton, and Charles Rice, for their efforts on the identification of HCV[37]. The discovery of HCV was a remarkable achievement, which saved millions of lives. It enabled the development of highly sensitive diagnostic blood tests and the rapid expansion of the pool of antiviral drugs directed at hepatitis C. Approximately 71 million people worldwide live with HCV and nearly half of them are currently unaware due to suboptimal screening programmes[5].

Hepatitis C infection is a silent systemic disease secondary to a hepatotropic and lymphotropic virus with a high chronicity rate. It promotes chronic systemic inflammation due to direct and indirect viral activities, characterised by increased levels of pro-inflammatory cytokines and chemokines. Chronic systemic inflammation is a well-known risk factor for insulin resistance; thus, it increases the risk for type 2 diabetes

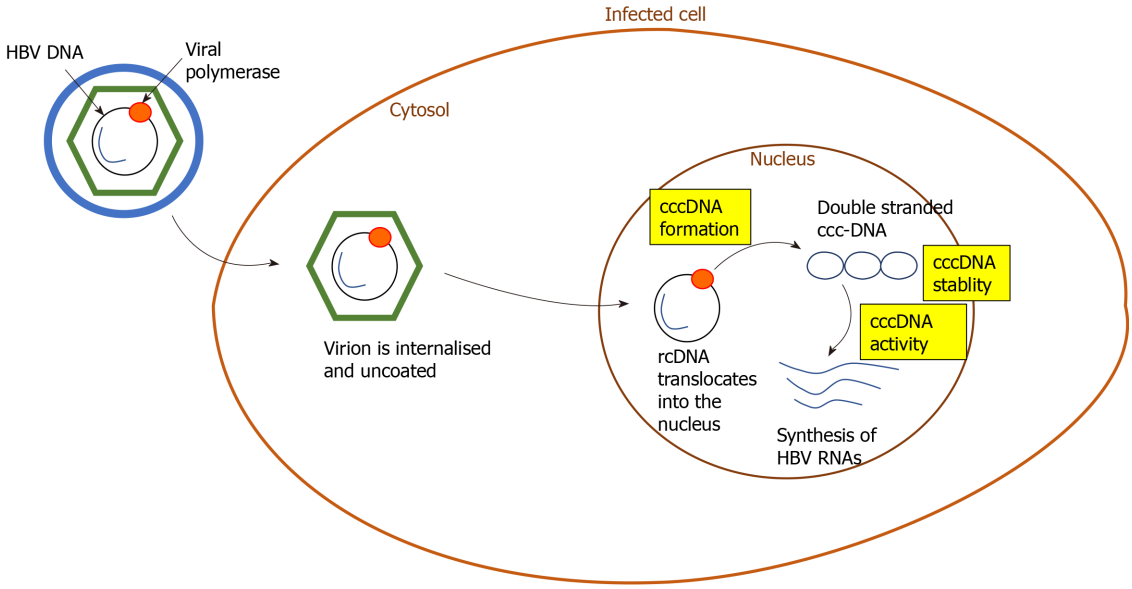


Figure 1 Diagrammatic summary of the therapeutic options targeting covalently closed circular deoxyribonucleic acid to prevent hepatitis B virus persistence. After entering the cell, the virion is uncoated and the relaxed circular deoxyribonucleic acid (DNA) genome (rcDNA) translocates into the cell nucleus. Once there, the covalently closed circular DNA (cccDNA) formed resides in the nucleus of infected cells as a minichromosome and originate the new viruses. Drugs that prevent cccDNA formation, that affect its stability, or even cccDNA activity must stop hepatitis B virus persistence. cccDNA: covalently closed circular deoxyribonucleic acid; HBV: Hepatitis B virus; RNA: Ribonucleic acid.

mellitus and, for cardiovascular events[38].

Hepatic manifestations include steatosis, fibrosis, and, finally, cirrhosis. The complications of cirrhosis and the occurrence of HCC compose the indications for liver transplantation in this disease[39]. Due to its lymphotropic property, HCV is able to multiply inside B lymphocytes and cause chronic stimulation of these cells by the viral infection. This stimulation possibly triggers autoimmune disorders, such as cryoglobulinemia vasculitis, purpura or necrotizing acrodermatitis, membranoproliferative glomerulonephritis, peripheral neuropathies, and polyarthritis[39,40]. Ultimately, B lymphocyte infection or chronic antigenic stimulation may be associated with lymphoma, mainly non-Hodgkin, splenic lymphoma type, or diffuse lymphomas[39,40]. Thus, chronic hepatitis C can enter the consulting rooms of several medical specialties because it is a systemic disease with manifestations affecting different organs and systems.

In the last decades, there have been significant advances in the treatment of hepatitis C, which motivated the WHO in 2017 to set targets to eradicate HCV by 2030 [7]. For more than 20 years, IFN has been used to treat chronic HCV infection. Pegylation (Peg-IFN) allowed a reduction in the frequency of subcutaneous injections from three to once a week[41]. Whereas the combination of Peg-IFN with ribavirin significantly increased the effectiveness of the treatment, it was poorly tolerated and resulted in a cure rate of at most 50% in 24 to 48 wk[41,42].

In 2011, the first protease inhibitors (telaprevir and boceprevir) demonstrated significant benefits, but they were not well tolerated and resulted in a suboptimal cure rate. Later, the development of the first polymerase inhibitor (sofosbuvir) and the first inhibitor of nonstructural protein (NS) 5A (daclatasvir) changed hepatitis C history due to the excellent tolerance and a cure rate of approximately 95%[43].

Direct-acting antivirals (DAAs) are highly effective agents, regardless of genotype and high barrier to resistance, which revolutionized HCV treatment[44]. Multiple combinations of DAAs with high pangenotypic efficacy result in high SVR rates, excellent safety, and good tolerance, even for patients with advanced fibrosis and cirrhosis[44]. The strong antiviral potency of these pangenotypic treatments has withdrawn the factors of poor response and developed a 'simplified route', which allowed general practitioners to treat patients without hepatic comorbidity and liver dysfunction[45]. This HCV treatment decentralization strategy was shown to be effective and safe for most patients. For example, multiple combinations of drugs with high pangenotypic efficacy, easy to use (one to three capsules per day) for 8 to 12 wk provide a cure for the vast majority of patients; these include the combinations glecaprevir/pibrentasvir, sofosbuvir/velpatasvir with or without voxilaprevir[45,46].

Combinations of DAAs are also available for specific genotypes. For example, ledipasvir/sofosbuvir (Harvoni™, Gilead Sciences) is approved for genotypes 1, 4, 5, and 6; and elbasvir/grazoprevir (Zepatier™, Merck Sharp and Dohme) for genotypes 1 and 4[44,47-49].

Therapeutic failures occur in approximately 3%-5% of cases, secondary to non-adherence to treatment or drug resistance. Resistance-associated variants of HCV have been identified and they are mainly a consequence of mutations in the nonstructural proteins NS3 and especially NS5. Only in cases of therapeutic failure in the first regimen is it advisable to perform resistance genotyping[50].

Despite the existence of effective treatments, HCV still remains a threat to public health. Albeit differences between the effectiveness of the medicines in clinical trials and real-life being a contributing factor, the main challenges are the low awareness of the disease, lack of screening programs, loss of follow-up in health services, and high rate of reinfection in certain populations[51].

Extensive efforts are being made to create efficient HCV care programmes around the world, respecting the particularities of each country. Macro-elimination based on mass testing and treatment has started in several American and European countries. Other countries, aiming to improve the efficiency of the therapy and considering cost-effectiveness, have chosen to adopt micro-elimination, targeting smaller population groups at high risk of infection, such as those in hyper-endemic areas, prisons, and haemodialysis centres[52,53].

In the DAA era, optimisation of their use must be a top priority. Identifying factors predicting a high chance of SVR with an ultra-short DAA regimen could be of great value in the global goal of HCV eradication[54]. Also, specific care needs to be taken in the post-RVS phase: (1) surveillance every six months for both HCC and hepatic decompensation remains imperative in patients with advanced fibrosis, especially in those with comorbidities that increase the risk of fibrosis progression, such as obesity, diabetes mellitus, and alcohol abuse; (2) close monitoring of extrahepatic complications, such as cardiovascular diseases, diabetes, lymphoma, and cryoglobulinemia, the once beneficial effects of HCV elimination on these complications are not clear; and (3) annual screening for HCV reinfection, mainly for those at high risk, such as people who inject drugs and those in prisons[55,56]. Recent analyses investigated the effects of eliminating a long-term persistent infection on the immune system. Persistent HCV infection is known to cause profound changes in the immune system, which do not appear to be fully reversible after viral elimination[57].

It is expected that the efforts of several countries in extensive testing for HCV and the availability of oral treatments of acceptable cost and with few side effects will result in the successful elimination of HCV. Hopes for an eventual preventive HCV vaccine remain.

HEPATITIS D

The hepatitis D virus (HDV) is a single-stranded circular RNA virus, first reported in 1977[58]. This is a defective virus, so HDV does not produce an envelope or capsid, requiring the use of HBV envelopes. Therefore, HBV infection is necessary for productive HDV infection in humans[58,59]. Although HDV infection is chronic in less than 5% of coinfected patients in adulthood, chronic infection is more common in the neonatal period[60].

An estimated 15-20 million people are infected worldwide[60]. Due to the dependence of HDV on HBV, the presence of HBsAg is necessary for the diagnosis of HDV infection. Serum HDV RNA and the presence of serum delta antigen are useful for diagnosis[61,62]. HDV infection can be acute or chronic[60].

Acute HDV infection can occur through HBV coinfection (simultaneous infection with both viruses during the same exposure) or superinfection (HDV infection in an HBsAg-positive individual). The clinical course of an acute HDV/HBV coinfection resembles an acute HBV infection, but with an increased risk of ALF[60]. Characteristically, there is a biphasic course with two peaks of alanine aminotransferase, sometimes separated by weeks, since HBV infection must be established first to allow for subsequent HDV infection. Whereas acute HDV superinfection can be mistaken for an HBV flare in patients with previous HBV infection, in undiagnosed patients it can be misinterpreted as acute HBV infection[60,63]. Therefore, high suspicion of HDV infection is required in patients with identified risk factors, such as a history of intravenous drug use, high-risk sexual behaviour, first-degree relative infection, and immigration from HDV-endemic regions[63].

Chronic HDV/HBV coinfection commonly results in the most rapidly progressive form of hepatitis, with a higher likelihood of cirrhosis and its complications. Compared to HBV monoinfected patients, HDV/HBV coinfected patients have a risk of HCC up to 3 times higher and that of liver decompensation up to 2 times higher[63,64].

Although the guidelines recommend Peg-IFN alpha for the treatment of chronic HDV infection, this therapy is limited by poor tolerance. Also, it is usually avoided in patients with cirrhosis, active autoimmune disease, or certain psychiatric disorders[27,63,64].

Novel therapeutic options targeting HDV life cycle are currently under clinical investigation. HDV cell entry, replication, and viral assembly and release are targets for medications such as bulevirtide, telafarnibe, and REP3702139, respectively[65]. Among all the agents studied, bulevirtide (formerly known as Myrcludex-B) received conditional marketing authorization under the trade name Hepcludex® by the European Medicines Agency in 2020. The agency warns that administration should continue 'as long as the patient benefits' and until future clinical trial data indicate different therapeutic actions. Hepcludex® blocks the entry of viruses into hepatocytes and should be administered at a dose of 2 mg once daily by subcutaneous injection as monotherapy or co-administered with a nucleoside/nucleotide analogue for the treatment of underlying HBV infection. The ideal duration of treatment is unknown. Hepcludex® has also been tested in combination therapy with Peg-IFN[66].

Recently, Peg-IFN lambda has also been studied against HDV[65]. Despite having an antiviral effect equivalent to Peg-IFN alpha, patients had better tolerability to the drug[67]. The combination of Peg-IFN lambda and other drugs is also under clinical investigation[65].

HEPATITIS E

Hepatitis E virus (HEV) is responsible for outbreaks in developing countries and zoonotic cases in both developing and developed countries, mainly transmitted enterically[68]. This virus is a member of the Hepeviridae family; within the genus Orthohepevirus, species Orthohepevirus A, which includes eight recognised HEV genotypes. Genotypes 1 and 2 HEV have only been detected in humans, and these infections frequently result in outbreaks of jaundice in areas traditionally considered endemic, which are resource-poor, where HEV is spread by the faecal-oral route often *via* contaminated water[68]. Other genotypes, including HEV3 and HEV4, have been detected in both humans and animals, with pigs being the main reservoir[68,69].

Whilst most infections are acute and self-limiting or asymptomatic, there are situations wherein it can progress to ALF and even become chronic. Immunocompromised patients are at risk of developing chronic HEV infection, such as solid organ transplant recipients, patients with haematologic malignancy undergoing chemotherapy, and those with human immunodeficiency virus infection[68,69]. Extrahepatic manifestations, mostly neurological and renal diseases, have also been described. Acute icteric hepatitis is a classic presentation that occurs in 5%–30% of infected patients. Pregnant women are particularly at risk and a large proportion of those in their second and third trimester of pregnancy can progress to ALF. Patients with underlying liver disease have a poor prognosis in developing and developed countries [68-70].

The mechanisms of pathogenesis appear to be substantially immune-mediated[71]. Several studies have suggested that the immune response, rather than viral damage to hepatocytes, may drive clinical manifestations of hepatitis E, including both self-limiting acute viral hepatitis and ALF. One of the reasons that pathogenesis may be mediated by the immune system rather than by the virus itself is that the onset of icteric symptoms typically coincides with a rise in antibodies and a decline in viral load[71]. Chronic HEV infections, which are rarely seen in otherwise healthy individuals, are increasingly being recognized in patients with impaired immune function[72].

Diagnostic assays with good sensitivity and specificity have only recently become commercially available. To facilitate global access to the tools necessary is vital to identify and respond to HEV infections, whether sporadic cases or nascent outbreaks. Clinical and field surveillance, coupled with laboratory investigations of viral strains isolated from human cases, will help advance our understanding of HEV genotypes' relative virulence, intergenotypic variation, and other features of the HEV global epidemiology[73].

There is no recommended treatment for acute HEV infections, which are usually self-limiting with spontaneous HEV clearance. Although a recent study suggested that ribavirin is effective in treating immunocompetent patients with severe hepatitis E, it is difficult to claim that the drug improved the course of the infection due to study limitations (*e.g.*, the absence of a control group)[74]. Sofosbuvir demonstrated antiviral activity against HEV *in vitro,* however, it had limited clinical efficacy. There are some studies on new anti-HEV drugs: NITD008, a broad-spectrum chain-terminating adenosine nucleoside analogue initially developed to treat the dengue virus; and GPC-N114, which binds to the RNA channels of picornavirus polymerases. These compounds are promising HEV antiviral candidates. Lastly, T cell therapy may be an alternative to conventional medicines[75,76].

Vaccines to combat HEV have been developed and tested, and one highly efficacious vaccine is now available to consumers in China[77]. Understanding the determinants of susceptibility and resistance to repeat infection and clinical disease is imperative. Identifying environmental factors, such as regional climatic patterns, water, and sanitation practices, farming, and food processing practices, which affect lifetime exposures to HEV may help both to explain regional differences in the age-specific incidence of the infection and the severity of the disease, which cannot be explained solely by genotypic variability. Identification of these determinants also may help to provide risk-based strategies for intervention.

HURDLES AND OPPORTUNITIES IN VIRAL HEPATITIS TREATMENT

In the last decade, rapid and significant advances in diagnosing and managing viral hepatitis were made and changed its treatment. These advances include the development of DAAs for the treatment of chronic hepatitis caused by HCV[78]-with SVR rates greater than 95%, the improvement of HBV vaccination as well as enhancement of the immunogenicity of HBV vaccines[79,80], and the identification of antiviral therapies with low rates of viral resistance[27]. Table 1 summarises the current clinical management of viral hepatitis and areas of development for future treatments.

Immunomodulators have been investigated to strengthen the immune system to fight HBV. Medications that stimulate both innate and adaptive immune systems, overcome CD8+ T cell exhaustion by checkpoint blockade, and transfer HBV-specific engineered CD8+ T cells are some of the therapies under investigation[36]. Immunomodulators may present a future treatment to cure hepatitis B infection, even though further research is necessary for this treatment.

Acute hepatitis A and E, frequently self-limiting or asymptomatic, still have no treatment recommendations, although the development and enhancement of vaccines improved its prevention. A vaccine to combat HEV is already available and consistent indications for HAV vaccination are now defined[12,77,81].

Despite these advances, issues with screening, diagnosis, referral, and treatment of viral hepatitis still persist. Problems in accessing treatment are reported in the published literature and reinforce the need to establish appropriate public policies for patient referral[82]. In addition, the identification of patients with viral resistance to the new treatment regimens and those with a satisfactory viral response and liver fibrosis, who might need close monitoring, deserve further investigation[83].

CONCLUSION

The treatment of viral hepatitis has evolved rapidly over the last decade with the remarkable introduction of curative therapies for hepatitis C. The development and improvement of HAV and HEV vaccination also constitute substantial advances in this field. Despite these advances, drugs that also cure hepatitis B, going beyond viral suppression, are so far not available. Targeted antiviral drugs against HBV are encouraging future treatments and immunomodulatory therapies and gene silencing technologies are the most promising approaches to eradicate the virus. The increase in the frequency of HDV cases leads to the development of targeted antiviral agents against HDV, currently under clinical investigation. Finally, optimal screening *via* extensive testing allied to broad vaccination and treatment coverage are fundamental goals to eliminate viral hepatitis and reduce the public health burden of these infections.

Table 1 Current clinical management of viral hepatitis and areas of development for future therapies

Type	Current management	Areas of development
Hepatitis A	No specific drugs against HAV infection are available so far; thus treatment consists of supportive care; Prevention of HAV infection includes vaccination, immune globulin, and attention to hygienic practices	Public health campaigns to promote the prevention of hepatitis A; Raise awareness of indications for hepatitis A vaccination
Hepatitis B	Entecavir, tenofovir disoproxil fumarate, tenofovir alafenamide fumarate, and pegylated interferon alpha are currently the first-line anti-HBV agents recommended for chronic hepatitis B treatment; Prevention of HBV infection is focused on vaccination;	Elimination or inactivation of HBV cccDNA is the major focus of HBV research; Targeted therapies to HBV (immunomodulatory therapies and gene silencing technologies are promising approaches); Need to increase hepatitis B vaccination coverage
Hepatitis C	Multiple combinations of direct-acting antivirals with high pangenotypic efficacy result in high sustained virological response rates, excellent safety, and good tolerance, even for patients with advanced fibrosis and cirrhosis;	Increase awareness of the disease, develop screening programmes; Optimization of direct-acting antivirals use; Attention to specific care needs to be taken in the post-treatment phase
Hepatitis D	There are no satisfactory drugs for this disease; Pegylated interferon alpha recommended for the treatment of chronic HDV infection, although limited by poor tolerance is usually avoided in patients with cirrhosis, active autoimmune disease, or certain psychiatric disorders	Further research on novel targeted HDV antiviral medications is necessary due to the lack of effective therapeutic options
Hepatitis E	There is no recommended treatment for acute HEV infections because it is usually self-limiting with spontaneous HEV clearance	Ribavirin is suggested to be an effective treatment for immunocompetent patients with severe hepatitis E; New anti-HEV drugs are under investigation; T cell therapy may be an alternative to conventional medicines; Vaccines to combat HEV have been developed and tested

HAV: Hepatitis A virus; HBV: Hepatitis B virus; cccDNA: Covalently closed circular deoxyribonucleic acid; HDV: Hepatitis D virus; HEV: Hepatitis E virus.

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REFERENCES

1 **Ringelhan M**, McKeating JA, Protzer U. Viral hepatitis and liver cancer. *Philos Trans R Soc Lond B Biol Sci* 2017; **372**: 20160274 [PMID: 28893941 DOI: 10.1098/rstb.2016.0274]
2 **Ben Ari Z**, Weitzman E, Safran M. Oncogenic viruses and hepatocellular carcinoma. *Clin Liver Dis* 2015; **19**: 341-360 [PMID: 25921667 DOI: 10.1016/j.cld.2015.01.006]
3 **Santopaolo F**, Lenci I, Milana M, Manzia TM, Baiocchi L. Liver transplantation for hepatocellular carcinoma: Where do we stand? *World J Gastroenterol* 2019; **25**: 2591-2602 [PMID: 31210712 DOI: 10.3748/wjg.v25.i21.2591]
4 **Summerfield JA**. Virus hepatitis update. *J R Coll Physicians Lond* 2000; **34**: 381-385 [PMID: 11005078]
5 **World Health Organization**. WHO estimates of the prevalence and incidence of hepatitis C virus infection by WHO region, 2015. In: Global hepatitis report 2017 [cited 16 March 2021]. Available from: https://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/
6 **Schweitzer A**, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *Lancet* 2015; **386**: 1546-1555 [PMID: 26231459 DOI: 10.1016/S0140-6736(15)61412-X]
7 **World Health Organization**. Combating Hepatitis B and C to Reach Elimination by 2030: Advocacy Brief. WHO 2016 [cited 16 March 2021]. Available from: WHO_HIV_2016.04_eng.pdf.
8 **Shin EC**, Jeong SH. Natural History, Clinical Manifestations, and Pathogenesis of Hepatitis A. *Cold Spring Harb Perspect Med* 2018; **8** [PMID: 29440324 DOI: 10.1101/cshperspect.a031708]
9 **Daniels D**, Grytdal S, Wasley A; Centers for Disease Control and Prevention (CDC). Surveillance for acute viral hepatitis - United States, 2007. *MMWR Surveill Summ* 2009; **58**: 1-27 [PMID: 19478727]
10 **Foster MA**, Hofmeister MG, Kupronis BA, Lin Y, Xia GL, Yin S, Teshale E. Increase in Hepatitis A Virus Infections - United States, 2013-2018. *MMWR Morb Mortal Wkly Rep* 2019; **68**: 413-415

[PMID: 31071072 DOI: 10.15585/mmwr.mm6818a2]
11 **Matheny SC**, Kingery JE. Hepatitis A. *Am Fam Physician* 2012; **86**: 1027-34; quiz 1010 [PMID: 23198670]
12 **Nelson NP**, Weng MK, Hofmeister MG, Moore KL, Doshani M, Kamili S, Koneru A, Haber P, Hagan L, Romero JR, Schillie S, Harris AM. Prevention of Hepatitis A Virus Infection in the United States: Recommendations of the Advisory Committee on Immunization Practices, 2020. *MMWR Recomm Rep* 2020; **69**: 1-38 [PMID: 32614811 DOI: 10.15585/mmwr.rr6905a1]
13 **Nelson NP**, Easterbrook PJ, McMahon BJ. Epidemiology of Hepatitis B Virus Infection and Impact of Vaccination on Disease. *Clin Liver Dis* 2016; **20**: 607-628 [PMID: 27742003 DOI: 10.1016/j.cld.2016.06.006]
14 **Trépo C**, Chan HL, Lok A. Hepatitis B virus infection. *Lancet* 2014; **384**: 2053-2063 [PMID: 24954675 DOI: 10.1016/S0140-6736(14)60220-8]
15 **Nassal M**. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. *Gut* 2015; **64**: 1972-1984 [PMID: 26048673 DOI: 10.1136/gutjnl-2015-309809]
16 **Seeger C**, Mason WS. Molecular biology of hepatitis B virus infection. *Virology* 2015; **479-480**: 672-686 [PMID: 25759099 DOI: 10.1016/j.virol.2015.02.031]
17 **Shire NJ**. Cure Strategies for Hepatitis B Virus: The Promise of Immunotherapy. *Clin Pharmacol Drug Dev* 2017; **6**: 186-194 [PMID: 28263466 DOI: 10.1002/cpdd.317]
18 **Mendy ME**, Welzel T, Lesi OA, Hainaut P, Hall AJ, Kuniholm MH, McConkey S, Goedert JJ, Kaye S, Rowland-Jones S, Whittle H, Kirk GD. Hepatitis B viral load and risk for liver cirrhosis and hepatocellular carcinoma in The Gambia, West Africa. *J Viral Hepat* 2010; **17**: 115-122 [PMID: 19874478 DOI: 10.1111/j.1365-2893.2009.01168.x]
19 **Liaw YF**, Jia JD, Chan HL, Han KH, Tanwandee T, Chuang WL, Tan DM, Chen XY, Gane E, Piratvisuth T, Chen L, Xie Q, Sung JJ, Wat C, Bernaards C, Cui Y, Marcellin P. Shorter durations and lower doses of peginterferon alfa-2a are associated with inferior hepatitis B e antigen seroconversion rates in hepatitis B virus genotypes B or C. *Hepatology* 2011; **54**: 1591-1599 [PMID: 22045673 DOI: 10.1002/hep.24555]
20 **Wang YC**, Yang SS, Su CW, Wang YJ, Lee KC, Huo TI, Lin HC, Huang YH. Predictors of response to pegylated interferon in chronic hepatitis B: a real-world hospital-based analysis. *Sci Rep* 2016; **6**: 29605 [PMID: 27405043 DOI: 10.1038/srep29605]
21 **Yang HC**, Kao JH. Viral hepatitis. HBV cure--can we pin our hopes on immunotherapy? *Nat Rev Gastroenterol Hepatol* 2015; **12**: 129-131 [PMID: 25623202 DOI: 10.1038/nrgastro.2015.8]
22 **Voican CS**, Mir O, Loulergue P, Dhooge M, Brezault C, Dréanic J, Chaussade S, Pol S, Coriat R. Hepatitis B virus reactivation in patients with solid tumors receiving systemic anticancer treatment. *Ann Oncol* 2016; **27**: 2172-2184 [PMID: 27803003 DOI: 10.1093/annonc/mdw414]
23 **Seto WK**, Chan TS, Hwang YY, Wong DK, Fung J, Liu KS, Gill H, Lam YF, Lau EHY, Cheung KS, Lie AKW, Lai CL, Kwong YL, Yuen MF. Hepatitis B reactivation in occult viral carriers undergoing hematopoietic stem cell transplantation: A prospective study. *Hepatology* 2017; **65**: 1451-1461 [PMID: 28027590 DOI: 10.1002/hep.29022]
24 **Yeh ML**, Huang JF, Dai CY, Yu ML, Chuang WL. Pharmacokinetics and pharmacodynamics of pegylated interferon for the treatment of hepatitis B. *Expert Opin Drug Metab Toxicol* 2019; **15**: 779-785 [PMID: 31593639 DOI: 10.1080/17425255.2019.1678584]
25 **Vittal A**, Ghany MG. WHO Guidelines for Prevention, Care and Treatment of Individuals Infected with HBV: A US Perspective. *Clin Liver Dis* 2019; **23**: 417-432 [PMID: 31266617 DOI: 10.1016/j.cld.2019.04.008]
26 **Terrault NA**, Bzowej NH, Chang KM, Hwang JP, Jonas MM, Murad MH; American Association for the Study of Liver Diseases. AASLD guidelines for treatment of chronic hepatitis B. *Hepatology* 2016; **63**: 261-283 [PMID: 26566064 DOI: 10.1002/hep.28156]
27 **Terrault NA**, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM, Brown RS Jr, Bzowej NH, Wong JB. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. *Hepatology* 2018; **67**: 1560-1599 [PMID: 29405329 DOI: 10.1002/hep.29800]
28 **Labarga P**, Barreiro P, Martin-Carbonero L, Rodriguez-Novoa S, Solera C, Medrano J, Rivas P, Albalater M, Blanco F, Moreno V, Vispo E, Soriano V. Kidney tubular abnormalities in the absence of impaired glomerular function in HIV patients treated with tenofovir. *AIDS* 2009; **23**: 689-696 [PMID: 19262355 DOI: 10.1097/QAD.0b013e3283262a64]
29 **Bedimo R**, Maalouf NM, Zhang S, Drechsler H, Tebas P. Osteoporotic fracture risk associated with cumulative exposure to tenofovir and other antiretroviral agents. *AIDS* 2012; **26**: 825-831 [PMID: 22301411 DOI: 10.1097/QAD.0b013e32835192ae]
30 **De Clercq E**. Tenofovir alafenamide (TAF) as the successor of tenofovir disoproxil fumarate (TDF). *Biochem Pharmacol* 2016; **119**: 1-7 [PMID: 27133890 DOI: 10.1016/j.bcp.2016.04.015]
31 **Buster EH**, Hansen BE, Lau GK, Piratvisuth T, Zeuzem S, Steyerberg EW, Janssen HL. Factors that predict response of patients with hepatitis B e antigen-positive chronic hepatitis B to peginterferon-alfa. *Gastroenterology* 2009; **137**: 2002-2009 [PMID: 19737568 DOI: 10.1053/j.gastro.2009.08.061]
32 **Lampertico P**, Brunetto MR, Craxì A, Gaeta GB, Rizzetto M, Rozzi A, Colombo M; HERMES Study Group. Add-on peginterferon alfa-2a to nucleos(t)ide analogue therapy for Caucasian patients with hepatitis B 'e' antigen-negative chronic hepatitis B genotype D. *J Viral Hepat* 2019; **26**: 118-125 [PMID: 30187599 DOI: 10.1111/jvh.12999]
33 **Ning Q**, Han M, Sun Y, Jiang J, Tan D, Hou J, Tang H, Sheng J, Zhao M. Switching from entecavir to PegIFN alfa-2a in patients with HBeAg-positive chronic hepatitis B: a randomised open-label trial

(OSST trial). *J Hepatol* 2014; **61**: 777-784 [PMID: 24915612 DOI: 10.1016/j.jhep.2014.05.044]

34 **Matsumoto A**, Nishiguchi S, Enomoto H, Kang JH, Tanaka Y, Shinkai N, Kurosaki M, Enomoto M, Kanda T, Yokosuka O, Yatsuhashi H, Nagaoka S, Okuse C, Kagawa T, Mine T, Takaguchi K, Saito S, Hino K, Ikeda F, Sakisaka S, Morihara D, Miyase S, Tsuge M, Chayama K, Hiramatsu N, Suzuki Y, Murata K, Tanaka E. Combinational use of hepatitis B viral antigens predicts responses to nucleos(t)ide analogue/peg-interferon sequential therapy. *J Gastroenterol* 2018; **53**: 247-257 [PMID: 28634723 DOI: 10.1007/s00535-017-1360-z]

35 **Gane EJ**, Lim YS, Gordon SC, Visvanathan K, Sicard E, Fedorak RN, Roberts S, Massetto B, Ye Z, Pflanz S, Garrison KL, Gaggar A, Mani Subramanian G, McHutchison JG, Kottilil S, Freilich B, Coffin CS, Cheng W, Kim YJ. The oral toll-like receptor-7 agonist GS-9620 in patients with chronic hepatitis B virus infection. *J Hepatol* 2015; **63**: 320-328 [PMID: 25733157 DOI: 10.1016/j.jhep.2015.02.037]

36 **Binder B**, Hofmann M, Thimme R. Role of Immunomodulators in Functional Cure Strategies for HBV. *Curr Hepatology Rep* 2020; **19**: 337-44 [DOI: 10.1007/s11901-020-00538-6]

37 **Afdhal NH**. The natural history of hepatitis C. *Semin Liver Dis* 2004; **24** Suppl 2: 3-8 [PMID: 15346240 DOI: 10.1055/s-2004-832922]

38 **El-Serag HB**, Christie IC, Puenpatom A, Castillo D, Kanwal F, Kramer JR. The effects of sustained virological response to direct-acting anti-viral therapy on the risk of extrahepatic manifestations of hepatitis C infection. *Aliment Pharmacol Ther* 2019; **49**: 1442-1447 [PMID: 30932218 DOI: 10.1111/apt.15240]

39 **Pol S**, Vallet-Pichard A, Hermine O. Extrahepatic cancers and chronic HCV infection. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 283-290 [PMID: 29339810 DOI: 10.1038/nrgastro.2017.172]

40 **Cacoub P**, Desbois AC, Comarmond C, Saadoun D. Impact of sustained virological response on the extrahepatic manifestations of chronic hepatitis C: a meta-analysis. *Gut* 2018; **67**: 2025-2034 [PMID: 29703790 DOI: 10.1136/gutjnl-2018-316234]

41 **McHutchison JG**, Lawitz EJ, Shiffman ML, Muir AJ, Galler GW, McCone J, Nyberg LM, Lee WM, Ghalib RH, Schiff ER, Galati JS, Bacon BR, Davis MN, Mukhopadhyay P, Koury K, Noviello S, Pedicone LD, Brass CA, Albrecht JK, Sulkowski MS; IDEAL Study Team. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N Engl J Med* 2009; **361**: 580-593 [PMID: 19625712 DOI: 10.1056/NEJMoa0808010]

42 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965 [PMID: 11583749 DOI: 10.1016/s0140-6736(01)06102-5]

43 **Gitto S**, Gamal N, Andreone P. NS5A inhibitors for the treatment of hepatitis C infection. *J Viral Hepat* 2017; **24**: 180-186 [PMID: 27925362 DOI: 10.1111/jvh.12657]

44 **Asselah T**, Boyer N, Saadoun D, Martinot-Peignoux M, Marcellin P. Direct-acting antivirals for the treatment of hepatitis C virus infection: optimizing current IFN-free treatment and future perspectives. *Liver Int* 2016; **36** Suppl 1: 47-57 [PMID: 26725897 DOI: 10.1111/Liv.13027]

45 **Zeuzem S**, Foster GR, Wang S, Asatryan A, Gane E, Feld JJ, Asselah T, Bourlière M, Ruane PJ, Wedemeyer H, Pol S, Flisiak R, Poordad F, Chuang WL, Stedman CA, Flamm S, Kwo P, Dore GJ, Sepulveda-Arzola G, Roberts SK, Soto-Malave R, Kaita K, Puoti M, Vierling J, Tam E, Vargas HE, Bruck R, Fuster F, Paik SW, Felizarta F, Kort J, Fu B, Liu R, Ng TI, Pilot-Matias T, Lin CW, Trinh R, Mensa FJ. Glecaprevir-Pibrentasvir for 8 or 12 Weeks in HCV Genotype 1 or 3 Infection. *N Engl J Med* 2018; **378**: 354-369 [PMID: 29365309 DOI: 10.1056/NEJMoa1702417]

46 **Gane E**, Lawitz E, Pugatch D, Papatheodoridis G, Bräu N, Brown A, Pol S, Leroy V, Persico M, Moreno C, Colombo M, Yoshida EM, Nelson DR, Collins C, Lei Y, Kosloski M, Mensa FJ. Glecaprevir and Pibrentasvir in Patients with HCV and Severe Renal Impairment. *N Engl J Med* 2017; **377**: 1448-1455 [PMID: 29020583 DOI: 10.1056/NEJMoa1704053]

47 **Karaoui LR**, Mansour H, Chahine EB. Elbasvir-grazoprevir: A new direct-acting antiviral combination for hepatitis C. *Am J Health Syst Pharm* 2017; **74**: 1533-1540 [PMID: 28947524 DOI: 10.2146/ajhp160558]

48 **Keating GM**. Ombitasvir/Paritaprevir/Ritonavir: A Review in Chronic HCV Genotype 4 Infection. *Drugs* 2016; **76**: 1203-1211 [PMID: 27401997 DOI: 10.1007/s40265-016-0612-1]

49 **Sandmann L**, Schulte B, Manns MP, Maasoumy B. Treatment of Chronic Hepatitis C: Efficacy, Side Effects and Complications. *Visc Med* 2019; **35**: 161-170 [PMID: 31367613 DOI: 10.1159/000500963]

50 **Liang TJ**, Ghany MG. Therapy of hepatitis C--back to the future. *N Engl J Med* 2014; **370**: 2043-2047 [PMID: 24795199 DOI: 10.1056/NEJMe1403619]

51 **Thursz M**, Fontanet A. HCV transmission in industrialized countries and resource-constrained areas. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 28-35 [PMID: 24080775 DOI: 10.1038/nrgastro.2013.179]

52 **Huang CF**, Chiu YW, Yu ML. Patient-centered outreach treatment toward micro-elimination of hepatitis C virus infection in hemodialysis patients. *Kidney Int* 2020; **97**: 421 [PMID: 31980077 DOI: 10.1016/j.kint.2019.10.030]

53 **Yang TH**, Fang YJ, Hsu SJ, Lee JY, Chiu MC, Yu JJ, Kuo CC, Chen CH. Microelimination of Chronic Hepatitis C by Universal Screening Plus Direct-Acting Antivirals for Incarcerated Persons in Taiwan. *Open Forum Infect Dis* 2020; **7**: ofaa301 [PMID: 32818142 DOI: 10.1093/ofid/ofaa301]

54 **Volpicelli L**, Biliotti E, Milito C, Cruciata A, Spaziante M, Rivano Capparuccia M, Taliani G, Mezzaroma I. Glecaprevir/pibrentasvir ultra-short treatment to cure HCV infection: case report and literature review. *Infez Med* 2020; **28**: 616-620 [PMID: 33257639]

55 **Huang CF**, Dai CY, Yeh ML, Huang CI, Lee HC, Lai WT, Liang PC, Lin YH, Hsieh MY, Hou NJ, Lin ZY, Chen SC, Huang JF, Chuang WL, Yu ML. Cure or curd: Modification of lipid profiles and cardio-cerebrovascular events after hepatitis C virus eradication. *Kaohsiung J Med Sci* 2020; **36**: 920-928 [PMID: 32643842 DOI: 10.1002/kjm2.12275]

56 **Muzica CM**, Stanciu C, Huiban L, Singeap AM, Sfarti C, Zenovia S, Cojocariu C, Trifan A. Hepatocellular carcinoma after direct-acting antiviral hepatitis C virus therapy: A debate near the end. *World J Gastroenterol* 2020; **26**: 6770-6781 [PMID: 33268960 DOI: 10.3748/wjg.v26.i43.6770]

57 **Hensel N**, Gu Z, Sagar, Wieland D, Jechow K, Kemming J, Llewellyn-Lacey S, Gostick E, Sogukpinar O, Emmerich F, Price DA, Bengsch B, Boettler T, Neumann-Haefelin C, Eils R, Conrad C, Bartenschlager R, Grün D, Ishaque N, Thimme R, Hofmann M. Memory-like HCV-specific $CD8^+$ T cells retain a molecular scar after cure of chronic HCV infection. *Nat Immunol* 2021; **22**: 229-239 [PMID: 33398179 DOI: 10.1038/s41590-020-00817-w]

58 **Rizzetto M**, Hoyer B, Canese MG, Shih JW, Purcell RH, Gerin JL. delta Agent: association of delta antigen with hepatitis B surface antigen and RNA in serum of delta-infected chimpanzees. *Proc Natl Acad Sci USA* 1980; **77**: 6124-6128 [PMID: 6934539 DOI: 10.1073/pnas.77.10.6124]

59 **Bonino F**, Hoyer B, Shih JW, Rizzetto M, Purcell RH, Gerin JL. Delta hepatitis agent: structural and antigenic properties of the delta-associated particle. *Infect Immun* 1984; **43**: 1000-1005 [PMID: 6698598 DOI: 10.1128/IAI.43.3.1000-1005.1984]

60 **Noureddin M**, Gish R. Hepatitis delta: epidemiology, diagnosis and management 36 years after discovery. *Curr Gastroenterol Rep* 2014; **16**: 365 [PMID: 24293018 DOI: 10.1007/s11894-013-0365-x]

61 **Safaie P**, Razeghi S, Rouster SD, Privitera I, Sherman KE. Hepatitis D diagnostics:Utilization and testing in the United States. *Virus Res* 2018; **250**: 114-117 [PMID: 29596839 DOI: 10.1016/j.virusres.2018.03.013]

62 **Kucirka LM**, Farzadegan H, Feld JJ, Mehta SH, Winters M, Glenn JS, Kirk GD, Segev DL, Nelson KE, Marks M, Heller T, Golub ET. Prevalence, correlates, and viral dynamics of hepatitis delta among injection drug users. *J Infect Dis* 2010; **202**: 845-852 [PMID: 20701536 DOI: 10.1086/655808]

63 **European Association for the Study of the Liver**. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]

64 **Sarin SK**, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS, Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int* 2016; **10**: 1-98 [PMID: 26563120 DOI: 10.1007/s12072-015-9675-4]

65 **Mentha N**, Clément S, Negro F, Alfaiate D. A review on hepatitis D: From virology to new therapies. *J Adv Res* 2019; **17**: 3-15 [PMID: 31193285 DOI: 10.1016/j.jare.2019.03.009]

66 **Rizzetto M**, Hamid S, Negro F. The changing context of hepatitis D. *J Hepatol* 2021 [PMID: 33484770 DOI: 10.1016/j.jhep.2021.01.014]

67 **Chan HLY**, Ahn SH, Chang TT, Peng CY, Wong D, Coffin CS, Lim SG, Chen PJ, Janssen HLA, Marcellin P, Serfaty L, Zeuzem S, Cohen D, Critelli L, Xu D, Wind-Rotolo M, Cooney E; LIRA-B Study Team. Peginterferon lambda for the treatment of HBeAg-positive chronic hepatitis B: A randomized phase 2b study (LIRA-B). *J Hepatol* 2016; **64**: 1011-1019 [PMID: 26739688 DOI: 10.1016/j.jhep.2015.12.018]

68 **Kamar N**, Izopet J, Pavio N, Aggarwal R, Labrique A, Wedemeyer H, Dalton HR. Hepatitis E virus infection. *Nat Rev Dis Primers* 2017; **3**: 17086 [PMID: 29154369 DOI: 10.1038/nrdp.2017.86]

69 **Wedemeyer H**, Pischke S, Manns MP. Pathogenesis and treatment of hepatitis e virus infection. *Gastroenterology* 2012; **142**: 1388-1397. e1 [PMID: 22537448 DOI: 10.1053/j.gastro.2012.02.014]

70 **Dalton HR**, Hazeldine S, Banks M, Ijaz S, Bendall R. Locally acquired hepatitis E in chronic liver disease. *Lancet* 2007; **369**: 1260 [PMID: 17434400 DOI: 10.1016/S0140-6736(07)60595-9]

71 **Zhang JZ**, Im SW, Lau SH, Chau TN, Lai ST, Ng SP, Peiris M, Tse C, Ng TK, Ng MH. Occurrence of hepatitis E virus IgM, low avidity IgG serum antibodies, and viremia in sporadic cases of non-A, -B, and -C acute hepatitis. *J Med Virol* 2002; **66**: 40-48 [PMID: 11748657 DOI: 10.1002/jmv.2109]

72 **Lhomme S**, Marion O, Abravanel F, Izopet J, Kamar N. Clinical Manifestations, Pathogenesis and Treatment of Hepatitis E Virus Infections. *J Clin Med* 2020; **9** [PMID: 31991629 DOI: 10.3390/jcm9020331]

73 **Krain LJ**, Nelson KE, Labrique AB. Host immune status and response to hepatitis E virus infection. *Clin Microbiol Rev* 2014; **27**: 139-165 [PMID: 24396140 DOI: 10.1128/CMR.00062-13]

74 **Péron JM**, Dalton H, Izopet J, Kamar N. Acute autochthonous hepatitis E in western patients with underlying chronic liver disease: a role for ribavirin? *J Hepatol* 2011; **54**: 1323-4; author reply 1324 [PMID: 21281681 DOI: 10.1016/j.jhep.2011.01.009]

75 **Netzler NE**, Enosi Tuipulotu D, Vasudevan SG, Mackenzie JM, White PA. Antiviral Candidates for Treating Hepatitis E Virus Infection. *Antimicrob Agents Chemother* 2019; **63** [PMID: 30885901 DOI: 10.1128/AAC.00003-19]

76 **Nishiyama T**, Kobayashi T, Jirintai S, Kii I, Nagashima S, Prathiwi Primadharsini P, Nishizawa T, Okamoto H. Screening of novel drugs for inhibiting hepatitis E virus replication. *J Virol Methods* 2019; **270**: 1-11 [PMID: 31004661 DOI: 10.1016/j.jviromet.2019.04.017]

77 **Huang WJ**, Zhang HY, Harrison TJ, Lan HY, Huang GY, Wang YC. Immunogenicity and protective efficacy in rhesus monkeys of a recombinant ORF2 protein from hepatitis E virus genotype 4. *Arch Virol* 2009; **154**: 481-488 [PMID: 19240977 DOI: 10.1007/s00705-009-0335-7]

78 **AASLD/IDSA HCV Guidance Panel**. Hepatitis C guidance: AASLD-IDSA recommendations for testing, managing, and treating adults infected with hepatitis C virus. *Hepatology* 2015; **62**: 932-954 [PMID: 26111063 DOI: 10.1002/hep.27950]

79 **Chang MH**, You SL, Chen CJ, Liu CJ, Lai MW, Wu TC, Wu SF, Lee CM, Yang SS, Chu HC, Wang TE, Chen BW, Chuang WL, Soon MS, Lin CY, Chiou ST, Kuo HS, Chen DS; Taiwan Hepatoma Study Group. Long-term Effects of Hepatitis B Immunization of Infants in Preventing Liver Cancer. *Gastroenterology* 2016; **151**: 472-480. e1 [PMID: 27269245 DOI: 10.1053/j.gastro.2016.05.048]

80 **Heyward WL**, Kyle M, Blumenau J, Davis M, Reisinger K, Kabongo ML, Bennett S, Janssen RS, Namini H, Martin JT. Immunogenicity and safety of an investigational hepatitis B vaccine with a Toll-like receptor 9 agonist adjuvant (HBsAg-1018) compared to a licensed hepatitis B vaccine in healthy adults 40-70 years of age. *Vaccine* 2013; **31**: 5300-5305 [PMID: 23727002 DOI: 10.1016/j.vaccine.2013.05.068]

81 **Shrestha MP**, Scott RM, Joshi DM, Mammen MP Jr, Thapa GB, Thapa N, Myint KS, Fourneau M, Kuschner RA, Shrestha SK, David MP, Seriwatana J, Vaughn DW, Safary A, Endy TP, Innis BL. Safety and efficacy of a recombinant hepatitis E vaccine. *N Engl J Med* 2007; **356**: 895-903 [PMID: 17329696 DOI: 10.1056/NEJMoa061847]

82 **Rege S**, Gonzalez YS, Marx S, Reau N. 964 Patient Flow Across Physician Specialties Over the Course of the Hepatitis C Care Cascade: A Real World Analysis From the United States. *Am J Gastroenterol* 2019; **114** [DOI: 10.14309/01.ajg.0000593392.14697.54]

83 **Farhang Zangneh H**, Wong WWL, Sander B, Bell CM, Mumtaz K, Kowgier M, van der Meer AJ, Cleary SP, Janssen HLA, Chan KKW, Feld JJ. Cost Effectiveness of Hepatocellular Carcinoma Surveillance After a Sustained Virologic Response to Therapy in Patients With Hepatitis C Virus Infection and Advanced Fibrosis. *Clin Gastroenterol Hepatol* 2019; **17**: 1840-1849. e16 [PMID: 30580095 DOI: 10.1016/j.cgh.2018.12.018]

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MINIREVIEWS

Large-duct pattern invasive adenocarcinoma of the pancreas–a variant mimicking pancreatic cystic neoplasms: A minireview

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Abstract

Pancreatic cancer currently has no subtypes that inform clinical decisions; hence, there exists an opportunity to rearrange the morphological and molecular taxonomy that guides a better understanding of tumor characteristics. Nonetheless, accumulating studies to date have revealed the large-duct type variant, a unique subtype of pancreatic ductal adenocarcinoma (PDA) with cystic features. This subtype often radiographically mimics intraductal papillary mucinous neoplasms (IPMNs) and involves multiple small cysts occasionally associated with solid masses. The "bunch-of-grapes" sign, an imaging characteristic of IPMNs, is absent in large-duct PDA. Large-duct PDA defines the mucin profile, and genetic alterations are useful in distinguishing large-duct PDA from IPMNs. Histologically, neoplastic ducts measure over 0.5 mm, forming large ductal elements. Similar to classic PDAs, this subtype is frequently accompanied by perineural invasion and abundant desmoplastic reactions, and *KRAS* mutations in codon 12 are nearly ubiquitous. Despite such morphological similarities with IPMNs, the prognosis of large-duct PDA is equivalent to that of classic PDA. Differential diagnosis is therefore essential.

Key Words: Large-duct pattern invasive carcinoma of the pancreas; Pancreatic ductal adenocarcinoma; Pancreatic cystic disease; Clinicopathological features of pancreatic cancer; Pancreatic cancer subtype

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Core Tip: This review integrates the current knowledge about large-duct pattern invasive adenocarcinoma of the pancreas [large-duct pancreatic ductal adenocarcinoma (PDA)]. This subtype is a rare exocrine pancreatic neoplasm and often mimics intraductal papillary mucinous neoplasms (IPMNs). However, its prognosis is notably different from that of IPMNs, and distinguishing this subtype from IPMNs preoperatively is crucial. We summarized the morphological features and genetic landscape of large-duct PDA, with a primary focus on its differences from other types of pancreatic cystic neoplasms. The information aid in making appropriate decisions when tackling atypical pancreatic cystic neoplasms.

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INTRODUCTION

Pancreatic cancer is one of the most devastating and fatal diseases in humans, with pancreatic ductal adenocarcinoma (PDA) being the most common exocrine pancreatic neoplasm[1]. With the increase in the number of patients, PDA is expected to become the second leading cause of cancer-related mortality worldwide[2,3]. Advances in multidisciplinary treatment have improved patient survival and have enhanced the effectiveness of modalities for the early detection and identification of cancer subtypes. Despite all efforts to prolong survival, PDA represents one of the worst tumors, with a 5-year overall survival rate of only 2%-9%[4]. Molecular subtyping of this tumor based on genetic signatures has been developed over the past 10 years; nevertheless, the correlation of such information with clinical phenotypes has been somewhat evasive.

Macroscopically, PDA is characterized by yellowish-white masses, usually without hemorrhagic necrosis[5,6]. On histological examination, typical PDA exhibits duct-like glandular structures infiltrating the pancreatic parenchyma, eliciting a strong desmoplastic stromal response[7,8]. The neoplastic cells are columnar to cuboidal and produce various mucins, depending on differentiation and histotypes. Several genetic alterations such as *KRAS* (chromosome 12p), *SMAD4* (chromosome 18q), *CDKN2A* (chromosome 9p), and *TP53* (chromosome 17p) are responsible for causing PDA[9-11] that progresses from low-grade pancreatic intraepithelial neoplasia (PanIN) to higher-grade PanIN and invasive PDA[12-16].

PDA accounts for up to 90% of pancreatic neoplasms and is typically visualized as a solid mass accompanied by abundant desmoplasia[17]. However, tumors with cystic components occasionally compose a small subset in approximately 4%-5% of patients. Colloid carcinoma, a subtype of PDA that is often conjugated to intraductal papillary mucinous neoplasms (IPMNs), is characterized by extracellular mucin pools. Morphologically, colloid carcinoma tends to exhibit a large, well-demarcated cystic lesion[18]. This subtype of PDA originates, at least in part, from IPMNs, which are well-known pancreatic cystic neoplasms[19-21]. IPMNs can be categorized into two morphologically distinct types — namely, the branched type and main duct type[22,23]. Furthermore, multiple subsets, including gastric, intestinal, and pancreatobiliary types, are histologically defined[24]. These types have unique paths during development and progression to invasive tumors[25], and specific genetic alterations observed in IPMNs may be responsible for tumor phenotypes and ultimately influence the patients' outcome[26,27]. In most cases, other pancreatic lesions with cystic features, such as solid cystic neoplasm (SCN)[28,29], mucinous cystic neoplasm (MCN)[30], and solid pseudopapillary neoplasm (SPN)[28], can be easily distinguished from classic PDA[31]; however, atypical imaging findings of patients occasionally impair proper diagnosis. Knowledge and use of relevant genetic alterations unique to these cystic neoplasms may aid patients and physicians in making appropriate decisions.

Recent studies have revealed a histological subtype of PDA with cystic features mimicking pancreatic cystic diseases[32]. This PDA variant is referred to as large-duct pattern invasive adenocarcinoma of the pancreas (large-duct type) and is characterized by invasive PDA forming large, sometimes dilatated glands. Nevertheless, this entity

is considered rare and often leads to the misdiagnosis of several pancreatic cystic neoplasms, including IPMNs. This review compares the clinical characteristics, gross morphology and histopathology, and genetic alterations of large-duct PDA and other pancreatic cystic neoplasms.

CLINICAL MANIFESTATIONS

Epidemiology

Large-duct PDA has been estimated to occur in < 7% of all PDA cases. It has been reported in only 63 patients in the past literature[33-35]. Large-duct PDA has been initially diagnosed as IPMN in most cases[34]; hence, the incidence of large-duct PDA has not been clearly recognized. Thus, regarding incidence, large-duct PDA of the pancreas is considered rare[36]. The median age of these patients with large-duct PDA was 63.0 years, and the sex ratio was female-dominant, with a female-to-male ratio of 1.74 (Table 1). The female-to-male ratio was higher in patients with large-duct PDA than in those without large-duct PDA[18]. A previous study reported no specific and unique risk factors.

Tumor location and symptoms

Generally, 60% of classic PDAs develop in the pancreatic head[37], and the majority of the patients exhibit symptoms associated with obstructive jaundice. A summary of previously reported cases with large-duct PDA indicated similar tumor locations (Table 1). Kelly *et al*[34] reported that 2 out of 9 patients with large-duct PDA had jaundice. Other patients had acute pancreatitis (n = 1) and abdominal pain (n = 5).

IMAGING FINDINGS

Computed tomography imaging

Dynamic contrast-enhanced computed tomography imaging is an essential modality for assessing cystic lesions in the pancreas. Typical PDA presents as a solid and hypovascular tumor, and the most common radiographic finding of PDA is dilatation or stricture of the main pancreatic duct. In contrast, large-duct PDA usually presents as a cluster of small cystic lesions. The diameter of cysts generally measures 5-7 mm, although they sometimes exceed 10 mm[38]. However, large-duct PDA does not have a "bunch-of-grapes" appearance[39], commonly detected in IPMNs. Given the multiple small cysts visualized on cross-sectional images, distinguishing them from SCN is more complicated, particularly in cases with macrocystic-type cysts[40]. SCN typically has a spongy or honeycomb-like appearance; one previous report described a large-duct PDA case showing a honeycomb-like morphology[36]. Large-duct PDA lacks either a star-shaped scar or calcification at the center of the cyst, commonly observed in SCN[38,41,42]. Considering the female-dominated epidemiology, MCN is also a significant differential diagnosis. MCN has an orange-like appearance and sometimes forms cystic lesions measuring over 10 cm; these features are not typical in large-duct PDA[42,43].

Another critical finding suggestive of the large-duct pattern is the slightly diminished enhancement of the parenchyma surrounding cystic components (Figure 1). Similar to typical PDA, such enhancement gradually increases with time after contrast media injection. Furthermore, walled-off necrosis (WON) should be excluded. WON often contains debris and hemorrhage in cysts, resulting in a mixed density change in the fluid[44,45]. These findings are not usually observed in large-duct PDA. Retention cysts often occur along with PDA and are also important in distinguishing it from large-duct PDA. Retention cysts are typically unilocular and tend to be larger than the cysts observed in large-duct PDA[46,47] (Table 2).

Magnetic resonance imaging

Magnetic resonance cholangiopancreatography is another essential modality for diagnosing pancreatic cystic diseases, except in claustrophobic individuals and those with implanted magnetic resonance imaging-unsafe foreign bodies. Crucial findings for large-duct PDA include small cysts in the pancreas[48], with each cyst usually having a diameter of 0.5-0.7 cm[49]. It should be noted that pancreatic duct stricture is more intense than that observed in other cystic neoplasms. Stricture of the main pancreatic duct and bile duct closely associated with a cluster of small cysts is another

Table 1 Patient characteristics of large-duct pancreatic ductal adenocarcinoma

Ref.	Number of cases	Median age	Sex		Tumor location	
			Female	Male	Head	Body or tail
Kelly *et al*[34], 2012	10	67	6	4	9	1
Bagci *et al*[33], 2012	28	67	19[1]	8[1]	16	11
Kosmahl *et al*[35], 2005	24	58	15	9	12[2]	9[2]
Total	63	63.0	40	23	37	21

[1]One case with missing information on sex.
[2]Three cases with missing information on tumor location.

finding that we may consider when diagnosing large-duct PDA (Figure 2). In making a differential diagnosis of IPMNs, communication with the main pancreatic duct is crucial. Both typical main duct-type and branched duct-type IPMNs communicate with the main pancreatic duct and often show dilatation of the main pancreatic duct [41,50,51].

Moreover, diffusion-weighted imaging and apparent diffusion coefficient map on magnetic resonance cholangiopancreatography is helpful in diagnosing PDA. Considering the high intensity of cystic lesions on T2-weighted images[52,53], diffusion-weighted imaging may not help distinguish large-duct PDA from other pancreatic cystic diseases (Figure 2).

Endoscopic findings

Endoscopic ultrasonography (EUS) has the highest sensitivity for detecting PDA lesions[54], specifically small-sized tumors. Irrespective of the size of PDA, non-neoplastic cystic changes associated with PDA include retention cysts caused by ductal obstruction, and pseudocysts attributable to tumor-associated pancreatitis may be considered to distinguish it from large-duct type tumors[55,56]. A "mucinous clot," which is a hyperechoic lesion inside cysts, is sometimes visualized using EUS in patients with IPMNs, but not in those with large-duct PDAs[57,58]. Furthermore, large-duct PDAs typically lack mural nodules and papillary growth, signatures of high-risk stigmata, and worrisome features in the Fukuoka criteria[41,59]. Youn *et al* [38] described a case of large-duct PDA with a solid mass inside a dilatated glandular cyst[38]. Such atypical findings make a differential diagnosis between large-duct PDAs and IPMNs with high-risk stigmata confusing among endoscopists; nonetheless, surgical resection should be performed for either disease. Considering the multifocality of IPMNs, performing EUS for multifocal lesions, which are not observed in large-duct PDA, is crucial (Table 2)[41,51]. The morphology of "cyst by cyst" appearance can be observed in typical IPMNs using EUS[41].

No report has specifically described EUS-guided fine-needle aspiration (EUS-FNA) in patients with large-duct PDA. In the case of pancreatic tumors with cystic components, concerns related to tumor cell dissemination from the cystic fluid (needle tract seeding)[60] may need to be pointed out (Figure 3). Thus, drawing a distinction between large-duct PDA and IPMN-associated cancer using EUS-FNA can become highly challenging, which will be explained well in the "Pathology" section. The requirements for preoperative histological diagnosis of PDA have increased in the neoadjuvant chemotherapy era; therefore, an ingenious solution for the safe and accurate diagnosis of large-duct PDA using EUS-FNA needs to be considered. No report regarding endoscopic retrograde cholangiopancreatography has described specific findings for large-duct PDA. For tumors developing in the pancreatic head, transpapillary biopsy for histological diagnosis can be useful if massive bile duct invasion is evident.

PATHOLOGY

Macroscopic features

Bagci *et al*[33] described 28 cases of large-duct PDA. Macroscopically, 10 patients had a cyst measuring < 10 mm, whereas 1 patient had a large cyst over 70 mm[33]. Pathologically, each small cyst is a dilatated glandular carcinoma. Similarly, Kosmahl *et al*[35].

Table 2 Clinical and morphological characteristics of pancreatic diseases

Ref.	Diseases	Median age	Sex (%, females)	Tumor location (%, pancreatic head)	Multifocality	Gross appearance	Cyst diameter	Main pancreatic duct communication	Internal structure	Other features	Key genetic alterations
Bagci *et al*[33], 2012 Kelly *et al*[34], 2012 Kosmahl *et al*[35], 2005 Youn *et al*[38], 2018	Large-duct PDA	63	68.3	56.2	No	Multiple small cysts	0.5–0.7 cm in each cyst	No	Dilatated glandular cyst (duct)	Dilatated glandular cyst (duct) is > 0.5 mm in diameter. Easily invades to the perineural plexus and shows desmoplastic reaction	*KRAS* (codon 12)
Seidel *et al*[18], 2002 Adsay *et al*[24], 2016	Colloid carcinoma	61	52.9	66.7	Infrequent	Single or multiple cysts, well demarcated	1.2–16 cm	No	Well-defined pools of mucin, contained scanty malignant epithelial cells	Associated with intestinal-type IPMN	*KRAS*, *BRAF*, *PIK3CA*; MSIs are more frequently observed than non-colloid cancer PDA
Tanaka *et al*[41], 2012 Kim and Cho[46], 2015 Laurent *et al*[51], 2016	Branched duct-type IPMN	65–70	55.2	62.3	Yes	Bunch of grapes	Up to 30 mm for non-invasive IPMN; 30 mm or greater for IPMN with worrisome features	Yes	Cyst by cyst	Main pancreatic duct; normal or dilatated to > 5 mm, suggesting combined type with main duct IPMN	*KRAS* (codon 12), *GNAS*, *RNF43*
Hecht *et al*[19], 2021 Tanaka *et al*[41], 2012 Salvia *et al*[50], 2010	Main duct-type IPMN	60 s	41.3	59.6	Yes	Bunch of grapes	Up to 30 mm for non-invasive IPMN; 30 mm or greater for IPMN with worrisome features	Yes	Cyst by cyst	Main pancreatic duct; partial or diffuse dilatation > 5 mm	*KRAS* (codon 12), *GNAS*, *RNF43*, *TP53*
Ånonsen *et al*[42], 2019 Wu *et al*[80], 2011	SCN	60–70	70	50	No	Spongy or honeycomb-like	3.7–5.1 cm	No	Microcystic or macrocystic		*VHL*
Ånonsen *et al*[42], 2019 Yamao *et al*	MCN	40–50	95	5	Infrequent	Orange-like	1.0–26.4 cm	No	Cyst by cyst	Ovarian-like stroma	*KRAS*, *RNF43*

[43], 2011 Wu *et al*[80], 2011											
Kim and Cho [46], 2015 Assifi *et al*[47], 2014	Retention cyst	60 s	?	?	No	Unilocular	2.8–12 cm	Yes	No cellular dysplasia	Main pancreatic obstruction in should be observed downstream	?
Singhi *et al* [61], 2012 Halfdanarson *et al*[62], 2008 Scarpa *et al* [86], 2017	Pancreatic neuroendocrine neoplasms with cystic changes	50 s	42	24	Infrequent, except MEN1-related neuroendocrine neoplasms	Pinkish-tan to yellowish in color, well demarcated	0.8–18.0 cm	No	Well circumscribed and surrounded by a thin-to-thick fibrous capsule	Larger cysts tend to show hemorrhage	*MEN1, PTEN, DAXX, ATRX, MUTYH, CHEK2*
Tanaka *et al* [41], 2012 Cohen *et al* [45], 2003	Walled-off necrosis	40–50	25	45	Rare	Variable	Variable	Yes	Unilocular	Main pancreatic duct; normal or irregularly dilatated. Observed no later than 6 wk after the occurrence of acute pancreatitis	

PDA: Pancreatic ductal adenocarcinoma; IPMN: Intraductal papillary mucinous neoplasms; MCN: Mucinous cystic neoplasm; SCN: Solid cystic neoplasm.

described 24 cases of large-duct PDA and reported a diameter ranging from 4 to 18 mm for each cyst; the gross tumor size was not different from ordinary PDA[35]. The entire cystic lesion size is crucial, and the cystic component of large-duct PDA is less than 100 mm generally. In comparison, the size of cysts in MCN, SCN, or pancreatic neuroendocrine neoplasms (PanNENs) sometimes exceeds 100 mm (Table 2)[61,62].

Microscopic features

Microscopically, large-duct PDA shows dilatated glands that range in size from 5 to 10 mm. The term "large duct" is defined as dilatation greater than 0.5 mm among ≥ 50% of glands in PDA[33]. Hematoxylin–eosin staining has revealed some essential pathological findings for large-duct PDA. The nuclei of the epithelial carcinoma (dilatated glands) are irregular, wrinkled, and basally located. The cytoplasm exhibits a foamy or microvesicular pattern, and the glands are jagged and irregular[34]. While a papillary growth pattern is typically absent, discrimination of large-duct PDA from other cystic neoplasms, especially gastric-type IPMNs, is crucial[63,64]. In contrast to mucinous carcinoma, large-duct PDA lacks signet ring cells or mucinous lakes[65,66].

Perineural invasion is another important finding for diagnosing large-duct PDA to discriminate from other cystic neoplasms. Bagci *et al*[33] reported that perineural invasion occurred in 88% of large-duct PDAs[33] but was less commonly observed in

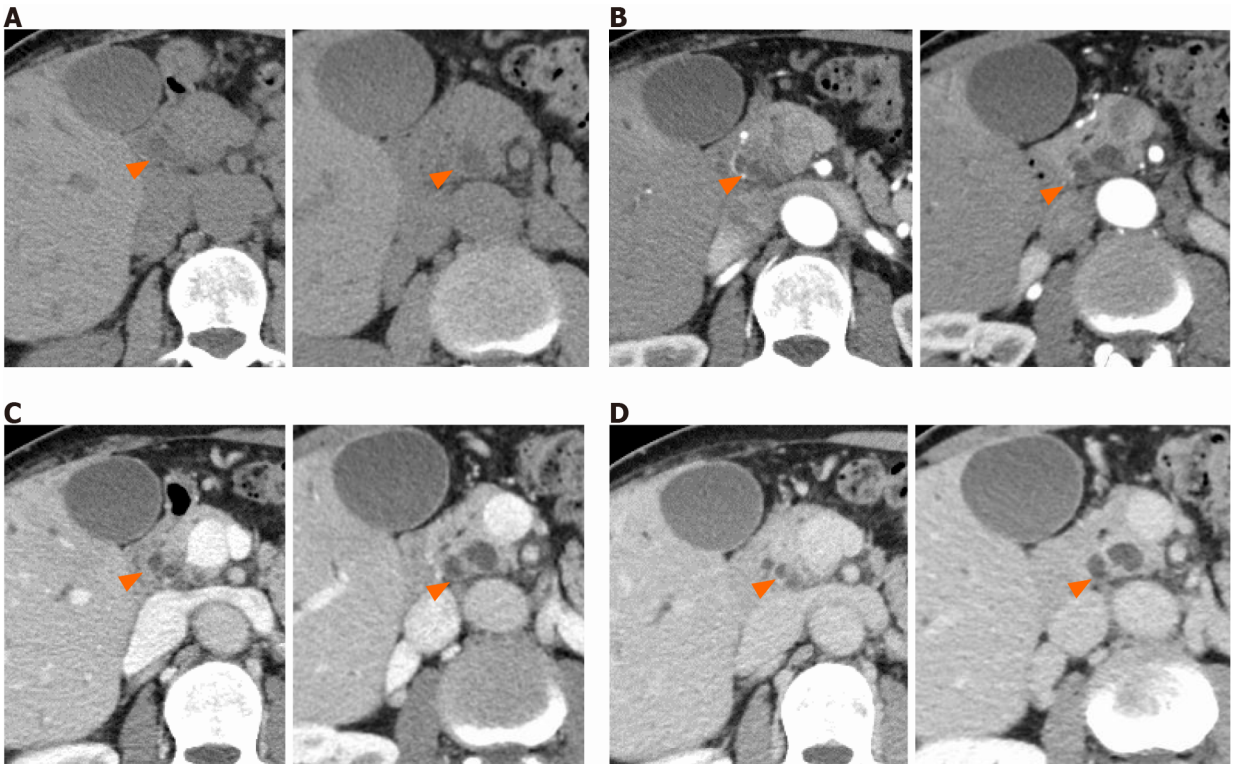


Figure 1 Typical computed tomography imaging of large-duct pancreatic ductal adenocarcinoma. The orange arrowheads show the cystic lesion in large-duct pancreatic ductal adenocarcinoma. A: Plain computed tomography indicates low attenuation area in the pancreas; B: Arterial phase reveals multiple cystic lesions in the pancreatic head without enhancement; C: Portal phase shows multiple cystic lesions in the pancreatic head with parenchymal enhancement; D: Equilibrium phase shows slight ring enhancement of the lesion.

IPMNs, even though the tumor had invasive compartments. In large-duct PDA, the stroma has a rich desmoplastic appearance[33,34]. These features are commonly observed in PDA compared to IPMNs; however, they are more remarkable in large-duct PDA. Additionally, hypercellular stroma or ovarian-like stroma is present in some cases. As for the differential diagnosis of colloid carcinoma or PanNENs, the cysts in these lesions are relatively well-demarcated relative to those in large-duct PDA. PanNENs sometimes have a thick fibrous capsule with hemorrhage[61]. WON usually shows necrotic debris in cysts. Macroscopically, this finding is usually not observed in large-duct PDA and can be a point to distinguish one from other cystic lesions[67].

Immunohistochemistry

Immunohistochemistry (IHC) may be a crucial diagnostic tool that can be utilized to discriminate large-duct PDA from other pancreatic cystic neoplasms[36]. Among the immunostaining widely used in the diagnosis of tumors, mucin (MUC) staining is particularly important and may play a key role in diagnosing large-duct PDA. Gastric-type IPMN exhibits relatively low papillary growth[64] and is typically low grade. However, in tumors with high-grade gastric IPMN, distinction from the large-duct type PDA is sometimes uncertain pathologically. IPMNs present unique MUC staining patterns, depending on the epithelial subtypes[63]. Gastric-type IPMNs show MUC5AC-positive staining patterns and are negative for MUC1, MUC2, and MUC6. Kelly *et al*[34] and Kosmahl *et al*[35] reported MUC staining for large-duct PDAs (Table 3)[34,35]; the positivity rates of MUC1, MUC2, MUC5AC, and MUC6 immunostaining were 79.4%, 8.8%, 78.8%, and 60.6%, respectively. Thus, MUC1 and MUC6 IHC may help make to a differential diagnosis from IPMNs[68].

Furthermore, p53 IHC is beneficial in diagnosing lesions with malignant potential. Bagci *et al*[33] showed that 73% of all large-duct PDAs positively stained for p53. Another point of IHC for large-duct PDA is whether the dilatated glands are dilatated glandular carcinomas or dilatated glands resulting from the occlusion of upper-stream pancreatic ducts. To presume the origin of anatomical localization of the dilated pancreatic ducts, Elastica–Masson staining may be suitable (Figure 4). In addition to the main pancreatic duct, elastin fibers in these normal pancreatic ducts can be stained with Elastica–Masson. Typically, the dilatated glands in large-duct PDA are negative

Table 3 Mucin staining profiles of large-duct pancreatic ductal adenocarcinoma

Ref.	MUC1	MUC2	MUC5AC	MUC6
Kelly *et al*[34], 2012	10/10 (100.0%)	1/10 (90.0%)	9/10 (90.0%)	8/10 (80.0%)
Kosmahl *et al*[35], 2005	17/24 (70.8%)	2/24 (8.3%)	17/23 (73.1%)	12/23 (52.2%)
Total	27/37 (73.0%)	3/37 (8.1%)	26/36 (72.2%)	20/36 (55.6%)

No. of staining-positive cases/available cases. MUC: Mucin.

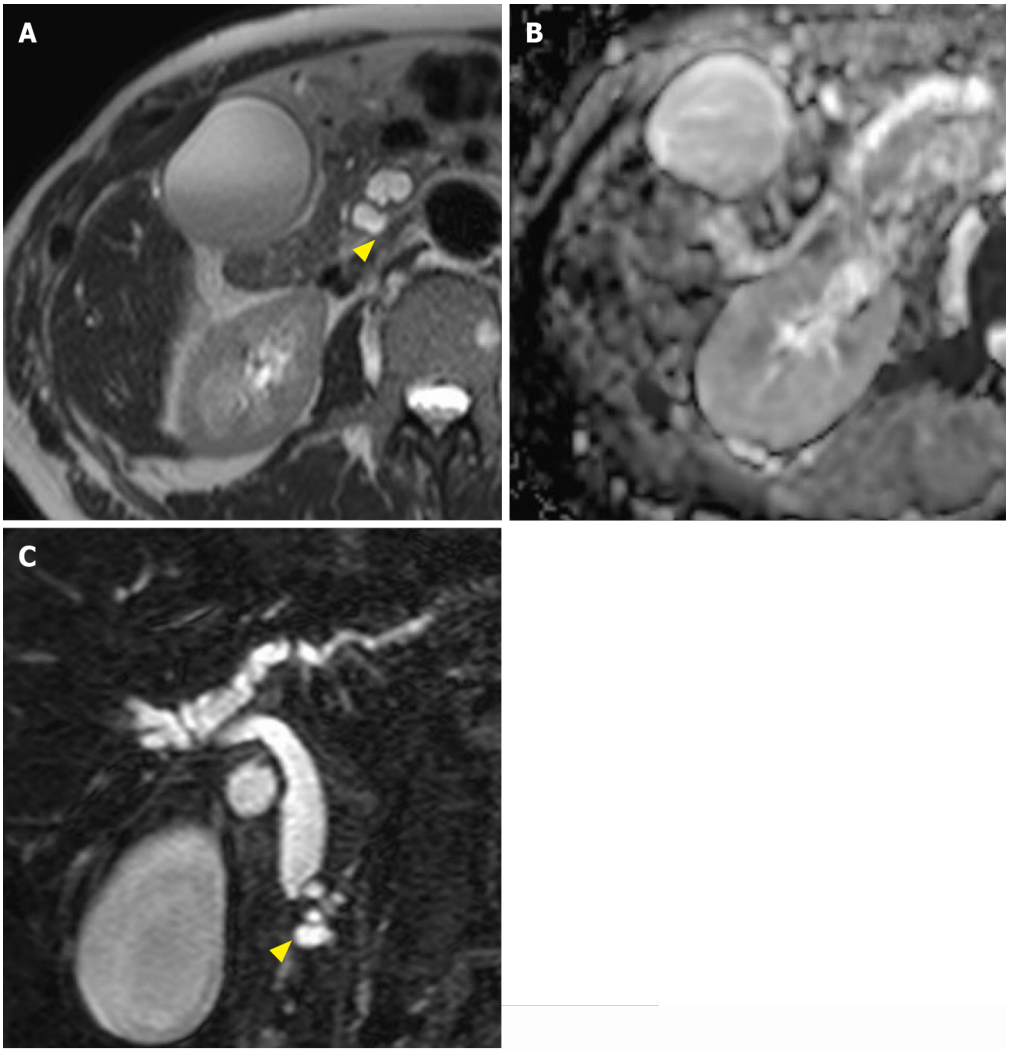


Figure 2 Typical magnetic resonance imaging of large-duct pancreatic ductal adenocarcinoma. The yellow arrowheads show the cystic lesion in large-duct pancreatic ductal adenocarcinoma (PDA). A: T2-weighted imaging reveals high intensity in the large-duct PDA lesion; B: Diffusion-weighted imaging shows no significant signal increase/decrease in the lesion; C: Magnetic resonance cholangiopancreatography (coronal view) reveals multiple cystic lesions in the pancreatic head and compressed bile duct.

for the immunostaining, suggesting they were derived from tumor glands rather than the secondary dilation of the normal main pancreatic duct and associated large branch duct[69].

GENETIC ALTERATIONS

KRAS is mutated in the great majority of PDA, and approximately 90%-95% of PDA patients have major hot spot mutations in codons 12, 13, and 61[9]. This genetic event has been identified at the earliest stages of PanIN, and studies in genetically

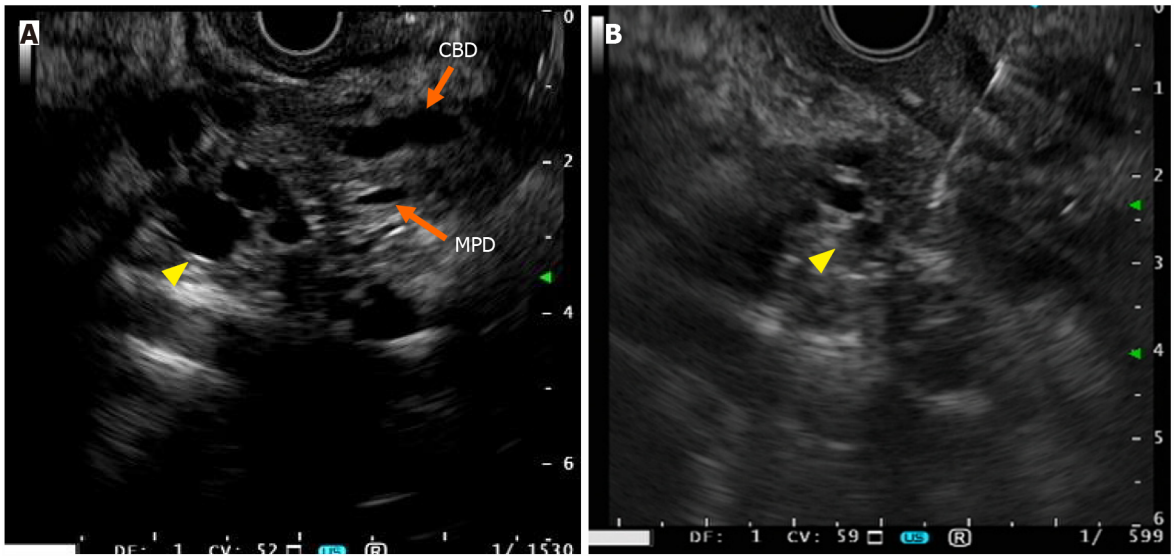


Figure 3 Endoscopic ultrasonography findings of large-duct pancreatic ductal adenocarcinoma. The yellow arrowhead shows the cystic lesion in large-duct pancreatic ductal adenocarcinoma (PDA). A: Endoscopic ultrasonography (EUS) reveals multiple echoic lesions in the pancreatic head; B: EUS-fine-needle aspiration was performed in the parenchyma of the cystic lesion in large-duct PDA. The EUS model used was GF-UE260-AL5 (Olympus, Tokyo, Japan). The ultrasonic diagnostic equipment used was EU-ME2 (Olympus, Tokyo, Japan). CBD: Common bile duct; MPD: Main pancreatic duct.

engineered mice models (GEMMs) indicate that PanINs arise from pancreatic acinar cells that incur Kras mutations (Ptf1a-induced Kras activation). In the $Kras^{G12D}$-expressing mice, PanIN formation coincides with acinar-to-ductal metaplasia (ADM), characterized by the replacement of acinar cells with cells expressing ductal markers, such as CK19 and the ductal fate determinant Sox9[70]. Further, propensity of ductal and centroacinar cells to give rise to PanINs is demonstrated by GEMM with $Sox9^{CreER}$-mediated Kras activation, although considerably less frequent relative to $Ptf1a^{CreER}$-induced Kras activation[71].

Although observations in these mice models have not been validated in humans, multiple PanIN-like lesions with various grades of atypia can often be observed in the "normal area" of the resected specimens of pancreatic cancer. These lesions are usually solitary and localized in the normal acinar compartment without pancreatitis. However, PanINs associated with a cluster of ADM can also be seen[9]. Nevertheless, it remains to be determined if the PDA, including large-duct type, originated from either differentiated duct or duct-like cells associated with ADM. Such hypothesis may be clarified based on the particular mutation profiles and molecular signature related to cellular origin.

Moreover, genetic analysis is valuable in establishing a differential diagnosis between large-duct PDA and IPMNs. Bagci *et al*[33] conducted a mutation analysis of *KRAS* codon 12, which is the most frequent mutation point in PDA, using specimens from 28 large-duct PDA patients compared to ordinary ductal adenocarcinoma of the pancreas[33]. The results indicated 82% of large-duct PDAs harbored *KRAS* codon 12 mutations, a bit less frequency observed in classic PDAs (generally over 90%)[33,72]. Since the authors utilized slot-blot Southern analysis of polymerase chain reaction-amplified samples, *KRAS* mutation in codon 12 might be missed in case of the tumor with low tumor cellularity, and latest methods may also identify other hotspots at codons 13 and 61. Additionally, IPMN can be initiated by *KRAS* mutation[9], although less frequently than PanIN[73,74] . *KRAS* mutations are observed in 53%-87% of gastric-type IPMNs and approximately 40% in intestinal-type IPMNs. In contrast to PDAs including large-duct type, IPMNs frequently harbor multiple different types of *KRAS* mutations, suggesting poly-clonal diseases[25]. Therefore, specifying the variation in *KRAS* mutation types may be valuable in determining a subset of patients with large-duct PDA.

TP53 gene is also frequently mutated around 75% of pancreatic carcinoma patients [75], and Bagci *et al*[33] reported that abnormal immunostaining of p53 was observed in 73% of large-duct type PDA, a higher frequency relative to classic PDA (60%). Moreover, *TP53* mutation can be seen in 10%-40% of high-grade IPMNs and 40%-60% of IPMN-associated invasive carcinomas[73,76]. Given the strong gain-of-function of particular missense mutations in *TP53* relative to the truncating mutations[77,78], further studies will be required if such *TP53* mutation types are associated with the

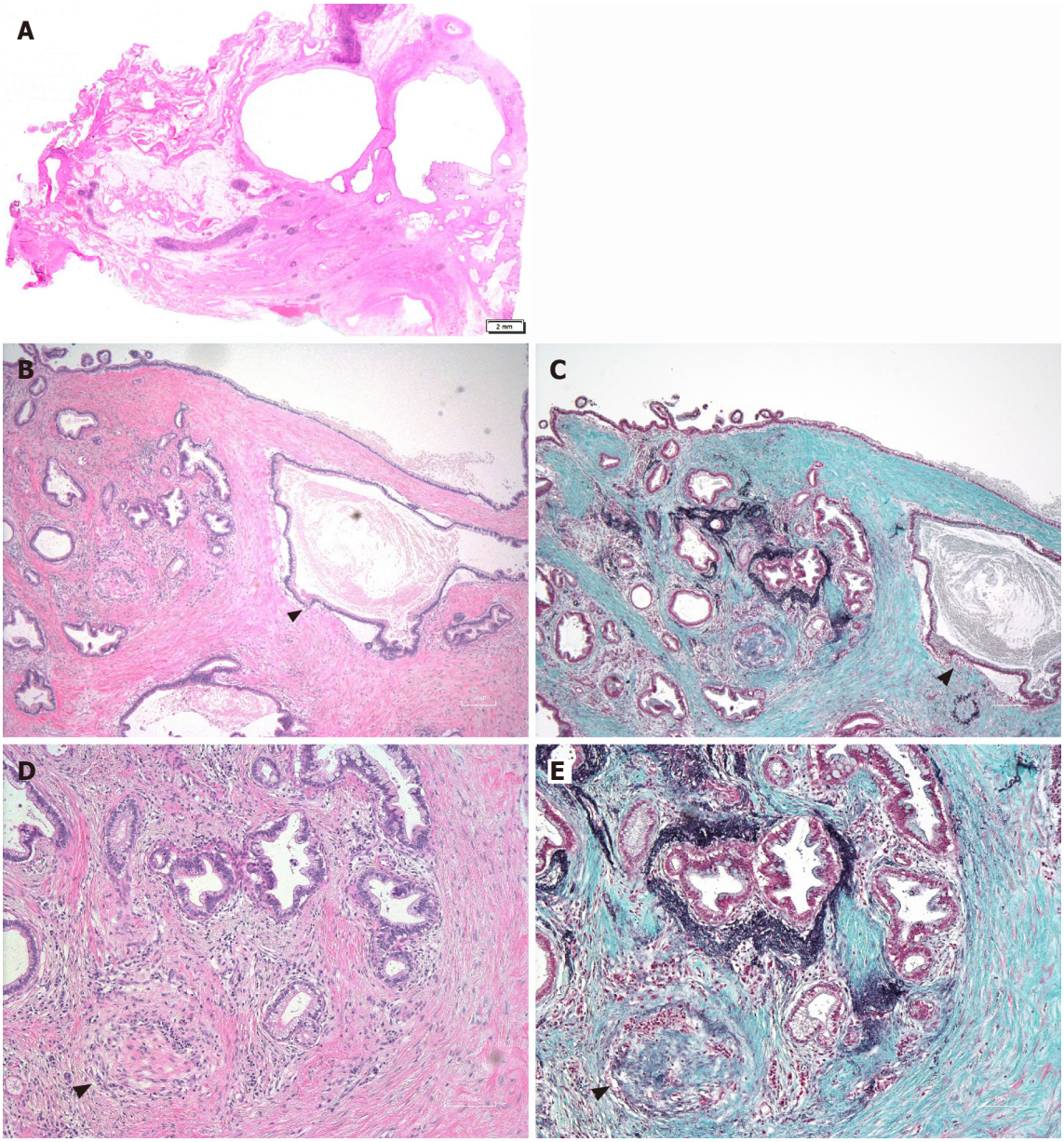


Figure 4 Pathological findings of large-duct pancreatic ductal adenocarcinoma. A: Hematoxylin–eosin (HE) staining of the lesion (× 4); B: HE staining of the lesion (× 200) shows a dilatated glandular carcinoma > 0.5 mm in diameter (black arrowhead); C: Elastica–Masson immunohistochemistry (× 200) reveals that the dilatated glands lack elastic fibers (black arrowhead); D: HE staining of the carcinoma invading the myelin sheath (× 200; black arrowhead); E: Elastica–Masson immunohistochemistry (× 200) reveals that the dilatated glands invading the myelin sheath lack elastic fibers (black arrowheads show the myelin fiber).

tumor phenotypes. *SMAD4* is also inactivated in 55% of PDA cases; of these, 35% are inactivated by homozygous deletion, whereas 20% show loss of one allele[10]. Inactivation of *SMAD4* enhances the rapid progression of $Kras^{G12D}$-initiated mice pancreatic neoplasms, showing IPMN-like phenotypes[79], and a significant fraction of IPMN-associated human pancreatic cancer harbors *SMAD4* mutation and the abnormal immunostaining[25]. Whether *SMAD4* inactivation can influence the progression or promotion of large-duct PDA remains unclear.

GNAS mutations are unique to IPMNs[80,81] and are more frequently identified in intestinal-type IPMNs than in gastric- and pancreatobiliary-type IPMNs[9]. The mutation can progress to IPMN-associated PDA, and recent studies demonstrated that about 8%-11% of pancreatic cancers[9], including cases considered not related explicitly to IPMN, harbor *GNAS* mutations or amplifications. Since *GNAS* mutation can lead to entirely distinct transcriptional and metabolic reprogramming to *KRAS*-driven circuitry[82], identification of *GNAS* mutations may discriminate large-duct PDA from IPMN-related pancreatic cancer.

RNF43 encodes an E3 ubiquitin ligase. *RNF43* mutations are observed in IPMNs and MCNs and are detected in approximately 50% of all IPMNs[83]. Nonetheless, a recent

meta-analysis has suggested that *RNF43* mutations are not associated with clinicopathologic parameters in patients with IPMN[83,84]. Approximately 50% of MCNs present *RNF43* mutations, including loss of heterozygosity[85]. Other cystic neoplasms in the pancreas exhibit specific genetic mutations. Genetic alterations such as *VHL* mutations and loss of heterozygosity in SCN, *CTNNB1*, and *PIK3CA* mutations in SPN and PanNEN-associated mutations (*MEN1*, *PTEN*, *DAXX*, and *ATRX*) have been specifically identified in SCNs and PanNENs, particularly in cases with cystic components resembling retention cysts[85,86] (Table 2).

Further genetic analysis should be performed in the large-duct PDA, considering whether the cellular origin of the large-duct PDA was the branch or main pancreatic duct and pathways of carcinogenesis[87,88]. Whole-genome sequencing may be helpful in determining the genes responsible for forming large-duct glands[87,89].

TREATMENT AND PROGNOSIS

Large-duct PDA has been recognized as an uncommon subtype of PDA. Hence, in previous reports, large-duct PDA was mainly diagnosed from surgically resected specimens[33,34]. Preoperative diagnosis was variable, including IPMN, MCN, and "PDA with solid and cystic mass." Furthermore, the patients in these reports were diagnosed at an advanced stage, and the period of survival after resections was about 7-16 mo. These reports were published in the era when adjuvant or neoadjuvant chemotherapy for PDA was not established. The outcome is worse than that of surgically resected IPMNs, reported as 37.0 mo[90], and chemotherapy is not generally considered if the invasive component in IPMN-associated cancer is not evident. Therefore, distinguishing large-duct PDA from other cystic neoplasms, including IPMNs, is essential. Accurate histological and genomic information related to the tumor will help decide between appropriate therapeutic options before surgery[91-93]. Currently, how standard chemotherapy against PDAs, including both adjuvant and neoadjuvant chemotherapy, can effectively eradicate large-duct PDAs remains unclear.

CONCLUSION

Large-duct PDA is a subtype of PDA that mimics IPMNs or other pancreatic cystic neoplasms. Given the rarity of this disease, the diagnostic approach is sometimes challenging. Considering the estimated prognosis of the patients, it is crucial to distinguish large-duct PDAs from IPMNs using macroscopic and pathological findings. When an atypical cystic lesion is identified in the pancreas, both symptomatic and asymptomatic, the differential diagnosis should include large-duct PDA. Given their advantages, genetic analysis of PDAs and IPMNs, exploration of *KRAS* mutations, and mutation profiling of other cancer-related genes may help establish an accurate diagnosis.

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REFERENCES

1 **Fitzgerald TL**, Hickner ZJ, Schmitz M, Kort EJ. Changing incidence of pancreatic neoplasms: a 16-year review of statewide tumor registry. *Pancreas* 2008; **37**: 134-138 [PMID: 18665072 DOI: 10.1097/MPA.0b013e318163a329]

2 **Rahib L**, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; **74**: 2913-2921 [PMID: 24840647 DOI: 10.1158/0008-5472.CAN-14-0155]

3 **Goess R**, Friess H. A look at the progress of treating pancreatic cancer over the past 20 years. *Expert*

Rev Anticancer Ther 2018; **18**: 295-304 [PMID: 29334794 DOI: 10.1080/14737140.2018.1428093]
4 **McGuigan A**, Kelly P, Turkington RC, Jones C, Coleman HG, McCain RS. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J Gastroenterol* 2018; **24**: 4846-4861 [PMID: 30487695 DOI: 10.3748/wjg.v24.i43.4846]
5 **Schlitter AM**, Jesinghaus M, Jäger C, Konukiewitz B, Muckenhuber A, Demir IE, Bahra M, Denkert C, Friess H, Kloeppel G, Ceyhan GO, Weichert W. pT but not pN stage of the 8th TNM classification significantly improves prognostication in pancreatic ductal adenocarcinoma. *Eur J Cancer* 2017; **84**: 121-129 [PMID: 28802189 DOI: 10.1016/j.ejca.2017.06.034]
6 **He J**, Ahuja N, Makary MA, Cameron JL, Eckhauser FE, Choti MA, Hruban RH, Pawlik TM, Wolfgang CL. 2564 resected periampullary adenocarcinomas at a single institution: trends over three decades. *HPB (Oxford)* 2014; **16**: 83-90 [PMID: 23472829 DOI: 10.1111/hpb.12078]
7 **Schlitter AM**, Segler A, Steiger K, Michalski CW, Jäger C, Konukiewitz B, Pfarr N, Endris V, Bettstetter M, Kong B, Regel I, Kleeff J, Klöppel G, Esposito I. Molecular, morphological and survival analysis of 177 resected pancreatic ductal adenocarcinomas (PDACs): Identification of prognostic subtypes. *Sci Rep* 2017; **7**: 41064 [PMID: 28145465 DOI: 10.1038/srep41064]
8 **Hruban RH**, Maitra A, Kern SE, Goggins M. Precursors to pancreatic cancer. *Gastroenterol Clin North Am* 2007; **36**: 831-849, vi [PMID: 17996793 DOI: 10.1016/j.gtc.2007.08.012]
9 **Patra KC**, Bardeesy N, Mizukami Y. Diversity of Precursor Lesions For Pancreatic Cancer: The Genetics and Biology of Intraductal Papillary Mucinous Neoplasm. *Clin Transl Gastroenterol* 2017; **8**: e86 [PMID: 28383565 DOI: 10.1038/ctg.2017.3]
10 **Hahn SA**, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996; **271**: 350-353 [PMID: 8553070 DOI: 10.1126/science.271.5247.350]
11 **Caldas C**, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, Weinstein CL, Hruban RH, Yeo CJ, Kern SE. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet* 1994; **8**: 27-32 [PMID: 7726912 DOI: 10.1038/ng0994-27]
12 **Real FX**. A "catastrophic hypothesis" for pancreas cancer progression. *Gastroenterology* 2003; **124**: 1958-1964 [PMID: 12806629 DOI: 10.1016/s0016-5085(03)00389-5]
13 **Hata T**, Suenaga M, Marchionni L, Macgregor-Das A, Yu J, Shindo K, Tamura K, Hruban RH, Goggins M. Genome-Wide Somatic Copy Number Alterations and Mutations in High-Grade Pancreatic Intraepithelial Neoplasia. *Am J Pathol* 2018; **188**: 1723-1733 [PMID: 29684357 DOI: 10.1016/j.ajpath.2018.03.012]
14 **Wilentz RE**, Iacobuzio-Donahue CA, Argani P, McCarthy DM, Parsons JL, Yeo CJ, Kern SE, Hruban RH. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res* 2000; **60**: 2002-2006 [PMID: 10766191]
15 **Cáceres M**, Quesada R, Iglesias M, Real FX, Villamonte M, de Villarreal JM, Pérez M, Andaluz A, Moll X, Berjano E, Dorcaratto D, Sánchez-Velázquez P, Grande L, Burdío F. Pancreatic duct ligation reduces premalignant pancreatic lesions in a Kras model of pancreatic adenocarcinoma in mice. *Sci Rep* 2020; **10**: 18344 [PMID: 33110094 DOI: 10.1038/s41598-020-74947-4]
16 **Kaneda A**, Matsusaka K, Aburatani H, Fukayama M. Epstein-Barr virus infection as an epigenetic driver of tumorigenesis. *Cancer Res* 2012; **72**: 3445-3450 [PMID: 22761333 DOI: 10.1158/0008-5472.CAN-11-3919]
17 **Di Maggio F**, El-Shakankery KH. Desmoplasia and Biophysics in Pancreatic Ductal Adenocarcinoma: Can We Learn From Breast Cancer? *Pancreas* 2020; **49**: 313-325 [PMID: 32168249 DOI: 10.1097/MPA.0000000000001504]
18 **Seidel G**, Zahurak M, Iacobuzio-Donahue C, Sohn TA, Adsay NV, Yeo CJ, Lillemoe KD, Cameron JL, Hruban RH, Wilentz RE. Almost all infiltrating colloid carcinomas of the pancreas and periampullary region arise from in situ papillary neoplasms: a study of 39 cases. *Am J Surg Pathol* 2002; **26**: 56-63 [PMID: 11756769 DOI: 10.1097/00000478-200201000-00006]
19 **Hecht EM**, Khatri G, Morgan D, Kang S, Bhosale PR, Francis IR, Gandhi NS, Hough DM, Huang C, Luk L, Megibow A, Ream JM, Sahani D, Yaghmai V, Zaheer A, Kaza R. Intraductal papillary mucinous neoplasm (IPMN) of the pancreas: recommendations for Standardized Imaging and Reporting from the Society of Abdominal Radiology IPMN disease focused panel. *Abdom Radiol (NY)* 2021; **46**: 1586-1606 [PMID: 33185741 DOI: 10.1007/s00261-020-02853-4]
20 **Nasca V**, Chiaravalli M, Piro G, Esposito A, Salvatore L, Tortora G, Corbo V, Carbone C. Intraductal Pancreatic Mucinous Neoplasms: A Tumor-Biology Based Approach for Risk Stratification. *Int J Mol Sci* 2020; **21** [PMID: 32887490 DOI: 10.3390/ijms21176386]
21 **Zaccari P**, Cardinale V, Severi C, Pedica F, Carpino G, Gaudio E, Doglioni C, Petrone MC, Alvaro D, Arcidiacono PG, Capurso G. Common features between neoplastic and preneoplastic lesions of the biliary tract and the pancreas. *World J Gastroenterol* 2019; **25**: 4343-4359 [PMID: 31496617 DOI: 10.3748/wjg.v25.i31.4343]
22 **Lu C**, Xu CF, Wan XY, Zhu HT, Yu CH, Li YM. Screening for pancreatic cancer in familial high-risk individuals: A systematic review. *World J Gastroenterol* 2015; **21**: 8678-8686 [PMID: 26229410 DOI: 10.3748/wjg.v21.i28.8678]
23 **Chang YR**, Park JK, Jang JY, Kwon W, Yoon JH, Kim SW. Incidental pancreatic cystic neoplasms in an asymptomatic healthy population of 21,745 individuals: Large-scale, single-center cohort study. *Medicine (Baltimore)* 2016; **95**: e5535 [PMID: 28002329 DOI: 10.1097/MD.0000000000005535]
24 **Adsay V**, Mino-Kenudson M, Furukawa T, Basturk O, Zamboni G, Marchegiani G, Bassi C, Salvia

R, Malleo G, Paiella S, Wolfgang CL, Matthaei H, Offerhaus GJ, Adham M, Bruno MJ, Reid MD, Krasinskas A, Klöppel G, Ohike N, Tajiri T, Jang KT, Roa JC, Allen P, Fernández-del Castillo C, Jang JY, Klimstra DS, Hruban RH; Members of Verona Consensus Meeting, 2013. Pathologic Evaluation and Reporting of Intraductal Papillary Mucinous Neoplasms of the Pancreas and Other Tumoral Intraepithelial Neoplasms of Pancreatobiliary Tract: Recommendations of Verona Consensus Meeting. *Ann Surg* 2016; **263**: 162-177 [PMID: 25775066 DOI: 10.1097/SLA.0000000000001173]

25 **Omori Y**, Ono Y, Tanino M, Karasaki H, Yamaguchi H, Furukawa T, Enomoto K, Ueda J, Sumi A, Katayama J, Muraki M, Taniue K, Takahashi K, Ambo Y, Shinohara T, Nishihara H, Sasajima J, Maguchi H, Mizukami Y, Okumura T, Tanaka S. Pathways of Progression From Intraductal Papillary Mucinous Neoplasm to Pancreatic Ductal Adenocarcinoma Based on Molecular Features. *Gastroenterology* 2019; **156**: 647-661. e2 [PMID: 30342036 DOI: 10.1053/j.gastro.2018.10.029]

26 **Schönleben F**, Qiu W, Bruckman KC, Ciau NT, Li X, Lauerman MH, Frucht H, Chabot JA, Allendorf JD, Remotti HE, Su GH. BRAF and KRAS gene mutations in intraductal papillary mucinous neoplasm/carcinoma (IPMN/IPMC) of the pancreas. *Cancer Lett* 2007; **249**: 242-248 [PMID: 17097223 DOI: 10.1016/j.canlet.2006.09.007]

27 **Tan MC**, Basturk O, Brannon AR, Bhanot U, Scott SN, Bouvier N, LaFemina J, Jarnagin WR, Berger MF, Klimstra D, Allen PJ. GNAS and KRAS Mutations Define Separate Progression Pathways in Intraductal Papillary Mucinous Neoplasm-Associated Carcinoma. *J Am Coll Surg* 2015; **220**: 845-854. e1 [PMID: 25840541 DOI: 10.1016/j.jamcollsurg.2014.11.029]

28 **Chagas VL**, Rosman FC, Carvalho MDGDC. Solid pseudopapillary neoplasia of the pancreas: a review. *Rev Assoc Med Bras (1992)* 2020; **66**: 87-94 [PMID: 32130387 DOI: 10.1590/1806-9282.66.1.87]

29 **Gandhi D**, Sharma P, Parashar K, Kochar PS, Ahuja K, Sawhney H, Sharma S. Solid pseudopapillary Tumor of the Pancreas: Radiological and surgical review. *Clin Imaging* 2020; **67**: 101-107 [PMID: 32559679 DOI: 10.1016/j.clinimag.2020.06.008]

30 **Nilsson LN**, Keane MG, Shamali A, Millastre Bocos J, Marijinissen van Zanten M, Antila A, Verdejo Gil C, Del Chiaro M, Laukkarinen J. Nature and management of pancreatic mucinous cystic neoplasm (MCN): A systematic review of the literature. *Pancreatology* 2016; **16**: 1028-1036 [PMID: 27681503 DOI: 10.1016/j.pan.2016.09.011]

31 **Hasan A**, Visrodia K, Farrell JJ, Gonda TA. Overview and comparison of guidelines for management of pancreatic cystic neoplasms. *World J Gastroenterol* 2019; **25**: 4405-4413 [PMID: 31496620 DOI: 10.3748/wjg.v25.i31.4405]

32 **Nitta T**, Mitsuhashi T, Hatanaka Y, Hirano S, Matsuno Y. Pancreatic ductal adenocarcinomas with multiple large cystic structures: a clinicopathologic and immunohistochemical study of seven cases. *Pancreatology* 2013; **13**: 401-408 [PMID: 23890139 DOI: 10.1016/j.pan.2013.05.004]

33 **Bagci P**, Andea AA, Basturk O, Jang KT, Erbarut I, Adsay V. Large duct type invasive adenocarcinoma of the pancreas with microcystic and papillary patterns: a potential microscopic mimic of non-invasive ductal neoplasia. *Mod Pathol* 2012; **25**: 439-448 [PMID: 22056954 DOI: 10.1038/modpathol.2011.181]

34 **Kelly PJ**, Shinagare S, Sainani N, Hong X, Ferrone C, Yilmaz O, Fernández-del Castillo C, Lauwers GY, Deshpande V. Cystic papillary pattern in pancreatic ductal adenocarcinoma: a heretofore undescribed morphologic pattern that mimics intraductal papillary mucinous carcinoma. *Am J Surg Pathol* 2012; **36**: 696-701 [PMID: 22367300 DOI: 10.1097/PAS.0b013e318249ce1c]

35 **Kosmahl M**, Pauser U, Anlauf M, Klöppel G. Pancreatic ductal adenocarcinomas with cystic features: neither rare nor uniform. *Mod Pathol* 2005; **18**: 1157-1164 [PMID: 15920540 DOI: 10.1038/modpathol.3800446]

36 **Adsay V**, Logani S, Sarkar F, Crissman J, Vaitkevicius V. Foamy gland pattern of pancreatic ductal adenocarcinoma: a deceptively benign-appearing variant. *Am J Surg Pathol* 2000; **24**: 493-504 [PMID: 10757396 DOI: 10.1097/00000478-200004000-00003]

37 **Greenlee RT**, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. *CA Cancer J Clin* 2000; **50**: 7-33 [PMID: 10735013 DOI: 10.3322/canjclin.50.1.7]

38 **Youn SY**, Rha SE, Jung ES, Lee IS. Pancreas ductal adenocarcinoma with cystic features on cross-sectional imaging: radiologic-pathologic correlation. *Diagn Interv Radiol* 2018; **24**: 5-11 [PMID: 29317372 DOI: 10.5152/dir.2018.17250]

39 **Kovač JD**, Janković A, Mašulović D. The "bunch of grapes" pattern of branch-duct IPMN. *Abdom Radiol (NY)* 2019; **45**: 249-250 [PMID: 31605187 DOI: 10.1007/s00261-019-02266-y]

40 **Chakravarthy S**, Chakravarty R, Sarkar N, Chitrotpala R. Radiological diagnosis of rare pancreatic serous cystadenoma. *J Family Med Prim Care* 2019; **8**: 2744-2746 [PMID: 31548970 DOI: 10.4103/jfmpc.jfmpc_454_19]

41 **Tanaka M**, Fernández-del Castillo C, Adsay V, Chari S, Falconi M, Jang JY, Kimura W, Levy P, Pitman MB, Schmidt CM, Shimizu M, Wolfgang CL, Yamaguchi K, Yamao K; International Association of Pancreatology. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. *Pancreatology* 2012; **12**: 183-197 [PMID: 22687371 DOI: 10.1016/j.pan.2012.04.004]

42 **Ånonsen K**, Sahakyan MA, Kleive D, Waage A, Verbeke C, Hauge T, Buanes T, Edwin B, Labori KJ. Trends in management and outcome of cystic pancreatic lesions - analysis of 322 cases undergoing surgical resection. *Scand J Gastroenterol* 2019; **54**: 1051-1057 [PMID: 31322457 DOI: 10.1080/00365521.2019.1642379]

43 **Yamao K**, Yanagisawa A, Takahashi K, Kimura W, Doi R, Fukushima N, Ohike N, Shimizu M,

Hatori T, Nobukawa B, Hifumi M, Kobayashi Y, Tobita K, Tanno S, Sugiyama M, Miyasaka Y, Nakagohri T, Yamaguchi T, Hanada K, Abe H, Tada M, Fujita N, Tanaka M. Clinicopathological features and prognosis of mucinous cystic neoplasm with ovarian-type stroma: a multi-institutional study of the Japan pancreas society. *Pancreas* 2011; **40**: 67-71 [PMID: 20924309 DOI: 10.1097/MPA.0b013e3181f749d3]

44 **Rana SS**, Bhasin DK, Sharma R, Gupta R. Factors determining recurrence of fluid collections following migration of intended long term transmural stents in patients with walled off pancreatic necrosis and disconnected pancreatic duct syndrome. *Endosc Ultrasound* 2015; **4**: 208-212 [PMID: 26374578 DOI: 10.4103/2303-9027.162999]

45 **Cohen-Scali F**, Vilgrain V, Brancatelli G, Hammel P, Vullierme MP, Sauvanet A, Menu Y. Discrimination of unilocular macrocystic serous cystadenoma from pancreatic pseudocyst and mucinous cystadenoma with CT: initial observations. *Radiology* 2003; **228**: 727-733 [PMID: 12954892 DOI: 10.1148/radiol.2283020973]

46 **Kim YS**, Cho JH. Rare nonneoplastic cysts of pancreas. *Clin Endosc* 2015; **48**: 31-38 [PMID: 25674524 DOI: 10.5946/ce.2015.48.1.31]

47 **Assifi MM**, Nguyen PD, Agrawal N, Dedania N, Kennedy EP, Sauter PK, Prestipino A, Winter JM, Yeo CJ, Lavu H. Non-neoplastic epithelial cysts of the pancreas: a rare, benign entity. *J Gastrointest Surg* 2014; **18**: 523-531 [PMID: 24449000 DOI: 10.1007/s11605-014-2459-7]

48 **Young MR**, Wagner PD, Ghosh S, Rinaudo JA, Baker SG, Zaret KS, Goggins M, Srivastava S. Validation of Biomarkers for Early Detection of Pancreatic Cancer: Summary of The Alliance of Pancreatic Cancer Consortia for Biomarkers for Early Detection Workshop. *Pancreas* 2018; **47**: 135-141 [PMID: 29346214 DOI: 10.1097/MPA.0000000000000973]

49 **Yoon SE**, Byun JH, Kim KA, Kim HJ, Lee SS, Jang SJ, Jang YJ, Lee MG. Pancreatic ductal adenocarcinoma with intratumoral cystic lesions on MRI: correlation with histopathological findings. *Br J Radiol* 2010; **83**: 318-326 [PMID: 19620175 DOI: 10.1259/bjr/69770140]

50 **Salvia R**, Crippa S, Partelli S, Armatura G, Malleo G, Paini M, Pea A, Bassi C. Differences between main-duct and branch-duct intraductal papillary mucinous neoplasms of the pancreas. *World J Gastrointest Surg* 2010; **2**: 342-346 [PMID: 21160841 DOI: 10.4240/wjgs.v2.i10.342]

51 **Laurent L**, Vullierme MP, Rebours V, Maire F, Hentic O, Francoz C, Durand F, Ruszniewski P, Levy P. Estimation of the prevalence of intraductal papillary mucinous neoplasm of the pancreas in the French population through patients waiting for liver transplantation. *United European Gastroenterol J* 2017; **5**: 499-503 [PMID: 28588880 DOI: 10.1177/2050640616664842]

52 **Wang Y**, Miller FH, Chen ZE, Merrick L, Mortele KJ, Hoff FL, Hammond NA, Yaghmai V, Nikolaidis P. Diffusion-weighted MR imaging of solid and cystic lesions of the pancreas. *Radiographics* 2011; **31**: E47-E64 [PMID: 21721197 DOI: 10.1148/rg.313105174]

53 **Morelli L**, Guadagni S, Borrelli V, Pisano R, Di Franco G, Palmeri M, Furbetta N, Gambaccini D, Marchi S, Boraschi P, Bastiani L, Campatelli A, Mosca F, Di Candio G. Role of abdominal ultrasound for the surveillance follow-up of pancreatic cystic neoplasms: a cost-effective safe alternative to the routine use of magnetic resonance imaging. *World J Gastroenterol* 2019; **25**: 2217-2228 [PMID: 31143072 DOI: 10.3748/wjg.v25.i18.2217]

54 **Kitano M**, Yoshida T, Itonaga M, Tamura T, Hatamaru K, Yamashita Y. Impact of endoscopic ultrasonography on diagnosis of pancreatic cancer. *J Gastroenterol* 2019; **54**: 19-32 [PMID: 30406288 DOI: 10.1007/s00535-018-1519-2]

55 **Kadiyala V**, Lee LS. Endosonography in the diagnosis and management of pancreatic cysts. *World J Gastrointest Endosc* 2015; **7**: 213-223 [PMID: 25789091 DOI: 10.4253/wjge.v7.i3.213]

56 **Will U**, Reichel A, Fueldner F, Meyer F. Endoscopic ultrasonography-guided drainage for patients with symptomatic obstruction and enlargement of the pancreatic duct. *World J Gastroenterol* 2015; **21**: 13140-13151 [PMID: 26674313 DOI: 10.3748/wjg.v21.i46.13140]

57 **Tsujino T**, Chang KJ. Cyst-Within-Cyst Found on EUS-Guided Cystoscopy of a Pancreatic Mucinous Cystic Neoplasia. *Am J Gastroenterol* 2016; **111**: 1089 [PMID: 27481409 DOI: 10.1038/ajg.2016.149]

58 **Sbeit W**, Kadah A, Farraj M, Shiller M. Pancreatic Cyst Mucin Ball Aspiration as a Diagnostic Maneuver. *Isr Med Assoc J* 2018; **20**: 394 [PMID: 29911766]

59 **Tanaka M**, Fernández-Del Castillo C, Kamisawa T, Jang JY, Levy P, Ohtsuka T, Salvia R, Shimizu Y, Tada M, Wolfgang CL. Revisions of international consensus Fukuoka guidelines for the management of IPMN of the pancreas. *Pancreatology* 2017; **17**: 738-753 [PMID: 28735806 DOI: 10.1016/j.pan.2017.07.007]

60 **Gao RY**, Wu BH, Shen XY, Peng TL, Li DF, Wei C, Yu ZC, Luo MH, Xiong F, Wang LS, Yao J. Overlooked risk for needle tract seeding following endoscopic ultrasound-guided minimally invasive tissue acquisition. *World J Gastroenterol* 2020; **26**: 6182-6194 [PMID: 33177792 DOI: 10.3748/wjg.v26.i40.6182]

61 **Singhi AD**, Chu LC, Tatsas AD, Shi C, Ellison TA, Fishman EK, Kawamoto S, Schulick RD, Wolfgang CL, Hruban RH, Edil BH. Cystic pancreatic neuroendocrine tumors: a clinicopathologic study. *Am J Surg Pathol* 2012; **36**: 1666-1673 [PMID: 23073325 DOI: 10.1097/PAS.0b013e31826a0048]

62 **Halfdanarson TR**, Rubin J, Farnell MB, Grant CS, Petersen GM. Pancreatic endocrine neoplasms: epidemiology and prognosis of pancreatic endocrine tumors. *Endocr Relat Cancer* 2008; **15**: 409-427 [PMID: 18508996 DOI: 10.1677/ERC-07-0221]

63 **Furukawa T**, Klöppel G, Volkan Adsay N, Albores-Saavedra J, Fukushima N, Horii A, Hruban RH,

Kato Y, Klimstra DS, Longnecker DS, Lüttges J, Offerhaus GJ, Shimizu M, Sunamura M, Suriawinata A, Takaori K, Yonezawa S. Classification of types of intraductal papillary-mucinous neoplasm of the pancreas: a consensus study. *Virchows Arch* 2005; **447**: 794-799 [PMID: 16088402 DOI: 10.1007/s00428-005-0039-7]

64 **Furukawa T**, Hatori T, Fujita I, Yamamoto M, Kobayashi M, Ohike N, Morohoshi T, Egawa S, Unno M, Takao S, Osako M, Yonezawa S, Mino-Kenudson M, Lauwers GY, Yamaguchi H, Ban S, Shimizu M. Prognostic relevance of morphological types of intraductal papillary mucinous neoplasms of the pancreas. *Gut* 2011; **60**: 509-516 [PMID: 21193453 DOI: 10.1136/gut.2010.210567]

65 **Adsay NV**, Merati K, Nassar H, Shia J, Sarkar F, Pierson CR, Cheng JD, Visscher DW, Hruban RH, Klimstra DS. Pathogenesis of colloid (pure mucinous) carcinoma of exocrine organs: Coupling of gel-forming mucin (MUC2) production with altered cell polarity and abnormal cell-stroma interaction may be the key factor in the morphogenesis and indolent behavior of colloid carcinoma in the breast and pancreas. *Am J Surg Pathol* 2003; **27**: 571-578 [PMID: 12717243 DOI: 10.1097/00000478-200305000-00002]

66 **Adsay NV**, Pierson C, Sarkar F, Abrams J, Weaver D, Conlon KC, Brennan MF, Klimstra DS. Colloid (mucinous noncystic) carcinoma of the pancreas. *Am J Surg Pathol* 2001; **25**: 26-42 [PMID: 11145249 DOI: 10.1097/00000478-200101000-00003]

67 **Rana SS**, Sharma V, Sharma R, Gupta R, Bhasin DK. Endoscopic ultrasound guided transmural drainage of walled off pancreatic necrosis using a "step - up" approach: A single centre experience. *Pancreatology* 2017; **17**: 203-208 [PMID: 28202234 DOI: 10.1016/j.pan.2017.02.007]

68 **Lau SK**, Weiss LM, Chu PG. Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study. *Am J Clin Pathol* 2004; **122**: 61-69 [PMID: 15272531 DOI: 10.1309/9R66-73QE-C06D-86Y4]

69 **Haeberle L**, Esposito I. Pathology of pancreatic cancer. *Transl Gastroenterol Hepatol* 2019; **4**: 50 [PMID: 31304427 DOI: 10.21037/tgh.2019.06.02]

70 **Morris JP 4th**, Cano DA, Sekine S, Wang SC, Hebrok M. Beta-catenin blocks Kras-dependent reprogramming of acini into pancreatic cancer precursor lesions in mice. *J Clin Invest* 2010; **120**: 508-520 [PMID: 20071774 DOI: 10.1172/JCI40045]

71 **Kopp JL**, von Figura G, Mayes E, Liu FF, Dubois CL, Morris JP 4th, Pan FC, Akiyama H, Wright CV, Jensen K, Hebrok M, Sander M. Identification of Sox9-dependent acinar-to-ductal reprogramming as the principal mechanism for initiation of pancreatic ductal adenocarcinoma. *Cancer Cell* 2012; **22**: 737-750 [PMID: 23201164 DOI: 10.1016/j.ccr.2012.10.025]

72 **Kanda M**, Matthaei H, Wu J, Hong SM, Yu J, Borges M, Hruban RH, Maitra A, Kinzler K, Vogelstein B, Goggins M. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology* 2012; **142**: 730-733. e9 [PMID: 22226782 DOI: 10.1053/j.gastro.2011.12.042]

73 **Kuboki Y**, Shimizu K, Hatori T, Yamamoto M, Shibata N, Shiratori K, Furukawa T. Molecular biomarkers for progression of intraductal papillary mucinous neoplasm of the pancreas. *Pancreas* 2015; **44**: 227-235 [PMID: 25423558 DOI: 10.1097/MPA.0000000000000253]

74 **Tsuda M**, Fukuda A, Roy N, Hiramatsu Y, Leonhardt L, Kakiuchi N, Hoyer K, Ogawa S, Goto N, Ikuta K, Kimura Y, Matsumoto Y, Takada Y, Yoshioka T, Maruno T, Yamaga Y, Kim GE, Akiyama H, Wright CV, Saur D, Takaori K, Uemoto S, Hebrok M, Chiba T, Seno H. The BRG1/SOX9 axis is critical for acinar cell-derived pancreatic tumorigenesis. *J Clin Invest* 2018; **128**: 3475-3489 [PMID: 30010625 DOI: 10.1172/JCI94287]

75 **Redston MS**, Caldas C, Seymour AB, Hruban RH, da Costa L, Yeo CJ, Kern SE. p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. *Cancer Res* 1994; **54**: 3025-3033 [PMID: 8187092]

76 **Furukawa T**, Fujisaki R, Yoshida Y, Kanai N, Sunamura M, Abe T, Takeda K, Matsuno S, Horii A. Distinct progression pathways involving the dysfunction of DUSP6/MKP-3 in pancreatic intraepithelial neoplasia and intraductal papillary-mucinous neoplasms of the pancreas. *Mod Pathol* 2005; **18**: 1034-1042 [PMID: 15832194 DOI: 10.1038/modpathol.3800383]

77 **Walerych D**, Lisek K, Sommaggio R, Piazza S, Ciani Y, Dalla E, Rajkowska K, Gaweda-Walerych K, Ingallina E, Tonelli C, Morelli MJ, Amato A, Eterno V, Zambelli A, Rosato A, Amati B, Wiśniewski JR, Del Sal G. Proteasome machinery is instrumental in a common gain-of-function program of the p53 missense mutants in cancer. *Nat Cell Biol* 2016; **18**: 897-909 [PMID: 27347849 DOI: 10.1038/ncb3380]

78 **Escobar-Hoyos LF**, Penson A, Kannan R, Cho H, Pan CH, Singh RK, Apken LH, Hobbs GA, Luo R, Lecomte N, Babu S, Pan FC, Alonso-Curbelo D, Morris JP 4th, Askan G, Grbovic-Huezo O, Ogrodowski P, Bermeo J, Saglimbeni J, Cruz CD, Ho YJ, Lawrence SA, Melchor JP, Goda GA, Bai K, Pastore A, Hogg SJ, Raghavan S, Bailey P, Chang DK, Biankin A, Shroyer KR, Wolpin BM, Aguirre AJ, Ventura A, Taylor B, Der CJ, Dominguez D, Kümmel D, Oeckinghaus A, Lowe SW, Bradley RK, Abdel-Wahab O, Leach SD. Altered RNA Splicing by Mutant p53 Activates Oncogenic RAS Signaling in Pancreatic Cancer. *Cancer Cell* 2020; **38**: 198-211. e8 [PMID: 32559497 DOI: 10.1016/j.ccell.2020.05.010]

79 **Bardeesy N**, Cheng KH, Berger JH, Chu GC, Pahler J, Olson P, Hezel AF, Horner J, Lauwers GY, Hanahan D, DePinho RA. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. *Genes Dev* 2006; **20**: 3130-3146 [PMID: 17114584 DOI: 10.1101/gad.1478706]

80 **Wu J**, Matthaei H, Maitra A, Dal Molin M, Wood LD, Eshleman JR, Goggins M, Canto MI, Schulick

RD, Edil BH, Wolfgang CL, Klein AP, Diaz LA Jr, Allen PJ, Schmidt CM, Kinzler KW, Papadopoulos N, Hruban RH, Vogelstein B. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. *Sci Transl Med* 2011; **3**: 92ra66 [PMID: 21775669 DOI: 10.1126/scitranslmed.3002543]

81 **Furukawa T**, Kuboki Y, Tanji E, Yoshida S, Hatori T, Yamamoto M, Shibata N, Shimizu K, Kamatani N, Shiratori K. Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. *Sci Rep* 2011; **1**: 161 [PMID: 22355676 DOI: 10.1038/srep00161]

82 **Patra KC**, Kato Y, Mizukami Y, Widholz S, Boukhali M, Revenco I, Grossman EA, Ji F, Sadreyev RI, Liss AS, Screaton RA, Sakamoto K, Ryan DP, Mino-Kenudson M, Castillo CF, Nomura DK, Haas W, Bardeesy N. Mutant GNAS drives pancreatic tumourigenesis by inducing PKA-mediated SIK suppression and reprogramming lipid metabolism. *Nat Cell Biol* 2018; **20**: 811-822 [PMID: 29941929 DOI: 10.1038/s41556-018-0122-3]

83 **Amato E**, Molin MD, Mafficini A, Yu J, Malleo G, Rusev B, Fassan M, Antonello D, Sadakari Y, Castelli P, Zamboni G, Maitra A, Salvia R, Hruban RH, Bassi C, Capelli P, Lawlor RT, Goggins M, Scarpa A. Targeted next-generation sequencing of cancer genes dissects the molecular profiles of intraductal papillary neoplasms of the pancreas. *J Pathol* 2014; **233**: 217-227 [PMID: 24604757 DOI: 10.1002/path.4344]

84 **Lee JH**, Kim Y, Choi JW, Kim YS. KRAS, GNAS, and RNF43 mutations in intraductal papillary mucinous neoplasm of the pancreas: a meta-analysis. *Springerplus* 2016; **5**: 1172 [PMID: 27512631 DOI: 10.1186/s40064-016-2847-4]

85 **Wu J**, Jiao Y, Dal Molin M, Maitra A, de Wilde RF, Wood LD, Eshleman JR, Goggins MG, Wolfgang CL, Canto MI, Schulick RD, Edil BH, Choti MA, Adsay V, Klimstra DS, Offerhaus GJ, Klein AP, Kopelovich L, Carter H, Karchin R, Allen PJ, Schmidt CM, Naito Y, Diaz LA Jr, Kinzler KW, Papadopoulos N, Hruban RH, Vogelstein B. Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. *Proc Natl Acad Sci USA* 2011; **108**: 21188-21193 [PMID: 22158988 DOI: 10.1073/pnas.1118046108]

86 **Scarpa A**, Chang DK, Nones K, Corbo V, Patch AM, Bailey P, Lawlor RT, Johns AL, Miller DK, Mafficini A, Rusev B, Scardoni M, Antonello D, Barbi S, Sikora KO, Cingarlini S, Vicentini C, McKay S, Quinn MCJ, Bruxner TJC, Christ AN, Harliwong I, Idrisoglu S, McLean S, Nourse C, Nourbakhsh E, Wilson PJ, Anderson MJ, Fink JL, Newell F, Waddell N, Holmes O, Kazakoff SH, Leonard C, Wood S, Xu Q, Hiriyur Nagaraj S, Amato E, Dalai I, Bersani S, Cataldo I, Dei Tos AP, Capelli P, Vittoria Davì M, Landoni L, Malpaga A, Miotto M, Whitehall VLJ, Leggett BA, Harris JL, Harris J, Jones MD, Humphris J, Chantrill LA, Chin V, Nagrial AM, Pajic M, Scarlett CJ, Pinho A, Rooman I, Toon C, Wu J, Pinese M, Cowley M, Barbour A, Mawson A, Humphrey ES, Colvin EK, Chou A, Lovell JA, Jamieson NB, Duthie F, Gingras MC, Fisher WE, Dagg RA, Lau LMS, Lee M, Pickett HA, Reddel RR, Samra JS, Kench JG, Merrett ND, Epari K, Nguyen NQ, Zeps N, Falconi M, Simbolo M, Butturini G, Van Buren G, Partelli S, Fassan M; Australian Pancreatic Cancer Genome Initiative, Khanna KK, Gill AJ, Wheeler DA, Gibbs RA, Musgrove EA, Bassi C, Tortora G, Pederzoli P, Pearson JV, Biankin AV, Grimmond SM. Corrigendum: Whole-genome landscape of pancreatic neuroendocrine tumours. *Nature* 2017; **550**: 548 [PMID: 28953865 DOI: 10.1038/nature24026]

87 **Pelosi E**, Castelli G, Testa U. Pancreatic Cancer: Molecular Characterization, Clonal Evolution and Cancer Stem Cells. *Biomedicines* 2017; **5** [PMID: 29156578 DOI: 10.3390/biomedicines5040065]

88 **Lytle NK**, Ferguson LP, Rajbhandari N, Gilroy K, Fox RG, Deshpande A, Schürch CM, Hamilton M, Robertson N, Lin W, Noel P, Wartenberg M, Zlobec I, Eichmann M, Galván JA, Karamitopoulou E, Gilderman T, Esparza LA, Shima Y, Spahn P, French R, Lewis NE, Fisch KM, Sasik R, Rosenthal SB, Kritzik M, Von Hoff D, Han H, Ideker T, Deshpande AJ, Lowy AM, Adams PD, Reya T. A Multiscale Map of the Stem Cell State in Pancreatic Adenocarcinoma. *Cell* 2019; **177**: 572-586. e22 [PMID: 30955884 DOI: 10.1016/j.cell.2019.03.010]

89 **Jones S**, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008; **321**: 1801-1806 [PMID: 18772397 DOI: 10.1126/science.1164368]

90 **Okabayashi T**, Shima Y, Kosaki T, Sumiyoshi T, Kozuki A, Iiyama T, Takezaki Y, Kobayashi M, Nishimori I, Ogawa Y, Hanazaki K. Invasive carcinoma derived from branch duct-type IPMN may be a more aggressive neoplasm than that derived from main duct-type IPMN. *Oncol Lett* 2013; **5**: 1819-1825 [PMID: 23833648 DOI: 10.3892/ol.2013.1268]

91 **Oettle H**, Neuhaus P, Hochhaus A, Hartmann JT, Gellert K, Ridwelski K, Niedergethmann M, Zülke C, Fahlke J, Arning MB, Sinn M, Hinke A, Riess H. Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: the CONKO-001 randomized trial. *JAMA* 2013; **310**: 1473-1481 [PMID: 24104372 DOI: 10.1001/jama.2013.279201]

92 **Uesaka K**, Boku N, Fukutomi A, Okamura Y, Konishi M, Matsumoto I, Kaneoka Y, Shimizu Y, Nakamori S, Sakamoto H, Morinaga S, Kainuma O, Imai K, Sata N, Hishinuma S, Ojima H, Yamaguchi R, Hirano S, Sudo T, Ohashi Y; JASPAC 01 Study Group. Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: a phase 3, open-label, randomised, non-inferiority trial (JASPAC 01). *Lancet* 2016; **388**: 248-257 [PMID: 27265347 DOI:

10.1016/S0140-6736(16)30583-9]

93 **Eguchi H**, Takeda Y, Takahashi H, Nakahira S, Kashiwazaki M, Shimizu J, Sakai D, Isohashi F, Nagano H, Mori M, Doki Y. A Prospective, Open-Label, Multicenter Phase 2 Trial of Neoadjuvant Therapy Using Full-Dose Gemcitabine and S-1 Concurrent with Radiation for Resectable Pancreatic Ductal Adenocarcinoma. *Ann Surg Oncol* 2019; **26**: 4498-4505 [PMID: 31440928 DOI: 10.1245/s10434-019-07735-8]

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MINIREVIEWS

Chronic hepatitis B in pregnant women: Current trends and approaches

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Abstract

Chronic hepatitis B (CHB) is a significant public health problem worldwide. The aim of the present review is to summarize the actual trends in the management of CHB in pregnant women. The prevalence of hepatitis B virus (HBV) infection in pregnant women is usually comparable to that in the general population in the corresponding geographic area. All women have to be screened for hepatitis B surface antigen (HBsAg) during pregnancy. Additional examinations of pregnant women with CHB may include maternal hepatitis B e antigen, HBV viral load, alanine aminotransferase level, and HBsAg level. The management of pregnancy depends on the phase of the HBV infection, which has to be determined before pregnancy. In women of childbearing age with CHB, antiviral therapy can pursue two main goals: Treatment of active CHB, and vertical transmission prevention. During pregnancy, tenofovir is the drug of choice in both cases. A combination of hepatitis B immunoglobulin and vaccine against hepatitis B should be administered within the first 12 h to all infants born to mothers with CHB. In such cases, there are no contraindications to breastfeeding.

Key Words: Chronic hepatitis B; Hepatitis B viral load; Pregnancy; Antiviral treatment;

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Core Tip: All women have to be screened for hepatitis B surface antigen (HBsAg) during pregnancy. Additional examinations of pregnant women with chronic hepatitis B (CHB) may include maternal hepatitis B e antigen, hepatitis B virus (HBV) viral load, alanine aminotransferase level, and HBsAg level. The management of pregnancy depends on the phase of the HBV infection, which has to be determined before pregnancy. During pregnancy, tenofovir is the drug of choice both for active CHB treatment and vertical transmission prevention. A combination of hepatitis B immunoglobulin and vaccine against hepatitis B should be administered within the first 12 h to all infants born to mothers with CHB.

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INTRODUCTION

Chronic hepatitis B (CHB) is a significant public health problem worldwide. According to the current estimation by the World Health Organization (WHO), in 2015 about 257 million people in the world were living with CHB[1,2]. The geographic distribution of CHB is highly heterogeneous. There are regions with high (more than 8%), medium (2%-8%) and low (less than 2%) levels of hepatitis B (HB) prevalence. The course of CHB varies from asymptomatic carriage of hepatitis B surface antigen (HBsAg) to severe, active variants with progression of fibrosis, formation of liver cirrhosis, and the development of hepatocellular carcinoma (HCC). Despite the successes achieved by the introduction of mass vaccination against hepatitis B, the vertical route of transmission remains an important factor. Every year, 4-5 million children in the world are infected from mothers with CHB[3]. In endemic regions, more than 50% of patients with CHB become infected at birth or in early childhood[4]. The problem of HBV mother-to-child transmission (MTCT) is important because patients infected in early childhood develop CHB in most cases, while the risk of CHB development in patients infected in adulthood is not higher than 20%. Without prophylaxis, MTCT rates vary significantly depending on the mother's hepatitis B e antigen (HBeAg) status: the transmission rate for HBeAg-positive mothers is about 70%-90%, *vs* 10%-40% for HBeAg-negative mothers[5]. In 2016, the WHO set the goal of eliminating viral hepatitis as a major public health threat by 2030[6]. However, this goal cannot be achieved without solving the problem of vertical transmission of HBV. In this context, in order to reduce the HBV MTCT risk, it is important to apply different approaches to the management of pregnancy in women with CHB.

CURRENT LIMITATION ON SCREENING FOR HBSAG IN PREGNANT WOMEN

In most developed and developing countries, all pregnant women are screened for HBsAg. Examining pregnant women only from the so-called risk groups (intravenous drug use, promiscuous sex, work in sex industry, sexual contact with HBsAg carriers) was not enough, since such an examination leaves up to 50% of pregnant women with CHB undetected[7].

Particular attention should be given to women who are diagnosed with CHB for the first time during pregnancy. In these patients, acute hepatitis B has to be excluded. Additional examinations of pregnant women with CHB may differ depending on the region. Table 1 presents the recommendations of the main hepatological communities

Table 1 Examination of pregnant women

	APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
All pregnant women	Pregnant female (preferably during the first trimester to vaccinate unprotected mothers) should be tested for HBV infection	Screening for HBsAg in the first trimester of pregnancy is strongly recommended	All pregnant women should be screened for HBV infection
Examination of HBsAg-positive women during pregnancy	Maternal HBeAg, HBV DNA status, and ALT level should be checked during pregnancy	ALT, HBV DNA level, and HBsAg level	ALT level, HBV DNA or imaging for HCC surveillance if indicated

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; HCC: Hepatocellular carcinoma.

for the examination of pregnant women with CHB[8-10].

Most recommendations agree that viral load determination is necessary to understand the advisability of antiviral treatment during pregnancy. Recommendations differ as to the timing of therapy initiation and timing of the examination. The viral load determination should be performed no later than week 30 of gestation.

Determination of the HBsAg level during pregnancy is currently prescribed only in the European clinical guidelines for the management of patients with CHB[9]. Meanwhile, available studies indicate a significant correlation between the level of HBsAg during pregnancy and the risk of vertical transmission[11-13]. During pregnancy, HBsAg level is a more stable parameter than viral load, and its measurement is cheaper. Therefore, it can be recommended as a predictor of the vertical transmission of HBV infection, especially in a resource-limited setting. In a pregnant woman with a low HBsAg level, HBV viral load testing is not necessary.

PREVALENCE OF HEPATITIS B IN PREGNANT WOMEN

The prevalence of HBV infection in pregnant women is usually comparable to that in the general population in the same geographic area. In China the prevalence of HBV infection among women of childbearing age is 2%-8%[14,15], while in the United States it is only 0.4%[16].

The prevalence of HBsAg positive patients among pregnant women in several countries is shown in Table 2.

At present, a high HBV prevalence among pregnant women persists in African countries, while the rate of HBsAg-positive pregnant women in Europe and America is low. Even in China, where the prevalence of HBV was very high in the past, a significant reduction in the rate of HBsAg-positive pregnant women is now observed.

COURSE OF CHB AND VERTICAL TRANSMISSION RISK

As agreed by most researchers, there are five phases of the natural course of CHB.

The first phase, called the phase of immune tolerance, usually occurs during perinatal infection and is characterized by a prolonged and low-symptom course, normal serum alanine aminotransferase (ALT) level and minimal changes in liver tissue. As shown in Table 3, patients in this phase of CHB are seropositive for HBeAg and have mostly a high viral load (10^8-10^9 IU/mL HBV DNA)[30,31]. In patients infected in adulthood, the duration of this phase is usually short[32].

The second phase, known as the immunoreactive phase, occurs in patients infected at birth or in early childhood. It starts after two or three decades and is characterized by occasionally increasing ALT values. The anti-HBV immune response results in a moderate (as compared to the first phase) decrease in HBV DNA level. The age of patients when this phase occurs depends on the HBV genotype and varies by geographic region. In Taiwan, 90% of HBeAg seroconversion occurs in patients under the age of 40 years, with genotype B seroconversion occurring earlier than with genotype C[30]. In the European region, no more than 30% of patients remain HBeAg-positive after the age of 40 years[30]. This is important, because the earlier pregnancy occurs, the higher the chances that the woman is in the first phase of CHB, with high viral replication, and, accordingly, a high risk of vertical transmission of HBV

Table 2 Prevalence of hepatitis B surface antigen among pregnant women

Ref.	Country	Years	Number	HBsAg-positive (%)
Kirbak *et al*[17], 2017	Republic of South Sudan	2013-2014	280	11
Fouelifack *et al*[18], 2018	Cameroon	2016	360	9.4
Bittaye *et al*[19], 2019	Gambia	2015	426	9.2
Tanga *et al*[20], 2019	South Western Ethiopia	2017	253	7.9
Kishk *et al*[21], 2020	Egypt	2018-2019	600	5
Fessehaye *et al*[22], 2018	Eritrea	2016	5009	3.2
Sheng *et al*[23], 2018	China	2016	14314	3.1
Cetin *et al*[24], 2018	Turkey	2016	475	2.1
Mishra *et al*[25], 2017	India	2016	3567	1.09
Biondi *et al*[26], 2020	Canada	2012-2016	651745	0.63
Lembo *et al*[27], 2017	Italy	2010-2015	7558	0.5
Ruiz-Extremera *et al*[28], 2020	Spain	2015	21870	0.42
Harris *et al*[29], 2018	United States	2011-2014	870888	0.14

HBsAg: Hepatitis B surface antigen.

Table 3 Clinical features and vertical transmission risk in different phases of chronic hepatitis B

Phase of CHB	ALT	Fibrosis (Metavir score)	HBV DNA level	Markers of HBV-infection	Vertical transmission risk
Phase of immune tolerance	Normal	F0	Very high (10^8-10^9 IU/mL)	HBsAg+; HBeAg+; HBeAb-; HBcorAb+	Very high
Immunoreactive phase	Elevated	F1-F4	High (10^6-10^7 IU/mL)	HBsAg+; HBeAg+/-; HBeAb-/+; HBcorAb+	High
Inactive carriage of HBsAg	Normal	F0	Less than 2000 IU/mL	HBsAg+; HBeAg-; HBeAb+; HBcorAb+	Low
Phase of HBeAg-negative CHB	Elevated	F1-F4	Middle (10^3-10^7 IU/mL)	HBsAg+; HBeAg-; HBeAb+; HBcorAb+	Depends on HBV viral load
Occult CHB	Normal	F1-F4	+/-, HBV DNA in liver+	HBsAg-; HBeAg-; HBeAb-; HBcorAb+/-	Low

CHB: Chronic hepatitis B; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBeAB: Hepatitis B e antibody; HBcorAb: Hepatitis B core antibody.

infection.

The third phase—the phase of inactive carriage of HBsAg—is characterized by the presence of HBsAg, the absence of HBeAg, and a low (less than 2000 IU/mL) or undetectable HBV viral load. The ALT level is normal in this phase, and no fibrosis progression is observed. Spontaneous HBsAg seroconversion is possible. This phase can continue for decades. The risk of vertical transmission at this stage is low.

The fourth phase, referred to as the HBeAg-negative CHB phase, is characterized by an undulating course, with periodic ALT increases. The HBV viral load can vary significantly, while HBsAg level is a more stable indicator[33]. HBeAg is absent during this phase. There is a gradual progression of fibrosis, and the risk of developing HCC increases. In this phase, the vertical transmission risk depends on HBV viral load.

The fifth phase, called the HBsAg-negative phase or "occult" CHB, is characterized by the disappearance of HBsAg, although the virus continues to replicate in the liver. Clinical symptoms are usually not pronounced, the ALT level remains normal. There is a possibility of CHB reactivation, especially due to immunosuppression, for example a physiological immunosuppression during pregnancy. A few cases of CHB reactivation during pregnancy are reported[34,35]. The vertical transmission risk in

such situations is low.

Management of pregnancy depends on the phase of HBV-infection. Unfortunately, women frequently only learn about their CHB diagnosis during pregnancy. Thus, it is advisable to examine all women for markers of viral hepatitis before pregnancy. During pregnancy, there are limitations for reliably determining the stage of CHB, since several indicators change significantly from the beginning of pregnancy. The level of alpha-fetoprotein increases as early as in the first weeks of pregnancy. Some pathological conditions (toxicosis of the first half of pregnancy, excessive vomiting of pregnant women, *etc.*) can lead to significant changes in cytolytic indicators. In such cases, it is sometimes difficult to determine whether an increase in ALT is caused by these conditions or by CHB activity. Some standard examinations are unreliable during pregnancy. For example, a significant change in circulating blood volume during pregnancy can lead to inaccurate data on liver fibrosis obtained using transient elastography. For this reason, it is preferable to determine the stage of CHB before pregnancy.

Typically, women of childbearing age do not have significant liver fibrosis and cirrhosis. However, due to the increasing age of primiparous women and to the fact that before mass vaccination of newborns against hepatitis B was introduced, one of the main routes of transmission was the vertical route, CHB with advanced fibrosis is not unique. Pregnancy at the stage of liver cirrhosis is also associated with an increased risks of complications for the mother[36].

EFFECT OF PREGNANCY ON THE COURSE OF CHB

In most cases, no exacerbation of CHB occurs during pregnancy, and the cytolytic activity indicators are usually normalized. Nevertheless, a few cases of CHB exacerbation during pregnancy, including development of fulminant liver failure[37,38]. The level of HBV viral load during pregnancy may vary. Cases of CHB reactivation during pregnancy have been known. In one study, in mothers without detectable HBV DNA in the first trimester, HBV DNA was detected in 19.6% of cases in the second trimester and in 30.4% of cases in the third trimester[39]. In another study, the viral load in women with CHB increased during pregnancy and decreased after childbirth[34]. In addition, some studies describe exacerbation of hepatitis in the first months after childbirth[34,40,41]. In the majority of women, the ALT level decreases during pregnancy, but after childbirth there is a significant increase in the cytolytic activity. For example, an increase in ALT level of three times or more was observed in 45% of women within 6 mo after childbirth[34]. Cases of HBeAg seroconversion during pregnancy have also been described in 12.5%-17% of patients[40,41].

Clinical manifestations of CHB in pregnant women are characterized by the predominance of asthenic and dyspeptic syndromes (63%). Hemorrhagic syndrome, such as bleeding gums, was observed in 15% of pregnant women, and hepatomegaly occurred in 10% of cases[42].

PREGNANCY OUTCOMES IN HBV INFECTED WOMEN

The effect of chronic maternal HBV infection on pregnancy outcome has not been well studied. Published works on this topic contradict each other. Some studies show that there is no association between pregnancy outcomes and maternal CHB[43]. Other studies have shown that chronic HBV infection does not result in negative perinatal outcomes, except for lower Apgar scores in newborns[44,45]. However, some studies indicate a high rate of diseases such as fetal distress syndrome, preterm labor and meconium peritonitis among HBV infected women and their newborns[41,46]. A large cohort study carried out in China showed that HBsAg positive pregnant women had a higher risk of gestational diabetes mellitus, postpartum hemorrhage, and intrahepatic cholestasis[44]. A recent study showed a significant correlation between HBV viral load and blood glucose level (fasting blood glucose, 2-h postprandial blood glucose and hemoglobin A1c)[47]. No statistical associations were found between HBsAg positivity and pre-eclampsia, as well as between HBsAg positivity and placenta previa. HBsAg positivity during pregnancy was associated with a higher risk of multiple adverse maternal outcomes.

In a large case-control study in China[48], it was shown that maternal HBsAg carriage was associated with several adverse pregnancy outcomes. In particular, it was correlated with an increased risk of pregnancy-induced hypertension, fetal distress,

cesarean delivery and macrosomia. This study also demonstrated a statistically significant association between high maternal viral load in the second trimester and a high risk of preterm birth. Other previous studies have also reported that maternal HBV infection was associated with an increased risk of preterm birth[49,50], although there are also studies showing the opposite results[51,52].

Some studies indicate a more frequent development of bleeding during childbirth in women with CHB[53]. It was also reported that women with CHB are less likely to have hypertension and pre-eclampsia during pregnancy[51].

CHB THERAPY DURING PREGNANCY

At present, the therapy of CHB cannot yet achieve complete HBV elimination in patients. Therefore, depending on the status of the patient, the goals of CHB therapy may be the following: (1) Suppression of virus replication; (2) Reduction of the inflammatory process in the liver; (3) Reverse the development of fibrosis; (4) Prevention of cirrhosis and HCC development; and (5) Reduction of the HBV vertical transmission risk.

When choosing a therapy, it is necessary to take into account the safety and effectiveness of antiviral drugs, as well as the possibility of drug resistance developing. In women of childbearing age with CHB, antiviral therapy can pursue two main goals: the treatment of women with active CHB and the prevention of vertical transmission (see Table 4). At present, the necessity to treat inactive HBsAg carriers[54] is being discussed, but currently it is recommended only by the Asia-Pacific Association for the Study of the Liver (APASL)[8], while the European Association for the Study of the Liver (EASL)[9] and American Association for the Study of Liver Diseases (AASLD)[10] societies refrain from such recommendations.

A large trial[55] has reported reduced HBV transmission and HBsAg-positivity in infants born to telbivudine or lamivudine treated HBsAg-positive mothers. A systematic review[56] has shown that antiviral therapy of pregnant women with nucleoside analogues (NAs), such as lamivudine, telbivudine or tenofovir, significantly decreases maternal HBV viral load. During pregnancy, tenofovir is the drug of choice, due to its profile of antiviral activity and a low risk of developing resistance. Tenofovir in pregnancy is well tolerated and reduces viral load prior to parturition[57].

NA prophylaxis is also useful in HBeAg-negative women with a high HBV DNA level but normal ALT level[11,55].

The administration of NAs at 28-30 wk of gestation leads to a rapid decrease in the viral load by the time of delivery[58], and, as a consequence, to a significant reduction in vertical transmission risk. However, if the drug intake is discontinued, the viral load quickly returns to its original level. It is reported[58] that a prescription of telbivudine in the third trimester to women with a high viral load leads to an HBV DNA decrease up to an undetectable level at the time of delivery in 33% of patients. In the control group, no such decrease was observed. In the same study, it was shown that there were no cases of vertical transmission in the group of women who received telbivudine in the third trimester, while in the control group, 8% of children 7 mo after delivery were HBsAg-positive. Another large prospective study of 450 HBeAg-positive women with high viral load also showed no vertical transmission in women receiving telbivudine, while in the control group HBsAg was detected in 14.7% of newborns 6 mo after birth[59].

If antiviral therapy was administered in order to prevent MTCT, it is usually discontinued after delivery. However, there is no common opinion how soon after delivery this can be done. As shown in Table 5, according to the AASLD recommendations, the drug can be discontinued soon after delivery; according to EASL — at delivery or within the first 3 mo; while APASL recommends continuing drug intake for 4-12 wk.

HBV PROPHYLAXIS IN NEWBORNS

HBV vaccination reduces the vertical transmission risk from 90% to 21% in HBeAg-positive women and from 30% to 2.6% in HBeAg-negative women[60]. With the addition of hepatitis B immunoglobulin (HBIG), the risk of MTCT is decreased to 6% in HBeAg-positive women and to 1% in HBeAg-negative women[60]. This prophylaxis has to be administered within 12 h after birth (see Table 6).

Table 4 Treatment of pregnant women with chronic hepatitis B

	APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
Therapy	In pregnant females with chronic HBV infection who need antiviral therapy, tenofovir is the drug of choice for mothers indicated for antiviral treatment during the first through third trimester of pregnancy	Tenofovir is recommended for pregnant women with CHB and advanced fibrosis. Therapy with tenofovir should be continued, and if the woman was receiving other drugs, these other drugs should be replaced with tenofovir	Women who meet standard indications for HBV therapy should be treated. HBV-infected pregnant women with cirrhosis should be managed in high-risk obstetrical practices and treated with tenofovir to prevent decompensation
To prevent vertical transmission	For reduction of risk of mother-to-infant transmission that occurs during the perinatal period, short-term maternal NAs starting from 28 wk to 32 wk of gestation is recommended using either tenofovir or telbuvidine for those mothers with HBV DNA above 6-7 log10 IU/mL. Since, the HBV transmission could occur even with lower maternal HBV DNA level, NAs could be administered after discussion with the patient, even in patients with lower DNA level. The NA could be stopped at birth and when breastfeeding starts, if there is no contraindication to stopping NA	In all pregnant women with high HBV DNA level (> 200000 IU/mL) or HBsAg level > 4 log10 IU/mL, antiviral prophylaxis with tenofovir disoproxil fumarate should start at week 24-28 of gestation and continue for up to 12 wk after delivery	Women without standard indications but who have HBV DNA > 200000 IU/mL in the second trimester should consider treatment to prevent mother-to-child transmission

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; HBV: Hepatitis B virus; CHB: Chronic hepatitis B; NA: Nucleoside analogues; HBsAg: Hepatitis B surface antigen.

Table 5 Cessation of nucleoside analogues treatment after delivery

APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
Cessation of NA therapy (at delivery or 4-12 wk after delivery) is recommended in females without ALT flares and without pre-existing advanced liver fibrosis/cirrhosis. Continuation of NA treatment after delivery may be necessary according to maternal liver disease status	If NA therapy is given as prophylaxis, *i.e.*, only for the prevention of perinatal transmission, its duration is not well defined (stopping at delivery or within the first 3 mo after delivery)	HBV-infected pregnant women who are not on antiviral therapy as well as those who stop antiviral at or early after delivery should be monitored closely for up to 6 mo after delivery for hepatitis flares and seroconversion. Long-term follow-up should be continued to assess need for future therapy

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; NA: Nucleoside analogues; ALT: Alanine aminotransferase.

Table 6 Hepatitis B virus prophylaxis in newborns

APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
HBIG and hepatitis B vaccine can be given to newborns from HBsAg-positive mothers immediately after delivery	The combination of HBIG and vaccination is administered within 12 h of birth	HBIG and HBV vaccine should be administered to the newborn < 12 h after delivery

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; HBIG: Hepatitis B immunoglobulin; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

The 3-dose vaccine against hepatitis B produces a protective antibody response (anti-HBs ≥ 10 mIU/mL) in approximately 95% of healthy infants[61].

BREASTFEEDING

It is known that in many women infected by HBV, HBsAg can be detected in the breast milk[62]. Moreover, there is evidence that HBV DNA can also be found in breast milk and colostrum[63]. As a result, there are frequent concerns that breastfeeding may facilitate MTCT, although the studies available so far have not confirmed this. No statistically significant differences between breastfed and artificially fed perinatally infected children were detected, and provided a timely vaccination[64-66]. A recent study showed that the frequency of vertical transmission in mothers with similar HBV DNA level is independent of the type of feeding[67]. Thus, HBV infection is not currently considered to be a contraindication to breastfeeding infants receiving HBIG

Table 7 Breastfeeding of newborns

APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
Breastfeeding is not recommended while the woman is receiving antiviral therapy	Breastfeeding is not contraindicated in women not receiving antiviral therapy and during treatment with tenofovir	Breastfeeding is not prohibited for women with or without antiviral therapy

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases.

and HBV vaccine. In addition, there are several studies showing that breastfeeding does not affect the child's immune response to vaccination[68]. The current recommendations of major societies are shown in Table 7.

CONCLUSION

Despite the continuously decreasing prevalence of CHB achieved after the introduction of vaccination against hepatitis B, this disease remains a significant public health problem worldwide. In the present study, we summarized the major trends in the management of CHB in pregnant women and provided recommendations for clinical practice necessary to achieve the elimination of hepatitis B as a public health threat, as proposed by the WHO. The most important of these recommendations are: (1) All women have to be screened for HBsAg during pregnancy. Additional examinations of pregnant women with CHB may include maternal HBeAg, HBV viral load, ALT level, and HBsAg level; (2) The management of pregnancy depends on the phase of the HBV infection, which has to be determined before pregnancy; (3) In women of childbearing age with CHB, antiviral therapy can pursue two main goals: treatment of active CHB, and vertical transmission prevention. During pregnancy, tenofovir is the drug of choice in both cases; and (4) A combination of HBIG and vaccine against hepatitis B should be administered within the first 12 h to all infants born to mothers with CHB. In such cases, there are no contraindications to breastfeeding.

REFERENCES

1 **World Health Organization**. Global Hepatitis Report 2017. [cited 15 April 2021]. In: World Health Organization [Internet]. Available from: https://www.who.int/hepatitis/publications/global-hepatitis-report2017/en

2 **Aslam A**, Campoverde Reyes KJ, Malladi VR, Ishtiaq R, Lau DTY. Management of chronic hepatitis B during pregnancy. *Gastroenterol Rep (Oxf)* 2018; **6**: 257-262 [PMID: 30430013 DOI: 10.1093/gastro/goy025]

3 **Thio CL**, Guo N, Xie C, Nelson KE, Ehrhardt S. Global elimination of mother-to-child transmission of hepatitis B: revisiting the current strategy. *Lancet Infect Dis* 2015; **15**: 981-985 [PMID: 26145195 DOI: 10.1016/S1473-3099(15)00158-9]

4 **Alter MJ**. Epidemiology of hepatitis B in Europe and worldwide. *J Hepatol* 2003; **39** Suppl 1: S64-S69 [PMID: 14708680 DOI: 10.1016/s0168-8278(03)00141-7]

5 **Tran TT**. Hepatitis B in Pregnancy. *Clin Infect Dis* 2016; **62** Suppl 4: S314-S317 [PMID: 27190321 DOI: 10.1093/cid/ciw092]

6 **World Health Organization**. Global health sector strategy on viral hepatitis 2016–2021. [cited 15 April 2021]. In: World Health Organization [Internet]. Available from: https://apps.who.int/iris/bitstream/handle/10665/246177/WHO-HIV-2016.06-eng.pdf?sequence=1

7 **Jonas MM**. Hepatitis B and pregnancy: an underestimated issue. *Liver Int* 2009; **29** Suppl 1: 133-139 [PMID: 19207977 DOI: 10.1111/j.1478-3231.2008.01933.x]

8 **Sarin SK**, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS, Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int* 2016; **10**: 1-98 [PMID: 26563120 DOI: 10.1007/s12072-015-9675-4]

9 **European Association for the Study of the Liver**. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]

10 **Terrault NA**, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM, Brown RS Jr, Bzowej NH, Wong JB. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018

hepatitis B guidance. *Hepatology* 2018; **67**: 1560-1599 [PMID: 29405329 DOI: 10.1002/hep.29800]
11 **Sun KX**, Li J, Zhu FC, Liu JX, Li RC, Zhai XJ, Li YP, Chang ZJ, Nie JJ, Zhuang H. A predictive value of quantitative HBsAg for serum HBV DNA level among HBeAg-positive pregnant women. *Vaccine* 2012; **30**: 5335-5340 [PMID: 22749833 DOI: 10.1016/j.vaccine.2012.06.036]
12 **Wen WH**, Huang CW, Chie WC, Yeung CY, Zhao LL, Lin WT, Wu JF, Ni YH, Hsu HY, Chang MH, Lin LH, Chen HL. Quantitative maternal hepatitis B surface antigen predicts maternally transmitted hepatitis B virus infection. *Hepatology* 2016; **64**: 1451-1461 [PMID: 27044007 DOI: 10.1002/hep.28589]
13 **Belopolskaya M**, Avrutin V, Firsov S, Yakovlev A. HBsAg level and hepatitis B viral load correlation with focus on pregnancy. *Ann Gastroenterol* 2015; **28**: 379-384 [PMID: 26127004]
14 **Huang Y**, Li L, Sun X, Lu M, Liu H, Tang G, Wang D, Hutin YJ. Screening of pregnant women for hepatitis B virus surface antigen (HBsAg) and subsequent management, Qiandongnan prefecture, Guizhou, China, 2010. *Vaccine* 2013; **31** Suppl 9: J62-J65 [PMID: 24331022 DOI: 10.1016/j.vaccine.2013.05.103]
15 **Lao TT**, Sahota DS, Law LW, Cheng YK, Leung TY. Age-specific prevalence of hepatitis B virus infection in young pregnant women, Hong Kong Special Administrative Region of China. *Bull World Health Organ* 2014; **92**: 782-789 [PMID: 25378739 DOI: 10.2471/BLT.13.133413]
16 **Sorrell MF**, Belongia EA, Costa J, Gareen IF, Grem JL, Inadomi JM, Kern ER, McHugh JA, Petersen GM, Rein MF, Strader DB, Trotter HT. National Institutes of Health consensus development conference statement: management of hepatitis B. *Hepatology* 2009; **49**: S4-S12 [PMID: 19399804 DOI: 10.1002/hep.22946]
17 **Kirbak ALS**, Ng'ang'a Z, Omolo J, Idris H, Usman A, Mbabazi WB. Sero-prevalence for Hepatitis B virus among pregnant women attending antenatal clinic in Juba Teaching Hospital, Republic of South Sudan. *Pan Afr Med J* 2017; **26**: 72 [PMID: 28451049 DOI: 10.11604/pamj.2017.26.72.11410]
18 **Fouelifack FY**, Fouedjio JH, Fouogue JT, Fouelifa LD. Seroprevalences and Correlates of Hepatitis B and C Among Cameroonian Pregnant Women. *Clin Med Insights Reprod Health* 2018; **12**: 1179558118770671 [PMID: 29692639 DOI: 10.1177/1179558118770671]
19 **Bittaye M**, Idoko P, Ekele BA, Obed SA, Nyan O. Hepatitis B virus sero-prevalence amongst pregnant women in the Gambia. *BMC Infect Dis* 2019; **19**: 259 [PMID: 30876397 DOI: 10.1186/s12879-019-3883-9]
20 **Tanga AT**, Teshome MA, Hiko D, Fikru C, Jilo GK. Sero-prevalence of hepatitis B virus and associated factors among pregnant women in Gambella hospital, South Western Ethiopia: facility based cross-sectional study. *BMC Infect Dis* 2019; **19**: 602 [PMID: 31291901 DOI: 10.1186/s12879-019-4220-z]
21 **Kishk R**, Mandour M, Elprince M, Salem A, Nemr N, Eida M, Ragheb M. Pattern and interpretation of hepatitis B virus markers among pregnant women in North East Egypt. *Braz J Microbiol* 2020; **51**: 593-600 [PMID: 31677078 DOI: 10.1007/s42770-019-00174-3]
22 **Fessehaye N**, Berhane A, Ahmed H, Mohamed S, Tecle F, Gikunju J, Odari E. Prevalence of Hepatitis B Virus Infection and Associated Seromarkers among Pregnant Women in Eritrea. *J Hum Virol Retrovirol* 2018; **6**: 00191
23 **Sheng QJ**, Wang SJ, Wu YY, Dou XG, Ding Y. Hepatitis B virus serosurvey and awareness of mother-to-child transmission among pregnant women in Shenyang, China: An observational study. *Medicine (Baltimore)* 2018; **97**: e10931 [PMID: 29851831 DOI: 10.1097/MD.0000000000010931]
24 **Cetin S**, Cetin M, Turhan E, Dolapcioglu K. Seroprevalence of hepatitis B surface antigen and associated risk factors among pregnant women. *J Infect Dev Ctries* 2018; **12**: 904-909 [PMID: 32004160 DOI: 10.3855/jidc.10018]
25 **Mishra S**, Purandre P, Thakur R, Agrawal S, Alwani M. Study on prevalence of hepatitis B in pregnant women and its effect on maternal and fetal outcome at tertiary care centre. *IJRCOG* 2017; **6**: 2238-2240 [DOI: 10.18203/2320-1770.ijrcog20172069]
26 **Biondi MJ**, Marchand-Austin A, Cronin K, Nanwa N, Ravirajan V, Mandel E, Goneau LW, Mazzulli T, Shah H, Capraru C, Janssen HLA, Sander B, Feld JJ. Prenatal hepatitis B screening, and hepatitis B burden among children, in Ontario: a descriptive study. *CMAJ* 2020; **192**: E1299-E1305 [PMID: 33106301 DOI: 10.1503/cmaj.200290]
27 **Lembo T**, Saffioti F, Chiofalo B, Granese R, Filomia R, Grasso R, Triolo O, Raimondo G. Low prevalence of hepatitis B and hepatitis C virus serum markers in a cohort of pregnant women from Southern Italy. *Dig Liver Dis* 2017; **49**: 1368-1372 [PMID: 28818677 DOI: 10.1016/j.dld.2017.07.012]
28 **Ruiz-Extremera Á**, Díaz-Alcázar MDM, Muñoz-Gámez JA, Cabrera-Lafuente M, Martín E, Arias-Llorente RP, Carretero P, Gallo-Vallejo JL, Romero-Narbona F, Salmerón-Ruiz MA, Alonso-Diaz C, Maese-Heredia R, Cerrillos L, Fernández-Alonso AM, Camarena C, Aguayo J, Sánchez-Forte M, Rodríguez-Maresca M, Pérez-Rivilla A, Quiles-Pérez R, Muñoz de Rueda P, Expósito-Ruiz M, García F, Salmerón J. Seroprevalence and epidemiology of hepatitis B and C viruses in pregnant women in Spain. Risk factors for vertical transmission. *PLoS One* 2020; **15**: e0233528 [PMID: 32437468 DOI: 10.1371/journal.pone.0233528]
29 **Harris AM**, Isenhour C, Schillie S, Vellozzi C. Hepatitis B Virus Testing and Care among Pregnant Women Using Commercial Claims Data, United States, 2011-2014. *Infect Dis Obstet Gynecol* 2018; **2018**: 4107329 [PMID: 29805248 DOI: 10.1155/2018/4107329]
30 **Ott JJ**, Stevens GA, Wiersma ST. The risk of perinatal hepatitis B virus transmission: hepatitis B e antigen (HBeAg) prevalence estimates for all world regions. *BMC Infect Dis* 2012; **12**: 131 [PMID:

22682147 DOI: 10.1186/1471-2334-12-131]
31 **Liaw YF**, Brunetto MR, Hadziyannis S. The natural history of chronic HBV infection and geographical differences. *Antivir Ther* 2010; **15** Suppl 3: 25-33 [PMID: 21041901 DOI: 10.3851/IMP1621]
32 **Lao TT**. Hepatitis B - chronic carrier status and pregnancy outcomes: An obstetric perspective. *Best Pract Res Clin Obstet Gynaecol* 2020; **68**: 66-77 [PMID: 32312688 DOI: 10.1016/j.bpobgyn.2020.03.006]
33 **Belopolskaya MA**, Volokobinskaya TV, Firsov SL, Yakovlev AA. Using the quantitative determination of HBsAg to predict the course of chronic hepatitis B in women during pregnancy and after childbirth. *J Infectol* 2013; **5**: 50-54
34 **ter Borg MJ**, Leemans WF, de Man RA, Janssen HL. Exacerbation of chronic hepatitis B infection after delivery. *J Viral Hepat* 2008; **15**: 37-41 [PMID: 18088243 DOI: 10.1111/j.1365-2893.2007.00894.x]
35 **Giles M**, Visvanathan K, Lewin S, Bowden S, Locarnini S, Spelman T, Sasadeusz J. Clinical and virological predictors of hepatic flares in pregnant women with chronic hepatitis B. *Gut* 2015; **64**: 1810-1815 [PMID: 25431458 DOI: 10.1136/gutjnl-2014-308211]
36 **Shaheen AA**, Myers RP. The outcomes of pregnancy in patients with cirrhosis: a population-based study. *Liver Int* 2010; **30**: 275-283 [PMID: 19874491 DOI: 10.1111/j.1478-3231.2009.02153.x]
37 **Liu Y**, Hussain M, Wong S, Fung SK, Yim HJ, Lok AS. A genotype-independent real-time PCR assay for quantification of hepatitis B virus DNA. *J Clin Microbiol* 2007; **45**: 553-558 [PMID: 17182753 DOI: 10.1128/JCM.00709-06]
38 **Mahtab MA**, Rahman S, Khan M, Mamun AA, Afroz S. Etiology of fulminant hepatic failure: experience from a tertiary hospital in Bangladesh. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 161-164 [PMID: 18397851]
39 **Lao TT**, Leung TY, Chan HL, Wong VW. Effect of pregnancy on the activity and infectivity of hepatitis B virus in women with chronic hepatitis B infection. *Hong Kong Med J* 2015; **21** Suppl 7: S4-S7 [PMID: 26908264]
40 **Lin HH**, Wu WY, Kao JH, Chen DS. Hepatitis B post-partum e antigen clearance in hepatitis B carrier mothers: Correlation with viral characteristics. *J Gastroenterol Hepatol* 2006; **21**: 605-609 [PMID: 16638107 DOI: 10.1111/j.1440-1746.2006.04198.x]
41 **Nguyen G**, Garcia RT, Nguyen N, Trinh H, Keeffe EB, Nguyen MH. Clinical course of hepatitis B virus infection during pregnancy. *Aliment Pharmacol Ther* 2009; **29**: 755-764 [PMID: 19183158 DOI: 10.1111/j.1365-2036.2009.03932.x]
42 **Fedoseeva LR**, Alekseeva MN, Imeneva VI, Samsonov VK, Ivanova E. D. Clinical features of viral hepatitis in pregnant women in the Republic of Sakha (Yakutia). *Fundamental Res* 2004; **2**: 101-102
43 **Connell LE**, Salihu HM, Salemi JL, August EM, Weldeselasse H, Mbah AK. Maternal hepatitis B and hepatitis C carrier status and perinatal outcomes. *Liver Int* 2011; **31**: 1163-1170 [PMID: 21745298 DOI: 10.1111/j.1478-3231.2011.02556.x]
44 **Lao TT**, Chan BC, Leung WC, Ho LF, Tse KY. Maternal hepatitis B infection and gestational diabetes mellitus. *J Hepatol* 2007; **47**: 46-50 [PMID: 17434231 DOI: 10.1016/j.jhep.2007.02.014]
45 **Suen SS**, Lao TT, Sahota DS, Lau TK, Leung TY. Implications of the relationship between maternal age and parity with hepatitis B carrier status in a high endemicity area. *J Viral Hepat* 2010; **17**: 372-378 [PMID: 19780946 DOI: 10.1111/j.1365-2893.2009.01195.x]
46 **Potthoff A**, Rifai K, Wedemeyer H, Deterding K, Manns M, Strassburg C. Successful treatment of fulminant hepatitis B during pregnancy. *Z Gastroenterol* 2009; **47**: 667-670 [PMID: 19606409 DOI: 10.1055/s-0028-1109148]
47 **Wu D**. Correlation of viral load of Hepatitis B with the gestation period and the development of diabetes mellitus. *Saudi J Biol Sci* 2019; **26**: 2022-2025 [PMID: 31889788 DOI: 10.1016/j.sjbs.2019.08.009]
48 **Wan Z**, Zhou A, Zhu H, Lin X, Hu D, Peng S, Zhang B, Du Y. Maternal Hepatitis B Virus Infection and Pregnancy Outcomes: A Hospital-based Case-control Study in Wuhan, China. *J Clin Gastroenterol* 2018; **52**: 73-78 [PMID: 28723858 DOI: 10.1097/MCG.0000000000000842]
49 **Liu J**, Zhang S, Liu M, Wang Q, Shen H, Zhang Y. Maternal pre-pregnancy infection with hepatitis B virus and the risk of preterm birth: a population-based cohort study. *Lancet Glob Health* 2017; **5**: e624-e632 [PMID: 28495266 DOI: 10.1016/S2214-109X(17)30142-0]
50 **Huang QT**, Zhong M. Maternal hepatitis B virus infection and risk of preterm birth in China. *Lancet Glob Health* 2017; **5**: e563-e564 [PMID: 28495254 DOI: 10.1016/S2214-109X(17)30175-4]
51 **Lao TT**, Sahota DS, Cheng YK, Law LW, Leung TY. Maternal hepatitis B surface antigen status and incidence of pre-eclampsia. *J Viral Hepat* 2013; **20**: 343-349 [PMID: 23565617 DOI: 10.1111/jvh.12037]
52 **Chen J**, Zhang S, Zhou YH, Xu B, Hu Y. Minimal adverse influence of maternal hepatitis B carrier status on perinatal outcomes and child's growth. *J Matern Fetal Neonatal Med* 2015; **28**: 2192-2196 [PMID: 25354287 DOI: 10.3109/14767058.2014.981805]
53 **Tse KY**, Ho LF, Lao T. The impact of maternal HBsAg carrier status on pregnancy outcomes: a case-control study. *J Hepatol* 2005; **43**: 771-775 [PMID: 16139923 DOI: 10.1016/j.jhep.2005.05.023]
54 **Li MH**, Xie Y, Zhang L, Lu Y, Shen G, Wu SL, Chang M, Mu CQ, Hu LP, Hua WH, Song SJ, Zhang SF, Cheng J, Xu DZ. Hepatitis B surface antigen clearance in inactive hepatitis B surface antigen carriers treated with peginterferon alfa-2a. *World J Hepatol* 2016; **8**: 637-643 [PMID: 27239256 DOI: 10.4254/wjh.v8.i15.637]

55 **Zhang H**, Pan CQ, Pang Q, Tian R, Yan M, Liu X. Telbivudine or lamivudine use in late pregnancy safely reduces perinatal transmission of hepatitis B virus in real-life practice. *Hepatology* 2014; **60**: 468-476 [PMID: 25187919 DOI: 10.1002/hep.27034]

56 **Brown RS Jr**, McMahon BJ, Lok AS, Wong JB, Ahmed AT, Mouchli MA, Wang Z, Prokop LJ, Murad MH, Mohammed K. Antiviral therapy in chronic hepatitis B viral infection during pregnancy: A systematic review and meta-analysis. *Hepatology* 2016; **63**: 319-333 [PMID: 26565396 DOI: 10.1002/hep.28302]

57 **Samadi Kochaksaraei G**, Castillo E, Osman M, Simmonds K, Scott AN, Oshiomogho JI, Lee SS, Myers RP, Martin SR, Coffin CS. Clinical course of 161 untreated and tenofovir-treated chronic hepatitis B pregnant patients in a low hepatitis B virus endemic region. *J Viral Hepat* 2016; **23**: 15-22 [PMID: 26192022 DOI: 10.1111/jvh.12436]

58 **Han GR**, Cao MK, Zhao W, Jiang HX, Wang CM, Bai SF, Yue X, Wang GJ, Tang X, Fang ZX. A prospective and open-label study for the efficacy and safety of telbivudine in pregnancy for the prevention of perinatal transmission of hepatitis B virus infection. *J Hepatol* 2011; **55**: 1215-1221 [PMID: 21703206 DOI: 10.1016/j.jhep.2011.02.032]

59 **Wu Q**, Huang H, Sun X, Pan M, He Y, Tan S, Zeng Y, Li L, Deng G, Yan Z, He D, Li J, Wang Y. Telbivudine prevents vertical transmission of hepatitis B virus from women with high viral loads: a prospective long-term study. *Clin Gastroenterol Hepatol* 2015; **13**: 1170-1176 [PMID: 25251571 DOI: 10.1016/j.cgh.2014.08.043]

60 **Isaacs D**, Kilham HA, Alexander S, Wood N, Buckmaster A, Royle J. Ethical issues in preventing mother-to-child transmission of hepatitis B by immunisation. *Vaccine* 2011; **29**: 6159-6162 [PMID: 21723352 DOI: 10.1016/j.vaccine.2011.06.065]

61 **Schillie SF**, Murphy TV. Seroprotection after recombinant hepatitis B vaccination among newborn infants: a review. *Vaccine* 2013; **31**: 2506-2516 [PMID: 23257713 DOI: 10.1016/j.vaccine.2012.12.012]

62 **Chan OK**, Lao TT, Suen SS, Lau TK, Leung TY. Knowledge on hepatitis B infection among pregnant women in a high endemicity area. *Patient Educ Couns* 2011; **85**: 516-520 [PMID: 21167671 DOI: 10.1016/j.pec.2010.11.006]

63 **de Oliveira PR**, Yamamoto AY, de Souza CB, de Araújo NM, de Andrade Gomes S, Heck AR, de Castro Figueiredo JF, Mussi-Pinhata MM. Hepatitis B viral markers in banked human milk before and after Holder pasteurization. *J Clin Virol* 2009; **45**: 281-284 [PMID: 19473876 DOI: 10.1016/j.jcv.2009.04.003]

64 **Beasley RP**. Rocks along the road to the control of HBV and HCC. *Ann Epidemiol* 2009; **19**: 231-234 [PMID: 19344859 DOI: 10.1016/j.annepidem.2009.01.017]

65 **Petrova M**, Kamburov V. Breastfeeding and chronic HBV infection: clinical and social implications. *World J Gastroenterol* 2010; **16**: 5042-5046 [PMID: 20976840 DOI: 10.3748/wjg.v16.i40.5042]

66 **Hill JB**, Sheffield JS, Kim MJ, Alexander JM, Sercely B, Wendel GD. Risk of hepatitis B transmission in breast-fed infants of chronic hepatitis B carriers. *Obstet Gynecol* 2002; **99**: 1049-1052 [PMID: 12052598 DOI: 10.1016/s0029-7844(02)02000-8]

67 **Chen X**, Chen J, Wen J, Xu C, Zhang S, Zhou YH, Hu Y. Breastfeeding is not a risk factor for mother-to-child transmission of hepatitis B virus. *PLoS One* 2013; **8**: e55303 [PMID: 23383145 DOI: 10.1371/journal.pone.0055303]

68 **Chowdhury SD**, Eapen CE. Perinatal transmission of Hepatitis B. *Hep B Annual* 2009; **6**: 80-88

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MINIREVIEWS

Viscoelastic tests in liver disease: where do we stand now?

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Abstract

Hemostasis is a complex physiological process based on the balance between pro-coagulant and anticoagulant systems to avoid pathological bleeding or thrombosis. The changes in standard coagulation tests in liver disease were assumed to reflect an acquired bleeding disorder, and cirrhotic patients were considered naturally anticoagulated. In the light of the new evidence, the theory of rebalanced hemostasis replaced the old concept. According to this model, the hemostatic alteration leads to a unique balance between pro-coagulant, anticoagulant, and fibrinolytic systems. But the balance is fragile and may prone to bleeding or thrombosis depending on various risk factors. The standard coagulation tests [INR (international normalized ratio), platelet count and fibrinogen] only explore parts of the hemostasis, not offering an entire image of the process. Rotational thromboelastometry (ROTEM) and thromboelastography (TEG) are both point of care viscoelastic tests (VET) that provide real-time and dynamic information about the entire hemostasis process, including clot initiation (thrombin generation), clot kinetics, clot strength, and clot stability (lysis). Despite prolonged PT/INR (international normalized ratio of prothrombin time) and low platelet counts, VET is within the normal range in many patients with both acute and chronic liver disease. However, bleeding remains the dominant clinical issue in patients with liver diseases, especially when invasive interventions are required. VET has been shown to asses more appropriately the risk of bleeding than conventional laboratory tests, leading to decrial use of blood products transfusion. Inappropriate clotting is common but often subtle and may be challenging to predict even with the help of VET. Although VET has shown its benefit, more studies are needed to establish cut-off values for TEG and ROTEM in these populations and standardization of transfusion guidelines before invasive interventions in cirrhotic patients/orthotopic liver transplantation.

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Core Tip: Despite having specific alterations in all hemostasis phases and, thus, considered naturally anticoagulated, cirrhotic patients have, in fact, balanced hemostasis. However, this balance may be disturbed by different factors, and the result may vary from devastating bleeding to massive thrombosis. Conventional laboratory tests failed to predict these events. Viscoelastic tests appear to offer a better, global view of hemostasis in these patients. They have been used to assess bleeding risk before invasive interventions and for a precocious use of blood product transfusions.

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INTRODUCTION

Hemostasis in advanced liver disease

The hematological changes encountered in cirrhosis have shown a great interest in the last two decades. The misconception of the cirrhotic patient being naturally anticoagulated has changed with the new concept of balanced hemostasis.

The new cell-based model of hemostasis elaborated by Hoffman and Monroe[1] in 2001 Led to a better understanding of hemostasis's complex process. In Hoffman's conception, three phases simultaneously cooperate for adequate hemostasis: primary hemostasis, which involves the activated platelets with the formation of platelet-plug; coagulation with the fibrin mesh construction and clot fortification, involving plasma procoagulant proteins and, finally, clot fibrinolysis by plasma anticoagulant proteins.

In liver cirrhosis, all these three phases are affected by hepatic synthetic dysfunction and portal hypertension[2].

In hemostasis, platelets have a dual role[3,4]. Through the adhesive protein von Willebrand factor (vWF), they adhere to the subendothelium and aggregate to initiate thrombus formation and, by assembling vitamin K dependent coagulation factors on their surface, they support thrombin generation. The most common abnormality in cirrhotic patients is the thrombocytopenia-numerical decrease of circulating platelet count[2-4]. The etiology of thrombocytopenia is multifactorial: platelet spleen sequestration, low thrombopoietin levels from impaired hepatic synthesis, immune destruction.

However, there is controversy over the qualitative changes in platelet function in chronic liver disease[2,3]. vWF, activated by cleavage into smaller subunits (high molecular weight multimers) by the endothelial-derived metalloproteinase ADAMTS13, mediates platelet adhesion and aggregation[3]. Lisman *et al*[5] have shown *in vitro* that cirrhotic patients' plasma may support the adhesion of normal or cirrhotic platelets. This is possible due to the increased level of vWF and, at the same time, a decrease in vWF collagen binding capacity, as well as a reduction in vWF and ADAMTS13 multimers. These results indicate that increased levels of VWF contribute to the induction of primary hemostasis by maintaining the platelets adherence despite the functional or numerical alteration of them. Tripodi *et al*[6] found in an *in vitro* study that thrombocytes from cirrhotic patients were qualitatively able to support thrombin generation if their range was over 50-60 × 10^9/L.

A reduction in the synthesis of procoagulant factors (FII, FV, FVII, FIX, FX, FXI) characterizes chronic liver disease[2,4]. The exception makes FVIII, whose level is elevated secondary to synthesis induced by cytokines, released from necrotic tissue, and reduced clearance[2,7]. Fibrinogen level is normal or increased in most patients with cirrhosis, and dysfibrinogenemia occurs in 50%-78% of patients with chronic liver disease[4]. Despite the reduction in hepatic synthesis of procoagulant factors, patients with liver cirrhosis do not experience spontaneous bleeding similarly to those with congenital deficiency of coagulation factors do (*e.g.*, haemarthrosis)[2]. The decreased

protein C synthesis, a potent anticoagulant, protein S, and antithrombin III, also contributes to the coagulations' normality[2-4,7].

All profibrinolytic and antifibrinolytic factors are synthesized by hepatic cells, except tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI 1), which are produced by endothelial cells. Liver cirrhosis is associated with hyperfibrinolysis secondary to high levels of t-PA and low levels of plasma inhibitor and thrombin-activatable fibrinolysis inhibitor (TAFI), but also with hypofibrinolysis secondary to increased PAI and plasminogen levels[7]. Lisman *et al*[8] pointed out that a parallel decrease in antifibrinolytic factors counterbalances the low levels of profibrinolytic factors that occur in cirrhosis. However, Colucci *et al*[9] showed contradictory results, demonstrating that the reduction in TAFI level is associated with hyperfibrinolysis.

All of these changes (Figure 1) support the new theory of rebalanced hemostasis in patients with liver cirrhosis[2,4,7,10]. However, various circumstantial risk factors can quickly destabilize this balance, increasing the risk of bleeding or thrombosis[3,10].

HEMOSTASIS TESTING IN ADVANCED LIVER DISEASES

The problems appear when it comes to exploring hemostasis. The major inconvenience of conventional laboratory tests [platelet count, PT/INR (international normalized ratio of prothrombin time), procoagulant/anticoagulant factors, profibrinolytic/antifibrinolytic factors] is that they test parts of hemostasis, and they do not offer a global view of the process.

The INR appeared as a necessity in the standardization of anticoagulant therapy with vitamin K antagonists (VKA). Still, it is not calibrated to the specific changes of cirrhotic coagulation[2,3]. There were two attempts[11,12] to introduce a new liver dedicated INR (INR_{liver}) by recalibrating ISI (International Sensitivity Index). The method requires replacing plasma from the patients treated with VKA with cirrhotic patients' plasma. This technique has technological limits, so it remains more theoretical than a practical one[2]. Moreover, the usual lab tests are not useful for appreciating the hemorrhagic risk related to invasive maneuvers in cirrhotic patients[2].

There is no evidence that a prolonged PT/INR is an indicator of hemorrhagic risk during or following invasive procedures[13].

The platelet count seems to correlate better with the bleeding risk, but a cut-off value below which the risk is increased has not been demonstrated.

Some studies have associated values below 60.000-75.000 with increased hemorrhagic risk following invasive procedures[14].

The fibrinogen level is variable in hepatic diseases[15]. A correlation with bleeding risk has not been defined, except for evident disseminated intravascular coagulation syndrome, sepsis, and different liver transplant stages[16].

Thrombin generation tests measure the entire quantity of thrombin that is generated during hemostasis. Using this assay, several studies[17,18] have shown that compensated cirrhotic patient plasma can produce normal or increased quantities of thrombin, despite prolonged PT/INR.

Thromboelastography (TEG) and Rotational thromboelastometry (ROTEM) are tools based on Hartert's invention, which assesses overall hemostasis, reflecting the interaction between plasma, platelets, and blood cells[4].

ROTEM and TEG are both point of care viscoelastic tests (VET) of hemostasis in whole blood providing real-time, dynamic information about the entire coagulation process, including clot initiation (thrombin generation), clot kinetics, clot strength, and clot stability (lysis). The force exerted on a small metal pin suspended in whole blood during clot formation is measured while the cup (TEG) or the pin is rotated. Data are processed and analyzed with dedicated software and exposed as graphical and numerical values. Table 1 and Figure 2 represents the principal parameters from both VETs.

It should be highlighted that PT/INR correlates poorly with R/CT (reaction time/clotting time) VET parameters[19,20]. However, an excess of anticoagulants or low coagulation factors (less than 30%) would prolong the R/CT time. In contrast, a lot of tissue factor, high factor VIII, or low protein C would shorten these parameters[21]. An increase in maximum clot firmness (MCF) or amplitude could be explained by a combination of increased fibrinogen levels and platelet reactivity[22].

Since TEG/ROTEM are global hemostasis tests, they are more used to evaluate coagulopathy in chronic liver disease[23]. Consistent with the new vision of the rebalanced hemostasis, patients with compensated liver cirrhosis often have normal

Table 1 Parameters of viscoelastic tests numerical

	ROTEM	TEG	
Clotting initiation	CT (clotting time)	R (reaction time)	Enzymatic coagulation
Clot kinetics	CFT (clot formation time); α angle	K (K time); α angle	Speed to reach a certain level of clot strength; Rapidity of fibrin synthesis
Clot strength	MCF (maximum clot firmness)	MA (maximum amplitude)	Ultimate strength of the fibrin clot
Clot stability	CLI30 (clot lysis index at 30 min after MCF); CLI60 (clot lysis index at 60 min after MCF)	Ly30 (clot lysis at 30 min after MA); Ly60 (clot lysis at 60 min after MA)	Clot lysis

TEG: Thromboelastography; ROTEM: Rotational thromboelastometry; MCF: Maximum clot firmness; MA: Maximum amplitude; CT: Clotting time; CFT: Clot formation time.

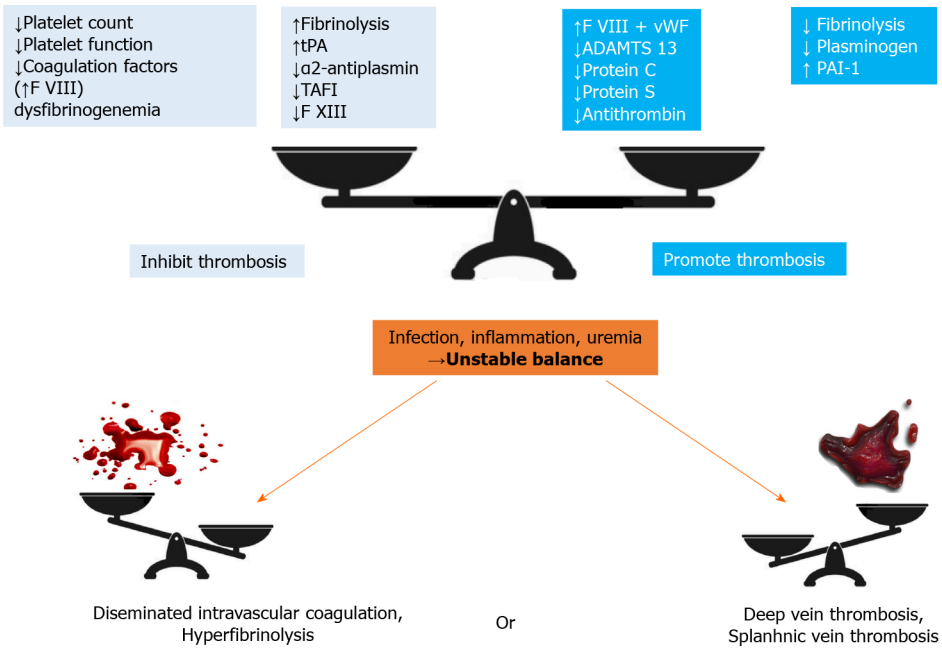


Figure 1 Rebalanced hemostasis in liver cirrhosis. In primary hemostasis, high levels of von Willebrand factor and low levels of disintegrin and metalloproteinase with a thrombospondin type 1 motif 13 counteract numerical or functional abnormalities of platelets. In the coagulation phase, low levels of procoagulant proteins are balanced by reduced synthesis of anticoagulant factors. In fibrinolysis, parallel changes are seen in profibrinolytic and antifibrinolytic proteins. The balance is though fragile, and various factors, as inflammation, infection, uremia may unstable it, leading to bleeding or thrombosis. tPA: Tissue plasminogen activator; TAFI: Thrombin-activatable fibrinolysis inhibitor; vWF: von Willebrand factor; ADAMTS13: Disintegrin and metalloproteinase with a thrombospondin type 1 motif 13; PAI-1: Plasminogen activator inhibitor-1.

TEG parameters[24].

We will discuss further the importance of VET in the most frequent settings from the hepatology field.

HYPERCOAGULABILITY, THROMBOSIS AND VET IN LIVER CIRRHOSIS

Despite the "natural anticoagulation" concept that marked the diagnosis of cirrhosis, portal vein thrombosis (PVT) is a relatively frequent complication of patients with cirrhosis (up to 25% of patients with decompensated cirrhosis)[25]. All the elements of Virchow's triad are present in patients with cirrhosis: decreased velocity (through the presence of portal hypertension), vessel-wall abnormalities (endothelial dysfunction, fibrotic mechanical distortion), and hypercoagulation[7]. However, using the conventional coagulation test, the hypercoagulation status is difficult to demonstrate.

Moreover, the hypercoagulation could vary among different etiologies of cirrhosis. Compared to only 5% of non-cholestatic cirrhosis, 28% of patients with primary biliary cholangitis (PBC) and 43% of patients with primary sclerosing cholangitis (PSC)

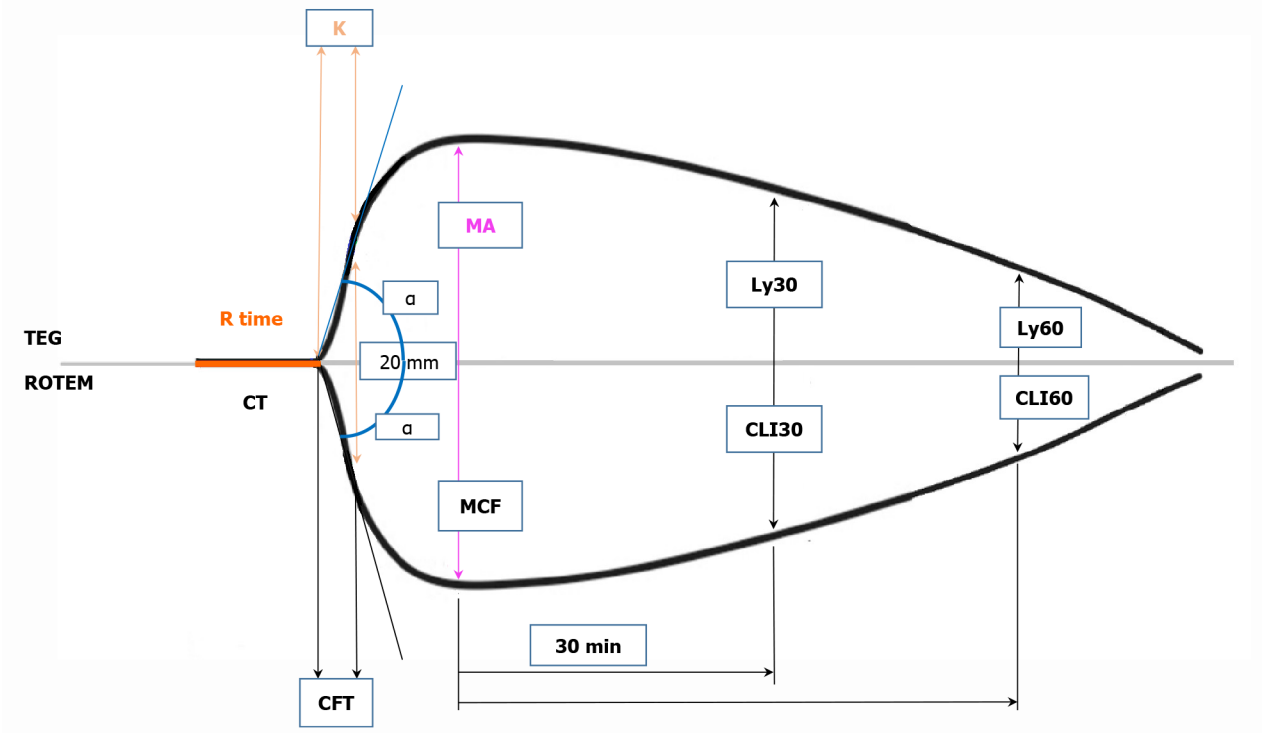


Figure 2 Parameters of viscoelastic tests graphical. TEG: Thromboelastography; ROTEM: Rotational thromboelastometry; CFT: Clot formation time; CT: Clotting time; R: Reaction time; K: K time; MA: Maximum amplitude; MCF: Maxmum clot firmness; CLI30: Clot lysis index at 30 min after maximum clot firmness; Ly30: Clot lysis at 30 min after maximum amplitude; CLI60: Clot lysis index at 60 min after maximum clot firmness; Ly60: Clot lysis at 60 min after maximum amplitude.

demonstrated hypercoagulation status on TEG parameters[26]. Notably, the conventional coagulation tests did not identify this hypercoagulability. Pihusch *et al*[27] also found a hypercoagulable state in noncirrhotic patients with PBC/PSC.

Among various etiologies, non-alcoholic fatty liver disease (NAFLD) has a higher risk of thrombosis. Using TEG, patients with NAFLD had a significantly stronger clot development than healthy controls [maximum amplitude (MA) 58.3 ± 6.3 *vs* 52 ± 10 mm, P = 0.01][28]. The platelet contribution to overall clot strength was higher in NAFLD patients with a trend to reduced inducible clot lysis (P = 0.03). Based on shortened TEG's R and increased net clot strength, Krzanicki *et al*[29] found a high rate of hypercoagulation in patients with PBC (42.9%), patients with PSC (85.7%), patients with fulminate hepatic failure (50%), and patients with NAFLD (37.5%). Similar findings were also reported by Hugenholtz *et al*[30] in a large (n = 270) prospective study where 43% of patients with cholestatic liver disease had hypercoagulability MA values beyond the normal range. Contrary to what would be expected, 80% of patients with obstructive jaundice had hypercoagulable status on TEG analysis (increased MA), which was independent of prolonged PT[31]. However, three weeks after a biliary drainage procedure, all TEG parameters had returned to normal range.

It is still not clear whether a hypercoagulable status increases the risk of PVT[32]. It is tempting to assume that hypercoagulability in cirrhotic patients puts them at a higher risk of thrombosis. However, scarce data is supporting this hypothesis. Moreover, among the thrombotic risk factors, the lower portal velocity is the only independent factor of PVT [odds ratio of 44.9 (95% confidence interval (CI): 5.3-382)][33]. The existing evidence is contradictory. Hugenholtz *et al*[30] found no difference in TEG parameters at baseline between patients who developed PVT (8 out of 270 patients followed almost three years). In another study[34], in patients with cirrhosis and gastroesophageal varices, TEG's R was significantly lower in the group with PVT (5.20 *vs* 6.00, P = 0.009), a sign of enhanced coagulation activity.

The evidence is more apparent in patients with hepatocellular carcinoma (HCC). In HCC patients, the fibrinogen and the MCF in the FIBTEM module were higher in patients who developed PVT during follow-up than those who did not (24 mm *vs* 16 mm, P = 0.04). An increased baseline MCF FIBTEM (0.25 mm) was linked to a higher risk of developing PVT in HCC patients [risk ratio: 4.8 (95%CI: 2-11.3), P = 0.0001][35]. These findings might be valid for cirrhosis patients and no HCC, which still needs to be proved.

Regarding the treatment of PVT, which is still a matter of debate, it would be ideal that the VET's parameters would predict which patients will recanalize with anticoagulation treatment and who will experience spontaneous recanalization. TEG use to guide antithrombotic therapy has been reported in Budd-Chiari syndrome (BCS)[36]. However, the problem is much more complicated as TEG's hemostasis proved to be heterogeneous in BCS. Contrary to the general belief that all patients with BCS have a hypercoagulant status, 20% of patients have a hypocoagulable status based on TEG [37]. However, large-scale prospective studies are mandatory to evaluate the impact of VET in managing PVT in cirrhotic patients.

Another real clinical dilemma is the ability to monitor the efficacy and safety of anticoagulant therapy in patients with liver diseases. First, patients with cirrhosis can develop vein thrombosis despite a prolonged INR[23]. Second, monitoring the efficacy of LMWH using anti-factor Xa is not readily available[38]. Third, TEG was shown to be a sensitive method for monitoring LMWH efficacy in non-cirrhotic patients[39]. And last, the use of LMWH in 70 patients with advanced cirrhosis completely abolished the risk of PVT compared to 17% in the control group[40]. Altogether, the use of point of care VET could be o solution for this scenario.

Coagulation in acute-on-chronic liver failure

When it comes to acute multisystem imbalances, such as acute-on-chronic liver failure (ACLF), available data is relatively scarce, and reliable reports are rare.

It is essential to recognize the distinctive features of ACLF, to understand better its impact on coagulation and why precise assessment is needed[41]. Along with the classic liver failure features, the clinics are dominated by a marked systemic inflammatory response syndrome, often associated with bacterial infections, sequentially leading to multiple organ failure and, ultimately, death[42]. The typical ACLF patient is either treated in a high-dependency or an intensive care unit, requiring multiple invasive procedures[41,42]. In this light, an adequate assessment of their coagulation status appears to be particularly important. Based on prior experience with compensated and decompensated liver disease, the validity of standard coagulation tests (SCTs) in accurately assessing coagulation and bleeding risk in this clinical setting may yet again stand on shaky grounds.

Most of the VETs' data is relatively recent. It comprises monocentric reports, typically including less than one hundred patients with ACLF, assessing coagulation *via* ROTEM or TEG.

To this point, three available published reports are assessing the role of TEG in ACLF, all on Asian populations. In 2018, Goyal *et al*[43], comparing the coagulation profile of 68 ACLF patients with non-ACLF acutely decompensated patients and healthy controls, revealed a stark increase in SCT alteration with liver disease severity. Yet, the dynamic assessment was mostly normal, except for the reduced MA in ACLF, entailing a minimally altered coagulation profile. These findings might suggest that SCTs better reflect liver failure, rather than *per se* coagulation failure, as the diagnostic criteria for ACLF would imply. However, conclusions drawn from this dataset are in relative discordance with the other two available reports.

The patients who developed sepsis had a worse coagulation profile, tilted towards hypocoagulation, expressed by a higher R time[44]. In addition, among the enrolled ACLF patients, those with a hypocoagulation TEG profile had a significantly higher risk of bleeding [hazard ratio (HR) 2.1; CI: 1.6-4.9; P = 0.050] and short-term mortality (HR 1.9; CI: 1.3-7.9; P = 0.043). A more recent Chinese report[45] compared 51 hepatitis B virus-related cases of ACLF with healthy controls and patients with fully compensated chronic hepatitis B. They found that the coagulation dynamics were significantly altered in ACLF, with higher R and K times and lower α angles and MAs, corresponding to a marked hypocoagulable state. However, in this case, the comparison groups may not be ideal because healthy subjects are at the opposite spectrum of the disease than ACLF patients. Here, SCTs and TEG variables appeared to follow a concordant trend line. Furthermore, 90-day mortality was significantly associated with hypocoagulation within the ACLF group, as patients with ACLF and low MA were prone to a worse outcome.

While the studies were significantly different in design, a fragile common ground seems to emerge. While not all patients appear to have a marked coagulation imbalance, those who do tend to be in a hypocoagulable state appear to have a worse outcome.

Studies using ROTEM for assessing the coagulation profile have reached similar conclusions to those using TEG. One report comparing 36 ACLF patients to 24 non-ACLF acutely decompensated patients estimated transfusion requirement, bleeding events, and short-term mortality[46]. On admission, patients with ACLF had a more

hypocoagulable state, and the parameters worsened at 72 h, contrasting to the control group, which had an improved coagulation profile. Hypocoagulation was associated with a marked pro-inflammatory status and led to increased 28-d mortality. However, there was no association with an increased risk of bleeding events or transfusion requirements despite a worse coagulation profile. Of note, while SCTs and ROTEM variables followed the same trend line, ROTEM was a better outcome predictor. A second study, published in 2020, compared 22 ACLF patients with a compensated control group[47]. In this small dataset, the agreement between SCTs and ROTEM was slightly better in the ACLF group, which had a more hypocoagulable state. Besides, bleeding events were more frequent among ACLF patients with a worse coagulation function.

VET in liver transplantation

Historically, orthotopic liver transplantation (OLT) was associated with significant blood loss and the need for massive blood product transfusions[48]. Recently, with the new concept of rebalanced hemostasis, a more conservative attitude towards transfusion of red blood cells (RBC), fresh frozen plasma (FFP), or platelets is proposed. VET-guided transfusion algorithms to treat coagulopathy in OLT were first proposed by Kang *et al*[16] in the 1980s. They evaluated the blood coagulation system of 66 consecutive patients undergoing liver transplantation using TEG or standard liver transplantation monitoring and assessed the first clinical use of TEG in OLT. The use of TEG contributed to a 33% reduction of RBC, FFP, and platelet transfusion, whereas blood loss was comparable in all patients.

Comparing the standard management with ROTEM-guided hemostatic control results in a significant reduction of transfused RBC, FFP, and platelets in the ROTEM group[49]. Moreover, the number of blood product-free transplantations increased from 5% to 24% ($P < 0.001$). Secondary endpoints like reintervention for bleeding, acute kidney failure, or hemodynamic instability were significantly lower in the ROTEM group.

During liver transplantation, enhanced physiological fibrinolysis can occur, especially during the anhepatic period due to lack of tPA clearance. Immediately after reperfusion, there is a substantial increase in tPA, which can lead to hazardous primary hyperfibrinolysis resulting in diffuse uncontrolled bleeding. Suppose the graft has a good function the hyperfibrinolysis after reperfusion is self-limiting and does not require treatment. Hyperfibrinolysis in OLT has been reported very frequently (range 5%-84%), mainly during the transplanted liver[50,51]. However, most fibrinolysis is self-limiting and shall only be treated when it occurs concomitantly with excessive bleeding[50].

In the context of OLT, VETs are particularly useful for detecting the presence of systemic fibrinolysis[52] and also to detect poor clot strength that is often the result of low fibrinogen levels[52,53]. Therefore, the VET parameters for fibrinogen (FIBTEM or TEG functional fibrinogen) are essential to avoid over transfusion of platelets to increase the MA or MCF[52], which is associated with higher mortality[54].

The risk-benefit balance of the routine use of prophylactic antifibrinolytic agents (*e.g.*, tranexamic acid 1-2 g) shifted to a more precocious use of antifibrinolytics, in high-risk patients or treatment only, since massive bleeding is less frequent. Because hyperfibrinolysis-induced bleeding may manifest in the postreperfusion stage of surgery and depends on the donor liver's quality, the assessment is more difficult[55]. Treatment with antifibrinolytics is recommended only when there is evidence of microvascular ooze or documented fibrinolysis (CLI > 15) on TEG/ROTEM[56].

Conventional coagulation tests give no information where the balance in the coagulation lies since they do not provide a composite picture of the interaction of plasma, blood cells, and platelets. Some data suggest that VET detected hypercoagulability increases individual patients' risk for both venous and arterial thrombotic events[57,58] and is associated with high morbidity and mortality rates[59].

In a systematic review[60] to predict postoperative thromboembolic events by TEG, the most relevant parameter was MA. However, there was significant inhomogeneity among the included studies regarding the definition of hypercoagulability, and the majority of them were underpowered. It seems that hypercoagulation is more common in alcoholic and viral cirrhosis, and most often during the anhepatic phase (28%)[61]. Moreover, there is an association between hypercoagulation TEG profile and intracardiac thrombi. Despite conventional tests proving hypocoagulation, more than 70% of cases demonstrated TEG parameters compatible with hypercoagulation[62].

THE HEMOSTASIS ASSESSMENT BEFORE INVASIVE PROCEDURES IN PATIENTS WITH CIRRHOSIS

Traditionally, due to the presence of thrombocytopenia and hypoprothrombinemia, it was considered that the patients with cirrhosis have an increased risk of bleeding after interventional procedures[14,63]. Consequently, guidelines have recommended the correction of INR and platelets deficits through FFP or platelet transfusion before invasive procedures to prevent bleeding complications[64]. Arguments against this fear were raised by liver transplantation, which can be easily performed without blood product replacement[63,65]. Moreover, recent studies show little evidence for a higher prevalence of post-procedural bleeding following invasive procedures[63]. In 6 trials studying the prevalence of severe bleeding after interventional procedures (of low, high, and intermediate-risk) was 0.69%, range 0%-2.75% (50 out of 7146)[63]. In one prospective Italian study, the incidence of bleeding among 380 cirrhotic with or without abnormal coagulation parameters (defined as an INR ≥ 1.5 and/or platelet count ≤ 50 × 10^9/L) was zero for low-risk procedures like paracentesis[66]. High-risk procedures like percutaneous liver biopsy and percutaneous ablation were associated with higher bleeding rates in the abnormal coagulation group than the normal coagulation group. When analyzing in detail, the presence of sepsis and Child-Pugh C cirrhosis was associated with a higher incidence of bleeding in the abnormal coagulation group[67]. One large retrospective single-center study from the United States analyzed bleeding complications from 3357 liver biopsies and found a bleeding rate of 0.6%[68]. The median pre-biopsy platelet count, PT, and APTT (activated partial thromboplastin time) did not differ between patients that experienced or not bleeding complications; however, multivariate logistic regression identified a combination of APTT > 35 s and platelet count ≤ 100 × 10^9/L, as independent predictors of bleeding risk[67]. Seeff *et al*[68] found a bleeding rate of 0.6% in 2740 cirrhotic patients undergoing liver biopsy. In this study, a platelet count of less than 60 × 10^9/L was associated with a higher risk of bleeding meanwhile, an INR above 1.5 was not[68].

Therefore, the INR is not an accurate predictor of bleeding events in patients with cirrhosis. However, most of the studies have shown that severe thrombocytopenia was associated with a higher risk of bleeding, although the cut-off values were different in several studies (50 × 10^9/L-75 × 10^9/L)[14,63]. Reflecting the recent findings showing that cirrhotic patients are more often on a procoagulant slope[14,63], conventional coagulation tests are limited in predicting bleeding risk in cirrhosis because they do not account for the true *in vivo* coagulation status[69,70]. A systematic review comparing cirrhotic patients with a prolonged INR to those with normal INR found no difference in bleeding between the groups[13].

Role of VET before invasive procedures in cirrhosis

Conventional SCTs (PT and aPTT) omit thrombomodulin, which activates protein C and, thus, downregulating *in vivo* the thrombin generation. Therefore, the SCTs are not suitable to investigate acquired deficiency of both pro-and anticoagulants as occurs in cirrhosis[64].

Four studies have assessed the role of TEG before invasive procedures in cirrhosis (Table 2). All four studies reported a statistically significant reduction in overall blood product use with TEG guided transfusion[64,71-73]. The trials reported different outcomes regarding the transfusion of specific blood products such as FFP, platelets, and cryoprecipitate. A statistically significant reduction in platelet transfusion was reported in all studies[64,65,71-73]. The most striking difference was in the study of Vuyyuru *et al*[71], where only 10.3% of patients with cirrhosis undergoing interventional procedures needed platelet transfusion when guided by TEG compared to 75.9% when guided by conventional methods. The number of platelets used for transfusion was significantly lower in 3 of 4 studies[64,24,73]. Interestingly the number of platelets transfused was significantly lower for high-risk procedures (6 units *vs* 78 units, P < 0.001) but not low risk[64].

Three out of four studies reported a statistically significant reduction in FFP use[64,72,73]. In the study of De Petri 0% required FFP in the TEG arm compared to 53.3% in the conventional arm (P = 0.001)[64]. The absolute volume of FFP transfused was also markedly reduced in the TEG arm, where 4400 mL of FFP was transfused compared with 17550 mL in the control arm[64]. The difference was also maintained in upper gastrointestinal bleeding (both variceal and non-variceal)[72,73]. The amount of cryoprecipitate transfused was also lower with TEG in non-variceal bleeding (4 units in the TEG group compared with 16 in the standard of care group)[73].

Table 2 Role of thromboelastography prior invasive procedures in cirrhosis

Ref.	Type of invasive procedure	Threshold for intervention	Transfusion	Blood products transfused (%)/total amount of FFP (mL) and PLT (units)	Bleeding complications (%)	No. of death
De Pietri *et al*[64] (2016)	All invasive procedures (low and high risk)	TEG: FFP R > 40 min; PLT MA < 30 mm. SOC: FFP INR > 1.8; PLT transfusion PLT < 50000/mmc	TEG guided (n = 30); SOC (n = 30)	TEG guided/SOC: All % 16/100; FFP % 0/53.3; PLT % 6.7/33.3; FP + PLT % 3/13.3. Low risk procedures: FFP (mL) 4000/11050; PLT (unit) 22/28. High risk procedures; FFP (mL) 0/6500; PLT (unit) 6/78	TEG 0; SOC 3.3	TEG 8; SOC 7; 90 d
Vuyyuru *et al*[71] (2020)	All invasive procedures (low and high risk)	TEG: FFP R > 14 min; PLT MA < 33 mm. SOC: FFP INR > 1.8; PLT transfusion PLT < 50000/mmc	TEG guided (n = 29); SOC (n = 29)	TEG guided/SOC: All % 27.6/96.6; FFP % 24/27; PLT % 10.3/75.9; FFP + PLT % 3.4/3.4	TEG 0; SOC 0	TEG 0; SOC 1; 28 d
Rout *et al* [72] (2020)	Procedures for treating variceal bleeding	TEG: FFP R > 15 min; PLT MA < 30 mm, SOC: FFP INR > 1.8; PLT transfusion PLT < 50000/mmc	TEG guided (n = 30); SOC (n = 30)	TEG guided/SOC: All % 13.3/100; FFP % 13.3/46.7; PLT% 10/70; FFP + PLT % 10/16.7; FFP (mL) 4000/11050; PLT (mL) 450/3450	Rebleeding 5 d; TEG 3.3; SOC 13.3. Rebleeding 42 d; TEG 10; SOC 36.7	TEG 13; SOC 26
Kumar *et al*[73] (2020)	Procedures for treating nonvariceal bleeding	TEG: FFP R > 10 min; PLT MA < 55 mm; CryoP angle < 45. SOC: FFP INR > 1.8; PLT transfusion PLT < 50000/mmc. CryoP Fibrinogen < 80 mg%	TEG guided (n = 49); SOC (n = 47)	TEG guided/SOC: All% 26.5/87.2; FFP 4.1/0; PLT% 4.1/0; FFP + PLT% 14.3/0; Cryo % 12.2/0; Cryo + PLT % 8.2/4.3; CryoP + FFP % 16.3/8.5; None% 14.3/0; FFP (mL) 440/880; PLT (unit) 1/2; CryoP (unit) 4/16	Failure to control bleeding at 5 d. TEG 22.4; SOC 29.8. Failure to prevent bleeding after 5 d. TEG 50; SOC 57	TEG 22.4; SOC 29.8; 5 d. TEG 55; SOC 66; 42 d

TEG: Thromboelastography; INR: International normalized ratio; SOC: Standard of care; FFP: Fresh frozen plasma; PLT: Platelets; CryoP: Cryoprecipitate.

There was no statistically significant difference in blood loss and bleeding events in the two trials, which examined the use of TEG before an invasive procedure[64,71]. It is important to emphasize that the bleeding rates were low in both arms[64,71].

There was no difference in the control of initial bleeding between the TEG and conventional hemostasis assessment in the two trials in cirrhotic patients with upper gastrointestinal bleeding[72,73]. In those with variceal bleeding, the re-bleeding rate at 42 d was lower in the TEG guided transfusion group (10% *vs* 26.7%, P = 0.012)[72]. This advantage is not surprising since the over transfusion was associated with worse bleeding control and prognostic in cirrhosis patients[74].

Still, when considering overall mortality[64,71-73], length of stay in the intensive care unit[73], and the number of days in the hospital, there is no difference between the two guiding modalities[73].

One of the main advantages of using TEG for hemostasis assessment may be reducing transfusion-related adverse effects, 30.6% in the TEG group *vs* 74.5% in the control arm[73].

CONCLUSION

Recently, VETs of hemostasis are increasingly used for "point-of-care" assessment of complex hemostatic abnormalities. These tests' advantages lie in providing real-time, dynamic information about the whole coagulation process, including clot initiation (thrombin generation), clot kinetics, clot strength, and clot stability (lysis). In cirrhosis, SCTs are reliable tools in assessing liver function, but they fail to evaluate the hemostasis correctly. VET based assessment of bleeding risk and VET-guided transfusion strategies have been shown to reduce blood product use in cirrhotic patients who require invasive procedures and those presenting with variceal and non-variceal gastrointestinal bleeding. The reduction in blood product use was not associated with an increased risk of bleeding, the difference in controlling bleeding, morbidity, or mortality compared to standard care. The main disadvantage is related to the lack of extended validation in cirrhosis using more robust endpoints. By now, in the majority of the interventional randomized validation studies, the primary endpoint was the transfusion reduction, with eventual benefit extended to clinical endpoints as bleeding or survival. Therefore, all these studies were underpowered for reliable validation of some robust endpoints, and, thus, the VETs use is not widely available.

However, the standardization of TEG cut-off is mandatory to ensure a more reproducible evaluation of bleeding risk in cirrhosis patients.

REFERENCES

1 **Hoffman M**, Monroe DM 3rd. A cell-based model of hemostasis. *Thromb Haemost* 2001; **85**: 958-965 [PMID: 11434702]
2 **Northup PG**, Caldwell SH. Coagulation in liver disease: a guide for the clinician. *Clin Gastroenterol Hepatol* 2013; **11**: 1064-1074 [PMID: 23506859 DOI: 10.1016/j.cgh.2013.02.026]
3 **Tripodi A**, Primignani M, Mannucci PM, Caldwell SH. Changing Concepts of Cirrhotic Coagulopathy. *Am J Gastroenterol* 2017; **112**: 274-281 [PMID: 27801884 DOI: 10.1038/ajg.2016.498]
4 **Kujovich JL**. Coagulopathy in liver disease: a balancing act. *Hematology Am Soc Hematol Educ Program* 2015; **2015**: 243-249 [PMID: 26637729 DOI: 10.1182/asheducation-2015.1.243]
5 **Lisman T**, Bongers TN, Adelmeijer J, Janssen HL, de Maat MP, de Groot PG, Leebeek FW. Elevated levels of von Willebrand Factor in cirrhosis support platelet adhesion despite reduced functional capacity. *Hepatology* 2006; **44**: 53-61 [PMID: 16799972 DOI: 10.1002/hep.21231]
6 **Tripodi A**, Primignani M, Chantarangkul V, Clerici M, Dell'Era A, Fabris F, Salerno F, Mannucci PM. Thrombin generation in patients with cirrhosis: the role of platelets. *Hepatology* 2006; **44**: 440-445 [PMID: 16871542 DOI: 10.1002/hep.21266]
7 **Tripodi A**, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med* 2011; **365**: 147-156 [PMID: 21751907 DOI: 10.1056/NEJMra1011170]
8 **Lisman T**, Leebeek FW, Mosnier LO, Bouma BN, Meijers JC, Janssen HL, Nieuwenhuis HK, De Groot PG. Thrombin-activatable fibrinolysis inhibitor deficiency in cirrhosis is not associated with increased plasma fibrinolysis. *Gastroenterology* 2001; **121**: 131-139 [PMID: 11438502 DOI: 10.1053/gast.2001.25481]
9 **Colucci M**, Binetti BM, Branca MG, Clerici C, Morelli A, Semeraro N, Gresele P. Deficiency of thrombin activatable fibrinolysis inhibitor in cirrhosis is associated with increased plasma fibrinolysis. *Hepatology* 2003; **38**: 230-237 [PMID: 12830006 DOI: 10.1053/jhep.2003.50277]
10 **Leonardi F**, Maria N, Villa E. Anticoagulation in cirrhosis: a new paradigm? *Clin Mol Hepatol* 2017; **23**: 13-21 [PMID: 28288507 DOI: 10.3350/cmh.2016.0110]
11 **Tripodi A**, Chantarangkul V, Primignani M, Fabris F, Dell'Era A, Sei C, Mannucci PM. The international normalized ratio calibrated for cirrhosis (INR(liver)) normalizes prothrombin time results for model for end-stage liver disease calculation. *Hepatology* 2007; **46**: 520-527 [PMID: 17659574 DOI: 10.1002/hep.21732]
12 **Bellest L**, Eschwège V, Poupon R, Chazouillères O, Robert A. A modified international normalized ratio as an effective way of prothrombin time standardization in hepatology. *Hepatology* 2007; **46**: 528-534 [PMID: 17654598 DOI: 10.1002/hep.21680]
13 **Segal JB**, Dzik WH; Transfusion Medicine/Hemostasis Clinical Trials Network. Paucity of studies to support that abnormal coagulation test results predict bleeding in the setting of invasive procedures: an evidence-based review. *Transfusion* 2005; **45**: 1413-1425 [PMID: 16131373 DOI: 10.1111/j.1537-2995.2005.00546.x]
14 **Giannini EG**, Greco A, Marenco S, Andorno E, Valente U, Savarino V. Incidence of bleeding following invasive procedures in patients with thrombocytopenia and advanced liver disease. *Clin Gastroenterol Hepatol* 2010; **8**: 899-902; quiz e109 [PMID: 20601131 DOI: 10.1016/j.cgh.2010.06.018]
15 **Arif S**, Khan AS, Khan AR. Changes in fibrinogen level in liver cirrhosis. *J Ayub Med Coll Abbottabad* 2002; **14**: 19-21 [PMID: 12238339]
16 **Kang YG**, Martin DJ, Marquez J, Lewis JH, Bontempo FA, Shaw BW Jr, Starzl TE, Winter PM. Intraoperative changes in blood coagulation and thrombelastographic monitoring in liver transplantation. *Anesth Analg* 1985; **64**: 888-896 [PMID: 3896028]
17 **Tripodi A**, Salerno F, Chantarangkul V, Clerici M, Cazzaniga M, Primignani M, Mannuccio Mannucci P. Evidence of normal thrombin generation in cirrhosis despite abnormal conventional coagulation tests. *Hepatology* 2005; **41**: 553-558 [PMID: 15726661 DOI: 10.1002/hep.20569]
18 **Gatt A**, Riddell A, Calvaruso V, Tuddenham EG, Makris M, Burroughs AK. Enhanced thrombin generation in patients with cirrhosis-induced coagulopathy. *J Thromb Haemost* 2010; **8**: 1994-2000 [PMID: 20546119 DOI: 10.1111/j.1538-7836.2010.03937.x]
19 **Haas T**, Spielmann N, Mauch J, Madjdpour C, Speer O, Schmugge M, Weiss M. Comparison of thromboelastometry (ROTEM®) with standard plasmatic coagulation testing in paediatric surgery. *Br J Anaesth* 2012; **108**: 36-41 [PMID: 22086509 DOI: 10.1093/bja/aer342]
20 **Görlinger K**, Saner FH. Prophylactic plasma and platelet transfusion in the critically Ill patient: just useless and expensive or even harmful? *BMC Anesthesiol* 2015; **15**: 86 [PMID: 26054337 DOI: 10.1186/s12871-015-0074-0]
21 **Golder M**, Mewburn J, Lillicrap D. In vitro and *in vivo* evaluation of the effect of elevated factor VIII on the thrombogenic process. *Thromb Haemost* 2013; **109**: 53-60 [PMID: 23178924 DOI: 10.1160/TH12-05-0316]
22 **Mallett SV**, Sugavanam A, Krzanicki DA, Patel S, Broomhead RH, Davidson BR, Riddell A, Gatt A,

Chowdary P. Alterations in coagulation following major liver resection. *Anaesthesia* 2016; **71**: 657-668 [PMID: 27030945 DOI: 10.1111/anae.13459]
23 **Mallett SV**, Chowdary P, Burroughs AK. Clinical utility of viscoelastic tests of coagulation in patients with liver disease. *Liver Int* 2013; **33**: 961-974 [PMID: 23638693 DOI: 10.1111/liv.12158]
24 **Stravitz RT**. Potential applications of thromboelastography in patients with acute and chronic liver disease. *Gastroenterol Hepatol (N Y)* 2012; **8**: 513-520 [PMID: 23293564]
25 **DeLeve LD**, Valla DC, Garcia-Tsao G; American Association for the Study Liver Diseases. Vascular disorders of the liver. *Hepatology* 2009; **49**: 1729-1764 [PMID: 19399912 DOI: 10.1002/hep.22772]
26 **Ben-Ari Z**, Panagou M, Patch D, Bates S, Osman E, Pasi J, Burroughs A. Hypercoagulability in patients with primary biliary cirrhosis and primary sclerosing cholangitis evaluated by thrombelastography. *J Hepatol* 1997; **26**: 554-559 [PMID: 9075662 DOI: 10.1016/s0168-8278(97)80420-5]
27 **Pihusch R**, Rank A, Göhring P, Pihusch M, Hiller E, Beuers U. Platelet function rather than plasmatic coagulation explains hypercoagulable state in cholestatic liver disease. *J Hepatol* 2002; **37**: 548-555 [PMID: 12399218 DOI: 10.1016/s0168-8278(02)00239-8]
28 **Hickman IJ**, Sullivan CM, Flight S, Campbell C, Crawford DH, Masci PP, O'Moore-Sullivan TM, Prins JB, Macdonald GA. Altered clot kinetics in patients with non-alcoholic fatty liver disease. *Ann Hepatol* 2009; **8**: 331-338 [PMID: 20009132]
29 **Krzanicki D**, Sugavanam A, Mallett S. Intraoperative hypercoagulability during liver transplantation as demonstrated by thromboelastography. *Liver Transpl* 2013; **19**: 852-861 [PMID: 23696318 DOI: 10.1002/lt.23668]
30 **Hugenholtz GCG**, Lisman T, Stravitz RT. Thromboelastography does not predict outcome in different etiologies of cirrhosis. *Res Pract Thromb Haemost* 2017; **1**: 275-285 [PMID: 30046697 DOI: 10.1002/rth2.12037]
31 **Cakir T**, Cingi A, Yeğen C. Coagulation dynamics and platelet functions in obstructive jaundiced patients. *J Gastroenterol Hepatol* 2009; **24**: 748-751 [PMID: 19646016 DOI: 10.1111/j.1440-1746.2009.05801.x]
32 **Janko N**, Majeed A, Kemp W, Roberts SK. Viscoelastic Tests as Point-of-Care Tests in the Assessment and Management of Bleeding and Thrombosis in Liver Disease. *Semin Thromb Hemost* 2020; **46**: 704-715 [PMID: 32932542 DOI: 10.1055/s-0040-1715475]
33 **Zocco MA**, Di Stasio E, De Cristofaro R, Novi M, Ainora ME, Ponziani F, Riccardi L, Lancellotti S, Santoliquido A, Flore R, Pompili M, Rapaccini GL, Tondi P, Gasbarrini GB, Landolfi R, Gasbarrini A. Thrombotic risk factors in patients with liver cirrhosis: correlation with MELD scoring system and portal vein thrombosis development. *J Hepatol* 2009; **51**: 682-689 [PMID: 19464747 DOI: 10.1016/j.jhep.2009.03.013]
34 **Huang X**, Fan X, Zhang R, Jiang S, Yang K, Chen S. Systemic inflammation and portal vein thrombosis in cirrhotic patients with gastroesophageal varices. *Eur J Gastroenterol Hepatol* 2020; **32**: 401-405 [PMID: 31356372 DOI: 10.1097/MEG.0000000000001526]
35 **Zanetto A**, Senzolo M, Vitale A, Cillo U, Radu C, Sartorello F, Spiezia L, Campello E, Rodriguez-Castro K, Ferrarese A, Farinati F, Burra P, Simioni P. Thromboelastometry hypercoagulable profiles and portal vein thrombosis in cirrhotic patients with hepatocellular carcinoma. *Dig Liver Dis* 2017; **49**: 440-445 [PMID: 28109767 DOI: 10.1016/j.dld.2016.12.019]
36 **James K**, Bertoja E, O'Beirne J, Mallett S. Use of thromboelastography PlateletMapping to monitor antithrombotic therapy in a patient with Budd-Chiari syndrome. *Liver Transpl* 2010; **16**: 38-41 [PMID: 20035517 DOI: 10.1002/lt.21933]
37 **Jain A**, Dhore P, Meshram M, Bhatia S, Shukla A. Patients With Budd-Chiari Syndrome Have Variable Coagulation Status on Thromboelastography at Diagnosis. *J Clin Exp Hepatol* 2019; **9**: 460-467 [PMID: 31516262 DOI: 10.1016/j.jceh.2018.10.002]
38 **Hirsh J**, Warkentin TE, Shaughnessy SG, Anand SS, Halperin JL, Raschke R, Granger C, Ohman EM, Dalen JE. Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety. *Chest* 2001; **119**: 64S-94S [PMID: 11157643 DOI: 10.1378/chest.119.1_suppl.64s]
39 **Van PY**, Cho SD, Underwood SJ, Morris MS, Watters JM, Schreiber MA. Thrombelastography *vs* AntiFactor Xa levels in the assessment of prophylactic-dose enoxaparin in critically ill patients. *J Trauma* 2009; **66**: 1509-15; discussion 1515 [PMID: 19509608 DOI: 10.1097/TA.0b013e3181a51e33]
40 **Villa E**, Cammà C, Marietta M, Luongo M, Critelli R, Colopi S, Tata C, Zecchini R, Gitto S, Petta S, Lei B, Bernabucci V, Vukotic R, De Maria N, Schepis F, Karampatou A, Caporali C, Simoni L, Del Buono M, Zambotto B, Turola E, Fornaciari G, Schianchi S, Ferrari A, Valla D. Enoxaparin prevents portal vein thrombosis and liver decompensation in patients with advanced cirrhosis. *Gastroenterology* 2012; **143**: 1253-1260. e4 [PMID: 22819864 DOI: 10.1053/j.gastro.2012.07.018]
41 **Jalan R**, Saliba F, Pavesi M, Amoros A, Moreau R, Ginès P, Levesque E, Durand F, Angeli P, Caraceni P, Hopf C, Alessandria C, Rodriguez E, Solis-Muñoz P, Laleman W, Trebicka J, Zeuzem S, Gustot T, Mookerjee R, Elkrief L, Soriano G, Cordoba J, Morando F, Gerbes A, Agarwal B, Samuel D, Bernardi M, Arroyo V; CANONIC study investigators of the EASL-CLIF Consortium. Development and validation of a prognostic score to predict mortality in patients with acute-on-chronic liver failure. *J Hepatol* 2014; **61**: 1038-1047 [PMID: 24950482 DOI: 10.1016/j.jhep.2014.06.012]
42 **Trebicka J**, Fernandez J, Papp M, Caraceni P, Laleman W, Gambino C, Giovo I, Uschner FE,

Jimenez C, Mookerjee R, Gustot T, Albillos A, Bañares R, Janicko M, Steib C, Reiberger T, Acevedo J, Gatti P, Bernal W, Zeuzem S, Zipprich A, Piano S, Berg T, Bruns T, Bendtsen F, Coenraad M, Merli M, Stauber R, Zoller H, Ramos JP, Solè C, Soriano G, de Gottardi A, Gronbaek H, Saliba F, Trautwein C, Özdogan OC, Francque S, Ryder S, Nahon P, Romero-Gomez M, Van Vlierberghe H, Francoz C, Manns M, Garcia E, Tufoni M, Amoros A, Pavesi M, Sanchez C, Curto A, Pitarch C, Putignano A, Moreno E, Shawcross D, Aguilar F, Clària J, Ponzo P, Jansen C, Vitalis Z, Zaccherini G, Balogh B, Vargas V, Montagnese S, Alessandria C, Bernardi M, Ginès P, Jalan R, Moreau R, Angeli P, Arroyo V; PREDICT STUDY group of the EASL-CLIF Consortium. The PREDICT study uncovers three clinical courses of acutely decompensated cirrhosis that have distinct pathophysiology. *J Hepatol* 2020; **73**: 842-854 [PMID: 32673741 DOI: 10.1016/j.jhep.2020.06.013]

43 **Goyal S**, Jadaun S, Kedia S, Kumar-Acharya S, Varma S, Nayak B, Thakur B, M D S. Thromboelastography Parameters in Patients with Acute on Chronic Liver Failure. *Ann Hepatol* 2018; **17**: 1042-1051 [PMID: 30600294 DOI: 10.5604/01.3001.0012.7205]

44 **Premkumar M**, Saxena P, Rangegowda D, Baweja S, Mirza R, Jain P, Bhatia P, Kumar G, Bihari C, Kalal C, Vyas T, Choudhury A, Sarin SK. Coagulation failure is associated with bleeding events and clinical outcome during systemic inflammatory response and sepsis in acute-on-chronic liver failure: An observational cohort study. *Liver Int* 2019; **39**: 694-704 [PMID: 30589495 DOI: 10.1111/liv.14034]

45 **Zhu Z**, Yu Y, Ke Y, Deng D, Zheng G, Hua X, Gao G. Thromboelastography maximum amplitude predicts short-term mortality in patients with hepatitis B virus-related acute-on-chronic liver failure. *Exp Ther Med* 2020; **20**: 2657-2664 [PMID: 32765759 DOI: 10.3892/etm.2020.8990]

46 **Blasi A**, Calvo A, Prado V, Reverter E, Reverter JC, Hernández-Tejero M, Aziz F, Amoros A, Cardenas A, Fernández J. Coagulation Failure in Patients With Acute-on-Chronic Liver Failure and Decompensated Cirrhosis: Beyond the International Normalized Ratio. *Hepatology* 2018; **68**: 2325-2337 [PMID: 29790188 DOI: 10.1002/hep.30103]

47 **Seeßle J**, Löhr J, Kirchner M, Michaelis J, Merle U. Rotational thrombelastometry (ROTEM) improves hemostasis assessment compared to conventional coagulation test in ACLF and Non-ACLF patients. *BMC Gastroenterol* 2020; **20**: 271 [PMID: 32807080 DOI: 10.1186/s12876-020-01413-w]

48 **Butler P**, Israel L, Nusbacher J, Jenkins DE Jr, Starzl TE. Blood transfusion in liver transplantation. *Transfusion* 1985; **25**: 120-123 [PMID: 3885484 DOI: 10.1046/j.1537-2995.1985.25285169201.x]

49 **Leon-Justel A**, Noval-Padillo JA, Alvarez-Rios AI, Mellado P, Gomez-Bravo MA, Álamo JM, Porras M, Barrero L, Hinojosa R, Carmona M, Vilches-Arenas A, Guerrero JM. Point-of-care haemostasis monitoring during liver transplantation reduces transfusion requirements and improves patient outcome. *Clin Chim Acta* 2015; **446**: 277-283 [PMID: 25916692 DOI: 10.1016/j.cca.2015.04.022]

50 **Poon KS**, Chen CC, Thorat A, Chiang YY, Jeng LB, Yang HR, Chen TH, Yeh CC, Chen KB. Fibrinolysis after reperfusion of liver graft. *Acta Anaesthesiol Taiwan* 2015; **53**: 41-43 [PMID: 25649273 DOI: 10.1016/j.aat.2014.12.001]

51 **Görlinger K**. [Coagulation management during liver transplantation]. *Hamostaseologie* 2006; **26**: S64-S76 [PMID: 16953295]

52 **Larsen OH**, Fenger-Eriksen C, Christiansen K, Ingerslev J, Sørensen B. Diagnostic performance and therapeutic consequence of thromboelastometry activated by kaolin *vs* a panel of specific reagents. *Anesthesiology* 2011; **115**: 294-302 [PMID: 21691196 DOI: 10.1097/ALN.0b013e318220755c]

53 **Gorlinger K**, Dirkmann D, Muller-Beissenhirtz H, Paul A, Hartmann M, Saner F. Thromboelastometry-based perioperative coagulation management in visceral surgery and liver transplantation: experience of 10 tears and 1105 LTX. *Liver Transpl* 2010; **16**: S86

54 **Pereboom IT**, de Boer MT, Haagsma EB, Hendriks HG, Lisman T, Porte RJ. Platelet transfusion during liver transplantation is associated with increased postoperative mortality due to acute lung injury. *Anesth Analg* 2009; **108**: 1083-1091 [PMID: 19299765 DOI: 10.1213/ane.0b013e3181948a59]

55 **Porte RJ**, Blauw E, Knot EA, de Maat MP, de Ruiter C, Minke Bakker C, Terpstra OT. Role of the donor liver in the origin of platelet disorders and hyperfibrinolysis in liver transplantation. *J Hepatol* 1994; **21**: 592-600 [PMID: 7814807 DOI: 10.1016/S0168-8278(94)80107-X]

56 **Schofield N**, Sugavanam A, Thompson K, Mallett SV. No increase in blood transfusions during liver transplantation since the withdrawal of aprotinin. *Liver Transpl* 2014; **20**: 584-590 [PMID: 24481770 DOI: 10.1002/lt.23839]

57 **McCrath DJ**, Cerboni E, Frumento RJ, Hirsh AL, Bennett-Guerrero E. Thromboelastography maximum amplitude predicts postoperative thrombotic complications including myocardial infarction. *Anesth Analg* 2005; **100**: 1576-1583 [PMID: 15920177 DOI: 10.1213/01.ANE.0000155290.86795.12]

58 **Kashuk JL**, Moore EE, Sabel A, Barnett C, Haenel J, Le T, Pezold M, Lawrence J, Biffl WL, Cothren CC, Johnson JL. Rapid thrombelastography (r-TEG) identifies hypercoagulability and predicts thromboembolic events in surgical patients. *Surgery* 2009; **146**: 764-72; discussion 772 [PMID: 19789037 DOI: 10.1016/j.surg.2009.06.054]

59 **Warnaar N**, Molenaar IQ, Colquhoun SD, Slooff MJ, Sherwani S, de Wolf AM, Porte RJ. Intraoperative pulmonary embolism and intracardiac thrombosis complicating liver transplantation: a systematic review. *J Thromb Haemost* 2008; **6**: 297-302 [PMID: 18005235 DOI: 10.1111/j.1538-7836.2007.02831.x]

60 **Dai Y**, Lee A, Critchley LA, White PF. Does thromboelastography predict postoperative thromboembolic events? *Anesth Analg* 2009; **108**: 734-742 [PMID: 19224777 DOI:

10.1213/ane.0b013e31818f8907]
61 **Schöchl H**, Solomon C, Schulz A, Voelckel W, Hanke A, Van Griensven M, Redl H, Bahrami S. Thromboelastometry (TEM) findings in disseminated intravascular coagulation in a pig model of endotoxinemia. *Mol Med* 2011; **17**: 266-272 [PMID: 21170471 DOI: 10.2119/molmed.2010.00159]
62 **Xia VW**, Ho JK, Nourmand H, Wray C, Busuttil RW, Steadman RH. Incidental intracardiac thromboemboli during liver transplantation: incidence, risk factors, and management. *Liver Transpl* 2010; **16**: 1421-1427 [PMID: 21117252 DOI: 10.1002/lt.22182]
63 **Zakeri N**, Tsochatzis EA. Bleeding Risk with Invasive Procedures in Patients with Cirrhosis and Coagulopathy. *Curr Gastroenterol Rep* 2017; **19**: 45 [PMID: 28752476 DOI: 10.1007/s11894-017-0585-6]
64 **De Pietri L**, Bianchini M, Montalti R, De Maria N, Di Maira T, Begliomini B, Gerunda GE, di Benedetto F, Garcia-Tsao G, Villa E. Thrombelastography-guided blood product use before invasive procedures in cirrhosis with severe coagulopathy: A randomized, controlled trial. *Hepatology* 2016; **63**: 566-573 [PMID: 26340411 DOI: 10.1002/hep.28148]
65 **Wei H**, Child LJ. Clinical utility of viscoelastic testing in chronic liver disease: A systematic review. *World J Hepatol* 2020; **12**: 1115-1127 [PMID: 33312434 DOI: 10.4254/wjh.v12.i11.1115]
66 **Napolitano G**, Iacobellis A, Merla A, Niro G, Valvano MR, Terracciano F, Siena D, Caruso M, Ippolito A, Mannuccio PM, Andriulli A. Bleeding after invasive procedures is rare and unpredicted by platelet counts in cirrhotic patients with thrombocytopenia. *Eur J Intern Med* 2017; **38**: 79-82 [PMID: 27989373 DOI: 10.1016/j.ejim.2016.11.007]
67 **Takyar V**, Etzion O, Heller T, Kleiner DE, Rotman Y, Ghany MG, Fryzek N, Williams VH, Rivera E, Auh S, Liang TJ, Hoofnagle JH, Koh C. Complications of percutaneous liver biopsy with Klatskin needles: a 36-year single-centre experience. *Aliment Pharmacol Ther* 2017; **45**: 744-753 [PMID: 28074540 DOI: 10.1111/apt.13939]
68 **Seeff LB**, Everson GT, Morgan TR, Curto TM, Lee WM, Ghany MG, Shiffman ML, Fontana RJ, Di Bisceglie AM, Bonkovsky HL, Dienstag JL; HALT–C Trial Group. Complication rate of percutaneous liver biopsies among persons with advanced chronic liver disease in the HALT-C trial. *Clin Gastroenterol Hepatol* 2010; **8**: 877-883 [PMID: 20362695 DOI: 10.1016/j.cgh.2010.03.025]
69 **Singh AD**, Shalimar. Use of Blood Products and Drugs Before Procedures in Patients With Cirrhosis. *Clin Liver Dis (Hoboken)* 2020; **16**: 153-157 [PMID: 33163168 DOI: 10.1002/cld.906]
70 **Shenoy A**, Intagliata NM. Thromboelastography and Utility in Hepatology Practice. *Clin Liver Dis (Hoboken)* 2020; **16**: 149-152 [PMID: 33163167 DOI: 10.1002/cld.947]
71 **Vuyyuru SK**, Singh AD, Gamanagatti SR, Rout G, Gunjan D, Shalimar. A Randomized Control Trial of Thromboelastography-Guided Transfusion in Cirrhosis for High-Risk Invasive Liver-Related Procedures. *Dig Dis Sci* 2020; **65**: 2104-2111 [PMID: 31720889 DOI: 10.1007/s10620-019-05939-2]
72 **Rout G**, Shalimar, Gunjan D, Mahapatra SJ, Kedia S, Garg PK, Nayak B. Thromboelastography-guided Blood Product Transfusion in Cirrhosis Patients With Variceal Bleeding: A Randomized Controlled Trial. *J Clin Gastroenterol* 2020; **54**: 255-262 [PMID: 31008867 DOI: 10.1097/MCG.0000000000001214]
73 **Kumar M**, Ahmad J, Maiwall R, Choudhury A, Bajpai M, Mitra LG, Saluja V, Mohan Agarwal P, Bihari C, Shasthry SM, Jindal A, Bhardwaj A, Kumar G, Sarin SK. Thromboelastography-Guided Blood Component Use in Patients With Cirrhosis With Nonvariceal Bleeding: A Randomized Controlled Trial. *Hepatology* 2020; **71**: 235-246 [PMID: 31148204 DOI: 10.1002/hep.30794]
74 **Villanueva C**, Colomo A, Bosch A. Transfusion for acute upper gastrointestinal bleeding. *N Engl J Med* 2013; **368**: 1362-1363 [PMID: 23550677 DOI: 10.1056/NEJMc1301256]

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MINIREVIEWS

Gastrointestinal involvement in paediatric COVID-19 — from pathogenesis to clinical management: A comprehensive review

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), is responsible for the first pandemic of the 21st century. As found in adults, signs and symptoms related to the disease mainly involve the respiratory tract in the paediatric population. However, a considerable number of children present with gastrointestinal symptoms such as vomiting, abdominal pain, and diarrhea. The purpose of this review is an accurate description, from pathogenesis to clinical presentation, diagnosis and treatment, of COVID-19 effects on the gastrointestinal system at a paediatric age. SARS-CoV-2 can be identified in stool specimens of affected children by real-time polymerase chain reaction techniques. Positivity can last for several weeks after the end of the symptomatic phase. Gastrointestinal signs and symptoms are generally self-limited, can correlate with blood tests and imaging alterations, and may require supportive treatment such as hydration. However, they can precede severe disease manifestations such as the COVID-19-related multisystem inflammatory syndrome. Children belonging to risk categories such as those affected by celiac disease, inflammatory bowel disease, and hepatic disease seem to not have a more severe course than the others, even if they are undergoing immunosuppressant treatment. Medical follow-ups of patients with chronic diseases need to be revised during the pandemic period in order to postpone unnecessary tests, mainly endoscopic ones.

Key Words: SARS-CoV-2; COVID-19; Paediatric; Gastrointestinal; Diarrhea; Hydration

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Core Tip: Gastrointestinal signs and symptoms seem to be more common in paediatric

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coronavirus disease 2019 (COVID-19) compared to adults. Manifestations are generally self-limited, and may only require supportive treatment. In a minority of children, gastrointestinal involvement may precede severe forms such as the multisystem inflammatory syndrome. Conversely to what is expected, the COVID-19 impact on paediatric patients with chronic gastrointestinal diseases is limited, with no need for therapeutic regimen changes. However, the severe acute respiratory syndrome coronavirus 2 pandemic determined multiple variations in routine practice. The use of telemedicine and telehealth can be a solution in order to continue to provide regular follow-up to chronic patients, avoiding the risk of viral transmission.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) is caused by single-stranded RNA severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The disease was first described in Wuhan, China, in December 2019. Then the virus spread rapidly worldwide, making the World Health Organization declare the first pandemic of the 21st century in March 2020[1]. On March 4, 2021, there were more than 114 million confirmed cases of COVID-19 worldwide, with nearly 3 million registered deaths[2].

The clinical spectrum of COVID-19 in children and adolescents ranges from asymptomatic/pauci symptomatic infection to severe disease. Digestive symptoms related to COVID-19 such as vomiting, diarrhea, and abdominal pain have emerged as extrapulmonary manifestations, with SARS-CoV-2 RNA detected in the faeces of people affected, suggesting faecal-oral transmission. Children with gastrointestinal (GI) chronic diseases, including those undergoing immunosuppressive or biological treatment, need to be strictly evaluated for the risk of developing severe forms of COVID-19. GI involvement may play a role in the presentation of symptoms in children affected by the multisystem inflammatory syndrome (MIS-C) associated with COVID-19[3].

The purpose of this review is to summarise what is known about COVID-19 GI manifestations in children, from diagnosis to symptoms and treatment, including the peculiar aspects of patients with GI chronic diseases.

PATHOGENESIS

There are many mechanisms through which SARS-CoV-2 can interact and damage the GI system.

The first is a virus-induced cytopathic effect. SARS-CoV-2 interacts with membrane receptors of the host cells through the spike protein, which mediates the fusion of the virus and the cell membrane[4]. Angiotensin-converting enzyme receptor 2 (ACE-2) and transmembrane protease serine 2 (TMPRSS2) are both essential for the cellular entry process of the virus. They are co-expressed at a high level both in the type II alveolar cells of the lung, in the glandular cells of the gastric, duodenal, and rectal epithelium, and in the enterocytes of ileum and colon[4]. After viral entry, new virions are synthesised in the cytoplasm of the GI cells and then are released in the GI tract, causing direct disruption of enterocytes and the viral shedding in the stool[5,6]. As absorptive enterocytes are destroyed by SARS-CoV-2, this changes the intestinal permeability, leading to malabsorption and unbalanced intestinal secretion, resulting in the genesis of diarrhea[6-8].

As ACE-2 is equally expressed in liver and pancreas, the virus cytopathic effect can also be detected in these organs. Hepatic distribution of ACE-2 is peculiar; it is highly expressed in the endothelial layer of small blood vessels but not in the sinusoidal

endothelium[9]. Its concentration on cholangiocytes' surface is higher than of the hepatocyte surface and is similar to the type II alveolar cells of the lungs. SARS-CoV-2 may have the ability to infect cholangiocytes *via* the ACE-2 receptor and directly dysregulate liver function[7,9]. However, there is no evidence of active virus replication in hepatocyte cells[6]. A histological examination of a liver biopsy obtained from a deceased COVID-19 patient showed no viral inclusions, but rather a microvesicular steatosis and mild lobular activity[7].

SARS-CoV-2 can affect both the exocrine and endocrine pancreas; an abnormal elevation of amylase and lipase, together with glucose dysregulation and acute diabetes are described in patients with severe COVID-19 pneumonia, with development of acute pancreatitis in a few cases[10].

The second mechanism depends on immune-system activation[11]. SARS-CoV-2-infected cells release a large number of inflammatory mediators and chemokines such as interleukin (IL)-2, IL-7, granulocyte colony-stimulating factor, interferon-γ inducible protein 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1-α and tumour necrosis factor-alpha (TNF-α). This "cytokine storm" causes neutrophil aggregation and activates type 1 helper cells. This promotes the accumulation of immune cells in the GI system[7]; a large number of infiltrating plasma cells and lymphocytes together with interstitial oedema have been found in the inherent layers of the stomach, duodenum, and rectum in adult COVID-19 patients[8]. The cytokine overproduction correlates with disease severity and multiple organ insufficiency development outside the lung, liver, and pancreas.

SARS-CoV-2 may alter the intestinal microbiome even when only the respiratory mucosa is involved (the "gut-lung axis")[5,6]. Increased inflammatory mediators lead to lung hyperpermeability so that the virus and inflammatory mediators migrate to the intestine *via* circulation. SARS-CoV-2 and the inflammatory mediators disrupt the intestinal permeability leading to the leakage of gut microbes and associated metabolites into circulation. The leaked microbes and products migrate to organs including lungs and produce abnormalities.

Other hypothesised mechanisms of liver and pancreatic damage include hypoxic injury due to respiratory distress and drug-induced injury[5-7,10]. Kidneys can also be involved, as they are fundamental in eliminating amylases and lipases from the circulatory stream: Their malfunction can lead to a transient increase in pancreatic enzymes[10].

CLINICAL MANIFESTATIONS

Children and adolescents with GI symptoms such as nausea, vomiting, or diarrhea should be seriously evaluated for COVID-19, as the faecal-oral route transmission of SARS-CoV-2 is extensively described (Figure 1). Oba *et al*[12] reported that GI signs and symptoms may affect 3% to 79% of children, adolescents and adults with COVID-19. Various paediatric systematic reviews evidenced similar results[4,6,13], curiously with different frequencies in the United States and Europe compared to China (21.1% *vs* 12.9%)[4]. Manifestations include diarrhea (2%-50%), anorexia (40%-50%), vomiting (4%-67%), nausea (1%-30%), abdominal pain (2%-6%) and GI bleeding (4%-14%). Diarrhea and vomiting are the most common GI symptoms described, sometimes as the first symptoms of disease, even before or in absence of respiratory manifestations. Diarrhea, often watery, occurs from 1 d to 8 d after the onset of COVID-19, with a median time of 3.3 d, and lasts for a mean of 4 d[12]. Vomiting is more often reported in the paediatric population than in the adult one.

GI symptoms may correlate with severe COVID-19 in children admitted to hospital. A Spanish multicentre study involving 101 paediatric inpatients noticed that patients presenting with GI symptoms tended to have higher C-reactive protein (CRP), procalcitonin (PCT), ferritin and aspartate aminotransferase values, and to receive antibiotics, lopinavir-ritonavir, corticosteroids and immunoglobulins more frequently than the others. Moreover, they had a higher risk of paediatric intensive care unit (ICU) admission, regardless of age, gender, immunosuppressive therapy and previous underlying conditions[14]. Similar findings were evidenced in studies among adults, where those with diarrhea had a higher risk of ICU admission regardless of the age, sex and comorbidities[15,16].

GI symptoms such as vomiting, abdominal pain and/or diarrhea are typically present and considered diagnostic criteria in children with the COVID-19-related MIS-C (71%-84% of the cases), along with fever lasting more than 3 d, evidence of mucocutaneous inflammation (rash, conjunctivitis, oromucosal changes), lymphopenia and

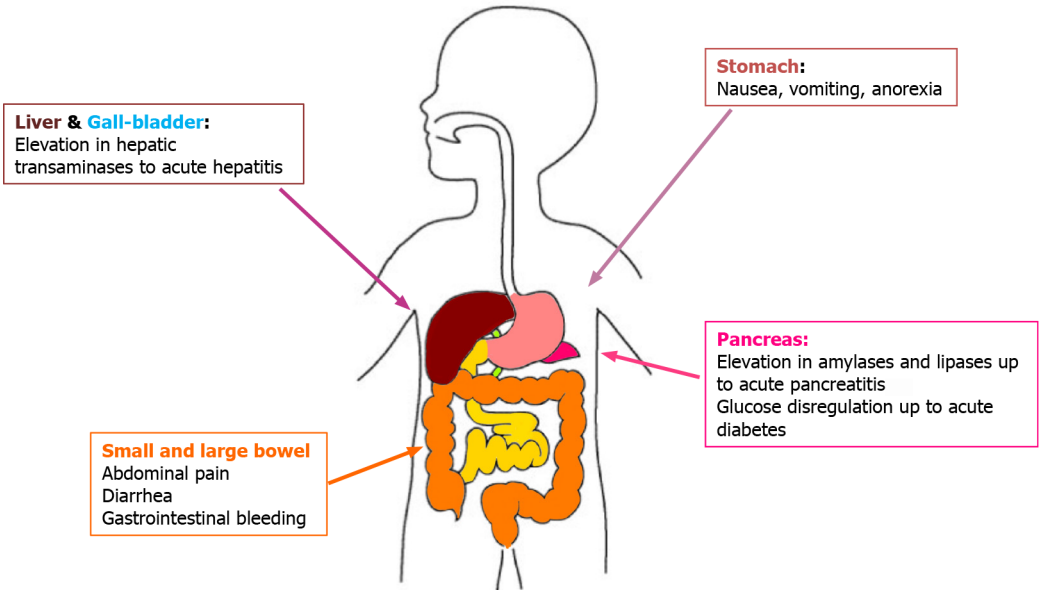


Figure 1 Gastrointestinal involvement in coronavirus disease 2019.

high levels of circulating inflammation[3,17-19]. Abdominal pain may be so relevant that few children with a final diagnosis of MIS-C initially present with acute surgical abdomen; they may undergo exploratory laparotomy with intra-operative findings of mesenteric lymphadenitis and peritonitis[20]. Evolution to severe disease including cardiac involvement, hypotension and shock is often described in children with MIS-C [19].

DIAGNOSIS

Prompt diagnosis of SARS-CoV-2 infection can determine treatment strategies and influence the outcome of the disease in children. Suggestive symptoms together with the history of a close contact with a COVID-19 patient are the most useful criteria for a suspicion of infection[21]. Diagnosis is confirmed by SARS-CoV-2 isolation in patient samples, while auxiliary examinations are useful to determine the severity of the disease and organ involvement in infected children.

Leukopenia with neutropenia and lymphocytosis or lymphopenia are the most common findings at blood count. Erythrocyte sedimentation rate is elevated in a great number of children, as well as IL-10 and IL-6. CRP and PCT levels can be normal. However, a PCT value > 0.5 ng/mL may be suggestive of bacterial coinfection or of a severe autoinflammatory state such as the MIS-C[22,23].

A mild increase in liver enzymes is well described in COVID-19, with various percentages among studies, ranging from 13% to 50% in paediatric patients; however, serious liver dysfunction is uncommon[6,24]. Elevated aspartate aminotransferase levels (> 50 UI/L; 20.4%-50% of cases) are observed more frequently than alanine aminotransferase levels (> 45 UI/L; 9%-35% of cases). Increased transaminases are often accompanied by high creatinine kinase and lactate dehydrogenase, suggesting the possibility of a viral myositis[25,26].

Considerable alteration in liver enzymes is common in those with severe spectrum of disease (40%-60%) compared to those who are asymptomatic or have mild manifestations (18%-25%). Nevertheless, every child with COVID-19 and raised transaminases should be investigated for other causes of liver pathology[5].

Patients with MIS-C present with a remarkable elevation in transaminases levels (52.3%) together with a mild decrease in albumin rates. Elevation of lipase levels > 3 times the normal limit was observed in one paediatric patient with MIS-C[3,25]. In fact, pancreatic involvement in adults with COVID-19 is well described, with a lipase increase in 7.9% of cases. Bilirubin levels are also more than doubled in those with severe infection, when compared to those with mild disease[10].

Nucleic acid testing is the method of choice for virus identification. SARS-CoV-2 RNA can be detected in sputum, lower respiratory tract secretions, urine, stool, tears,

and blood samples by real-time polymerase chain reaction (RT-PCR) technology or by viral gene sequencing. RT-PCR on nose-pharyngeal swab (NPS) is the diagnostic method of choice in children. However, paediatric patients tested for SARS-CoV-2 by RT-PCR on a rectal swab or stool returned a positive result in 89% of cases, despite not presenting with any GI symptoms. Moreover, COVID-19 children may have a stool SARS-CoV-2 RT-PCR positive result more frequently than adults in spite of a negative respiratory swab; RT-PCR on stool samples seems to be as accurate as those performed on the NPS in order to identify SARS-CoV-2[27,28].

As the faecal-oral route is confirmed as a way for SARS-CoV-2 transmission, all children with digestive tract symptoms should be tested for SARS-CoV-2 on faeces[12,29]. RT-PCR on stool becomes positive from 2 d to 2 wk after the respiratory specimen ones, and 23%-82% of patients continue to have positive faecal test for approximately 1-16 d after their NPS turn negative[4,28]. The interval to stool negativisation may be prolonged, exceeding 70 d in healthy children, longer in patients treated with corticosteroids[4].

Therefore, despite major evidence being necessary to consider a negative RT-PCR on faeces as one of the discharge criteria, it may be important to recommend isolation at home for at least 2 wk after hospital discharge[4].

Little is known about typical imaging findings in COVID-19 paediatric patients with GI symptoms. Abdominal ultrasonography to computed tomography or magnetic resonance imaging may be taken into consideration in patients with a severe course of disease or relevant blood test alterations (*e.g.*, increased transaminases and/or lipases). Miller *et al*[3] described a paediatric population of 44 cases with MIS-C and GI symptoms, with abdominal images collected in 15 patients. Common findings were mesenteric adenitis (2 patients), biliary sludge or acalculous cholecystis (6) and ascites (6). Normal abdominal imaging was found in 20% of cases. In 3 patients, ultrasonography or magnetic resonance imaging evidenced bowel wall thickening analogous to that of inflammatory bowel disease (IBD). One child had severe clinical manifestations (fever, abdominal pain, rash) with evidence of concentric mural thickening, oedema and hyperenhancement of a short segment of terminal ileum, with similar findings in the rectosigmoid colon. Two patients had nonspecific ultrasonography imaging, with thickened bowel loops on the right iliac site associated with highly elevated inflammatory index and mildly decreased albumin levels[3].

THERAPY

Generally, COVID-19 paediatric patients require symptomatic care, both because the great majority of them has mild symptoms and also because all virus-targeted therapies are employed exclusively in clinical trial settings[30]. Supportive care included fever treatment, oxygen therapy in patients with respiratory complications with or without airway management, and nasogastric or intravenous hydration in children unable to tolerate oral fluids such as those with severe GI symptoms[30].

COVID-19 children are at higher risk of developing malnourishment during critical illness, which has been associated with increased morbidity and mortality; therefore, nutritional therapy plays a significant role in these children[12]. The European Society of Paediatric and Neonatal Intensive Care cornerstones for nutrition recommend commencing early enteral feeding within 24 h of hospital admission in critically ill children unless contraindicated. Energy requirements need not exceed resting energy expenditure during the acute phase and an increase in enteral nutrition in a stepwise fashion is recommended until the goal for delivery is achieved. Overfeeding harms critically ill children, especially during the acute phase[31].

In children with severe GI COVID-19 and MIS-C, enteral nutrition support may be continued for a long time into the recovery phase until sufficient oral intake is consistently achieved to support physical and nutritional rehabilitation[32]. Enteral nutrition is also recommended in critically ill children on hemodynamic support with a stable clinical condition; parenteral nutrition has to be withheld during the first 7 d of admission[12,31]. However, in children who continue to require fluid resuscitation or escalating doses of vasoactive agents with evidence of severe GI dysfunction and MIS-C, enteral nutrition may be withheld for up to 7 d[32].

It may be useful to consider providing enteral feeds *via* a post-pyloric tube in critically ill children with COVID-19 with severe GI symptoms or cardiac manifestations or inotrope resistance shock in which early gastric enteral feeding is not possible [12,32].

There is no evidence to support supra-physiological doses of micronutrients supplementation, including zinc during the acute phase[32].

Recent literature suggests the role of probiotics in manipulating the gut microbiota, as they may play a fruitful role as a therapeutic strategy for GI COVID-19 and its comorbidities. Focused clinical trials are needed to support this hypothesis[33].

Based on currently available limited data, children with comorbidities or pre-existing chronic diseases, such as IBD or liver disease, do not seem to carry a higher risk of COVID-19 infection compared to the general population (see below)[34,35].

PARTICULAR CASES

Celiac disease

Celiac disease (CeD), also known as celiac sprue or gluten-sensitive enteropathy, is a common immune-mediated inflammatory disease of the small intestine resulting from sensitivity to dietary gluten and related proteins in genetically predisposed individuals[36]. It is estimated that CeD affects approximately 0.5% to 1% of the general population[37]. CeD therapy consists in a strict gluten-free diet (GFD) to achieve complete resolution of symptoms and mucosal healing for most individuals. It also reduces the risk of long-term adverse health outcomes including intestinal lymphoma[38].

During the COVID-19 pandemic, it has been postulated that some patients with chronic GI disease may be at an increased risk of a more severe illness due to COVID-19. Potential risk factors in these patients include their chronic inflammatory disease, comorbidities (*e.g.*, diabetes mellitus), and the use of glucocorticoids[39].

Considering that CeD subjects have an increased risk of both bacterial and viral infections, such as pneumococcal pneumonia[40], sepsis[41], and complications from influenza virus, probably due to malnutrition and increased mucosal permeability [42]. Various studies have investigated the possibility of a higher risk of SARS-CoV-2 infection in CeD patients compared to the general population.

Data on patients with chronic GI disease who have been infected by SARS-CoV-2 are accumulating, and disease-specific patient registries include Surveillance Epidemiology of Coronavirus Under Research Exclusion (SECURE-Celiac). This is an international, paediatric and adult database to monitor and report the outcomes of COVID-19 in patients with CeD. On January 17, 2021 a total number of 111 cases was reported; 4 cases < 18 years of age, nobody was hospitalised, and no death was registered (SECURE-Celiac Database, available at: https://covidceliac.org/data). Several studies evidenced that the risk of severe COVID-19 is not increased in patients with CeD both in adulthood and childhood[43-48]. A large-scale study, performed on 18000 participants from different Countries, including Argentina, Australia, Canada, Italy, Mexico, New Zealand, Spain, Uruguay, and the United States, examined the risk of COVID-19 in CeD compared with the nonceliac population. They found that patients with CeD had similar odds to contract SARS-CoV-2 infection compared to the control subjects. The presence of comorbidities, which were identified as an important predictor of morbidity and mortality associated with COVID-19, were more frequent in CeD than the control subjects. However, they did not determine higher odds for SARS-CoV-2 infection in CeD. The authors observed that the sole factor increasing the odds of a positive test was exposure to a COVID-19 contact. In this study, patients with CeD were less exposed to COVID-19 than the control subjects, probably due to a careful prevention related to their chronic condition[46]. Moreover, a cross-sectional study conducted by a paediatric celiac centre in Central Italy did not show any significant increase in SARS-CoV-2 infection prevalence among the group of children with CeD compared to the general population[48].

A relevant aspect of the COVID-19 pandemic is the impact of restrictive measures on primary health care. In this new scenario, all elective diagnostic procedures such as paediatric digestive endoscopy have been suspended and were allowed only in emergency cases[49,50].

As esophagogastroduodenoscopy is still necessary for CeD diagnosis in children with low antibodies titres and this elective procedure has been substantially shut down during the COVID-19 pandemic, many children remained undiagnosed and therefore untreated for a long time. The European Society of Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) analysed the feasibility and accuracy of a biopsy-free approach in suspected CeD children with tissue transglutaminase-immunoglobulin A (TGA-IgA) values < 10 time upper the normal limit (ULN) during the COVID-19 outbreak. A temporary reduction of the TGA-IgA threshold seems

feasible in antiendomysial antibodies (EMA) positive children with TGA-IgA between 5 and 10 ULN for a biopsy-sparing approach[51].

The global impact of the lockdown on the compliance to GFD in CeD patients was investigated in a cross-sectional survey conducted in Italy on a total of 1983 patients with CeD, 1614 (81.4%) adults and 369 (18.6%) children. The compliance to GFD was unchanged for 70% of the subjects, and even improved for 29%, in particular for those with a previous worse disease control, due to reduced opportunities for contamination and transgression and an increased use of naturally gluten-free ingredients[52].

An observational study on 71 paediatric patients with CeD examined the prevalence of functional GI disorders (FGIDs) in children during the COVID-19 lockdown. The Authors observed a reduced prevalence of FGIDs in these patients, probably due to a good quality of life and a low state of anxiety related to the positive effects of reducing stressful events, such as school, as well as to an increased parental closeness, which may have influenced the occurrence of GI symptoms and the related FGIDs. Psychosocial aspects may play a particularly important role in the genesis of the FGIDs, despite the presence of a residual low-grade chronic inflammatory process[53].

IBD

IBD, including Crohn's disease (CD) and ulcerative colitis, are chronic pathologies of the GI system characterised by a dysregulated immune response with an over production of pro-inflammatory cytokines. To control the disease, patients require frequent treatment with corticosteroids, immunosuppressants, and/or biological drugs with the consequence of an increased risk of infections. IBDs tend to be more extensive and severe in children than in adults, with a consistently greater need for immunomodulators and biological agents to maintain remission in this population [54].

The IBD-related immunosuppressive treatment has raised concerns regarding the management and the risk of SARS-CoV-2 infection severity. On the other hand, it can be assumed that immunosuppressive medications may be associated with a decreased risk of poor COVID-19 outcomes by limiting the cytokine storm typical of severe COVID-19.

The first document on the global impact of SARS-CoV-2 infection on paediatric IBD was published in March 2020 by the Paediatric IBD Porto Group of ESPGHAN. It was a survey among 102 Paediatric IBD centres affiliated with the Porto and Interest-group of ESPGHAN, with external paediatric experts from China and South Korea invited to participate. All cases of COVID-19 in IBD paediatric patients enrolled were mild despite they were under immunosuppressive treatment. The study expert group suggested that IBD children, with or without immunosuppressive and biological therapy, do not seem to have a greater risk of severe SARS-CoV-2 infection compared to the general population. Due to this, they recommend not stopping standard IBD treatments[35].

A total of 522 IBD patients were enrolled in one of the Italian regions with greater COVID-19 incidence and were followed for a 1-mo period between February and March 2020. Fifty-nine (11%) paediatric patients (7-18-years-old) were included. In this IBD cohort, the Authors did not report any case of COVID-19 despite the fact that the enrolled patients continued to receive immunosuppressive treatments such as thiopurines or methotrexate and steroids[55].

Surveillance Epidemiology of Coronavirus Under Research Exclusion (SECURE-IBD) is an international, paediatric and adult database to monitor and report on outcomes of COVID-19 occurring in IBD patients. On January 5, a total number of 4280 cases was reported; 441 cases < 19 years of age, only 22% hospitalised, and no death evidenced[56].

Brenner *et al*[57] described the course of the disease of COVID-19 in a sample of 209 paediatric IBD patients (age 18 years and younger) from the 2 international databases (The SECURE-IBD and the COVID-19 database of the Paediatric IBD Porto group of ESPGHAN). They reported a hospitalisation rate of 7%; only 2 patients (1%) required mechanical ventilation, one for a MIS-C and one for a secondary infection. There were no deaths. Risk factors for hospitalisation included other comorbidities, moderate/severe IBD disease activity, and GI symptoms.

In March 2020 Paediatric IBD Porto group of ESPGHAN generated guidance points for paediatric gastroenterologists in the era of the COVID-19 pandemic. These points are mainly focus on therapy indications. There is no evidence that any of the drugs used in IBDs, including immune-modulators and biological drugs, increases the severity of COVID-19. IBD patients should stay on IBD medications prescribed before the SARS-CoV-2 pandemic[35]. Newly diagnosed patients should be treated according to the standard protocols as before the spread of the virus[5,12].

SARS-CoV-2 enters cells *via* the ACE-2 receptor, widely expressed in the GI tract. Its expression is upregulated during inflammation and this overexpression may therefore increase host susceptibility. There are two forms of ACE-2: full-length ACE-2 with a structural transmembrane domain, which anchors its extracellular domain to the cellular membrane, and a soluble form of ACE-2 that lacks the membrane anchor and circulates in the blood. Some authors have speculated that the soluble form may act as a competitive interceptor for SARS-CoV-2, preventing the binding of the viral particle to the surface full-length ACE-2[58]. It can explain why children with IBD have a COVID-19 course similar to healthy individuals.

By contrast, immune dysfunction in untreated IBD children may increase the risk of a severe inflammatory response to SARS-CoV-2 infection. Pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6 are overproduced in IBD patients[59], and serum concentration of TNF-α and IL-6 are associated with severe COVID-19 illness[60]. Dolinger *et al*[61] described a case of MIS-C associated with COVID-19 in a recently diagnosed paediatric CD patient. He was treated with infliximab for both clinical entities with a successful result. The authors speculated that anti-TNF agents could play a role in the therapy of patients with active IBD and MIS-C temporally related to COVID-19.

A COVID-19 Risk Calculator is on the SECURE-IBD website and it is intended for use by physicians caring for patients with IBD to calculate the risk of hospitalisation, ICU admission, mechanical ventilation, or death in IBD patients with COVID-19. It is available at: https://covidibd.org/covid-19-risk-calculator/.

Regarding CeD, the COVID-19 pandemic determined essential healthcare changes, mainly during the lockdown period, which could alter IBD follow-up in children. These include postponed elective appointments, reduced access to diagnostic endoscopy, and difficulties in continuing infusion therapy, with potential clinical and psychological negative impact on the course of the disease.

A recent survey, conducted in the United Kingdom, outlined that more than 50% of children and young people presenting with a suspected diagnosis of IBD were diagnosed without a histological examination due to restrictions placed on endoscopy at over 90% of centres across the United Kingdom. The authors observed that "diagnosing children and young people with IBD, without a histological confirmation, is controversial and only acceptable given the special circumstances we are currently finding ourselves in"[62].

A multicentre investigation of the Italian Society of Paediatric Gastroenterology, Hepatology and Nutrition (SIGENP) analysed the impact of COVID-19 related lockdown on the levels of care offered to paediatric IBD patients all over the Italian territory[63]. In total, 2291 children affected by IBD were regularly followed by the 21 participating referral centres; a total of 6 cases of SARS-CoV-2 infections of 2291 (0.2%) patients were identified. In 5 of 6 (83.3%) cases, the clinical course of the SARS-CoV-2 infection resulted as mild, without the need for hospitalisation. The authors observed a reduction of hospitalisations for new diagnosis and endoscopic re-evaluation, while the number of hospitalisations for relapse and surgical procedure remained substantially unchanged. The number of outpatients' visits were significantly decreased. Biologics' infusions did not significantly vary and Italian paediatric IBD centres did not modify their therapeutic approach in the majority of cases, as recommended by the ESPGHAN guidelines.

Differently from the United Kingdom data, the multicentre Italian survey found a drop in the new diagnoses during the lockdown.

The fear of SARS-CoV-2 infection could determine the risk of inappropriate management of IBD with consequent significant impact on the health of IBD patients [35]. As the decrease of face-to-face consultations, telemedicine represents a promising opportunity. In the Italian survey, telemedicine services for children with IBD were activated in 52.3% of the participating centres.

Recently, members of the SIGENP IBD group study drafted a position paper with the aim of providing guidance for the management of paediatric IBD on the basis of the existing evidence[64].

In addition to specific recommendations regarding diagnostic procedures and therapies, the position paper provides general indications for a safer and gradual restarting of routine clinical activities after the COVID-19 peak, with particular attention also on psychological issues.

Chronic liver diseases

At present, there is no concrete evidence that the SARS-CoV-2 infection causes significant worsening in underlying chronic liver disease. Children undergoing treatment for pathologies like Wilson disease, autoimmune hepatitis, hepatitis B and C should continue their treatment protocols[5]. Elevated transaminases in COVID-19 are

not a contraindication for antiviral therapy in viral chronic hepatitis, even if regular monitoring of liver function is needed[65].

Solid organ transplantation

Reports of critical disease in adults and children with cancer have raised concerns about the risk of a severe COVID-19 course in patients with immune system impairment, including recipients of solid organ transplants on long-term immune suppression[66].

Data on the COVID-19 course and outcome in children needing liver solid organ transplantation (SOT), or already transplanted, are limited. Reports by the team of the Transplantation Unit of Papa Giovanni XXIII Hospital in Bergamo (Italy) are reassuring; liver-transplanted young patients did not experience any severe respiratory infection, despite residing in one of the earliest and hardest-hit areas world-wide, where first cases likely dated long before any distancing or isolation measure [67].

Six cases of children awaiting transplantation and eight cases of paediatric transplant recipients infected by SARS-CoV-2 were reported by Doná *et al*[68] None of the candidates in the SOT waiting list, nor any of the SOT recipients, presented severe COVID-19.

It seems that the immunosuppressive treatment in immunocompromised children may not significantly increase the risk of severe COVID-19, as its complications are mainly driven by a well-documented pro-inflammatory state[69].

Post-liver transplant patients need particular emphasis on preventive measures, such as frequent hand washing, frequent cleaning of touched surfaces and social distancing. Generally, as the cell injury in COVID-19 disease is thought to be immune-mediated, immunosuppression and mycophenolate should not be reduced or stopped in asymptomatic post-transplant patients. In an established COVID-19, the continuation of calcineurin inhibitors targeting a lower trough levels and lowering of the dose of mycophenolate or azathioprine is recommended. Patients on high-dose steroids should have them reduced to a minimum dose based on body weight in order to prevent adrenal insufficiency. At present, there is no recommendation for any antivirals or hydroxychloroquine prophylaxis either in post-liver transplant children or those with COVID-19 associated acute liver disease[5].

The emergence of COVID-19 has had a profound impact on transplantation worldwide, both for issues regarding donors and recipients viral transmission and for healthcare resources, as the magnitude of COVID-19 cases in certain regions exceeds the available capacity of the health system[70].

Members of the European Reference Network of Paediatric Transplantation investigated the impact of the COVID-19 outbreak on paediatric transplant activity and healthcare practices in both SOT and hematopoietic stem cell transplantation: transplantation activity as well as outpatient visits were negatively affected by the COVID-19 pandemic across Europe[68]. The risk of SARS-CoV-2 transmission and the shortage of hospital bed capacity and staff might be the main determinants of this reduction. Significant extension of these limitations in healthcare resources may have severe consequences both for children on the transplant waiting list and for transplanted patients as the access to close monitoring and diagnostic testing would be significantly reduced. In this emerging public health context, there are no reasons to delay or interrupt oncological treatments or withdraw immune suppression, to postpone life-saving treatments in liver-transplanted patients, or to suspend transplant programs as an a priori preventative measure[71].

Abdominal surgery implications in paediatrics during COVID-19 pandemic

The COVID-19 pandemic determined diagnosis delay and higher complication rates among common paediatric medical conditions[72].

An example may be represented by the increased incidence of complicated appendicitis among children: no changes in patient demographics nor in the rate of appendicitis itself could explain this phenomenon. A considerable extension in the average time between the onset of symptoms and surgery was identified as the causative agent: normally, surgical intervention time is influenced by other factors such as elective activity and availability of theatre and staff. The fear of contracting SARS-CoV-2 infection leads the parents to avoid hospital access of their child at the early stages of the disease, doubling the time before the patient's access to the Emergency Department (ED). Delayed access to ED may explain the higher prevalence of complicated appendicitis[73]. At the same time and for the same reasons, a statistically significant increased rate of appendiceal perforation during the COVID-19 pandemic was reported. In a cross-sectional study, appendiceal perforation also

resulted in pelvic abscess, bowel obstruction, and sepsis[74]. Delayed presentation of children with acute appendicitis at the ED may increase the morbidity related to a common childhood condition, leading to increased complications and poor outcomes [75,76].

Although complicated courses have arisen, an Italian study noticed a reduction in the total number of acute appendicitis cases. A decrease in social contacts during the lockdown period, a reduction of respiratory and GI infections, healthy food intake due to the permanence at home and increased domestic hygiene could have played a crucial role, bringing mild appendicitis to a spontaneous resolution cases with or without domestic treatments[77,78].

CONCLUSION

This review describes the spectrum of GI manifestations in paediatric COVID-19, from signs and symptoms of disease to laboratory and imaging tests alterations. All the organs can be involved, ranging from mild to severe alterations. Pathogenesis is outlined, however further clarifications of virus-induced damages are needed. Although generally self-limited, GI signs and symptoms are closely related to complicated courses of disease: As a matter of fact, GI involvement is a diagnostic-criteria for the MIS-C. Close monitoring of infected children will permit to delineate potential predictors of severe COVID-19 evolution.

Conversely to what is expected, the COVID-19 impact on children with chronic GI diseases appears to be limited, with no need for therapeutic regimen changes. Ongoing studies may confirm these initial observations. Certainly, the SARS-CoV-2 pandemic determined multiple variations in routine practice of these patients. The use of telemedicine and telehealth can be a solution in order to continue to provide regular follow-up to chronic patients, avoiding the risk of viral transmission.

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REFERENCES

1 **World Health Organization**. WHO Coronavirus Disease (COVID-19) pandemic. [cited 4 March 2021]. In: World Health Organization [Internet]. Available from: https:// covid-19.who.int/
2 **World Health Organization**. WHO Coronavirus Disease (COVID-19) dashboard. [cited 4 March 2021]. In: World Health Organization [Internet]. Available from: https://covid-19.who.int/
3 **Miller J**, Cantor A, Zachariah P, Ahn D, Martinez M, Margolis KG. Gastrointestinal Symptoms as a Major Presentation Component of a Novel Multisystem Inflammatory Syndrome in Children That Is Related to Coronavirus Disease 2019: A Single Center Experience of 44 Cases. *Gastroenterology* 2020; **159**: 1571-1574. e2 [PMID: 32505742 DOI: 10.1053/j.gastro.2020.05.079]
4 **Wang JG**, Cui HR, Tang HB, Deng XL. Gastrointestinal symptoms and fecal nucleic acid testing of children with 2019 coronavirus disease: a systematic review and meta-analysis. *Sci Rep* 2020; **10**: 17846 [PMID: 33082472 DOI: 10.1038/s41598-020-74913-0]
5 **Matthai J**, Shanmugam N, Sobhan P; Indian Society of Pediatric Gastroenterology, Hepatology and Nutrition; Pediatric Gastroenterology Chapter of Indian Academy of Pediatrics. Coronavirus Disease (COVID-19) and the Gastrointestinal System in Children. *Indian Pediatr* 2020; **57**: 533-535 [PMID: 32562397 DOI: 10.1007/s13312-020-1851-5]
6 **Chiappini E**, Licari A, Motisi MA, Manti S, Marseglia GL, Galli L, Lionetti P. Gastrointestinal involvement in children with SARS-COV-2 infection: An overview for the pediatrician. *Pediatr Allergy Immunol* 2020; **31** Suppl 26: 92-95 [PMID: 33236437 DOI: 10.1111/pai.13373]
7 **Patel KP**, Patel PA, Vunnam RR, Hewlett AT, Jain R, Jing R, Vunnam SR. Gastrointestinal, hepatobiliary, and pancreatic manifestations of COVID-19. *J Clin Virol* 2020; **128**: 104386 [PMID: 32388469 DOI: 10.1016/j.jcv.2020.104386]
8 **Xiao F**, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for Gastrointestinal Infection of SARS-CoV-2. *Gastroenterology* 2020; **158**: 1831-1833. e3 [PMID: 32142773 DOI: 10.1053/j.gastro.2020.02.055]
9 **Jothimani D**, Venugopal R, Abedin MF, Kaliamoorthy I, Rela M. COVID-19 and the liver. *J Hepatol* 2020; **73**: 1231-1240 [PMID: 32553666 DOI: 10.1016/j.jhep.2020.06.006]
10 **Zippi M**, Hong W, Traversa G, Maccioni F, De Biase D, Gallo C, Fiorino S. Involvement of the exocrine pancreas during COVID-19 infection and possible pathogenetic hypothesis: a concise

review. *Infez Med* 2020; **28**: 507-515 [PMID: 33257624]
11 **Lei HY**, Ding YH, Nie K, Dong YM, Xu JH, Yang ML, Liu MQ, Wei L, Nasser MI, Xu LY, Zhu P, Zhao MY. Potential effects of SARS-CoV-2 on the gastrointestinal tract and liver. *Biomed Pharmacother* 2021; **133**: 111064 [PMID: 33378966 DOI: 10.1016/j.biopha.2020.111064]
12 **Oba J**, Carvalho WB, Silva CA, Delgado AF. Gastrointestinal manifestations and nutritional therapy during COVID-19 pandemic: a practical guide for pediatricians. *Einstein (Sao Paulo)* 2020; **18**: eRW5774 [PMID: 32667418 DOI: 10.31744/einstein_journal/2020rw5774]
13 **Xu Y**, Li X, Zhu B, Liang H, Fang C, Gong Y, Guo Q, Sun X, Zhao D, Shen J, Zhang H, Liu H, Xia H, Tang J, Zhang K, Gong S. Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. *Nat Med* 2020; **26**: 502-505 [PMID: 32284613 DOI: 10.1038/s41591-020-0817-4]
14 **Gonzalez Jimenez D**, Velasco Rodríguez-Belvís M, Ferrer Gonzalez P, Domínguez Ortega G, Segarra O, Medina Benitez E, Garcia Tirado D, Garcia Romero R, Vecino López R, Crehuá-Gaudiza E, Queralt M, Palomino Pérez LM, Diaz Martin JJ. COVID-19 Gastrointestinal Manifestations Are Independent Predictors of PICU Admission in Hospitalized Pediatric Patients. *Pediatr Infect Dis J* 2020; **39**: e459-e462 [PMID: 33105340 DOI: 10.1097/INF.0000000000002935]
15 **Jin X**, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, Hao SR, Jia HY, Cai H, Zhang XL, Yu GD, Xu KJ, Wang XY, Gu JQ, Zhang SY, Ye CY, Jin CL, Lu YF, Yu X, Yu XP, Huang JR, Xu KL, Ni Q, Yu CB, Zhu B, Li YT, Liu J, Zhao H, Zhang X, Yu L, Guo YZ, Su JW, Tao JJ, Lang GJ, Wu XX, Wu WR, Qv TT, Xiang DR, Yi P, Shi D, Chen Y, Ren Y, Qiu YQ, Li LJ, Sheng J, Yang Y. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. *Gut* 2020; **69**: 1002-1009 [PMID: 32213556 DOI: 10.1136/gutjnl-2020-320926]
16 **Wan Y**, Li J, Shen L, Zou Y, Hou L, Zhu L, Faden HS, Tang Z, Shi M, Jiao N, Li Y, Cheng S, Huang Y, Wu D, Xu Z, Pan L, Zhu J, Yan G, Zhu R, Lan P. Enteric involvement in hospitalised patients with COVID-19 outside Wuhan. *Lancet Gastroenterol Hepatol* 2020; **5**: 534-535 [PMID: 32304638 DOI: 10.1016/S2468-1253(20)30118-7]
17 **Nakra NA**, Blumberg DA, Herrera-Guerra A, Lakshminrusimha S. Multi-System Inflammatory Syndrome in Children (MIS-C) Following SARS-CoV-2 Infection: Review of Clinical Presentation, Hypothetical Pathogenesis, and Proposed Management. *Children (Basel)* 2020; **7** [PMID: 32630212 DOI: 10.3390/children7070069]
18 **Radia T**, Williams N, Agrawal P, Harman K, Weale J, Cook J, Gupta A. Multi-system inflammatory syndrome in children & adolescents (MIS-C): A systematic review of clinical features and presentation. *Paediatr Respir Rev* 2020 [PMID: 32891582 DOI: 10.1016/j.prrv.2020.08.001]
19 **Vogel TP**, Top KA, Karatzios C, Hilmers DC, Tapia LI, Moceri P, Giovannini-Chami L, Wood N, Chandler RE, Klein NP, Schlaudecker EP, Poli MC, Muscal E, Munoz FM. Multisystem inflammatory syndrome in children and adults (MIS-C/A): Case definition & guidelines for data collection, analysis, and presentation of immunization safety data. *Vaccine* 2021 [PMID: 33640145 DOI: 10.1016/j.vaccine.2021.01.054]
20 **Guanà R**, Pagliara C, Delmonaco AG, Scottoni F, Bordese R, Pruccoli G, Gennari F. Multisystem inflammatory syndrome in SARS-CoV-2 infection mimicking acute appendicitis in children. *Pediatr Neonatol* 2021; **62**: 122-124 [PMID: 33036934 DOI: 10.1016/j.pedneo.2020.09.007]
21 **Miao H**, Li H, Yao Y, Wu M, Lu C, Wang J, Tian M, Li Y, Luo P, Gu J, Yuan B, Wang S, Zhao X, Gan W, Zhao D. Update on recommendations for the diagnosis and treatment of SARS-CoV-2 infection in children. *Eur J Clin Microbiol Infect Dis* 2020; **39**: 2211-2223 [PMID: 32761481 DOI: 10.1007/s10096-020-03973-x]
22 **Li B**, Zhang S, Zhang R, Chen X, Wang Y, Zhu C. Epidemiological and Clinical Characteristics of COVID-19 in Children: A Systematic Review and Meta-Analysis. *Front Pediatr* 2020; **8**: 591132 [PMID: 33224909 DOI: 10.3389/fped.2020.591132]
23 **Henry BM**, Lippi G, Plebani M. Laboratory abnormalities in children with novel coronavirus disease 2019. *Clin Chem Lab Med* 2020; **58**: 1135-1138 [PMID: 32172227 DOI: 10.1515/cclm-2020-0272]
24 **Esmaeili Dooki M**, Mehrabani S, Sorkhi H, Nikpour M, Tabatabaie M, Mohammadi M, Kiani M. COVID-19 and Digestive System in Children: A Retrospective Study. *Arch Iran Med* 2020; **23**: 782-786 [PMID: 33220697 DOI: 10.34172/aim.2020.104]
25 **Parri N**, Lenge M, Buonsenso D; Coronavirus Infection in Pediatric Emergency Departments (CONFIDENCE) Research Group. Children with Covid-19 in Pediatric Emergency Departments in Italy. *N Engl J Med* 2020; **383**: 187-190 [PMID: 32356945 DOI: 10.1056/NEJMc2007617]
26 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]
27 **Xu CLH**, Raval M, Schnall JA, Kwong JC, Holmes NE. Duration of Respiratory and Gastrointestinal Viral Shedding in Children With SARS-CoV-2: A Systematic Review and Synthesis of Data. *Pediatr Infect Dis J* 2020; **39**: e249-e256 [PMID: 32618932 DOI: 10.1097/INF.0000000000002814]
28 **Zhang L**, Peres TG, Silva MVF, Camargos P. What we know so far about Coronavirus Disease 2019 in children: A meta-analysis of 551 Laboratory-confirmed cases. *Pediatr Pulmonol* 2020; **55**: 2115-2127 [PMID: 32519809 DOI: 10.1002/ppul.24869]
29 **Tian Y**, Rong L, Nian W, He Y. Review article: gastrointestinal features in COVID-19 and the possibility of faecal transmission. *Aliment Pharmacol Ther* 2020; **51**: 843-851 [PMID: 32222988 DOI: 10.1111/apt.15731]
30 **Chan K**, Beck C, Chauvin-Kimoff L, Gripp K, Krmpotic K, Thakore S, Trottier ED; Acute Care

Committee. The acute management of COVID-19 in paediatrics. Posted on April 20, 2020. [cited 4 March 2021]. In: Canadian Paediatric Society [Internet]. Available from: https://www.cps.ca/en/documents/position/the-acute-management-of-paediatric-coronavirus-disease-2019covid-19

31 **Tume LN**, Valla FV, Joosten K, Jotterand Chaparro C, Latten L, Marino LV, Macleod I, Moullet C, Pathan N, Rooze S, van Rosmalen J, Verbruggen SCAT. Nutritional support for children during critical illness: European Society of Pediatric and Neonatal Intensive Care (ESPNIC) metabolism, endocrine and nutrition section position statement and clinical recommendations. *Intensive Care Med* 2020; **46**: 411-425 [PMID: 32077997 DOI: 10.1007/s00134-019-05922-5]

32 **Marino LV**, Valla FV, Tume LN, Jotterand-Chaparro C, Moullet C, Latten L, Joosten K, Verbruggen SCAT. Considerations for nutrition support in critically ill children with COVID-19 and paediatric inflammatory multisystem syndrome temporally associated with COVID-19. *Clin Nutr* 2021; **40**: 895-900 [PMID: 33097307 DOI: 10.1016/j.clnu.2020.10.007]

33 **Din AU**, Mazhar M, Waseem M, Ahmad W, Bibi A, Hassan A, Ali N, Gang W, Qian G, Ullah R, Shah T, Ullah M, Khan I, Nisar MF, Wu J. SARS-CoV-2 microbiome dysbiosis linked disorders and possible probiotics role. *Biomed Pharmacother* 2021; **133**: 110947 [PMID: 33197765 DOI: 10.1016/j.biopha.2020.110947]

34 **Murray KF**, Gold BD, Shamir R, Agostoni C, Pierre-Alvarez R, Kolacek S, Hsu EK, Chen J. Coronavirus Disease 2019 and the Pediatric Gastroenterologist. *J Pediatr Gastroenterol Nutr* 2020; **70**: 720-726 [PMID: 32443019 DOI: 10.1097/MPG.0000000000002730]

35 **Turner D**, Huang Y, Martín-de-Carpi J, Aloi M, Focht G, Kang B, Zhou Y, Sanchez C, Kappelman MD, Uhlig HH, Pujol-Muncunill G, Ledder O, Lionetti P, Dias JA, Ruemmele FM, Russell RK; Paediatric IBD Porto group of ESPGHAN. Corona Virus Disease 2019 and Paediatric Inflammatory Bowel Diseases: Global Experience and Provisional Guidance (March 2020) from the Paediatric IBD Porto Group of European Society of Paediatric Gastroenterology, Hepatology, and Nutrition. *J Pediatr Gastroenterol Nutr* 2020; **70**: 727-733 [PMID: 32443020 DOI: 10.1097/MPG.0000000000002729]

36 **Schuppan D**. Current concepts of celiac disease pathogenesis. *Gastroenterology* 2000; **119**: 234-242 [PMID: 10889174 DOI: 10.1053/gast.2000.8521]

37 **Gujral N**, Freeman HJ, Thomson AB. Celiac disease: prevalence, diagnosis, pathogenesis and treatment. *World J Gastroenterol* 2012; **18**: 6036-6059 [PMID: 23155333 DOI: 10.3748/wjg.v18.i42.6036]

38 Consensus Development Conference on Celiac Disease. *NIH Consens State Sci Statements* 2004; **21**: 1-23 [PMID: 17308551]

39 **Centers for Disease Control and Prevention**. People with certain medical conditions. [cited 29 December 2020]. In: Centers for Disease Control and Prevention [Internet]. Available from: https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/groups-at-higher-risk.html

40 **Thomas HJ**, Wotton CJ, Yeates D, Ahmad T, Jewell DP, Goldacre MJ. Pneumococcal infection in patients with coeliac disease. *Eur J Gastroenterol Hepatol* 2008; **20**: 624-628 [PMID: 18679063 DOI: 10.1097/MEG.0b013e3282f45764]

41 **Ludvigsson JF**, Olén O, Bell M, Ekbom A, Montgomery SM. Coeliac disease and risk of sepsis. *Gut* 2008; **57**: 1074-1080 [PMID: 18270242 DOI: 10.1136/gut.2007.133868]

42 **Mårild K**, Fredlund H, Ludvigsson JF. Increased risk of hospital admission for influenza in patients with celiac disease: a nationwide cohort study in Sweden. *Am J Gastroenterol* 2010; **105**: 2465-2473 [PMID: 20823839 DOI: 10.1038/ajg.2010.352]

43 **Gokden Y**, Hot S, Adas M, Ogutmen Koc D, Atak S, Hot AB. Celiac disease and COVID-19 pandemic: should we worry? *Acta Gastroenterol Belg* 2020; **83**: 517-525 [PMID: 33321006]

44 **Zingone F**, D'Odorico A, Lorenzon G, Marsilio I, Farinati F, Savarino EV. Risk of COVID-19 in celiac disease patients. *Autoimmun Rev* 2020; **19**: 102639 [PMID: 32801048 DOI: 10.1016/j.autrev.2020.102639]

45 **Emmi G**, Bettiol A, Mattioli I, Silvestri E, Di Scala G, Urban ML, Vaglio A, Prisco D. SARS-CoV-2 infection among patients with systemic autoimmune diseases. *Autoimmun Rev* 2020; **19**: 102575 [PMID: 32376395 DOI: 10.1016/j.autrev.2020.102575]

46 **Zhen J**, Stefanolo JP, Temprano MP, Tedesco S, Seiler C, Caminero AF, de-Madaria E, Huguet MM, Vivas S, Niveloni SI, Bercik P, Smecuol E, Uscanga L, Trucco E, Lopez V, Olano C, Mansueto P, Carroccio A, Green PHR, Day A, Tye-Din J, Bai JC, Ciacci C, Verdu EF, Lebwohl B, Pinto-Sanchez MI. The Risk of Contracting COVID-19 Is Not Increased in Patients With Celiac Disease. *Clin Gastroenterol Hepatol* 2021; **19**: 391-393 [PMID: 33059041 DOI: 10.1016/j.cgh.2020.10.009]

47 **Schiepatti A**, Alimenti E, Maimaris S, Nicolardi ML, Manzella La Barbera F, Baiardi P, Biagi F. Prevalence, incidence and clinical features of SARS-CoV-2 infection in adult coeliac patients. *Eur J Gastroenterol Hepatol* 2021; Publish Ahead of Print [PMID: 33399400 DOI: 10.1097/MEG.0000000000001969]

48 **Lionetti E**, Fabbrizi A, Catassi C. Prevalence of COVID-19 in Italian Children With Celiac Disease: A Cross-Sectional Study. *Clin Gastroenterol Hepatol* 2021; **19**: 1075 [PMID: 33279773 DOI: 10.1016/j.cgh.2020.11.035]

49 **Gralnek IM**, Hassan C, Beilenhoff U, Antonelli G, Ebigbo A, Pellisè M, Arvanitakis M, Bhandari P, Bisschops R, Van Hooft JE, Kaminski MF, Triantafyllou K, Webster G, Pohl H, Dunkley I, Fehrke B, Gazic M, Gjergek T, Maasen S, Waagenes W, de Pater M, Ponchon T, Siersema PD, Messmann H, Dinis-Ribeiro M. ESGE and ESGENA Position Statement on gastrointestinal endoscopy and the

COVID-19 pandemic. *Endoscopy* 2020; **52**: 483-490 [PMID: 32303090 DOI: 10.1055/a-1155-6229]
50 **Ruan W**, Fishman DS, Lerner DG, Engevik MA, Elmunzer BJ, Walsh CM; International Pediatric Endoscopy COVID-19 Alliance. Changes in Pediatric Endoscopic Practice During the Coronavirus Disease 2019 Pandemic: Results From an International Survey. *Gastroenterology* 2020; **159**: 1547-1550 [PMID: 32485178 DOI: 10.1053/j.gastro.2020.05.068]
51 **Trovato CM**, Montuori M, Cucchiara S, Oliva S. ESPGHAN 'biopsy-sparing' guidelines for celiac disease in children with low antitransglutaminase during COVID-19. *Eur J Gastroenterol Hepatol* 2020; **32**: 1523-1526 [PMID: 32956181 DOI: 10.1097/MEG.0000000000001924]
52 **Monzani A**, Lionetti E, Felici E, Fransos L, Azzolina D, Rabbone I, Catassi C. Adherence to the Gluten-Free Diet during the Lockdown for COVID-19 Pandemic: A Web-Based Survey of Italian Subjects with Celiac Disease. *Nutrients* 2020; **12** [PMID: 33198212 DOI: 10.3390/nu12113467]
53 **Nastro FF**, Tolone C, Serra MR, Pacella D, Campanozzi A, Strisciuglio C. Prevalence of functional gastrointestinal disorders in children with celiac disease during the COVID-19 Lockdown. *Dig Liver Dis* 2020; **52**: 1082-1084 [PMID: 32680759 DOI: 10.1016/j.dld.2020.06.030]
54 **Moon JS**. Clinical Aspects and Treatments for Pediatric Inflammatory Bowel Diseases. *Pediatr Gastroenterol Hepatol Nutr* 2019; **22**: 50-56 [PMID: 30671373 DOI: 10.5223/pghn.2019.22.1.50]
55 **Norsa L**, Indriolo A, Sansotta N, Cosimo P, Greco S, D'Antiga L. Uneventful Course in Patients With Inflammatory Bowel Disease During the Severe Acute Respiratory Syndrome Coronavirus 2 Outbreak in Northern Italy. *Gastroenterology* 2020; **159**: 371-372 [PMID: 32247695 DOI: 10.1053/j.gastro.2020.03.062]
56 **Brenner EJ**, Ungaro RC, Colombel JF, Kappelman MD. SECURE-IBD Database Public Data Update. [cited 7 Jan 2021]. In: SECURE-IBD Database [Internet]. Available from: https://covidibd.org/
57 **Brenner EJ**, Pigneur B, Focht G, Zhang X, Ungaro RC, Colombel JF, Turner D, Kappelman MD, Ruemmele FM. Benign Evolution of SARS-Cov2 Infections in Children With Inflammatory Bowel Disease: Results From Two International Databases. *Clin Gastroenterol Hepatol* 2021; **19**: 394-396.e5 [PMID: 33059040 DOI: 10.1016/j.cgh.2020.10.010]
58 **Batlle D**, Wysocki J, Satchell K. Soluble angiotensin-converting enzyme 2: a potential approach for coronavirus infection therapy? *Clin Sci (Lond)* 2020; **134**: 543-545 [PMID: 32167153 DOI: 10.1042/CS20200163]
59 **Singh UP**, Singh NP, Murphy EA, Price RL, Fayad R, Nagarkatti M, Nagarkatti PS. Chemokine and cytokine levels in inflammatory bowel disease patients. *Cytokine* 2016; **77**: 44-49 [PMID: 26520877 DOI: 10.1016/j.cyto.2015.10.008]
60 **Chen G**, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang M, Wu S, Song J, Chen T, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest* 2020; **130**: 2620-2629 [PMID: 32217835 DOI: 10.1172/JCI137244]
61 **Dolinger MT**, Person H, Smith R, Jarchin L, Pittman N, Dubinsky MC, Lai J. Pediatric Crohn Disease and Multisystem Inflammatory Syndrome in Children (MIS-C) and COVID-19 Treated With Infliximab. *J Pediatr Gastroenterol Nutr* 2020; **71**: 153-155 [PMID: 32452979 DOI: 10.1097/MPG.0000000000002809]
62 **Ashton JJ**, Kammermeier J, Spray C, Russell RK, Hansen R, Howarth LJ, Torrente F, Deb P, Renji E, Muhammed R, Paul T, Kiparissi F, Epstein J, Lawson M, Hope B, Zamvar V, Narula P, Kadir A, Devadason D, Bhavsar H, Beattie RM. Impact of COVID-19 on diagnosis and management of paediatric inflammatory bowel disease during lockdown: a UK nationwide study. *Arch Dis Child* 2020; **105**: 1186-1191 [PMID: 32732316 DOI: 10.1136/archdischild-2020-319751]
63 **Arrigo S**, Alvisi P, Banzato C, Bramuzzo M, Celano R, Civitelli F, D'Arcangelo G, Dilillo A, Dipasquale V, Felici E, Fuoti M, Gatti S, Knafelz D, Lionetti P, Mario F, Marseglia A, Martelossi S, Moretti C, Norsa L, Panceri R, Renzo S, Romano C, Romeo E, Strisciuglio C, Martinelli M. Impact of COVID-19 pandemic on the management of paediatric inflammatory bowel disease: An Italian multicentre study on behalf of the SIGENP IBD Group. *Dig Liver Dis* 2021; **53**: 283-288 [PMID: 33388247 DOI: 10.1016/j.dld.2020.12.011]
64 **Arrigo S**, Alvisi P, Banzato C, Bramuzzo M, Civitelli F, Corsello A, D'Arcangelo G, Dilillo A, Dipasquale V, Felici E, Fuoti M, Gatti S, Giusti Z, Knafelz D, Lionetti P, Mario F, Marseglia A, Martelossi S, Moretti C, Norsa L, Nuti F, Panceri R, Rampado S, Renzo S, Romano C, Romeo E, Strisciuglio C, Martinelli M. Management of paediatric IBD after the peak of COVID-19 pandemic in Italy: A position paper on behalf of the SIGENP IBD working group. *Dig Liver Dis* 2021; **53**: 183-189 [PMID: 33132063 DOI: 10.1016/j.dld.2020.10.024]
65 **Bangash MN**, Patel J, Parekh D. COVID-19 and the liver: little cause for concern. *Lancet Gastroenterol Hepatol* 2020; **5**: 529-530 [PMID: 32203680 DOI: 10.1016/S2468-1253(20)30084-4]
66 **Zhang L**, Zhu F, Xie L, Wang C, Wang J, Chen R, Jia P, Guan HQ, Peng L, Chen Y, Peng P, Zhang P, Chu Q, Shen Q, Wang Y, Xu SY, Zhao JP, Zhou M. Clinical characteristics of COVID-19-infected cancer patients: a retrospective case study in three hospitals within Wuhan, China. *Ann Oncol* 2020; **31**: 894-901 [PMID: 32224151 DOI: 10.1016/j.annonc.2020.03.296]
67 **D'Antiga L**. Coronaviruses and Immunosuppressed Patients: The Facts During the Third Epidemic. *Liver Transpl* 2020; **26**: 832-834 [PMID: 32196933 DOI: 10.1002/lt.25756]
68 **Doná D**, Torres Canizales J, Benetti E, Cananzi M, De Corti F, Calore E, Hierro L, Ramos Boluda E, Melgosa Hijosa M, Garcia Guereta L, Pérez Martínez A, Barrios M, Costa Reis P, Teixeira A, Lopes MF, Kaliciński P, Branchereau S, Boyer O, Debray D, Sciveres M, Wennberg L, Fischler B, Barany

P, Baker A, Baumann U, Schwerk N, Nicastro E, Candusso M, Toporski J, Sokal E, Stephenne X, Lindemans C, Miglinas M, Rascon J, Jara P; ERN TransplantChild. Pediatric transplantation in Europe during the COVID-19 pandemic: Early impact on activity and healthcare. *Clin Transplant* 2020; **34**: e14063 [PMID: 32786120 DOI: 10.1111/ctr.14063]

69 **Li X**, Geng M, Peng Y, Meng L, Lu S. Molecular immune pathogenesis and diagnosis of COVID-19. *J Pharm Anal* 2020; **10**: 102-108 [PMID: 32282863 DOI: 10.1016/j.jpha.2020.03.001]

70 **Aslam S**, Mehra MR. COVID-19: Yet another coronavirus challenge in transplantation. *J Heart Lung Transplant* 2020; **39**: 408-409 [PMID: 32253113 DOI: 10.1016/j.healun.2020.03.007]

71 **Nicastro E**, Di Giorgio A, Zambelli M, Ginammi M, Bravi M, Stroppa P, Casotti V, Palladino R, Colledan M, D'Antiga L. Impact of the Severe Acute Respiratory Syndrome Coronavirus 2 Outbreak on Pediatric Liver Transplant Recipients in Lombardy, Northern Italy. *Liver Transpl* 2020; **26**: 1359-1362 [PMID: 32614988 DOI: 10.1002/lt.25840]

72 **Snapiri O**, Rosenberg Danziger C, Krause I, Kravarusic D, Yulevich A, Balla U, Bilavsky E. Delayed diagnosis of paediatric appendicitis during the COVID-19 pandemic. *Acta Paediatr* 2020; **109**: 1672-1676 [PMID: 32460364 DOI: 10.1111/apa.15376]

73 **Raffaele A**, Cervone A, Ruffoli M, Cereda E, Avolio L, Parigi GB, Riccipetitoni G. Critical factors conditioning the management of appendicitis in children during COVID-19 Pandemic: experience from the outbreak area of Lombardy, Italy. *Br J Surg* 2020; **107**: e529-e530 [PMID: 32835410]

74 **Place R**, Lee J, Howell J. Rate of Pediatric Appendiceal Perforation at a Children's Hospital During the COVID-19 Pandemic Compared With the Previous Year. *JAMA Netw Open* 2020; **3**: e2027948 [PMID: 33275149 DOI: 10.1001/jamanetworkopen.2020.27948]

75 **Lee-Archer P**, Blackall S, Campbell H, Boyd D, Patel B, McBride C. Increased incidence of complicated appendicitis during the COVID-19 pandemic. *J Paediatr Child Health* 2020; **56**: 1313-1314 [PMID: 32830891 DOI: 10.1111/jpc.15058]

76 **Velayos M**, Muñoz-Serrano AJ, Estefanía-Fernández K, Sarmiento Caldas MC, Moratilla Lapeña L, López-Santamaría M, López-Gutiérrez JC. [Influence of the coronavirus 2 (SARS-Cov-2) pandemic on acute appendicitis]. *An Pediatr (Barc)* 2020; **93**: 118-122 [PMID: 32493604 DOI: 10.1016/j.anpedi.2020.04.022]

77 **Zampieri N**. Effect of SARS-CoV-2 on the Incidence of Appendicitis: The Role of Quarantine. *Pediatr Emerg Care* 2020; **36**: e482-e483 [PMID: 32740270 DOI: 10.1097/PEC.0000000000002182]

78 **Tankel J**, Keinan A, Blich O, Koussa M, Helou B, Shay S, Zugayar D, Pikarsky A, Mazeh H, Spira R, Reissman P. The Decreasing Incidence of Acute Appendicitis During COVID-19: A Retrospective Multi-centre Study. *World J Surg* 2020; **44**: 2458-2463 [PMID: 32458019 DOI: 10.1007/s00268-020-05599-8]

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MINIREVIEWS

Can control of gut microbiota be a future therapeutic option for inflammatory bowel disease?

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Abstract

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract encompassing two main clinical entities, Crohn's disease and ulcerative colitis. Accumulated evidence indicates that an aberrant immune activation caused by the interplay of genetic susceptibility and environmental impact on the gut microbiota may be involved in the pathogenesis of IBD. Rapid advances in next-generation sequencing technology have enabled a number of studies to identify the alteration of the gut microbiota, termed dysbiosis, in IBD. Moreover, the alteration in the metabolites derived from the gut microbiota in IBD has also been described in many studies. Therefore, microbiota-based interventions such as fecal microbiota transplantation (FMT) have attracted attention as a novel therapeutic option in IBD. However, in clinical trials, the efficacy of FMT for IBD remains controversial. Additional basic and clinical studies are required to validate whether FMT can assume a complementary role in the treatment of IBD. The present review provides a synopsis on dysbiosis in IBD and on the association between the gut microbiota and the pathogenesis of IBD. In addition, we summarize the use of probiotics in IBD and the results of current clinical trials of FMT for IBD.

Key Words: Inflammatory bowel disease; Dysbiosis; Fecal microbiota transplantation; Short chain fatty acid; Probiotics

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Core Tip: In this review, we discuss the gut microbiota in inflammatory bowel disease and gut microbiota-derived metabolites, especially short chain fatty acids. The anti-inflammatory function of short chain fatty acids on the mucosal immune system in the

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gastrointestinal tract is also discussed. In addition, we review the efficacy of probiotics on inflammatory bowel disease and the current clinical trials on the effectiveness of fecal microbiota transplantation on inflammatory bowel disease.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), which are known as inflammatory bowel diseases (IBD), are chronic and relapsing inflammatory disorders of the gastrointestinal tract[1,2]. The precise etiology and pathogenesis remain to be elucidated. Genome-wide association studies have identified over 200 IBD associated-susceptible genes, some of which are known to be involved or implicated in mediating host responses to the gut microbiota[3]. This has evoked the possibility that the gut microbiota is implicated in the pathogenesis of IBD[4-7].

The human digestive tract is inhabited by more than 100 trillion commensal bacteria, which exceeds the total number of human cells of 37 trillion[8,9]. The concentration of human intestinal bacteria has been estimated to be from 10^{11} to 10^{12} cells per gram of luminal contents[10,11]. The intestinal bacteria regulate the balance between each bacterium by interacting with each other, thereby maintaining the homeostasis of the intestinal environment. More than 99% of the bacteria that live in the human intestine belong to the four major phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, of which Firmicutes is the most predominant phylum followed by Bacteroidetes[10,12].

Recent studies have shown that the composition of human gut microbiota is closely linked to health and disease[13,14]. Moreover, the gut microbiota contributes to the differentiation and maturation of intestinal epithelial cells and immune cells, the supply of energy to the host through metabolic processes, and protection against infection by pathogens[10]. Humans, on the other hand, provide the gut microbiota with an anaerobic place to reside. In this way, a symbiotic relationship is established between the gut microbiota and human host[15].

Developments in gene sequencing technologies as well as the increased availability of powerful bioinformatic tools have enabled novel insights into the microbial composition of human gut microbiota and the effect of microbial communities on human physiology and disease[16,17]. Recent studies using these technologies have indicated that abnormal microbiota composition, known as "dysbiosis," and a decreased complexity of the gut microbial ecosystem are common features in patients with IBD. Moreover, it has been demonstrated that dysbiosis is involved in the pathophysiology of non-gastrointestinal diseases such as obesity and diabetes[17,18], in addition to gastrointestinal diseases such as IBD and irritable bowel syndrome [6,7,19].

Recently, it has been widely recognized that the gut microbiota plays a vital role in human immunity, metabolism, and diseases[4,6,20-22], leading to the idea of considering it as an organ. Based on this idea, the gut microbiota is sometimes called a "superorganism" or "forgotten organ"[23]. Considering these various functions of the gut microbiota on human biological functions, controlling its composition and diversity might allow us to treat or cure human diseases.

In this review, we will discuss the relationship between the gut microbiota and IBD, the efficacy of probiotics on IBD, and the current status of fecal microbiota transplantation (FMT) as a therapeutic option for IBD.

THE GUT MICROBIOTA IN IBD

Various alterations of the gut microbiota have been reported in patients with IBD. The most consistent findings of the gut microbiota in IBD patients are a reduction of

diversity and a reduction of Firmicutes compared to healthy individuals[6,7,16]. Some studies on IBD patients have reported an increase in the phyla Proteobacteria and Bacteroidetes, while others have reported a decrease in these phyla[5,7].

Faecalibacterium prausnitzii (*F. prausnitzii*), which belongs to *Clostridium* cluster IV, has been reported to have an anti-inflammatory effect by producing short-chain fatty acids (SCFAs: C2-C6), especially butyrate[24]. It has been reported that there is a reduced abundance of *F. prausnitzii* in patients with CD and that this deficiency is associated with postoperative recurrence of CD[25]. It has been demonstrated that *F. prausnitzii*, *Blautia faecis*, *Roseburia inulinivorans*, *Ruminococcus torques*, and *Clostridium lavalense* are decreased in patients with CD when compared to healthy subjects[25,26] and that the number of *F. prausnitzii* is correlated with the risk of relapse of ileal CD after surgery[27]. Deficient colonization of *F. prausnitzii* was observed in UC patients during remission, and the recovery of the *F. prausnitzii* population after relapse is associated with the maintenance of clinical remission[27]. Moreover, Sokol *et al*[28] showed that human peripheral blood mononuclear cells stimulated with *F. prausnitzii* induce the production of interleukin (IL)-10 and inhibit the production of inflammatory cytokines, such as IL-12 and interferon-γ. Furthermore, a significant decrease of *Roseburia spp*. was shown in the gut microbiota of healthy individuals with a high genetic risk for IBD[29].

Another consistent finding from a number of reports is the relative increase in the phylum Proteobacteria, especially *Escherichia coli (E. coli)*, in CD patients. The CD-associated[30] proinflammatory *E. coli* is known as adhesion-invasive *E. coli*, which is a bacterium isolated from CD patients. Adhesion-invasive *E. coli* has been reported to increase the permeability of the intestinal epithelium and induce intestinal inflammation by adhering directly to it[31].

Thus, in IBD enterobacteria, along with a decrease in diversity, a decrease in anti-inflammatory bacteria (Firmicutes phylum) and an increase in proinflammatory bacteria (Proteobacteria phylum) were observed, and these changes have also been observed in IBD. It is possible that these factors contribute to chronic inflammation of the intestinal tract. It is possible that it contributes to chronic inflammation of the intestinal tract.

ROLE OF SHORT-CHAIN FATTY ACIDS IN THE INTESTINAL TRACT

Although most nutrients are digested and absorbed in the duodenum and small intestine, dietary fiber remains intact until it reaches the colon. Dietary fibers are complex carbohydrates of plant origin broken down by specialized enzymes produced by gut bacteria but indigestible by the host[32]. They have recently been redefined as microbiota-accessible carbohydrates (MACs) and represent the major energy source for colonic bacteria[33]. MACs favor an increase in beneficial bacteria. The composition and function of the gut microbiota are dependent on the availability of MACs[34]. In preclinical studies, low dietary MACs have been shown to aggravate the development of inflammatory diseases, including autoimmune diseases, infections, and allergies [35-38] (Figure 1).

SCFAs, namely, acetate, propionate, and butyrate, are produced in the intestinal tract by the gut microbiota during fermentation of dietary fibers under anaerobic conditions[17]. Among these SCFAs, butyrate is the main energy source for the intestinal epithelial cells. Butyric acid positively modulates mitochondrial function, such as enhancing oxidative phosphorylation and β-oxidation, leading to an increase in oxygen consumption of colonic epithelial cells[32,33,39,40]. As a result, the concentration of oxygen in the intestinal tract decreases, and the number of obligate anaerobic bacteria, including those Firmicutes phylum that produce butyrate, increases[41]. As mentioned above, there is a decrease in butyrate-producing bacteria, such as *F. prausnitzii*, *Clostridium* cluster IV and XIVa, and a decrease in the concentration of butyrate in the gut microbiota in IBD patients[26,42]. Therefore, the decrease of both butyrate-producing bacteria and concentration of butyrate may be involved in the development of IBD and the persistence of chronic intestinal inflammation.

It has been reported that the genus *Clostridium* is important for the induction of regulatory T cells with an immunosuppressive function by producing butyrate from MACs, and in turn, the butyrate suppresses inflammatory cytokines *via* mucin and antimicrobial peptides from intestinal epithelial cells[22,43-45]. Moreover, it has been reported that the genus *Clostridium* promotes the differentiation and proliferation of regulatory T cells by enhancing the production of transforming growth factor β from intestinal epithelial cells[43]. In addition to the direct function of butyrate on T cells,

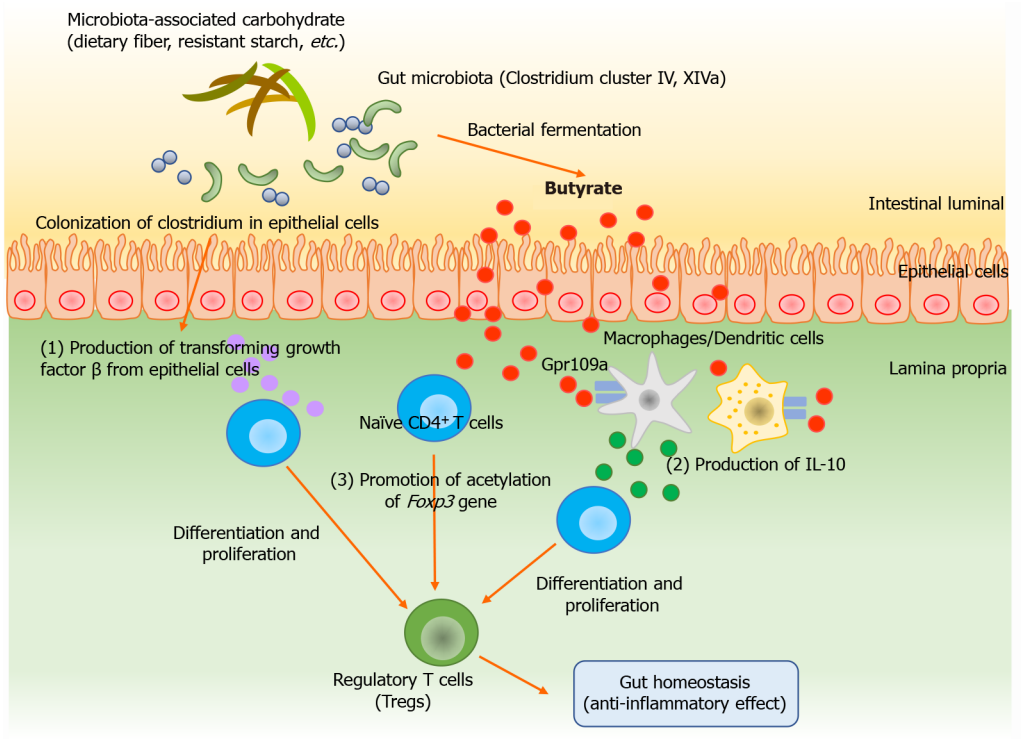


Figure 1 Function of butyrate in intestinal mucosa. Butyrate contributes to the maintenance of gut homeostasis by multiple mechanisms. Butyrate is mainly produced in the intestinal tract by bacteria of the Firmicutes phylum during fermentation of dietary fibers under anaerobic conditions. Butyrate is the main energy source for the intestinal epithelial cells. (1) The genus *Clostridium* promotes the differentiation and proliferation of regulatory T cells by enhancing the production of transforming growth factor β from intestinal epithelial cells; (2) Butyrate enhances the production of the anti-inflammatory cytokine, interleukin-10, produced by macrophages and dendritic cells through GPR109a, which is the G protein-coupled receptor for butyrate; and (3) Butyrate upregulates histone H3 acetylation at regulatory regions of the *Foxp3* gene and promotes the differentiation of naïve CD4$^+$ T cells into regulatory T cells. IL: Interleukin.

butyrate suppresses the induction of the inflammatory cytokine, IL-6, and enhances the production of the anti-inflammatory cytokine, IL-10, produced by macrophages and dendritic cells through GPR109a, which is the G protein-coupled receptor for butyrate[43,44]. Butyrate is also known to regulate gene expression epigenetically by inhibiting histone deacetylases. Recent studies have demonstrated that butyrate upregulates histone H3 acetylation at regulatory regions of the *Foxp3* gene and promotes the differentiation of naïve CD4$^+$ T cells into regulatory T cells[45].

Based on these findings, SCFAs play an important role in maintaining intestinal homeostasis through their anti-inflammatory properties. Thus, the decreased concentration of SCFAs in the feces of IBD patients may be involved in the pathophysiology of IBD through multiple points of action.

THE EFFECT OF PROBIOTICS ON IBD

Probiotics are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host"[46]. Over the past decade, there has been a great interest in the use of probiotics as a therapeutic option in IBD. However, highly reliable scientific evidence regarding the efficacy of probiotics in IBD has been lacking.

There is a large double-blind clinical trial to investigate the efficacy of *E. coli* Nissle 1917 on maintaining remission in comparison to mesalamine (1500 mg/d) in UC patients in clinical remission (n = 120). The clinical trial showed the similar relapse rate between *E. coli* Nissle 1917 and mesalamine (*E. coli* Nissle 1917 group: 14%, mesalamine group: 16%)[47]. This group conducted a second large trial to examine the efficacy of *E. coli* Nissle 1917 on maintaining UC remission compared to mesalamine (1500 mg/d). This study revealed a comparable clinical relapse rate (*E. coli* Nissle 1917 group: 36%, mesalamine group: 34%)[48].

There is one randomized clinical trial that examined whether the addition of *E. coli* Nissle 1917 to standard therapy increased the rate of remission of patients with active UC. While undergoing the induction therapy, subjects were randomized to *E. coli* Nissle 1917 group and mesalamine group (2400 mg/d). After remission, patients ware

maintained on either mesalamine or *E. coli* Nissle 1917. The remission rates were similar in the mesalamine group (75%) and *E. coli* Nissle 1917 group (68%). Moreover, the relapse rates were also similar in mesalamine group (73%) and *E. coli* Nissle 1917 group (67%). Notably, it is the only probiotic mentioned in the European Crohn's and Colitis Organization guidelines as an effective alternative to mesalamine in maintenance of remission in UC patients[49]. Collectively, the efficacy of *E. coli* Nissle 1917 on maintenance of remission was comparable to mesalamine.

To date, the evidence of the use of VSL#3 in UC patients has been accumulated. VAL#3 is a combination of four strains of *Lactobacillus* (*Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbruekii* subsp. *bulgaricus*), three strains of *Bifidobacteria* (*Bifidobacteria longim*, *Bifidobacteria breve*, and *Bifidobacteria infants*) and *Streptococcus salivarius* subsp. *Thermophilus*. Sood *et al*[50] conducted a randomized, double-blind clinical trial to investigate the efficacy of VSL#3 on mild-to-moderately active UC compared to placebo. By week 12, more patients given VSL#3 achieved remission (43%) as compared with those given placebo (15.7%; $P < 0.001$). Furthermore, by week 12, more patients in the VSL#3 group achieved mucosal healing (32%) as compared to the placebo group (15%; $P < 0.03$). Another study with similar design conducted by Tursi *et al*[51] also showed that the remission rate in VSL#3 group was higher than in the placebo group (44% *vs* 32%; $P = 0.13$). Collectively, these results suggest that the use of VSL#3 has a benefit in inducing remission in active UC.

There has been a lack of large clinical trials assessing the efficacy of probiotics in both inducing and maintaining the remission for patients with CD. A Cochrane review published in 2020 assessing the efficacy of probiotics to induce remission in CD patients found two studies that met criteria for inclusion[52]. One study had 11 subjects with mild-to-moderately active CD that randomized assignment to *Lactobacillus rhamnosus* strain GG or placebo. The other study had 35 subjects with active CD, whose CD activity index score of 150 to 450 randomized to receive a symbiotic treatment (freeze-dried *Bifidobacterium longum* and a commercial product) or placebo. The review concluded that there was no evidence to support the use of probiotics for the induction of remission in CD.

A Cochran systematic review published in 2006 assessing the efficacy of probiotics to maintain the remission in CD found seven small controlled studies worthy of inclusion in their review[53]. The review concluded that there was no evidence to suggest that probiotics are beneficial for the maintenance of remission in CD. In summary, large clinical trials in the efficacy of probiotics on active and quiescent CD should be conducted to change these conclusions in the future.

THE EFFECT OF FMT ON IBD

FMT aims to restore the intestinal microbiota in diseased individuals by transplanting intestinal microbiota from healthy donors[10]. FMT has been reported to be highly effective against recurrent *Clostridium difficile* infection[54]. The success of FMT in treating *Clostridium difficile* infection has attracted attention as a new therapeutic option for IBD. Several clinical studies have been conducted to examine the effect of FMT on IBD, but the results have been inconsistent so it cannot be stated with confidence whether or not the treatment is effective. Furthermore, in these studies, the protocols including donor selection, method of stool administration, and method of stool preparation are not consistent. Collectively, at present FMT has not yet been used clinically as a therapeutic option.

To date, the findings of four randomized controlled trials (RCTs) of FMT for UC patients have been published: two in Gastroenterology in 2015[55,56], one in The Lancet in 2017[57], and the other in the Journal of the American Medical Association in 2019[58] (Table 1). In a report from Canada in 2015, 50 mL of donor stool was administered six times by enema in an FMT group, and 50 mL of water was administered in the same manner in a placebo group. The remission rate in the FMT group was significantly higher than the placebo group [9/38 (24%) *vs* 2/37 (5%); $P = 0.03$][55]. On the other hand, according to a report from the Netherlands in 2015, donor stool was administered to patients at day 0 and 3 wk later using a nasoduodenal tube in an FMT group, and autologous stool was administered to patients in the same manner in a control group. In this study, there was no significant difference in the effect of FMT between the two groups[56]. More recently, two RCTs in patients with mild-to-moderately active UC were reported in 2017 and in 2019. Paramsothy *et al*[57] reported in 2017 that 81 UC patients were randomly assigned FMT or placebo and that the primary outcome was defined as steroid-free clinical remission with endoscopic

Table 1 Randomized controlled studies of fecal microbiota transplantation in ulcerative colitis

Ref.	Moayyedi *et al*[55]	Rossen *et al*[56]	Paramsothy *et al*[57]	Costello *et al*[58]
Date of publication	2015	2015	2017	2019
Reference number	55	56	57	58
Number of patients	38	23	41	38
Number of controls	37	25	40	35
Severity of UC	Mayo 4-12 (mild to severe)	SCCAI 4-11 (mild to moderate)	Mayo 4-10 (mild to moderate)	Mayo 3-10 (mild to moderate)
Donor and Donor stool	6 volunteers	15 donors	Multi-donors	Multi-donors
	fresh or frozen	fresh	(3-7 donors), frozen	(3-4 donors), frozen
Mode of FMT	Retention enema	Nasoduodenal tube	Colonoscopy and enema	Colonoscopy and enema
Number of FMT	6	2	41	3
	1/wk × 6 wk	0 and 3 wk	First infusion by colonoscopy + 5/wk for 8 wk by enema	3/wk (colonoscopy followed by 2 enemas)
Follow-up	6 wk	12 wk	8 wk	8 wk
Pretreatment with antibiotics	No	No	No	No
Primary endpoint	Remission (Mayo ≤ 2 with an endoscopic score of 0)	Remission (SCCAI ≤ 2) combined with ≥ 1-point decrease in Mayo endoscopic score	Steroid-free clinical remission with endoscopic remission or response (Mayo ≤ 2, all subscores ≤ 1, and ≥ 1-point reduction in endoscopy subscore)	Steroid-free remission with endoscopic remission (Mayo ≤ 2 with endoscopic subscore ≤ 1)
Subjects who achieved the primary endpoint	9/38 (24%) treated with FMT *vs* 2/37 (5%) control (P = 0.03)	7/23 (30.4%) treated with FMT *vs* 5/25 (20.0%) control (P = 0.51)	11/41 (27%) treated with FMT *vs* 3/40 (8%) control (P = 0.021)	12/38 (32%) treated with FMT *vs* 3/35 (9%) control (P = 0.03)

SCCAI: Simple Clinical Colitis Activity Index; FMT: Fecal microbiota transplantation; UC: Ulcerative colitis.

remission or response at week 8. The rate of primary outcome of the FMT group was significantly higher than the placebo group [11/41 (27%) *vs* 3/40 (8%); P = 0.02]. Costello *et al*[58] reported that 73 patients with active UC were enrolled to an FMT group or autologous FMT group (placebo group). The steroid-free remission rate of the FMT group was significantly higher than that of the placebo group [12/38 (32%) *vs* 3/35 (9%); P = 0.03].

Since these four RCTs differ in donor selection, method of fecal administration, and the number of fecal administrations, it is difficult to make a direct comparison. It is presumed that increasing the number of FMT will be effective for UC. Based on the clinical data, FMT for UC may remain in clinical trials but not be adopted in practice. More high-quality RCTs are needed to optimize the protocol for FMT.

Sood *et al*[59] reported the effect of FMT in maintenance of remission in UC patients who had achieved clinical remission by FMT. In this pilot study, 61 patients with UC in clinical remission achieved after multisession FMT were randomized to an FMT group (n = 31) or placebo group (n = 30). There was no significant difference in the rate of steroid-free clinical remission between the FMT group and placebo group [27/31 (87.1%) *vs* 20/30 (66.7%); P = 0.111]. Secondary endpoints of endoscopic remission [FMT group: 18/31 (58.1%) *vs* placebo group: 8/30 (26.7%); P = 0.026] and histological remission [FMT group: 14/31 (45.2%) *vs* placebo group: 5/30 (16.7%); P = 0. 033] were achieved in a significantly higher number of patients with FMT. This pilot study suggested that maintenance FMT therapy may be one of the therapeutic options for UC patients in clinical remission.

To date, one pilot randomized controlled study has reported the effects of a single FMT administered *via* colonoscopy in patients with colonic or ileo-colonic CD who achieved clinical remission with systemic corticosteroids[60]. In this pilot study, 8 patients received FMT, and 9 patients received sham transplantation. The primary endpoint was the implantation of the donor microbiota at week 6. None of patients reached the primary endpoint. There was no significant difference in the steroid-free remission rate at week 10 between the FMT group and the sham group Stood [7/8 (87.5%) *vs* 4/9 (44.4%); P = 0.13]. The CD Endoscopic Index of Severity decreased significantly 6 wk after FMT [8.5 (4.6; 13.0) *vs* 3.5 (1.0; 8.9); P = 0.03] but not after sham

[2.4 (0.0; 8.3) *vs* 2.7 (0.7; 10.0); $P = 0.8$]. Moreover, 6 wk after FMT, C-reactive protein levels remained stable [3.0 (3.0; 3.0) *vs* 3.0 (3.0; 14.2) mg/L; $P = 0.05$], while they had already started to increase in the sham group [3.0 (3.0; 4.2) *vs* 6.9 (4.0; 8.7) mg/L; $P = 0.008$). This pilot study indicated that the benefit of FMT over sham transplantation was observed for several clinically relevant endpoints, including CD Endoscopic Index of Severity and C-reactive protein level. Several reports have been published on the effects of FMT for CD on a small number of cases, but the results have been inconsistent. A recent systematic review and meta-analysis of 11 studies (four case reports and seven prospective uncontrolled cohort studies) on FMT for CD that included 83 CD patients showed an overall clinical remission rate of 50.5% (42/83) [61]. However, the result of one large study had a great influence on the remission rate. The authors indicated that their overall findings should therefore be treated with caution because of the potential bias from this one study[30].

CONCLUSION

A convergence of technology, data from clinical studies, and experimental insights have revealed that the gut microbiota and its metabolites markedly contribute to the pathogenesis of IBD. However, it remains unclear whether the dysbiosis of the gut microbiota observed in IBD is a cause or a consequence of chronic intestinal inflammation. To answer this question, more basic approaches to reveal the precise effects of the gut microbiota on intestinal functions, including immune system and barrier system, will be essential. Moreover, intestinal microorganisms other than bacteria, such as viruses, archaea, and fungi should be investigated. The results from these basic studies may help to clarify the situation.

Although there is great promise for novel probiotics, we need to be circumspect when there was a lack of proof in efficacy of probiotics on IBD. There are promising results for *E. coli* Nissle 1917 in maintenance of quiescent UC and VSL#3 in active UC. There is no evidence available to support the use of probiotics in CD. The same results were found in a recently published systematic review[62]. In the future, large clinical trials should be essential to determine the effect of probiotics on IBD.

FMT has attracted attention as a new therapeutic option for IBD. However, despite its significant effect in the treatment of *Clostridium difficile* infection, its therapeutic efficacy in IBD still remains limited. There are many factors that should be taken into consideration to increase the success rate of FMT in IBD, including disease state, selection of stool donors, route of fecal administration, number of infusions, and use of antibiotic pretreatment. Therefore, further basic research as well as clinical studies are required to understand the mechanisms of action of FMT and to improve FMT preparations, modes of administration, and donor selection.

Recently, the techniques of genetic engineering have been introduced into treatments with gut microbiota. Various genetic platforms to deliver therapeutic molecules have been developed and transformed into microorganisms[63]. For disease treatment, the microbial delivery of various therapeutic molecules has been tested[64]. Host proteins, bacterial therapeutic proteins, antigens, and synthetic metabolic pathways have been introduced into the host through heterogeneous production in microbes[64]. As part of another microbiome-based therapy, engineered microbes may open new avenues for the treatment for IBD.

In the future, the combination of gut microbiology, gastroenterology, and epidemiology with advances in the rapid analysis of the gut microbiota, metabolites, molecular signals, and genetic engineering promises the development of novel therapeutic strategies in IBD.

REFERENCES

1 **Kaser A**, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol* 2010; **28**: 573-621 [PMID: 20192811 DOI: 10.1146/annurev-immunol-030409-101225]

2 **Khor B**, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-317 [PMID: 21677747 DOI: 10.1038/nature10209]

3 **Liu JZ**, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, Ripke S, Lee JC, Jostins L, Shah T, Abedian S, Cheon JH, Cho J, Dayani NE, Franke L, Fuyuno Y, Hart A, Juyal RC, Juyal G, Kim WH, Morris AP, Poustchi H, Newman WG, Midha V, Orchard TR, Vahedi H, Sood A, Sung JY, Malekzadeh R, Westra HJ, Yamazaki K, Yang SK; International Multiple Sclerosis Genetics Consortium; International IBD Genetics Consortium, Barrett JC, Alizadeh BZ, Parkes M, Bk T, Daly

MJ, Kubo M, Anderson CA, Weersma RK. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet* 2015; **47**: 979-986 [PMID: 26192919 DOI: 10.1038/ng.3359]
4 **Thaiss CA**, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. *Nature* 2016; **535**: 65-74 [PMID: 27383981 DOI: 10.1038/nature18847]
5 **Matsuoka K**, Kanai T. The gut microbiota and inflammatory bowel disease. *Semin Immunopathol* 2015; **37**: 47-55 [PMID: 25420450 DOI: 10.1007/s00281-014-0454-4]
6 **Belkaid Y**, Hand TW. Role of the microbiota in immunity and inflammation. *Cell* 2014; **157**: 121-141 [PMID: 24679531 DOI: 10.1016/j.cell.2014.03.011]
7 **Kamada N**, Seo SU, Chen GY, Núñez G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol* 2013; **13**: 321-335 [PMID: 23618829 DOI: 10.1038/nri3430]
8 **Bianconi E**, Piovesan A, Facchin F, Beraudi A, Casadei R, Frabetti F, Vitale L, Pelleri MC, Tassani S, Piva F, Perez-Amodio S, Strippoli P, Canaider S. An estimation of the number of cells in the human body. *Ann Hum Biol* 2013; **40**: 463-471 [PMID: 23829164 DOI: 10.3109/03014460.2013.807878]
9 **Honda K**, Littman DR. The microbiome in infectious disease and inflammation. *Annu Rev Immunol* 2012; **30**: 759-795 [PMID: 22224764 DOI: 10.1146/annurev-immunol-020711-074937]
10 **Nishida A**, Inoue R, Inatomi O, Bamba S, Naito Y, Andoh A. Gut microbiota in the pathogenesis of inflammatory bowel disease. *Clin J Gastroenterol* 2018; **11**: 1-10 [PMID: 29285689 DOI: 10.1007/s12328-017-0813-5]
11 **Human Microbiome Project Consortium**. Structure, function and diversity of the healthy human microbiome. *Nature* 2012; **486**: 207-214 [PMID: 22699609 DOI: 10.1038/nature11234]
12 **Andoh A**. Physiological Role of Gut Microbiota for Maintaining Human Health. *Digestion* 2016; **93**: 176-181 [PMID: 26859303 DOI: 10.1159/000444066]
13 **Sommer F**, Anderson JM, Bharti R, Raes J, Rosenstiel P. The resilience of the intestinal microbiota influences health and disease. *Nat Rev Microbiol* 2017; **15**: 630-638 [PMID: 28626231 DOI: 10.1038/nrmicro.2017.58]
14 **Round JL**, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009; **9**: 313-323 [PMID: 19343057 DOI: 10.1038/nri2515]
15 **Mazmanian SK**, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 2008; **453**: 620-625 [PMID: 18509436 DOI: 10.1038/nature07008]
16 **Manichanh C**, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 599-608 [PMID: 22907164 DOI: 10.1038/nrgastro.2012.152]
17 **Flint HJ**, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 577-589 [PMID: 22945443 DOI: 10.1038/nrgastro.2012.156]
18 **Flint HJ**. Obesity and the gut microbiota. *J Clin Gastroenterol* 2011; **45** Suppl: S128-S132 [PMID: 21992951 DOI: 10.1097/MCG.0b013e31821f44c4]
19 **Brown EM**, Kenny DJ, Xavier RJ. Gut Microbiota Regulation of T Cells During Inflammation and Autoimmunity. *Annu Rev Immunol* 2019; **37**: 599-624 [PMID: 31026411 DOI: 10.1146/annurev-immunol-042718-041841]
20 **Lavelle A**, Sokol H. Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat Rev Gastroenterol Hepatol* 2020; **17**: 223-237 [PMID: 32076145 DOI: 10.1038/s41575-019-0258-z]
21 **Belkaid Y**, Harrison OJ. Homeostatic Immunity and the Microbiota. *Immunity* 2017; **46**: 562-576 [PMID: 28423337 DOI: 10.1016/j.immuni.2017.04.008]
22 **Honda K**, Littman DR. The microbiota in adaptive immune homeostasis and disease. *Nature* 2016; **535**: 75-84 [PMID: 27383982 DOI: 10.1038/nature18848]
23 **Dalal SR**, Chang EB. The microbial basis of inflammatory bowel diseases. *J Clin Invest* 2014; **124**: 4190-4196 [PMID: 25083986 DOI: 10.1172/JCI72330]
24 **Mentella MC**, Scaldaferri F, Pizzoferrato M, Gasbarrini A, Miggiano GAD. Nutrition, IBD and Gut Microbiota: A Review. *Nutrients* 2020; **12**: 944 [PMID: 32235316 DOI: 10.3390/nu12040944]
25 **Fujimoto T**, Imaeda H, Takahashi K, Kasumi E, Bamba S, Fujiyama Y, Andoh A. Decreased abundance of Faecalibacterium prausnitzii in the gut microbiota of Crohn's disease. *J Gastroenterol Hepatol* 2013; **28**: 613-619 [PMID: 23216550 DOI: 10.1111/jgh.12073]
26 **Takahashi K**, Nishida A, Fujimoto T, Fujii M, Shioya M, Imaeda H, Inatomi O, Bamba S, Sugimoto M, Andoh A. Reduced Abundance of Butyrate-Producing Bacteria Species in the Fecal Microbial Community in Crohn's Disease. *Digestion* 2016; **93**: 59-65 [PMID: 26789999 DOI: 10.1159/000441768]
27 **Varela E**, Manichanh C, Gallart M, Torrejón A, Borruel N, Casellas F, Guarner F, Antolin M. Colonisation by Faecalibacterium prausnitzii and maintenance of clinical remission in patients with ulcerative colitis. *Aliment Pharmacol Ther* 2013; **38**: 151-161 [PMID: 23725320 DOI: 10.1111/apt.12365]
28 **Sokol H**, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008; **105**: 16731-16736 [PMID: 18936492 DOI: 10.1073/pnas.0804812105]
29 **Imhann F**, Vich Vila A, Bonder MJ, Fu J, Gevers D, Visschedijk MC, Spekhorst LM, Alberts R,

Franke L, van Dullemen HM, Ter Steege RWF, Huttenhower C, Dijkstra G, Xavier RJ, Festen EAM, Wijmenga C, Zhernakova A, Weersma RK. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut* 2018; **67**: 108-119 [PMID: 27802154 DOI: 10.1136/gutjnl-2016-312135]

30 **Cui B**, Feng Q, Wang H, Wang M, Peng Z, Li P, Huang G, Liu Z, Wu P, Fan Z, Ji G, Wang X, Wu K, Fan D, Zhang F. Fecal microbiota transplantation through mid-gut for refractory Crohn's disease: safety, feasibility, and efficacy trial results. *J Gastroenterol Hepatol* 2015; **30**: 51-58 [PMID: 25168749 DOI: 10.1111/jgh.12727]

31 **Ahmed I**, Roy BC, Khan SA, Septer S, Umar S. Microbiome, Metabolome and Inflammatory Bowel Disease. *Microorganisms* 2016; **4**: 20 [PMID: 27681914 DOI: 10.3390/microorganisms4020020]

32 **El Kaoutari A**, Armougom F, Gordon JI, Raoult D, Henrissat B. The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nat Rev Microbiol* 2013; **11**: 497-504 [PMID: 23748339 DOI: 10.1038/nrmicro3050]

33 **Daïen CI**, Pinget GV, Tan JK, Macia L. Detrimental Impact of Microbiota-Accessible Carbohydrate-Deprived Diet on Gut and Immune Homeostasis: An Overview. *Front Immunol* 2017; **8**: 548 [PMID: 28553291 DOI: 10.3389/fimmu.2017.00548]

34 **Sonnenburg ED**, Sonnenburg JL. Starving our microbial self: the deleterious consequences of a diet deficient in microbiota-accessible carbohydrates. *Cell Metab* 2014; **20**: 779-786 [PMID: 25156449 DOI: 10.1016/j.cmet.2014.07.003]

35 **Sonnenburg ED**, Smits SA, Tikhonov M, Higginbottom SK, Wingreen NS, Sonnenburg JL. Diet-induced extinctions in the gut microbiota compound over generations. *Nature* 2016; **529**: 212-215 [PMID: 26762459 DOI: 10.1038/nature16504]

36 **Tan J**, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, Macia L, Mackay CR. Dietary Fiber and Bacterial SCFA Enhance Oral Tolerance and Protect against Food Allergy through Diverse Cellular Pathways. *Cell Rep* 2016; **15**: 2809-2824 [PMID: 27332875 DOI: 10.1016/j.celrep.2016.05.047]

37 **Thorburn AN**, McKenzie CI, Shen S, Stanley D, Macia L, Mason LJ, Roberts LK, Wong CH, Shim R, Robert R, Chevalier N, Tan JK, Mariño E, Moore RJ, Wong L, McConville MJ, Tull DL, Wood LG, Murphy VE, Mattes J, Gibson PG, Mackay CR. Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. *Nat Commun* 2015; **6**: 7320 [PMID: 26102221 DOI: 10.1038/ncomms8320]

38 **Macia L**, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C, Binge L, Thorburn AN, Chevalier N, Ang C, Marino E, Robert R, Offermanns S, Teixeira MM, Moore RJ, Flavell RA, Fagarasan S, Mackay CR. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat Commun* 2015; **6**: 6734 [PMID: 25828455 DOI: 10.1038/ncomms7734]

39 **Tingirikari JMR**. Microbiota-accessible pectic poly- and oligosaccharides in gut health. *Food Funct* 2018; **9**: 5059-5073 [PMID: 30280147 DOI: 10.1039/c8fo01296b]

40 **Sartor RB**. Gut microbiota: Diet promotes dysbiosis and colitis in susceptible hosts. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 561-562 [PMID: 22890110 DOI: 10.1038/nrgastro.2012.157]

41 **Kelly CJ**, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, Wilson KE, Glover LE, Kominsky DJ, Magnuson A, Weir TL, Ehrentraut SF, Pickel C, Kuhn KA, Lanis JM, Nguyen V, Taylor CT, Colgan SP. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. *Cell Host Microbe* 2015; **17**: 662-671 [PMID: 25865369 DOI: 10.1016/j.chom.2015.03.005]

42 **Wang W**, Chen L, Zhou R, Wang X, Song L, Huang S, Wang G, Xia B. Increased proportions of Bifidobacterium and the Lactobacillus group and loss of butyrate-producing bacteria in inflammatory bowel disease. *J Clin Microbiol* 2014; **52**: 398-406 [PMID: 24478468 DOI: 10.1128/JCM.01500-13]

43 **Atarashi K**, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, Honda K. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 2013; **500**: 232-236 [PMID: 23842501 DOI: 10.1038/nature12331]

44 **Atarashi K**, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov II, Umesaki Y, Itoh K, Honda K. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 2011; **331**: 337-341 [PMID: 21205640 DOI: 10.1126/science.1198469]

45 **Furusawa Y**, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 2013; **504**: 446-450 [PMID: 24226770 DOI: 10.1038/nature12721]

46 **Schrezenmeir J**, de Vrese M. Probiotics, prebiotics, and synbiotics--approaching a definition. *Am J Clin Nutr* 2001; **73**: 361S-364S [PMID: 11157342 DOI: 10.1093/ajcn/73.2.361s]

47 **Kruis W**, Schütz E, Fric P, Fixa B, Judmaier G, Stolte M. Double-blind comparison of an oral Escherichia coli preparation and mesalazine in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther* 1997; **11**: 853-858 [PMID: 9354192 DOI: 10.1046/j.1365-2036.1997.00225.x]

48 **Kruis W**, Fric P, Pokrotnieks J, Lukás M, Fixa B, Kascák M, Kamm MA, Weismueller J, Beglinger C, Stolte M, Wolff C, Schulze J. Maintaining remission of ulcerative colitis with the probiotic

Escherichia coli Nissle 1917 is as effective as with standard mesalazine. *Gut* 2004; **53**: 1617-1623 [PMID: 15479682 DOI: 10.1136/gut.2003.037747]

49 **Harbord M**, Eliakim R, Bettenworth D, Karmiris K, Katsanos K, Kopylov U, Kucharzik T, Molnár T, Raine T, Sebastian S, de Sousa HT, Dignass A, Carbonnel F, Crohn's ftE, Organisation C. Third European Evidence-based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 2: Current Management. *J Crohns Colitis* 2017; **11**: 769-784 [DOI: 10.1093/ecco-jcc/jjx009]

50 **Sood A**, Midha V, Makharia GK, Ahuja V, Singal D, Goswami P, Tandon RK. The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. *Clin Gastroenterol Hepatol* 2009; **7**: 1202-1209, 1209. e1 [PMID: 19631292 DOI: 10.1016/j.cgh.2009.07.016]

51 **Tursi A**, Brandimarte G, Papa A, Giglio A, Elisei W, Giorgetti GM, Forti G, Morini S, Hassan C, Pistoia MA, Modeo ME, Rodino' S, D'Amico T, Sebkova L, Sacca' N, Di Giulio E, Luzza F, Imeneo M, Larussa T, Di Rosa S, Annese V, Danese S, Gasbarrini A. Treatment of relapsing mild-to-moderate ulcerative colitis with the probiotic VSL#3 as adjunctive to a standard pharmaceutical treatment: a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol* 2010; **105**: 2218-2227 [PMID: 20517305 DOI: 10.1038/ajg.2010.218]

52 **Limketkai BN**, Akobeng AK, Gordon M, Adepoju AA. Probiotics for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* 2020; **7**: CD006634 [PMID: 32678465 DOI: 10.1002/14651858.CD006634.pub3]

53 **Rolfe VE**, Fortun PJ, Hawkey CJ, Bath-Hextall F. Probiotics for maintenance of remission in Crohn's disease. *Cochrane Database Syst Rev* 2006; **4**: CD004826 [PMID: 17054217 DOI: 10.1002/14651858.CD004826.pub2]

54 **van Nood E**, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, Visser CE, Kuijper EJ, Bartelsman JF, Tijssen JG, Speelman P, Dijkgraaf MG, Keller JJ. Duodenal infusion of donor feces for recurrent Clostridium difficile. *N Engl J Med* 2013; **368**: 407-415 [PMID: 23323867 DOI: 10.1056/NEJMoa1205037]

55 **Moayyedi P**, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, Armstrong D, Marshall JK, Kassam Z, Reinisch W, Lee CH. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. *Gastroenterology* 2015; **149**: 102-109. e6 [PMID: 25857665 DOI: 10.1053/j.gastro.2015.04.001]

56 **Rossen NG**, Fuentes S, van der Spek MJ, Tijssen JG, Hartman JH, Duflou A, Löwenberg M, van den Brink GR, Mathus-Vliegen EM, de Vos WM, Zoetendal EG, D'Haens GR, Ponsioen CY. Findings From a Randomized Controlled Trial of Fecal Transplantation for Patients With Ulcerative Colitis. *Gastroenterology* 2015; **149**: 110-118. e4 [PMID: 25836986 DOI: 10.1053/j.gastro.2015.03.045]

57 **Paramsothy S**, Kamm MA, Kaakoush NO, Walsh AJ, van den Bogaerde J, Samuel D, Leong RWL, Connor S, Ng W, Paramsothy R, Xuan W, Lin E, Mitchell HM, Borody TJ. Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet* 2017; **389**: 1218-1228 [PMID: 28214091 DOI: 10.1016/s0140-6736(17)30182-4]

58 **Costello SP**, Hughes PA, Waters O, Bryant RV, Vincent AD, Blatchford P, Katsikeros R, Makanyanga J, Campaniello MA, Mavrangelos C, Rosewarne CP, Bickley C, Peters C, Schoeman MN, Conlon MA, Roberts-Thomson IC, Andrews JM. Effect of Fecal Microbiota Transplantation on 8-Week Remission in Patients With Ulcerative Colitis: A Randomized Clinical Trial. *JAMA* 2019; **321**: 156-164 [PMID: 30644982 DOI: 10.1001/jama.2018.20046]

59 **Sood A**, Mahajan R, Singh A, Midha V, Mehta V, Narang V, Singh T, Singh Pannu A. Role of Faecal Microbiota Transplantation for Maintenance of Remission in Patients With Ulcerative Colitis: A Pilot Study. *J Crohns Colitis* 2019; **13**: 1311-1317 [PMID: 30873549 DOI: 10.1093/ecco-jcc/jjz060]

60 **Sokol H**, Landman C, Seksik P, Berard L, Montil M, Nion-Larmurier I, Bourrier A, Le Gall G, Lalande V, De Rougemont A, Kirchgesner J, Daguenel A, Cachanado M, Rousseau A, Drouet É, Rosenzwajg M, Hagege H, Dray X, Klatzman D, Marteau P; Saint-Antoine IBD Network, Beaugerie L, Simon T. Fecal microbiota transplantation to maintain remission in Crohn's disease: a pilot randomized controlled study. *Microbiome* 2020; **8**: 12 [PMID: 32014035 DOI: 10.1186/s40168-020-0792-5]

61 **Paramsothy S**, Paramsothy R, Rubin DT, Kamm MA, Kaakoush NO, Mitchell HM, Castaño-Rodríguez N. Faecal Microbiota Transplantation for Inflammatory Bowel Disease: A Systematic Review and Meta-analysis. *J Crohns Colitis* 2017; **11**: 1180-1199 [PMID: 28486648 DOI: 10.1093/ecco-jcc/jjx063]

62 **Zhang XF**, Guan XX, Tang YJ, Sun JF, Wang XK, Wang WD, Fan JM. Clinical effects and gut microbiota changes of using probiotics, prebiotics or synbiotics in inflammatory bowel disease: a systematic review and meta-analysis. *Eur J Nutr* 2021 [PMID: 33555375 DOI: 10.1007/s00394-021-02503-5]

63 **Riglar DT**, Silver PA. Engineering bacteria for diagnostic and therapeutic applications. *Nat Rev Microbiol* 2018; **16**: 214-225 [PMID: 29398705 DOI: 10.1038/nrmicro.2017.172]

64 **Kang M**, Choe D, Kim K, Cho BK, Cho S. Synthetic Biology Approaches in The Development of Engineered Therapeutic Microbes. *Int J Mol Sci* 2020; **21**: 8744 [PMID: 33228099 DOI: 10.3390/ijms21228744]

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ORIGINAL ARTICLE

Basic Study

Oncogenic tuftelin 1 as a potential molecular-targeted for inhibiting hepatocellular carcinoma growth

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Abstract

BACKGROUND

Abnormal tuftelin 1 (TUFT1) has been reported in multiple cancers and exhibits oncogenic roles in tumor progression. However, limited data are available on the relationship between TUFT1 and hepatocellular carcinoma (HCC), and the exact biological mechanism of TUFT1 is still poorly understood in HCC.

AIM

To investigate TUFT1 expression in HCC and how interfering *TUFT1* transcription affects HCC growth.

METHODS

TUFT1 in HCC and non-HCC tissues based on databases of the Cancer Genome Atlas and Oncomine were analyzed, and TUFT1 in human HCC tissues on microarray were detected by immunohistochemistry for clinicopathological features, overall survival, and disease-free survival. HCC cells were transfected with constructed vectors of *TUFT1* that interfere or over-express TUFT1 for analyzing the biological behaviors of HCC cells. Proliferation, invasion, migration, and apoptosis of cells were detected by cell counting kit-8, scratch assay, transwell

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tests, and flow cytometry and confirmed by Western blotting, respectively.

RESULTS

Abnormal TUFT1 levels in databases expressed in HCC at messenger RNA (mRNA) level and HCC tissues were mainly located in cytoplasm and membrane. The level of TUFT1 expression in the HCC group was significantly higher (χ^2 = 18.563, P < 0.001) than that in the non-cancerous group, closely related to clinical staging, size, vascular invasion of tumor, hepatitis B e-antigen positive, and ascites (P < 0.01) of HCC patients, and negatively to HCC patients' overall survival and disease-free survival (P < 0.001). After interfering with *TUFT1* transcription at mRNA level in the MHCC-97H cells by the specific TUFT1-short hairpin RNA, cell proliferation, invasion, and metastasis were significantly inhibited with increasing apoptosis rate. In contrast, proliferation, invasion, and migration were significantly enhanced after over-expression of TUFT1 mRNA in Hep3B cells *in vitro*.

CONCLUSION

Oncogenic TUFT1 was associated with the progression of HCC and could be a potential molecular-target for inhibiting HCC growth.

Key Words: Hepatocellular carcinoma; Tuftelin 1; Prognosis; Molecular-target; Growth

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Core Tip: Tuftelin1 (TUFT1) has been reported to be regulated by hypoxia and involved in the Hedgehog signaling pathway, with over-expression in hepatocellular carcinoma (HCC) tissues or cell lines. Abnormal TUFT1 level was significantly related to tumor size, vascular invasion, positive hepatitis B e-antigen, advanced tumor-node-metastasis stage of HCC, patients with ascites, and shorter overall survival and disease-free survival. Interfering *TUFT1* transcription could markedly suppress the growth and metastasis of high TUFT1 MHCC-97H cell lines *in vitro* through accelerating apoptosis. Moreover, increasing TUFT1 expression might promote the growth and metastasis of low TUFT1 Hep3B cell lines *in vitro*. The data suggested that TUFT1 is involved in HCC progression *via* the mechanism of inhibiting apoptosis and might serve as a potential therapeutic target for inhibiting HCC growth.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is usually lethal because of late diagnosis and early metastasis[1]. HCC is still one of the most common cancers with increasing incidence in the inshore area of the Yangtze River[2]. Aberrant up-regulated or down-regulated expression of many genes was identified during malignant transformation of hepatocytes in patients with chronic inflammation or damage by hepatitis B viruses (HBV), hepatitis C viruses (HCV), toxic intake, liver cirrhosis, and non-alcoholic fatty liver disease[3,4]. Given the limited effective therapies available for HCC patients with advanced stages, surgical resection and radiofrequency ablation are still the most common curative treatments for early stage patients, but the prognosis as overall survival (OS) or disease free survival (DFS) rate is very poor[5,6]. Therefore, the pathogenesis and feasible therapeutic treatments of HCC need urgent investigation[7-9]. Recently, tuftelin 1 (TUFT1) has been reported to be elevated during multiple cancer types, and the abnormal expression of TUFT1 has attracted medical attention [10,11]. TUFT1 was first identified and sequenced from a bovine ameloblast-enriched complementary DNA library with a highly conserved gene localized in chromosome

1q21-31 that contains 13 exons encoding an acidic, phosphorylated glycoprotein of 390 amino acids and plays a critical role in the development and mineralization of enamel [12].

TUFT1 belongs to the enamel associated teeth proteins and is thought to play a role in enamel mineralization. Also, TUFT1 is expressed in embryonic stem cells, neuronal cells, and non-mineralizing tissues like eyes, brain, adrenal gland, lung, liver, kidneys, testis, and tumor tissues[13-15]. TUFT1 can modulate the Rab GTPase-regulated process to activate the mammalian target of rapamycin complex 1 signaling that is activated in many cancers[16] and associated with poor prognosis in breast or pancreatic cancer and thyroid carcinoma[10,11,14]. In addition, TUFT1 is correlated with the metastasis and epithelial-mesenchymal transition[10], which could be regulated by hypoxia that enhanced TUFT1 by down-regulating miR-671-5p *via* interaction with 3'-ultranslated region of TUFT1 messenger RNA (mRNA) rather than by directly promoting hypoxia inducible factor-1α (HIF-1α) binding to TUFT1 promoter, and the Hedgehog signaling pathway[17-19]. Abnormal TUFT1 has been reported in multiple cancers and exhibits oncogenic roles in tumor progression[17,20]. However, limited data are available on the relationship between TUFT1 and HCC, and the exact biological mechanism of TUFT1 in HCC is still poorly understood. In this present study, the landscape of TUFT1 expression in human HCC tissues was investigated, and its molecular mechanism was confirmed through intervening or overexpressing TUFT1 activation *in vitro*.

MATERIALS AND METHODS

Bioinformatics analysis

Differential expressions of TUFT1 mRNA between HCC and normal liver tissues were collected according to the bioinformatics data from the Gene Expression Profiling Interactive Analysis (GEPIA) public Website[21] (http://GEPIA.cancer-pku.cn/detail.php) and the Oncomine database (https://www.Oncomine.org/resource).

Liver tissues and clinical data

This study was approved by the Ethics Committee permission (TDFY2018-025) at the Affiliated Hospital of Nantong University, China from Jan 2009 to Dec 2014, and the prior written informed consent was obtained from HCC patients according to the Helsinki Declaration of World Medical Association. A total 132 pairs of self-controlled HCC tissues (HCC group) and their para-cancerous tissues (2 cm to cancer, Para-C group) were collected from patients with HCC after post-operation, frozen in liquid nitrogen, and kept at -85 °C until used. According to their medical records, the patients were 111 males and 21 females with 21-79-years-old (average 60.04 ± 15.8 years). Serum alpha-fetoprotein (AFP) concentrations were 104 cases with ≥ 20 ng/mL and 28 cases with < 20 ng/mL. There were 120 cases with positive HBV surface antigen and 45 cases with positive hepatitis B e-antigen (HBeAg). There were 106 cases with tumor size ≥ 3.0 cm, 58 cases with vascular invasion, and 32 cases with ascites.

According to the 2019 edition of the diagnosis and treatment of primary liver cancer, there were three groups: Well (I), medium (II), and poor (III) differentiation. HCC patients were divided into I-IV stages, with 59 cases at I-II, and 73 cases at III-IV staging on the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. All patients underwent histopathology and did not receive radiation or chemotherapy prior to surgery. HCC patients had regular follow-up from operation to death until Jan 2020. Diagnosis of HCC was confirmed with the criteria set by the Chinese National Collaborative Cancer Research Group[22].

Tissue microarray

Tissue microarrays (TMA) were constructed by the Departments of Pathology, Affiliated Hospital of Nantong University, China, containing formalin-fixed and paraffin-embedded specimens from 132 HCC tissues and their non-cancerous tissues. Tissue cores (1.5 mm) from the representative areas were constructed into the TMA slices.

Immunohistochemistry

Immunohistochemistry staining was determined for assessing TUFT1 expression by GTVision™ III Detection System/Mo&Rb (GK600710) according to the manufacturer's protocol. TMA slices were dewaxed and rehydrated, underwent antigen retrieval, and were blocked endogenous peroxidase and antibody nonspecific binding with 3% H_2O_2

and 10% goat serum, respectively. Slices were incubated with the primary anti-human TUFT1 antibody (1: 250, Abcam, Cambridge, United Kingdom) overnight at 4 °C. The slices after incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (Everest Biotech, Oxford, United Kingdom) were stained with diamino-benzidine, and counterstained with hematoxylin, cleared in xylene, and covered. The levels of TUFT1 expression were calculated with the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, United States). Blinded evaluations of TUFT1 immunostaining and independent observation by two independent pathologists were carried out simultaneously, and the score was calculated by the sum of staining intensities and the rates of 100 positive cells. TUFT1 level in HCC tissues was divided into low and high expression. The expressing intensities of TUFT1 were divided into four categories: 0, negative; 1, weakly positive; 2, moderate positive; and 3, strongly positive.

Cell culture

All human HCC (Hep3B, MHCC-97L, MHCC-97H, BEL-7404, HepG2, HCCLM3, and SMMC-7721) cells and LO2 cell line were purchased from the Zhongqiao Xinzhou Science and Technology Co. (Shanghai, China). HCC cells were divided into three groups: Control, negative control (sh-NC or NC), and intervening (sh-TUFT1 or over-expressing) groups. HCC (MHCC-97L, MHCC-97H, BEL-7404, HepG2, and HCCLM3) cells in high glucose Dulbecco's modified Eagle's Medium with 10% fetal bovine serum (FBS, Gibco Laboratories, Gaithersburg, MD, United States), SMMC-7721 and LO2 cell lines in Roswell Park Memorial Institute-1640 medium with 10% FBS, and Hep3B cells in Minimal Essential Medium with 15% FBS were cultured containing 100 U/mL of penicillin/streptomycin at 37 °C with 5% of CO_2. Cell density to about 85% was used to next cell passage.

Plasmid coding short hairpin RNA against TUFT1

Specific short hairpin RNA (shRNA) targeting sites of *TUFT1* sequence (GenBank: AH009496.2)[23] were designed and synthesized (Guangzhou Cyagen Biotechnology Co., Ltd., China) and used to construct lentivirus LV-U6 > Scramble-shRNA-PGK > enhanced green fluorescent protein (EGFP)/T2A/Puro vector. Inserted interfering sequences in plasmids were TUFT1-shRNA1: 5'-AGAAGCTCCGG GAGGATA-TAACTCGAGTTATATCCTCCCGGAGCTCT-3', TUFT1-shRNA2: 5'-TGA GGTGGACACCTGTATAAACTCGAGTTTATACAGGTGTCCACCTCA-3', and TUFT1 -shRNA3: 5'-GATTCACGAGAAGAATATTAACTCGAGTTAATA-TTCTTCTCGTGAA TC-3', respectively. TUFT1-negative plasmid was constructed similarly with a shRNA sequence that does not suppress TUFT1 expression, and the Scramble vector (Cyagen, Santa Clara, CA, United States) as a control. All inserted sequences were confirmed by sequencing.

Expressing TUFT1 plasmid

The exon sequence of TUFT1 gene (GenBank: AH009496.2)[23] was inserted into the Scramble vector (GenePharma, Shanghai, China) to construct pEX-4 (pGCMV/MCS/ T2A/EGFP/ Neo) vector. Negative or control plasmids were equal to above vectors.

Cell transfection

Human MHCC-97H cell line in high glucose Dulbecco's modified Eagle's Medium with 10% of FBS or Hep3B cell line in MEM with 15% of FBS was cultured at 37 °C with 5% CO_2. When Hep3B cells up to optimal confluence over 70% were transfected at time, the cells of blank, negative control, and TUFT1 over-expressing groups were transfected with corresponding plasmids using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. MHCC-97H cells were also divided into blank, negative control, and sh-TUFT1 groups. They were transfected with corresponding plasmids using polybrene (Cyagen) according to the manufacturer's instructions.

Western blotting

Western blot analysis was performed as described previously[24]. Briefly, 50 μg of purified protein from transfected cells was separated on electrophoresis of 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, United States), blocked in 5% of nonfat dry milk in Tris-buffered saline (pH 7.5, 100 mmol/L NaCl, 50 mmol/L Tris, 0.1% Tween-20), and incubated with anti-human TUFT1 antibodies (Abcam) overnight at 4 °C, followed by HRP conjugated immunoglobulin G (Everest Biotech, Oxford, United

Kingdom). Alterations of proteins were analyzed by Quantity-one software (Bio-Rad, Laboratories, Hercules, CA, United States) with Immobilon ECL Chemiluminescence HRP Substrate (Millipore) and quantified using Gel-Pro Analyzer software.

CCK-8 assay

Cell counting kit (CCK-8) was employed to evaluate HCC cell proliferation. There were three groups in the experiment: Blank, negative control, sh-TUFT1 intervention or TUFT1 over-expressing groups. Cells from different treatment groups were counted, adjusted to concentration 4×10^4/mL, and seeded in a 96-well plate with 100 μL/per well. Cells were seeded in triplicate for each group. The 96-well plate was placed in the incubator (37 °C and 5% CO_2), and cells were cultured till the appropriate time. Then, 10 μL CCK-8 solution was added in each well, and the culture plate was incubated for 1-4 h. The absorbance (A) at 450 nm was detected using a plate reader.

Detection of apoptosis

Cell culture and experiment groups were set up as described above. Cells were cultured in a 6-well plate at about 70% confluence. Cells were aspirated and placed in a centrifuge tube, centrifuged at 1000 rpm for 5 min, rinsed with phosphate-buffered saline twice, and centrifuged again at 1000 rpm for 5 min. Cells (1×10^6) with 5 μL of Annexin V Alexa Fluor 647 staining solution were mixed in 100 μL binding buffer, collected, and placed in dark at room temperature for 10 min. Then, 400 μL binding buffer and 10 μL propidium iodide were mixed and placed in the dark at room temperature for 10 min. Fluorescent microscope observation or flow cytometry was conducted within 1 h. FL2 channel was used to detect the yellow fluorescence of propidium iodide with excitation wavelength of 488 nm. FL4 channel was used to detect the red fluorescence of Alexa Fluor 647 with emission wavelength of 640 nm. Cells without apoptotic induction were used as control for fluorescence compensation to remove spectral overlap and determine the position of cross-over.

Cell invasion

Transwell assays were performed to examine the invasion of cells transfected with small interfering RNA that targeted TUFT1 or a scrambled negative control. Cell invasion was defined using Transwell polycarbonate membrane inserts (Millipore). Cells (1×10^5) in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (TheWell Bioscience Inc., North Brunswick, NJ, United States). Medium containing 10% FBS was added to the lower chamber to stimulate invasion, and the cells remaining on the upper membrane were removed with cotton wool after cultivation for 48-72 h. Cells that had invaded through the membrane were stained with methanol and 0.1% crystal violet, imaged, and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan). Each assay was repeatedly conducted three times.

Scratch migration assay

To measure the logarithmic growth period, cells were seeded on plates for scratch analysis. When the cell growth density was about 90%, the scratch migration assay was performed to test cell migration. Using the ruler perpendicular to the ix-hole plate, with the 200 μL spear head along the ruler, a scratch was made, keeping strength as far as possible. A picture was taken, and the cells were allowed to continue to grow; a picture was taken every 24 h. The images were then compared, and the data analyzed.

Statistical analysis

Statistical analysis was performed by GraphPad Prism 8.0 software (GraphPad Co. Ltd., San Diego, CA, Unites States) and SPSS statistical package Version 25.0 (SPSS Inc., Armonk, NY, United States). The Student's *t* test, a rank-sum test, χ^2 test, and Fisher's exact probability test were used for categorical variables[25]. Cumulative survival curves of OS and DFS were calculated using the Kaplan–Meier method and compared by the log-rank test. The Cox proportional hazards model was used to determine independent factors of recurrence based on the variables selected by univariate analysis. Estimated 95% confidence intervals (CIs) with hazard ratio (HR) were combined to evaluate the prognostic and clinicopathologic values according to the Tierney method[26]. All the significant predictors of recurrence in the univariate analysis were analyzed in a logistic regression model to show an independent value at the multivariate analysis. A two-tailed value of $P < 0.05$ was considered statistically significant.

RESULTS

TUFT1 in bioinformatics database

The comparative analysis of TUFT1 mRNA box diagrams between HCC (n = 594) and normal liver (n = 270) tissues from the bioinformatics websites are shown in Figure 1. In the Cancer Genome Atlas database (Figure 1A) from the GEPIA public website, the levels of TUFT1 mRNA expression in the HCC group were significantly higher (P < 0.05) than those in the normal group. Based on the Oncomine database (Figure 1B), the levels of TUFT1 mRNA expression in the HCC group were also significantly higher (P < 0.05) than those in the normal group. The data from online databases indicated that hepatic TUFT1 should be over-expressing status in HCC progression.

TUFT1 expression in Human HCC tissues

The immunohistochemistry analysis of hepatic TUFT1 expression in 132 cases of HCC and their non- HCC tissue microarray with clinical staging and prognosis is shown in Figure 2. The cellular distribution of hepatic TUFT1 expression was mainly located in cytoplasm and cell membrane. The intensity of TUFT1 staining was stronger in the HCC group (Figure 2A and A1) than that in the non-HCC group (Figure 2B and B1). The positive rate of TUFT1 in the HCC group (87.1%, 115/132) was significantly higher (χ^2 = 18.563, P < 0.001) than that in the non-HCC group (64.4%, 85/132). Comparative analysis of TUFT1 expressions in the HCC or non-HCC tissues is summarized in Table 1. The significant difference (Z = 4.911, P < 0.001) of TUFT1 staining scores was found between HCC and non-HCC tissues by Z test, with higher expression in 50.0% HCC tissues (2-3 scores, 66/132), and low or no expression in 76.5% their non-HCC tissues (0-1 scores, 101/132). Regarding the relationship between TUFT1 and HCC staging (Figure 2C and D), there was lower/no TUFT1 staining in non-HCC tissues (Figure 2C1 and D1) with brown staining of TUFT1 gradually increasing from stage I to IV (Figure 2C2-5 or Figure 2D2-5).

Clinicopathologic features and prognostic value of TUFT1

The clinicopathological features of TUFT1 expression in 132 HCC patients are shown in Table 2. The higher TUFT1 level was associated with tumor size, vascular invasion, HBeAg, advanced TNM stage of HCC, and ascites of patients. However, no significant difference was found between TUFT1 and age, sex, AFP level, HBV surface antigen, and Edmondson-Steiner grading. Analysis of univariate or multivariate Cox regression of TUFT1 in HCC tissues (Table 3) was significantly correlated with HBeAg, tumor size, vascular invasion, ascites, TNM stage, and AFP level with OS of patients, and further confirmed that TUFT1, tumor size, vascular invasion, ascites, and TNM stage were independent predictive factors for OS. On the contrary, TUFT1 and TNM stage should be independent prognostic factors for DFS of HCC. The OS or DFS of HCC with high or low TUFT1 was analyzed at early or advanced HCC (Figure 3A or Figure 3B). Compared to low TUFT1 group, the cases with high TUFT1 expression had significantly decreased OS and DFS, suggesting that TUFT1 should be a promoter or prognostic marker for HCC.

Effects of interfering TUFT1 transcription on HCC cells

Screening TUFU1 and interfering TUFT1 mRNA transcription on effects of HCC cell lines are shown in Figure 4. Differential expressions of TUFT1 among different HCC cell lines were screened (Figure 4A). The relative ratio from TUFT1 to glyceraldehyde 3-phosphate dehydrogenase showed Hep3B cells with lowest and MHCC-97H cells with strongest level of TUFT1 expression (P < 0.001, Figure 4A1). Based on the status of TUFT1 expression in the MHCC-97H or Hep3B cells, the MHCC-97H cells were chosen to be used for interfering TUFT1 mRNA transcription with shRNA1-3 (Figure 4B left), and the most significantly inhibiting effect was shRNA3 (sh-3) plasmid for TUFT1 (Figure 4B1 left). The Hep3B cells were selected to over-express TUFT1 with constructed pEX-4 (pGCMV/MCS/T2A/EGFP/ Neo) plasmid (Figure 4B right) and showed markedly increasing TUFT1 expression (Figure 4B1 right). Using a different style of TUFT1 interference, the TUFT1-shRNA3 significantly suppressed proliferation of MHCC-97H cells (P < 0.001, Figure 4C), and TUFT1 activation markedly increased proliferation of Hep3B cells (P < 0.001, Figure 4D). The results confirmed that TUFT1 could promote HCC cell proliferation.

Effects of TUFT1 on healing, invasion, and migration of HCC cells

According to the above results, the effects of higher or lower level of TUFT1 on the healing, invasion, and migration of HCC cells were further observed. The effects of

Table 1 Comparative analysis of hepatic tuftelin 1 immunohistochemical staining and expressing intensity between hepatocellular carcinoma and their para-cancerous tissues

Group	*n*	TUFT1		χ^2 value	*P* value	TUFT1 score				Z value	*P* value
		Neg.	Pos.			0	1	2	3		
HCC	132	17	115	18.563	< 0.001	17	49	55	11	4.911	< 0.001
Non-HCC	132	47	85			47	54	23	8		

HCC: Hepatocellular carcinoma; Neg: Negative; Non-HCC: Non-cancerous tissue; Pos: Positive; TUFT1: Tuftelin 1.

Table 2 Relationship between tuftelin 1 and clinicopathological characteristics in hepatocellular carcinoma patients

Group	*n*	TUFT1 expression		Pearson χ^2	*P* value
		Low	High		
AFP (ng/mL)				2.901	0.089
< 20	28	18	10		
≥ 20	104	48	56		
HBsAg				3.300	0.069
Neg.	12	9	3		
Pos.	120	57	63		
HBeAg				4.080	0.043
Neg.	87	49	38		
Pos.	45	17	28		
Tumor size (cm)				9.388	0.002
< 3	26	20	6		
≥ 3	106	46	60		
Differentiation degree				3.013	0.083
Well-Med.	19	13	6		
Poor	113	53	60		
Vascular invasion				14.885	< 0.001
With	58	18	40		
Without	74	48	26		
Ascites				5.940	0.015
With	32	10	22		
Without	100	56	44		
TNM staging				13.516	< 0.001
I-II	59	40	19		
III-IV	73	26	47		

AFP: Alpha-fetoprotein; HBeAg: Hepatitis B virus e antigen; HBsAg: Hepatitis B virus surface antigen; HCC: Hepatocellular carcinoma; Neg: Negative expression; Pos: Positive expression; TUFT1: Tuftelin 1.

different TUFT1 levels on the healing, invasion, and migration of the MHCC-97H or Hep3B cells are shown in Figure 5. The healing abilities of the MHCC-97H cells transfected with the TUFT1-shRNA3 plasmid were averaged down to 50% in the sh-NC group (Figure 5A), and the healing abilities in the sh-RNA3 group were significantly inhibited compared with the sh-NC group ($P < 0.001$, Figure 5A1). However, the healing abilities of the Hep3B cells transfected with the over-expressing

Table 3 Univariate and multivariate analysis of tuftelin 1 in the prognosis of hepatocellular carcinoma

Group	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
TUFT1 expression, high *vs* low	3.026	1.927-4.752	< 0.001	1.743	1.044-2.910	0.034
AFP (ng/mL), ≥ 20 *vs* < 20	1.866	1.066-3.266	0.027	1.482	0.835-2.630	0.178
HBsAg, pos. *vs* neg.	1.495	0.652-3.431	0.339			
HBeAg, pos. *vs* neg.	1.670	1.084-2.574	0.019	1.070	0.659-1.739	0.784
Tumor size (cm), ≥ 3 *vs* < 3	3.680	1.837-7.370	< 0.001	2.235	1.043-4.485	0.039
Differentiation degree, Poor *vs* well-med.	1.642	0.869-3.100	0.123			
Vascular invasion, with *vs* without	3.297	2.133-5.094	< 0.001	2.018	1.186-3.434	0.010
Ascites, with *vs* without	2.592	1.648-4.076	< 0.001	1.929	1.177-3.161	0.009
TNM staging, III-IV *vs* I-II	2.411	1.532-3.796	< 0.001	1.110	0.614-2.005	0.731

AFP: Alpha-fetoprotein; HBeAg: hepatitis B virus e antigen; HBsAg: hepatitis B virus surface antigen; HR: Hazard ratio; Neg: Negative expression; Pos: Positive expression; TNM: Tumor-node-metastasis.

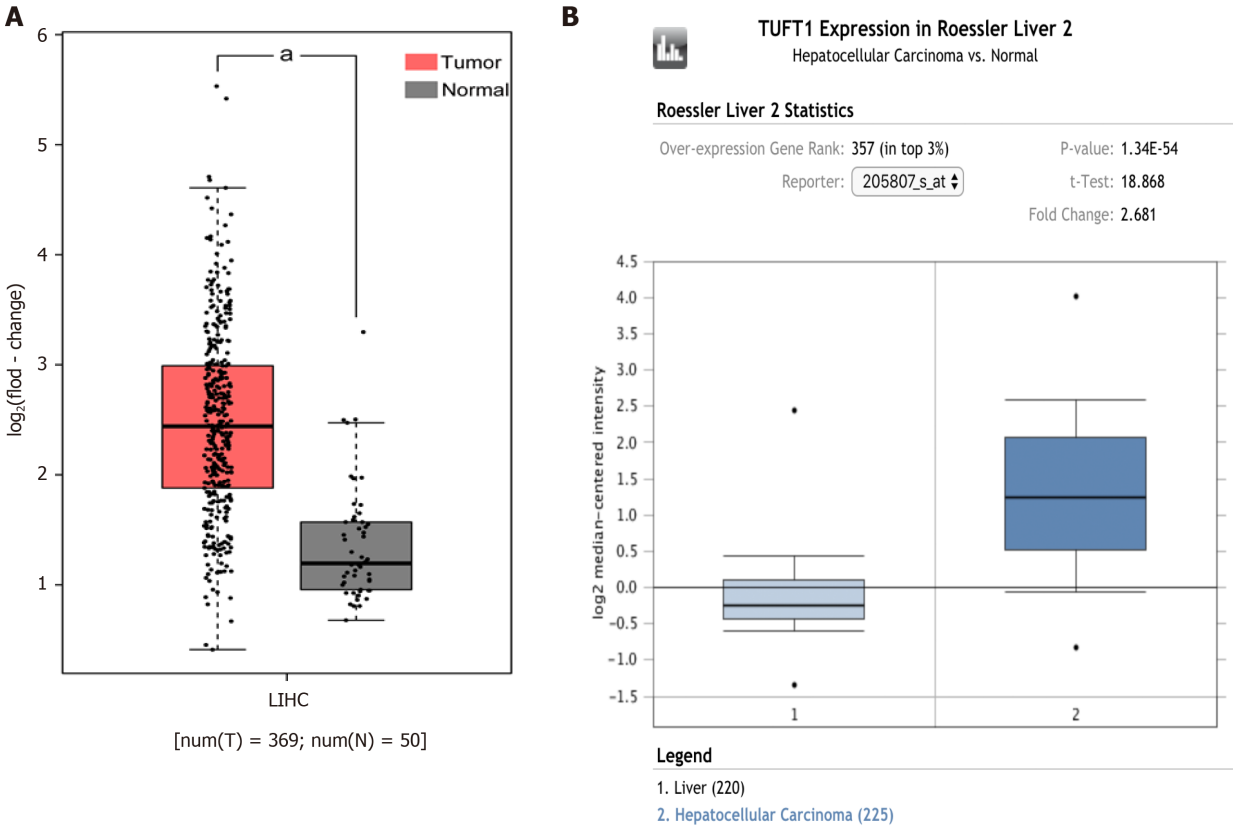


Figure 1 Comparative analysis of tuftelin 1 messenger RNA between hepatocellular carcinoma and normal livers based on bioinformatics databases. A: The expression of tuftelin 1 (TUFT1) messenger RNA in the cancerous tissues of hepatocellular carcinoma (HCC) patients (n = 369, red, $^aP < 0.05$) and normal liver tissues (n = 50, grey) from the Cancer Genome Atlas database; B: The expression of TUFT1 messenger RNA in the cancerous tissues of HCC patients (n = 225, dark blue, $^aP < 0.05$) and normal liver tissues (n = 220, light blue) from the Oncomine database. The horizontal line represents the median of the two groups. Vertical bars indicate the range of data. LIHC: Liver hepatocellular carcinoma.

TUFT1 plasmid were averaged up to 200% in the sh-NC group (Figure 5B); and the healing abilities of the Hep3B cells were significantly improved compared with the sh-NC group ($P < 0.001$, Figure 5B1). Compared with the sh-NC group, the migration or invasion abilities of the MHCC-97H cells were significantly suppressed (Figure 5C); and the migration or invasion abilities of the MHCC-97H cells were down to 50% or 30% less than those in the sh-NC group ($P < 0.001$, Figure 5C1). However, the

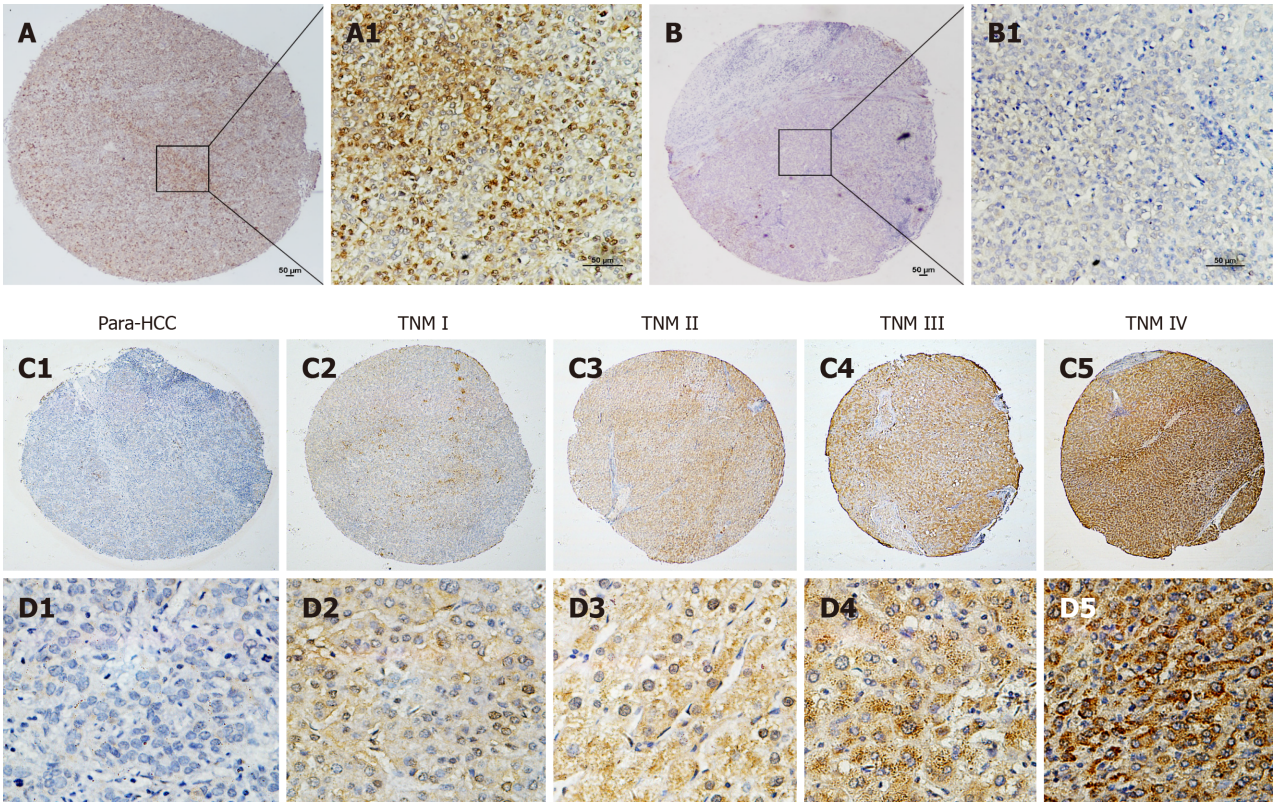


Figure 2 Tuftelin 1 expression with clinical staging of hepatocellular carcinoma. A, A1: Hepatocellular carcinoma (HCC) tissue with strongly positive staining; B, B1: Adjacent non-HCC tissue with negative staining, original magnifications of × 40 (scale bar: 50 μm) in A and B, and × 200 (scale bar: 50 μm) in A1 and B1; C, D: Tuftelin 1 (TUFT1) expression in different HCC staging; C1, D1: Low TUFT1 expression in the non-HCC tissues; C2-C5: The brown staining of TUFT1 gradually increases in the HCC tissues from stage I to IV (original magnification × 40); D2-D5: The staining of TUFT1 in the non-HCC tissues from stage I to IV (original magnification × 400). TNM: Tumor-node-metastasis.

migration or invasion abilities of the Hep3B cells were significantly increased compared with the sh-NC group (Figure 5D); and the migration and invasion abilities of the Hep3B cells were up to 200% or 300% more than those in the sh-NC group [P < 0.001, (Figure 5D1)].

Anti-apoptotic mechanism of TUFT1

After the MHCC-97H cells were transfected with the most effective TUFT1-shRNA3 plasmid, the relationship between TUFT1 expression and apoptosis of MHCC-97H cells was determined (Figure 6). After the MHCC-97H cells were transfected with the more specific TUFT1-shRNA3 plasmid, the numbers of the apoptotic cells in the sh-TUFT1 group (Figure 6A2) were markedly increased than those in the control (Figure 6A) or sh-NC groups (Figure 6A1). The ratio of the apoptotic cells in the sh-TUFT1 group was significantly higher (P < 0.001, Figure 6B) than those in the control or sh-NC groups.

Speculations based on the above data and the possible mechanism of TUFT1 promoting the progression of HCC are shown in Figure 6C. Aberrant up-regulating or down-regulating expressions of TUFT1 was associated with the biological features of HCC. Increased hepatic TUFT1 expression promoted the proliferation, growth, and metastasis of HCC by inhibiting tumor cell apoptosis.

DISCUSSION

Carcinogenesis of hepatocytes is a multi-factor, multi-step, and complex process with major risk factors including chronic infections with HBV or hepatitis C virus, exposure to dietary toxin, hereditary liver disease, and non-alcoholic fatty liver disease of any etiology[2,27]. Despite the urgent need for molecular targeted anti-HCC agents, the therapeutic strategies are difficult to be identified. Accumulating data have demonstrated that many kinds of abnormal signaling pathways are associated with HCC by interacting with highly complex genetic aberrations such as abnormal

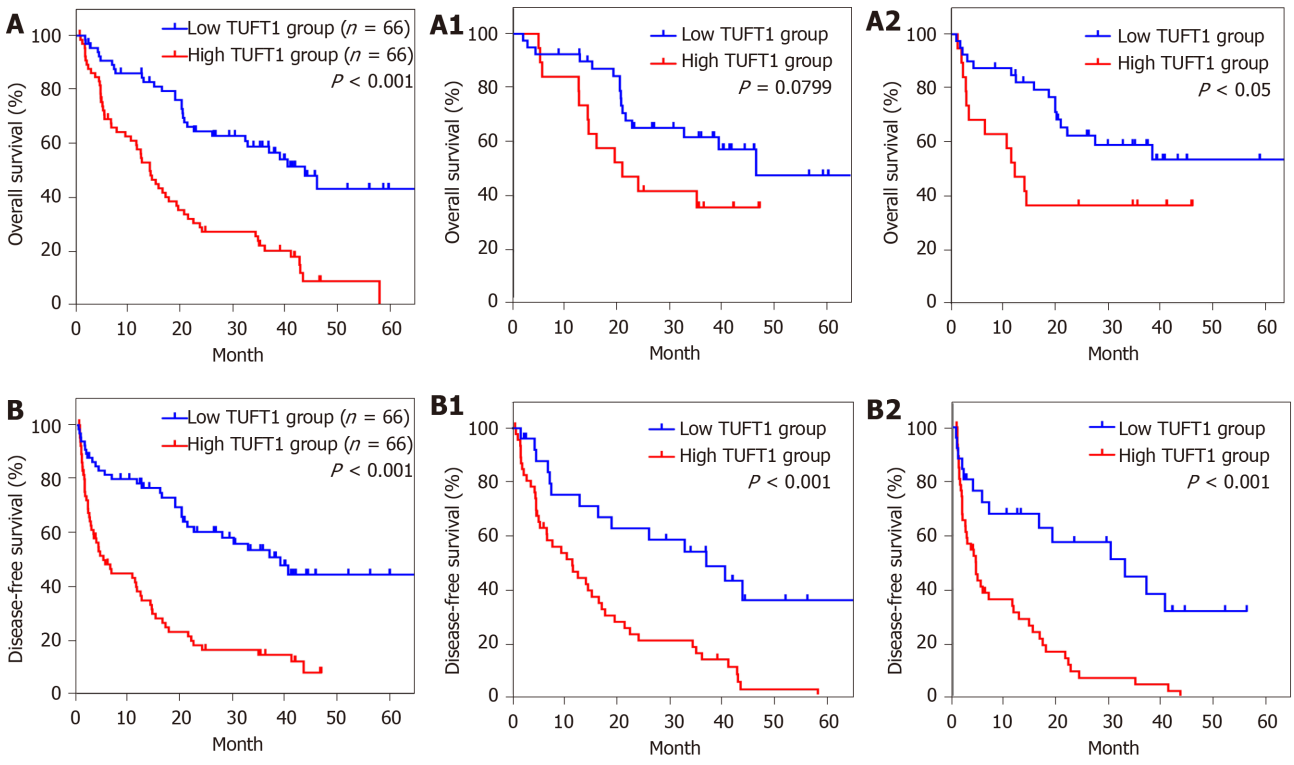


Figure 3 Relationship between tuftelin 1 expression and prognosis of hepatocellular carcinoma. A: Kaplan-Meier analysis was performed to compare the overall survival (OS) between hepatocellular carcinoma (HCC) with high (n = 66) and low (n = 66) TUFT1; A1: The high (n = 19) and low (n = 40) tuftelin 1 (TUFT1) with OS at early HCC; A2: The high (n = 47) and low (n = 26) TUFT1 with OS at advanced HCC; B: Kaplan-Meier analysis was performed to compare the disease-free survival (DFS) between HCC with high (n = 66) and low (n = 66) TUFT1; B1: The high (n = 19) and low (n = 40) TUFT1 with DFS at early HCC; B2: The high (n = 47) and low (n = 26) TUFT1 with DFS at advanced HCC.

methylation, histone modification, and chromosome remodeling[28]. Recently, TUFT1 has been shown to exist in many tissues and has been identified as a useful biomarker associated with poor prognosis in several cancers[29-31]. However, only limited studied were available on the relationship between TUFT1 and HCC. In this study, the alterations of TUFT1 expression in HCC tissues and if the oncogenic TUFT1 might promote HCC growth and metastasis *via* an anti-apoptotic mechanism were investigated.

TUFT1 was originally discovered and mostly studied in the tooth, where it plays an important role in the initial stages of the mineralization of ectodermal enamel[12,29]. Even though TUFT1 is widely distributed and expressed in different embryonic and adult tissues, TUFT1 has been shown to be associated with cellular adaptation to hypoxia and recently even with cell differentiation[32]. TUFT1 activation is related to the progression of tumors, and abnormal TUFT1 has been reported in breast, lung, pancreatic, and thyroid carcinomas[10,11,13]. TUFT1 expression was significantly higher in the HCC tissues than in non-HCC tissues, with invasion and metastasis of tumors[14]. In this study, the comparative analysis of TUFT1 in HCC and non-HCC tissues showed high TUFT1 expression related to tumor size, vascular invasion, advanced TNM stage of HCC, and HBV replication or ascites formation of HCC patients, suggesting that abnormal TUFT1 activation could play oncogenic roles in the progression of HCC.

TUFT1 expression in tissue hypoxia was associated with activations of signaling pathways and poor outcome of patients[18,19]. Previous studies have indicated high HIF-1α level in the hypoxic microenvironment of HCC[33,34] because of tissue hypoxia inducing HIF-1α expression and binding to TUFT1 promoter, thereby promoting tumor progression *via* the activation of the Rac1/β-catenin, mitogen activated protein kinase, calcium/phosphatidylinositol 3 kinase/AKT, Akt-mammalian target of rapamycin/glycogen synthase kinase 3β, and HIF1-SNAIL signaling pathway[11,14,18]. In this study, TUFT1 level was shown to be positively correlated with tumor size, histological grade, and lymph node metastasis rate, and elevated TUFT1 levels were related to unfavorable clinicopathologic characteristics and poor survival. Furthermore, the univariate and multivariate analysis of clinical data indicated that TUFT1 could be an independent prognostic marker for HCC.

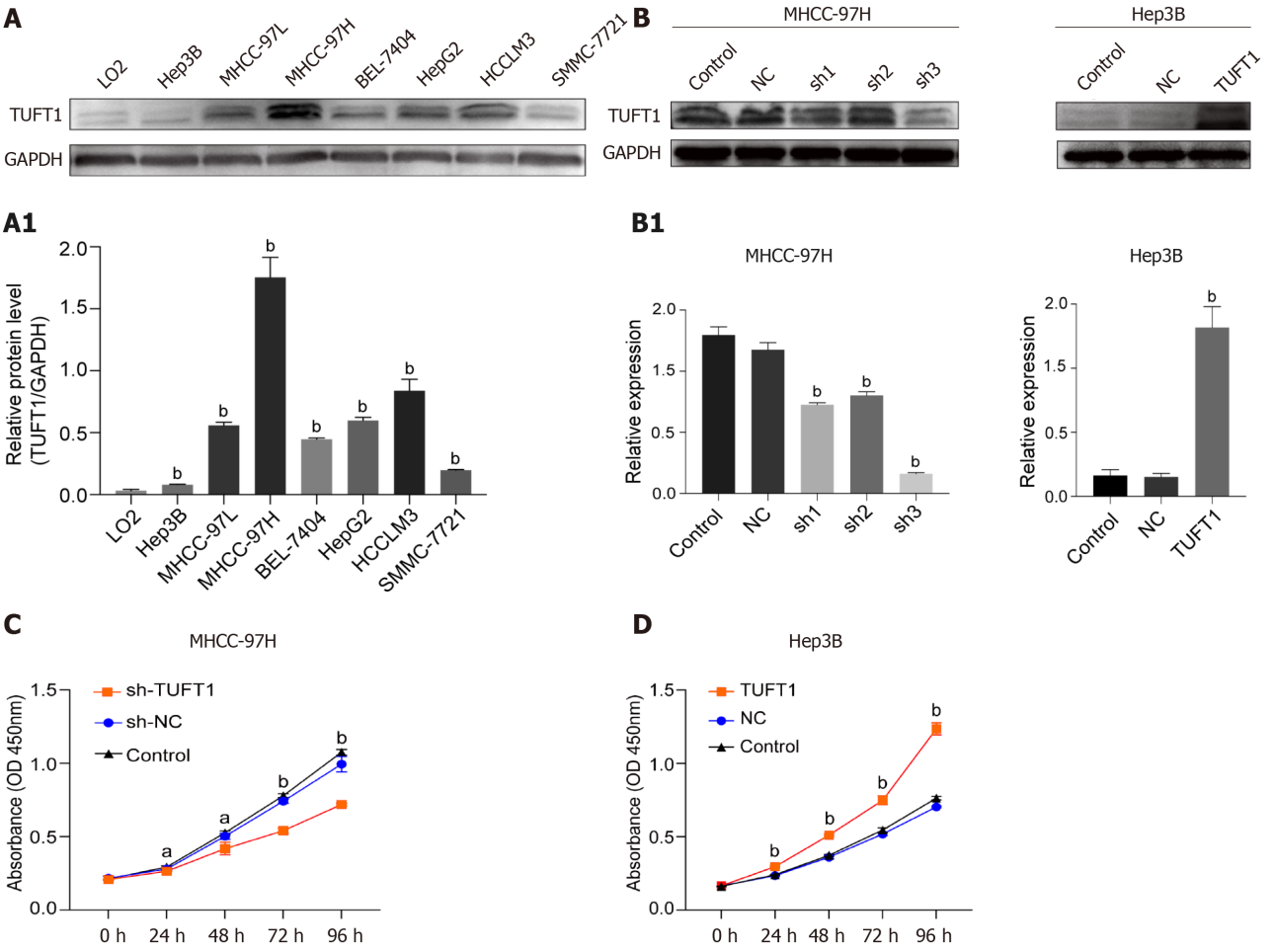


Figure 4 Expression, interfering of tuftelin 1 with proliferation of hepatocellular carcinoma cells. A: Expressions of tuftelin 1 (TUFT1) among different hepatocellular carcinoma (HCC) cell lines were detected by Western blotting, and the expressing levels of all HCC cells were significantly higher than that of the control LO2 cells ($^bP < 0.001$); A1: The relative ratio from TUFT1 to glyceraldehyde 3-phosphate dehydrogenase with the MHCC-97H cells showed the strongest TUFT1 expression, and the Hep3B cells showed the lowest TUFT1 expression; B: The MHCC-97H cells and interfering with TUFT1 short hairpin RNA (sh-RNA)1-3 (left); the TUFT1 was over-expressed after the Hep3B cells transfected with the constructed pEX-4 (pGCMV/MCS/T2A/EGFP/Neo) plasmid (Right); B1: The TUFT1 expression was significantly inhibited by the TUFT1-shRNA transfection ($^bP < 0.001$, left); the markedly increasing TUFT1 Level was compared with the control cells ($^bP < 0.001$, right); C: The TUFT1 activation interfering by TUFT1- shRNA3 significantly inhibited the proliferation of MHCC-97H cells ($^aP < 0.05$, $^bP < 0.001$); D: the over-expression of TUFT1 significantly enhanced the proliferation of the Hep3B cells ($^bP < 0.001$). Data are presented as mean ± standard error of the mean of at least three independent experiments. NC: Blank control; sh-NC: Negative sh-RNA for tuftelin 1 mRNA.

The levels of inhibiting or expressing TUFT1 might alter the biological features of HCC cells[12,23]. In this study, evidence from different cell line studies *in vitro* suggested that the higher or lower TUFT1 level could affect the biological behaviors of HCC cells. The MHCC-97H cells with high TUFT1 expression were transfected with the specific interfering shRNA3 plasmid, and the proliferation, healing, invasion, and migration abilities of the cells were significantly inhibited less than those in the sh-NC group. However, the Hep3B cells with low TUFT1 expression were transfected with the over-expressing TUFT1 plasmid, and those abilities of the cells were significant increased compared to those in the sh-NC group. These results have confirmed that TUFT1 could promote the growth and metastasis of HCC cells *in vitro via* an anti-apoptosis mechanism, suggesting that TUFT1 might function as a potential therapeutic target for inhibiting HCC growth.

CONCLUSION

In conclusion, high TUFT1 levels based on human specimen studies were associated with HCC progression. Further inhibiting TUFT1 *in vitro* could significantly alleviate the proliferation and metastasis of HCC cells. Moreover, TUFT1 over-expression could promote the proliferation and metastasis of HCC cells *in vitro*. Furthermore, TUFT1 inhibition led to significant changes of cell apoptosis rate. More studies in future are

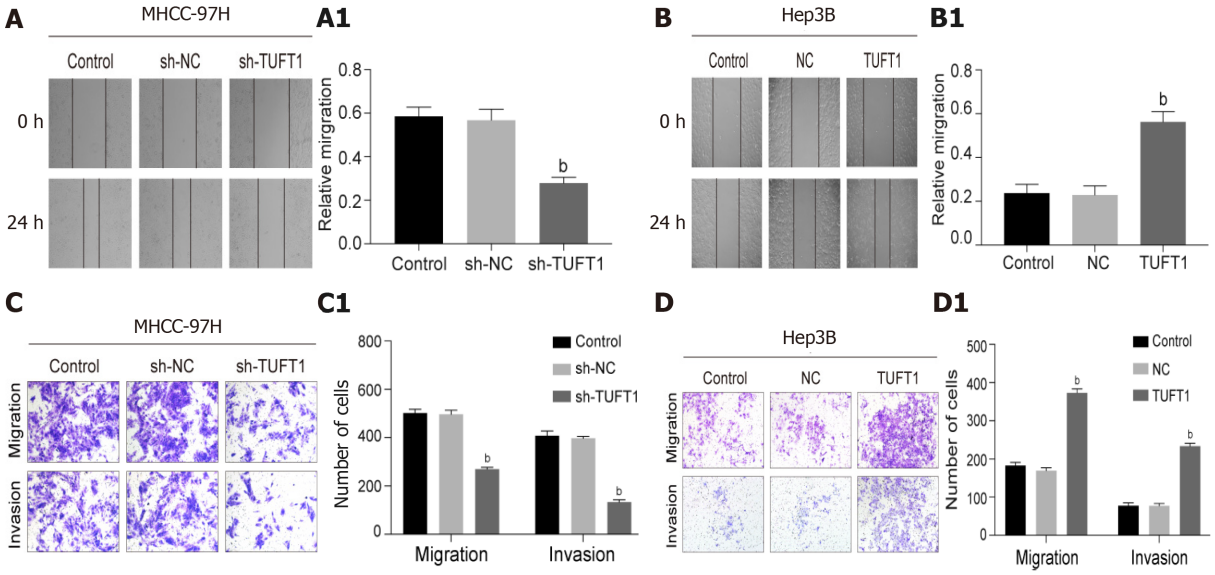


Figure 5 Effects of tuftelin 1 on healing, invasion, and migration of hepatocellular carcinoma cells. A: The scratch test of the MHCC-97H cells, the cell healing ability was decreasing after the high tuftelin 1 (TUFT1) cells were transfected with the TUFT1-shRNA3 plasmid, and A1, the relative cell healing ability was significantly inhibited compared with the control or negative sh-RNA for tuftelin 1 mRNA (sh-NC) cells ($^bP < 0.001$); B: The scratch test of the Hep3B cells, the cell healing ability was increased after the cells were transfected with the over-expressing TUFT1 plasmid; B1, the relative cell healing ability was significantly improved compared with the control or blank control (NC) cells ($^bP < 0.001$); C: The migration and invasion abilities of the MHCC-97H cells were analyzed by the transwell test; C1: The histograms of the cell migration and invasion abilities among the different groups ($^bP < 0.001$): D: The migration and invasion abilities of the Hep3B cells were analyzed by the transwell test; D1: The histograms of the cell migration and invasion abilities among the different groups ($^bP < 0.001$). Data are presented as mean ± standard error of the mean of at least three independent experiments. sh-RNA: sh-RNA3 for tuftelin 1 mRNA.

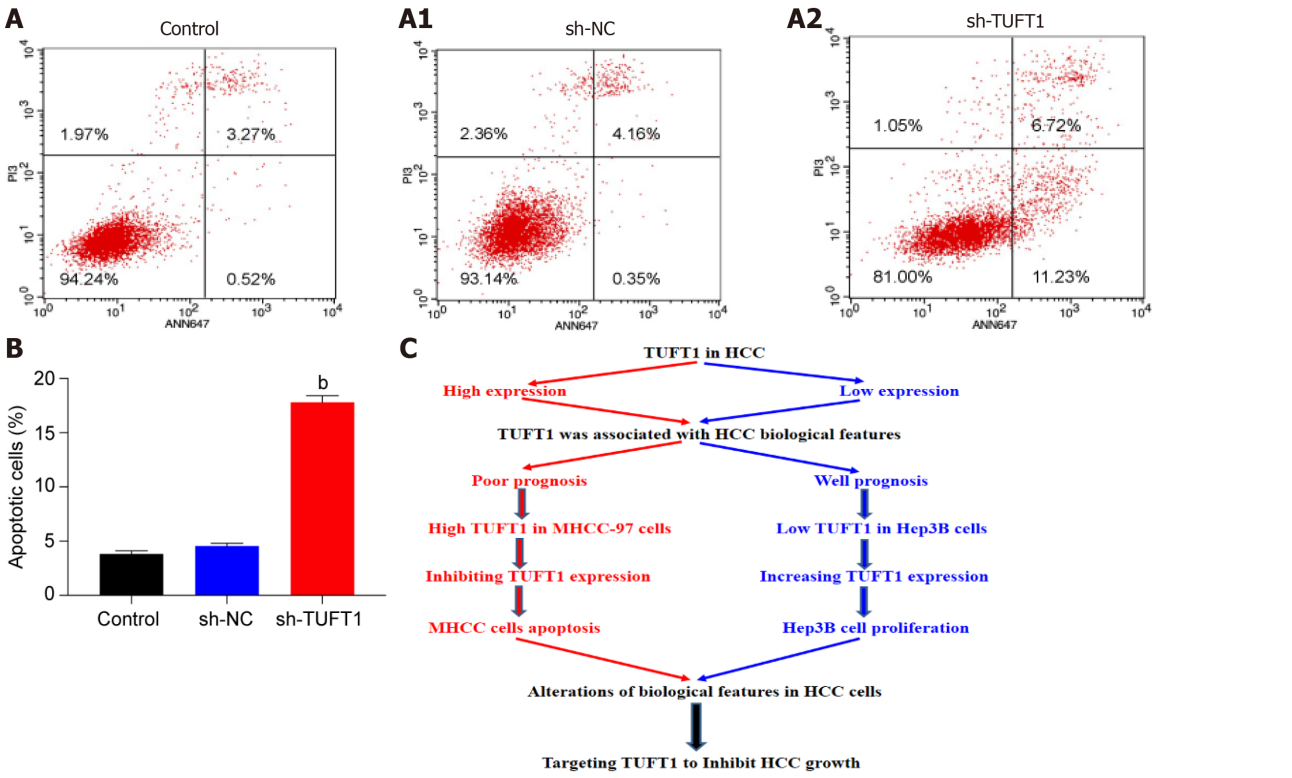


Figure 6 Possible mechanism of tuftelin 1 expression in hepatocellular carcinoma cells progression. A: The MHCC-97H cells were transfected with the tuftelin 1 (TUFT1)-short hairpin RNA (sh-RNA)3 plasmid, and the apoptotic cells in the sh-TUFT1 group (A2) were increased more than those in the blank control (NC) (A) or negative sh-RNA for tuftelin 1 mRNA (sh-NC) group (A1); B: The histograms of the apoptotic cells among the different groups, the rate of the MHCC-97H cell apoptosis in the sh-TUFT1 group was significantly higher ($^bP < 0.001$) than those in the NC or sh-NC group. Data are presented as mean ± standard error of the mean of at least three independent experiments; C: Speculation of possible mechanisms of TUFT1 promotion of the progression of hepatocellular carcinoma (HCC). Aberrant TUFT1 activation promoted the proliferation and metastasis *via* anti-apoptosis of HCC cells.

needed to clarify the molecular mechanisms underlying the TUFT1 up-regulation con

Research background

Tuftelin 1 (TUFT1) has been reported to be elevated in multiple cancer types, and its abnormal expression has attracted medical attention as a novel oncogenic biomarker. However, the relationship between TUFT1 and hepatocellular carcinoma (HCC) remains to be identified. In this study, the levels of TUFT1 in HCC tissues were investigated and the roles of TUFT1 expression were explored *in vitro* by intervening in *TUFT1* activation of HCC cell lines.

Research motivation

TUFT1 was first identified and sequenced from a bovine ameloblast-enriched complementary DNA library with a highly conserved gene localized in chromosome 1q21-31 that contained 13 exons, encoding an acidic, phosphorylated glycoprotein of 390 amino acids, and it plays a critical role in the development and mineralization of enamel. Recently, TUFT1 has been reported to be elevated in multiple cancers, and abnormal TUFT1 has attracted medical attention for HCC. However, limited data is available on the relationship between TUFT1 and HCC, and the exact biological mechanism of TUFT1 is still poorly understood in HCC.

Research objectives

The pathogenesis and feasible therapeutic treatments of HCC need urgent investigation. Abnormal TUFT1 has been reported in multiple cancers, and it exhibits oncogenic roles in tumor progression. In this study, the landscape of TUFT1 expression in human HCC tissues was investigated, and its mechanism was confirmed through intervening or over-expressing TUFT1 activation *in vitro*.

Research methods

TUFT1 on databases of the Cancer Genome Atlas and Oncomine or in HCC tissues were analyzed for clinicopathological features, overall survival, and disease-free survival. High and low expressing HCC cell lines were screened among different HCC cell lines and transfected with constructed vectors that interfere or over-express *TUFT1* to analyze biological behaviors. Proliferation, invasion, migration, and apoptosis of cells were detected by CCK-8, scratch assay, transwell tests, and flow cytometry, respectively, and confirmed by Western blotting.

Research results

The research results confirmed that TUFT1 was over-expressed in HCC tissues, and its expression was significantly related to tumor size, vascular invasion, positive hepatitis B e-antigen, advanced tumor-node-metastasis staging, ascites, shorter overall survival, and disease-free survival of HCC patients. Novel findings were that interfering with *TUFT1* gene transcription could markedly suppress the proliferation and metastasis of the higher TUFT1 MHCC-97H cell lines through an apoptosis mechanism. Moreover, over-expressing TUFT1 promoted the growth and metastasis of the lower TUFT1 Hep3B cell lines *in vitro*.

Research conclusions

Based on human specimen studies, high TUFT1 levels were associated with HCC progression, inhibiting TUFT1 affected proliferation and metastasis, over-expressing TUFT1 promoted proliferation and metastasis, and interfering activation of TUFT1 led to significant increase in cell apoptosis. TUFT1 could be a novel useful target for HCC effective therapy.

Research perspectives

Basic and clinical studies have confirmed that the alterations of oncogenic TUFT1 expression might promote HCC growth and metastasis *via* an anti-apoptotic mechanism, and abnormal TUFT1 level could be a new prognostic marker or potential molecular target for HCC therapy. More studies in the future are needed to clarify the molecular mechanisms underlying TUFT1 up-regulation contribution to the progression of HCC.

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REFERENCES

1 **Singal AG**, Lampertico P, Nahon P. Epidemiology and surveillance for hepatocellular carcinoma: New trends. *J Hepatol* 2020; **72**: 250-261 [PMID: 31954490 DOI: 10.1016/j.jhep.2019.08.025]

2 **Chen JG**, Zhu J, Zhang YH, Zhang YX, Yao DF, Chen YS, Lu JH, Ding LL, Chen HZ, Zhu CY, Yang LP, Zhu YR, Qiang FL. Cancer survival in Qidong between 1972 and 2011: A population-based analysis. *Mol Clin Oncol* 2017; **6**: 944-954 [PMID: 28588795 DOI: 10.3892/mco.2017.1234]

3 **Petruzziello A**. Epidemiology of Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) Related Hepatocellular Carcinoma. *Open Virol J* 2018; **12**: 26-32 [PMID: 29541276 DOI: 10.2174/1874357901812010026]

4 **Younossi Z**, Tacke F, Arrese M, Chander Sharma B, Mostafa I, Bugianesi E, Wai-Sun Wong V, Yilmaz Y, George J, Fan J, Vos MB. Global Perspectives on Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. *Hepatology* 2019; **69**: 2672-2682 [PMID: 30179269 DOI: 10.1002/hep.30251]

5 **Chen S**, Cao Q, Wen W, Wang H. Targeted therapy for hepatocellular carcinoma: Challenges and opportunities. *Cancer Lett* 2019; **460**: 1-9 [PMID: 31207320 DOI: 10.1016/j.canlet.2019.114428]

6 **Tang A**, Hallouch O, Chernyak V, Kamaya A, Sirlin CB. Epidemiology of hepatocellular carcinoma: target population for surveillance and diagnosis. *Abdom Radiol (NY)* 2018; **43**: 13-25 [PMID: 28647765 DOI: 10.1007/s00261-017-1209-1]

7 **Chung W**, Kim M, de la Monte S, Longato L, Carlson R, Slagle BL, Dong X, Wands JR. Activation of signal transduction pathways during hepatic oncogenesis. *Cancer Lett* 2016; **370**: 1-9 [PMID: 26433160 DOI: 10.1016/j.canlet.2015.09.016]

8 **Lin YH**, Wu MH, Yeh CT, Lin KH. Long Non-Coding RNAs as Mediators of Tumor Microenvironment and Liver Cancer Cell Communication. *Int J Mol Sci* 2018; **19** [PMID: 30477236 DOI: 10.3390/ijms19123742]

9 **Qin Y**, Liao P. Hepatitis B virus vaccine breakthrough infection: surveillance of S gene mutants of HBV. *Acta Virol* 2018; **62**: 115-121 [PMID: 29895151 DOI: 10.4149/av_2018_210]

10 **Zhou B**, Zhan H, Tin L, Liu S, Xu J, Dong Y, Li X, Wu L, Guo W. TUFT1 regulates metastasis of pancreatic cancer through HIF1-Snail pathway induced epithelial-mesenchymal transition. *Cancer Lett* 2016; **382**: 11-20 [PMID: 27566398 DOI: 10.1016/j.canlet.2016.08.017]

11 **Liu W**, Chen G, Sun L, Zhang Y, Han J, Dai Y, He J, Shi S, Chen B. TUFT1 Promotes Triple Negative Breast Cancer Metastasis, Stemness, and Chemoresistance by Up-Regulating the Rac1/β-Catenin Pathway. *Front Oncol* 2019; **9**: 617 [PMID: 31338333 DOI: 10.3389/fonc.2019.00617]

12 **Delgado S**, Deutsch D, Sire JY. Evolutionary Analysis of the Mammalian Tuftelin Sequence Reveals Features of Functional Importance. *J Mol Evol* 2017; **84**: 214-224 [PMID: 28409196 DOI: 10.1007/s00239-017-9789-5]

13 **Liu W**, Zhang L, Jin Z, Zhao M, Li Z, Chen G, Sun L, Chen B. TUFT1 is expressed in breast cancer and involved in cancer cell proliferation and survival. *Oncotarget* 2017; **8**: 74962-74974 [PMID: 29088838 DOI: 10.18632/oncotarget.20472]

14 **Liu H**, Zhu J, Mao Z, Zhang G, Hu X, Chen F. Tuft1 promotes thyroid carcinoma cell invasion and proliferation and suppresses apoptosis through the Akt-mTOR/GSK3β signaling pathway. *Am J Transl Res* 2018; **10**: 4376-4384 [PMID: 30662679]

15 **Shilo D**, Cohen G, Blumenfeld A, Goren K, Hanhan S, Sharon S, Haze A, Deutsch D, Lazarovici P. Tuftelin Is Required for NGF-Induced Differentiation of PC12 Cells. *J Mol Neurosci* 2019; **68**: 135-143 [PMID: 30903486 DOI: 10.1007/s12031-019-01292-1]

16 **Kawasaki N**, Isogaya K, Dan S, Yamori T, Takano H, Yao R, Morishita Y, Taguchi L, Morikawa M, Heldin CH, Noda T, Ehata S, Miyazono K, Koinuma D. TUFT1 interacts with RABGAP1 and regulates mTORC1 signaling. *Cell Discov* 2018; **4**: 1 [PMID: 29423269 DOI: 10.1038/s41421-017-0001-2]

17 **Ma C**, Nie ZK, Guo HM, Kong Y. MiR-671-5p plays a promising role in restraining osteosarcoma cell characteristics through targeting TUFT1. *J Biochem Mol Toxicol* 2020; **34**: e22490 [PMID: 32115852 DOI: 10.1002/jbt.22490]

18 **Dou C**, Zhou Z, Xu Q, Liu Z, Zeng Y, Wang Y, Li Q, Wang L, Yang W, Liu Q, Tu K. Hypoxia-induced TUFT1 promotes the growth and metastasis of hepatocellular carcinoma by activating the Ca^{2+}/PI3K/AKT pathway. *Oncogene* 2019; **38**: 1239-1255 [PMID: 30250300 DOI: 10.1038/s41388-018-0505-8]

19 **Qin Y**, Liu HJ, Li M, Zhai DH, Tang YH, Yang L, Qiao KL, Yang JH, Zhong WL, Zhang Q, Liu YR, Yang G, Sun T, Yang C. Salidroside improves the hypoxic tumor microenvironment and reverses the drug resistance of platinum drugs *via* HIF-1α signaling pathway. *EBioMedicine* 2018; **38**: 25-36 [PMID: 30396856 DOI: 10.1016/j.ebiom.2018.10.069]

20 **Li ZJ**, Li JS, Li DY, Pu T. [Effect of TUTF1 expression on the proliferation, apoptosis and prognosis of hepatocellular carcinoma cells]. *Zhonghua Gan Zang Bing Za Zhi* 2019; **27**: 879-884 [PMID: 31941243 DOI: 10.3760/cma.j.issn.1007-3418.2019.11.011]

21 **Tang Z**, Li C, Kang B, Gao G, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res* 2017; **45**: W98-W102 [PMID: 28407145 DOI: 10.1093/nar/gkx247]

22 **Xie DY**, Ren ZG, Zhou J, Fan J, Gao Q. 2019 Chinese clinical guidelines for the management of hepatocellular carcinoma: updates and insights. *Hepatobiliary Surg Nutr* 2020; **9**: 452-463 [PMID: 32832496 DOI: 10.21037/hbsn-20-480]

23 **Mao Z**, Shay B, Hekmati M, Fermon E, Taylor A, Dafni L, Heikinheimo K, Lustmann J, Fisher LW, Young MF, Deutsch D. The human tuftelin gene: cloning and characterization. *Gene* 2001; **279**: 181-196 [PMID: 11733143 DOI: 10.1016/s0378-1119(01)00749-1]

24 **Fang M**, Yao M, Yang J, Zheng WJ, Wang L, Yao DF. Abnormal CD44 activation of hepatocytes with nonalcoholic fatty accumulation in rat hepatocarcinogenesis. *World J Gastrointest Oncol* 2020; **12**: 66-76 [PMID: 31966914 DOI: 10.4251/wjgo.v12.i1.66]

25 **Qian J**, Yao D, Dong Z, Wu W, Qiu L, Yao N, Li S, Bian Y, Wang Z, Shi G. Characteristics of hepatic igf-ii expression and monitored levels of circulating igf-ii mRNA in metastasis of hepatocellular carcinoma. *Am J Clin Pathol* 2010; **134**: 799-806 [PMID: 20959664 DOI: 10.1309/AJCPTFDSE2V3LCZP]

26 **Tierney JF**, Stewart LA, Ghersi D, Burdett S, Sydes MR. Practical methods for incorporating summary time-to-event data into meta-analysis. *Trials* 2007; **8**: 16 [PMID: 17555582 DOI: 10.1186/1745-6215-8-16]

27 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019; **69**: 7-34 [PMID: 30620402 DOI: 10.3322/caac.21551]

28 **Kanda T**, Goto T, Hirotsu Y, Moriyama M, Omata M. Molecular Mechanisms Driving Progression of Liver Cirrhosis towards Hepatocellular Carcinoma in Chronic Hepatitis B and C Infections: A Review. *Int J Mol Sci* 2019; **20** [PMID: 30889843 DOI: 10.3390/ijms]

29 **Januszyk P**, Januszyk K, Wierzbik-Strońska M, Boroń D, Grabarek B. Analysis of the differences in the Expression of mRNAs and miRNAs Asso-ciated with Drug Resistance in Endometrial Cancer Cells Treated with Salinomycin. *Curr Pharm Biotechnol* 2020 [PMID: 32598254 DOI: 10.2174/1389201021666200629151008]

30 **Dou C**, Sun L, Wang L, Cheng J, Wu W, Zhang C, Xu Q, Tu K, Liu J. Bromodomain-containing protein 9 promotes the growth and metastasis of human hepatocellular carcinoma by activating the TUFT1/AKT pathway. *Cell Death Dis* 2020; **11**: 730 [PMID: 32908135 DOI: 10.1038/s41419-020-02943-7]

31 **Bobek J**, Oralova V, Kratochvilova A, Zvackova I, Lesot H, Matalova E. Tuftelin and HIFs expression in osteogenesis. *Histochem Cell Biol* 2019; **152**: 355-363 [PMID: 31520138 DOI: 10.1007/s00418-019-01813-4]

32 **Shilo D**, Blumenfeld A, Haze A, Sharon S, Goren K, Hanhan S, Gruenbaum-Cohen Y, Ornoy A, Deutsch D. Tuftelin's involvement in embryonic development. *J Exp Zool B Mol Dev Evol* 2019; **332**: 125-135 [PMID: 31045321 DOI: 10.1002/jez.b.22855]

33 **Dong ZZ**, Yao M, Wang L, Wu W, Gu X, Yao DF. Hypoxia-inducible factor-1alpha: molecular-targeted therapy for hepatocellular carcinoma. *Mini Rev Med Chem* 2013; **13**: 1295-1304 [PMID: 22512580 DOI: 10.2174/1389557511313090004]

34 **Li S**, Yao D, Wang L, Wu W, Qiu L, Yao M, Yao N, Zhang H, Yu D, Ni Q. Expression characteristics of hypoxia-inducible factor-1α and its clinical values in diagnosis and prognosis of hepatocellular carcinoma. *Hepat Mon* 2011; **11**: 821-828 [PMID: 22224081 DOI: 10.5812/kowsar.1735143X.771]

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ORIGINAL ARTICLE

Basic Study

Conditioned secretome of adipose-derived stem cells improves dextran sulfate sodium-induced colitis in mice

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Abstract

BACKGROUND

Inflammatory bowel diseases (IBD) is related to uncontrolled immune response. Currently, there is no successful treatment for significant improvement in IBD. Stem cells display their therapeutic effects through their repopulating capacity or secreting factors.

AIM

To investigate the effects of conditioned mouse adipose-derived stem cells (mADSCs) secretome on colitis-induced mice.

METHODS

mADSCs were isolated from adipose tissue of C57BL/6 mice. Conditioned mADSCs secretome was obtained by culturing of mADSCs with lipopolysaccharides (LPS, 1 μg/mL) for 24 h. Acute colitis was induced by 2% dextran sulfate

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sodium (DSS) drinking water for 7 d and then normal drinking water for 4 d. The mice were treated with normal culture medium (NM group), conditioned mADSCs secretome (CM group) or mADSCs (SC group). The length of colon and histopatholgy of colon tissues were evaluated. The mRNA expression levels of inflammatory cytokines in colon tissue and the serum interleukin (IL)-6 levels were determined.

RESULTS

The isolated mADSCs maintained the mADSCs specific gene expression profiles during experiment. The conditioned mADSCs secretome released by the treatment of mADSCs with LPS contained mainly inflammatory chemokines, colony-stimulating factors and inflammatory cytokines. The loss of body weight and reduction in colon length were ameliorated in the CM group. The conditioned mADSCs secretome reduced the histological score in colon tissue. The expression of IL-1b and IL-6 mRNAs in colon tissues significantly inhibited in the CM group compared to SC group and NM group, respectively. The elevation of serum IL-6 levels was also ameliorated in the CM group. These results indicate that the conditioned mADSCs secretome suppressed the synthesis of inflammatory cytokines in damaged colon tissue and the elevation of serum IL-6 concentration in DSS-induced mice

CONCLUSION

Conditioned mADSCs secretome might play regenerative roles by the suppression of IL-6 in serum and tissue during acute colitis, and may be more effective than stem cells themselves in the regeneration of colon tissue.

Key Words: Adipose-derived stem cells; Conditioned secretome; Cytokines; Interleukin-6; Colitis; Regeneration

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Core Tip: The therapeutic ability of mesenchymal stem cells (MSCs) is mostly mediated by their paracrine effects. Cell free therapy using MSCs secretome could be more promising strategy than stem cells based therapy. The present study demonstrates that the conditioned secretome of adipose–derived stem cells (ADSCs) has more efficient effects for improving acute colitis in dextran sulfate-sodium-induced mouse model. The effects by the conditioned secretome of ADSCs were mediated by suppression of interleukin (IL)-6 mRNA synthesis in colon tissue and serum IL-6 protein levels, which suggests that the stem cell secretome may have efficient therapeutic potential for incurable inflammatory bowel diseases.

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INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease (CD), are multifactorial disorders characterized by chronic inflammation, visceral hypersensitivity and diarrhea in the gastrointestinal tract[1]. Although the etiology of IBD remains unknown, it is well accepted that IBD is related to a dysregulated immune response, genetic susceptibility, and the environment[2]. Uncontrolled production of inflammatory cytokines and chemokines by infiltrating immune cells ultimately lead to damage of colon tissue. Current treatments for IBD are intended to control the inflammatory intestinal process using immunosuppressive agents; however, these current treatments are not entirely effective to maintain remission of intestinal inflammation and can result in multiple adverse effects due to their

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inefficacy or toxicity[3]. The use of certolizumab perol in patients with moderate-to-severe CD triggered a mild improvement in therapeutic response rates but no significant improvement in remission rates[4]. Therefore, it is necessary to develop novel therapeutic approaches that are effective, feasible and safe for IBD patients.

Stem cells are immature tissue precursor cells that have the ability to self-renew and to differentiate into various cell lineages[5]. Due to these properties, stem cells have emerged as attractive therapeutic tools for incurable diseases such as IBD[6]. Mesenchymal stem cells (MSCs) are mesoderm-derived fibroblast-like stem cells that have been isolated from various adult tissues including adipose tissue[7], skin[8], muscle[9], and peripheral blood[10]. The characteristics of MSCs are identified by the adherence of cells to plastic dishes in standard culture conditions, the expression profiles of specific cell surface molecules and their differentiation potential into osteogenic, chondrogenic and adipogenic lineage[11,12]. Besides their differentiation potential, MSCs also regulate the immune response including *in vitro* suppression of T-cell proliferation, B-cell function and dendritic cell maturation[13,14]. The three main actions of MSCs with regard to therapeutic potential are their homing action for migration of MSCs into damaged sites[15], differentiation action for replacing damaged tissues[16], and paracrine actions for secreting bioactive factors[17]. Generally, the protocol of stem cell therapy requires hundreds of millions of MSCs *per* treatment. However, the overall quantity of MSCs in the body is scarce, and *in vitro* cell expansion is necessary for obtaining enough cells before implantation. *In vitro* manipulation of MSCs for expansion or differentiation affects the quality of the cells such as their senescence, differentiation capacity and survival of the administered MSCs *in vivo*, which may influence homing and engraftment of MSCs into injured sites [18,19]. Although MSCs have become a promising therapeutic strategy for IBD because of their immunosuppressive and tissue healing ability[20], MSCs transplantation in IBD patients had yield inconsistent results, which may be due to the different sources of MSCs with distinct differentiation and regenerative potential, and the variety of protocols for treatments[21].

It has been known that the therapeutic ability of MSCs is mostly mediated by their paracrine effects. MSCs secrete a variety of protective bio-active factors (it is called secretome) such as cytokines, chemokines, growth factors, lipid mediators, hormones, and exosomes[22], which play important roles in the cross-talk between cells and the surrounding tissues for tissue repair and regeneration by their paracrine actions[23]. Many studies have shown that a range of bioactive factors play an important role in the modulation of the immune response. interleukin (IL)-6 and IL-10 secreted by MSCs inhibited the differentiation of macrophage into dendritic cells[24]. Therefore, MSCs secretome has received attention for concerning its potential use in tissue repair and regeneration[25]. The use of MSCs secretome as cell-free therapy may provide several advantages over direct stem cell-based therapy such as safety associated with immunocompatibility and tumorigenicity by stem cells, evaluation for dosage in a manner analogous to conventional pharmaceutical agents, storage for a long time without loss of potency, avoidance of invasive cell collection procedures, and mass production with less time and cost[26,27]. The effectiveness of MSCs secretome as therapeutic applications has been demonstrated in a variety of diseases such as colitis, gastric mucosal injury, osteoarthritis, spinal cord injury and cardiovascular disease[28-31]. It has been suggested that the therapeutic effectiveness of MSCs secretome has been attributed to the mixture of bioactive factors with their attendant paracrine activities[32]. These studies suggest that cell free therapy using the MSCs secretome could be more promising strategy than stem cell based therapy in regenerative medicine. Therefore, the purpose of this study was to investigate whether the conditioned mouse adipose-derived stem cells (ADSCs) secretome has a regenerative effect on damaged colon tissue in an acute colitis mouse model. The results showed that the conditioned mouse ADSCs (mADSCs) secretome recovered the colon tissue damaged by acute colitis in the mouse model. Moreover, the effects of the conditioned ADSCs secretome were more potent than those of ADSCs themselves in the regeneration of damaged colon tissue in acute colitis.

MATERIALS AND METHODS

Animals

C57BL/6 mice (female, 8-10 wk-old) were purchased from the 'KOATECH' laboratory animal company. The animals were kept in in pathogen-free facility at controlled conditions (20-23 °C, 12 h/12 h light/dark cycle, 50% humidity) with free access of

sterilized regular mouse chow and drinking water. Mice were housed 2 wk before the experiment for adaptation. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at Kosin University College of Medicine (IACUC protocol No. Kosin15-12).

Isolation and identification of mADSCs

mADSCs were isolated from subcutaneous, inguinal, epididymal and mesenteric fat of mice. Briefly, animals were euthanized with using CO_2. Fresh adipose tissues were washed with phosphate-buffered saline (PBS; Hyclone, Logan, UT, United States) and minced for 5 min with the sterilized fine scissors. The minced adipose tissues were digested with 0.1% collagenase type I (Invitrogen, Carlsbad, CA, United States) for 40 min at 37 °C in a shaking bath and then centrifuged at 260 × g for 7 min to obtain a pellet. The pellet was incubated in a 1:1 ratio of culture medium (StemX VIVO; R&D systems, Minneapolis, MN, United States) and DMEM/F12 (Invitrogen) along with 5% FBS (Hyclone, Logan, UT, United States) and 1 × penicillin-streptomycin (Invitrogen) overnight at 37 °C and 5% CO_2. The residual non-adherent red blood cells were removed by washing with the medium after 24 h. The cells grown until 80%-90% confluence were subcultured. The medium was changed every 2 d. For all experiments, cells at passage 4 to 6 were used.

To identify the isolated mADSCs, fluorescence-activated cell sorting analysis was performed. Approximately 5 × 10^5 cells were incubated with the conjugated antibodies, CD29-PE, CD31-FITC, CD34-PE, CD45-FITC, CD73-PE and CD90-FITC (Beckman Coulter, Brea, CA, United States), in the dark for 30 min at room temperature. After washing with PBS twice, the cells were resuspended in PBS and analyzed using a flow cytometer (Beckman Coulter, Brea, CA, United States).

Preparation of mADSCs secretome

To prepare the mADSCs secretome, mADSCs (1 × 10^5 cells/well) were seeded onto 6-well plates in culture medium and incubated for 24 h. And then serum-free medium with or without 1 μg/mL lipopolysaccharides (LPS; Sigma, Saint. Louis, MO, United States) replaced the culture medium of the cells. After 24 h, the supernatants were filtered using a 0.45 μm sieve and frozen in -80 °C. To concentrate the supernatants, the frozen supernatants were lyophilized using the lyophilizer (SFDSM12; SamWon Freezing Engineering Co., Busan, South Korea). The lyophilized powders were resuspended in PBS solution, transferred into Amicon Ultra-15 Centrifugal Filter Devices (3000 MWCO; Millipore, Burlington, MA, United States) and then centrifuged at 4000 × g for 50 min in 4 °C condition. The aliquots of concentrated mADSCs secretome were stored in -80 °C deep freezer until use. The conditioned mADSCs secretome stimulated with LPS were used for the animal experiment. Untreated mADSCs secretome were used as a control for the analysis of cytokine antibody array.

Cytokine antibody array analysis of mADSCs secretome

To characterize the mADSCs secretome, cytokine antibody array was performed with a Proteome Profiler mouse cytokine array kit (R&D systems), following the manufacturer instructions. Briefly, the mADSCs secretome were mixed with reconstituted detection antibody cocktail and incubated at room temperature for 1 h. Then the mixture of sample/antibody was incubated with the supplied membrane overnight at 4 °C on a rocking platform. After washing the membrane in wash buffer for 10 min on a rocking platform, Streptavidin-horse radish peroxidase (HRP) was added to the membrane and incubated for 30 min at room temperature. After washing, the membrane was labeled with Chemi Reagent Mix and exposed to X-ray film. The intensity of each spot was analyzed using Gel Documentation System Software (FluorChem HD2; Alpha Innotech, Santa Clara, CA, United States). The intensity of each spot was normalized with the intensity of positive reference spot on each membrane. The normalized intensity values were used to compare the cytokine profiles in untreated mADSCs secretome and the conditioned mADSCs secretome used in this study.

Induction of mouse colitis and treatment

To induce acute colitis, C57BL/6 mice (female, 8 wk old) were supplied with 2% dextran sodium sulfate (DSS; MW = 36000-50000; MP Biomedicals, Solon, OH, United States) for 7 d and then with normal drinking water for 4 d. Animals were divided into 4 groups, control (CON) (n = 4), normal culture medium (NM) (n = 6), mADSCs (SC) (n = 6) and conditioned mADSCs secretome (CM) (n = 6) groups. Animals in CON group received normal drinking water during the experimental period, and animals in

the NM, SC and CM groups received 2% DSS with intraperitoneal injection of 100 μL nomal medium solution three times (on 4, 6 and 8 d; NM group), 1 × 10^5 cells/100 μL ADSCs once (on 4 d; SC group) or 100 μL mADSCs secretome three times (on days 4, 6, and 8; CM group), and then received normal drinking water ad libitum. Animals were monitored daily for the body weight, the appearance of diarrhea, and the presence of blood in the stool. The length of the colon was measured after sacrificing animals on 11 d.

Histopathological analysis

To evaluate the histopathology of colon tissues, colons were incised longitudinally, fixed with 10% buffered formalin and embedded in paraffin. The paraffin-embedded colon tissue sections (4 μm-thickness) were stained with hematoxylin-eosin (H&E) to evaluate the damage of colon tissues. The damage of colon tissues was determined by four histological scoring parameters including inflammation severity (score 0-3), inflammation extent (score 0-3), tissue injury (score 0-4) and crypt damage (score 0-4) as described previously[33]. The histological score was defined as the sum of the four parameter scores.

Quantitative real-time polymerase chain reaction assay

To determine the expression levels of inflammatory cytokines in colon tissue, total RNA was extracted from each colon segment using Trizol reagent (Invitrogen). After assessing the quality and concentration, 3 μg of total RNA was subjected to cDNA synthesis using the TOPscript™ cDNA synthesis Kit (Enzynomics, Daejeon, Korea). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, United States) and 500 nM of each primer as follows: IL-1b, F: 5′-GAAATGCCACCTTTTGACAGTG-3′, R: 5′-TGGATGCTCTCATCAGGACAG-3′; IL-6, F: 5′-CTGCAAGAGACTTCCATCCAG-3′. R: 5′-AGTGGTATAGACAGGTCTGTTGG-3′; tumor necrosis factor-α (TNF-α), F: 5′-CTGAACTTCGGGGTGATCGG-3′, R: 5′-GGCTTGTCACTCGAATTTTGAGA-3′; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), F: 5′- TGGCCTTCCGTGTTCCTAC-3′, R: 5′-GAGTTGCTGTTGAAGTCGCA-3′. The following conditions were used: 95 °C/15 min, followed by 40 cycles of 95 °C/30 s, 60 °C/30 s, and 72 °C/30 s in the RT-PCR (7300 RT-PCR System; Applied Biosystems, Foster City, CA, United States). The levels of mRNA expression were normalized according to the internal control of the housekeeping gene GAPDH and represented as the levels of mRNA expression in NM, SC and The CM groups relative to those in CON group.

Enzyme-linked immunosorbent assay for serum IL-6

To determine the concentration of serum IL-6, blood was obtained from the inferior vena cava using a 21-gauge syringe and centrifuged at 100 × g for 3 min, then serum was collected and stored at -20 °C until use. The concentration of IL-6 in serum was measured using mouse IL-6 enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go (eBiosciences, San Diego, CA, United States) according to the manufacturer's protocols. Briefly, an ELISA plate was coated with anti-mouse IL-6 and incubated overnight at 4 °C. After washing 3 times with wash buffer, the plate was blocked with diluent solution at room temperature for 1 h. Then, serum sample was added to the plate and incubated overnight at 4 °C. After washing, the plate was incubated with detection antibody at room temperature for 1 h and then with Avidin-HRP for 30 min. After incubating with TMB solution for 5 min, the reaction was stopped with stop solution. The absorbance was measured at 450 and 570 nm. The concentrations of serum IL-6 were determined with the standard curve and represented as pg/mL.

Statistical analyses

Data values were expressed as mean ± SEM for each group. Statistical analysis was performed with the Kruscal-Wallis test using SPSS version 18. Differences with $P < 0.05$ or $P < 0.005$ were considered to be statistically significant.

RESULTS

Characterization of isolated mADSCs

mADSCs were isolated from abdominal adipose tissue of 7 week-old C57BL/6 mice. To characterize the isolated mADSCs, flow cytometry analysis was performed to

validate the surface antigens of mADSCs at passage 3 or 4. The isolated mADSCs were positive for CD90, CD73, and CD29, but negative for CD45, CD34. CD31 (Figure 1A). The expression profiles of the surface antigens of mADSCs were not changed during passages 3-7 (data not shown). Therefore, mADSCs at passage 3 through 5 were used for all experiments. These results indicate that the isolated mADSCs maintain the gene expression profile specific for mADSCs during the experiment.

Cytokine profiles in conditioned mADSCs secretome

Conditioned mADSCs secretome were prepared from the conditioned medium in which mADSCs were cultured with 1 μg/mL of LPS for 24 h and compared with mADSCs secretome cultured without LPS. Cytokine profiles in mADSCs secretome and the conditioned mADSCs secretome were determined using mouse cytokine array kit containing 40 kinds of cytokine antibody (Figure 1B). 21 antibodies out of 40 antibodies were detected as strong positive spots with more than 1000 intensity in the conditioned mADSCs secretome (Table 1). The conditioned mADSCs secretome contained a high amount of colony-stimulating factors [granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage-colony stimulating factor], inflammatory chemokines [(C-X-C motif) ligand (CXCL) 1, CXCL2, carbon tetrachloride (CCL)3, CCL5, and CCL2] and inflammatory cytokines (TNF-α and IL-6). The amount of CCL4, G-CSF, CXCL2 and CXCL10 increased more than 10-folds in the conditioned mADSCs secretome compared to mADSCs secretome. These results indicate that the conditioned mADSCs secretome by the stimulation of LPS contains high amount of colony-stimulating factors, inflammatory chemokines and cytokines.

Effects of the conditioned mADSCs secretome on body weight and colon length in DSS-induced mice

To investigate the effects of conditioned mADSCs secretome in the DSS-induced colitis model of mice, mice in the NM, SC, and CM groups were treated with 2% DSS in drinking water from days 0 to 7 and then with normal drinking water thereafter until sacrifice. The NM mice and CM mice were injected intraperitoneally on days 4, 6, and 8 with normal medium and conditioned mADSCs secretome, respectively. SC mice were injected with mADSCs (5×10^5 cells/100 μL/mouse) through the tail vein on day 4. In all treatment groups, body weight started to decrease on day 5 (Figure 2A). The NM and SC groups kept the loss of body weights until sacrificed on day 11. However, the body weight in the CM group started to be recovered on day 7 and similar to body weight in untreated CON group on day 11. On day 11 mice were sacrificed and colon length was measured without tension from cecum to rectum (Figure 2B). Colon length in NM and SC groups significantly ($P < 0.05$) decreased compared to that in the CON group, but colon length in the CM were longer than that in NM and SC groups (Figure 2C). These results indicate that the conditioned mADSCs secretome improved the recovery of body weight and colon length in DSS-induced mice.

Effects of the conditioned mADSCs secretome on damage of colon tissues in DSS-induced mice

To investigate the effects of conditioned mADSCs secretome on the damage of colon tissues in DSS-induced mice, the damage of colon tissues was assessed by histological score using H&E stained colon tissue sections. To assess the overall damage to colon tissue, macroscopic appearances of the opened whole colon tissues were examined after sacrificing the mice on day 11 (Figure 3A). Colon tissues from the NM and SC groups showed striking hyperemia and a little hyperemia, respectively. Meanwhile, colon tissues from the CM group did not show hyperemia. In H&E stained tissues (Figure 3B), the CM group showed no loss of mucosa without crypt damage whereas the NM and SC groups showed severe loss of mucosa with crypt damage. The NM and SC group also showed the significant infiltration of immune cells, but the CM group showed a little infiltration of immune cells. The damage to colon tissues was assessed using histological scores determined by the severity of inflammation, tissue injury and crypt damage (Figure 3C). Histological scores in the CM group were significantly lower than in the NM group (14.4 ± 1.2 in the NM group *vs* 6.3 ± 1.5 in the CM group, $P < 0.05$). These results indicate that the conditioned mADSCs secretome improved the recovery of damaged colon tissue in DSS-induced mice.

Effects of the conditioned mADSCs secretome on expression of inflammatory cytokines

The mRNA expression levels of inflammatory cytokines IL-1b, IL-6, and TNF-α were

Table 1 Cytokine expression profiles in the secretomes of mouse adipose-derived stem cells and mouse adipose-derived stem cells stimulated with lipopolysaccharides (μg/mL) for 24 h

Cytokine	mADSCs	MADSCs w/LPS	Ratio[1]
CCL4	218	4516	20.8
G-CSF	218	4138	19.0
CXCL2	467	4811	10.3
CXCL10	433	4342	10.0
GM-CSF	335	3198	9.5
CCL3	629	4986	7.9
TNF-α	836	5863	7.0
IL-1ra	515	3610	7.0
CXCL1	1583	6263	4.0
CCL1	307	1178	3.8
CCL5	1137	4194	3.7
CD54	243	796	3.3
CXCL9	399	1180	3.0
M-CSF	1530	3147	2.1
IL-6	3197	4642	1.5
IFN-γ	512	559	1.1
CXCL12	4711	4798	1.0
TIMP-1	3735	3553	1.0
CCL2	5228	3646	0.7
IL-1α	852	482	0.6

[1]Ratio represents the intensity value of mouse adipose-derived stem cells (mADSCs) w/lipopolysaccharides relative to the intensity value of mADSCs used as a control. mADSCs: Mouse adipose-derived stem cells; LPS: Lipopolysaccharides; CCL: Carbon tetrachloride; G-CSF: Granulocyte colony-stimulating factor; CXCL: (C-X-C motif) ligand; TNF-α: Tumor necrosis factor-α; IL: Interleukin; M-CSF: Macrophage-colony stimulating factor; IFN-γ: Interferon-γ; TIMP-1: Tissue inhibitor of metalloproteinase 1.

evaluated in colon tissues of DSS-induced mice on day 11 after treatment using qRT-PCR (Figure 4). The expression levels of mRNAs in all groups were expressed as the relative levels compared to the CON group. The expression of IL-1b mRNAs increased in the NM group, SC group and CM group compared to the CON group. The expression of IL-1b mRNA in the CM group was significantly lower ($P < 0.05$) than in the SC group. The expression of IL-6 mRNA increased in the NM group and SC group. The expression levels of IL-6 in the CM group were significantly lower than in both of the NM group ($P < 0.005$) and SC group ($P < 0.05$). The expression level of TNF-α mRNAs was significantly higher in the SC group than in the CON group ($P < 0.005$) and the CM group ($P < 0.05$). However, the expression level of TNF-α mRNAs in the NM and CM groups did not differ from that in the CON group. These results indicate that the conditioned mADSCs secretome suppressed the upregulation of IL-6 mRNA expression in damaged colon tissue of DSS-induced mice.

Effects of the conditioned mADSCs secretome on serum IL-6 concentration of DSS-induced mice

To investigate the effects of conditioned mADSCs secretome on serum IL-6 concentration of DSS-induced mice, the DSS-induced mice were sacrificed on 11 d after DSS treatment. The concentration of IL-6 protein in serum was determined by ELISA (Figure 5). The concentration of serum IL-6 in DSS-induced mice was significantly higher in NM group (207.40 pg/mL) than in the SC group (38.01 pg/mL, $P < 0.05$) and the CM group (5.94 pg/mL, $P < 0.05$). The concentration of serum IL-6 in the CM group was not much different from that in the CON group compared to the NM group and SC group. These results indicated that the conditioned mADSCs secretome

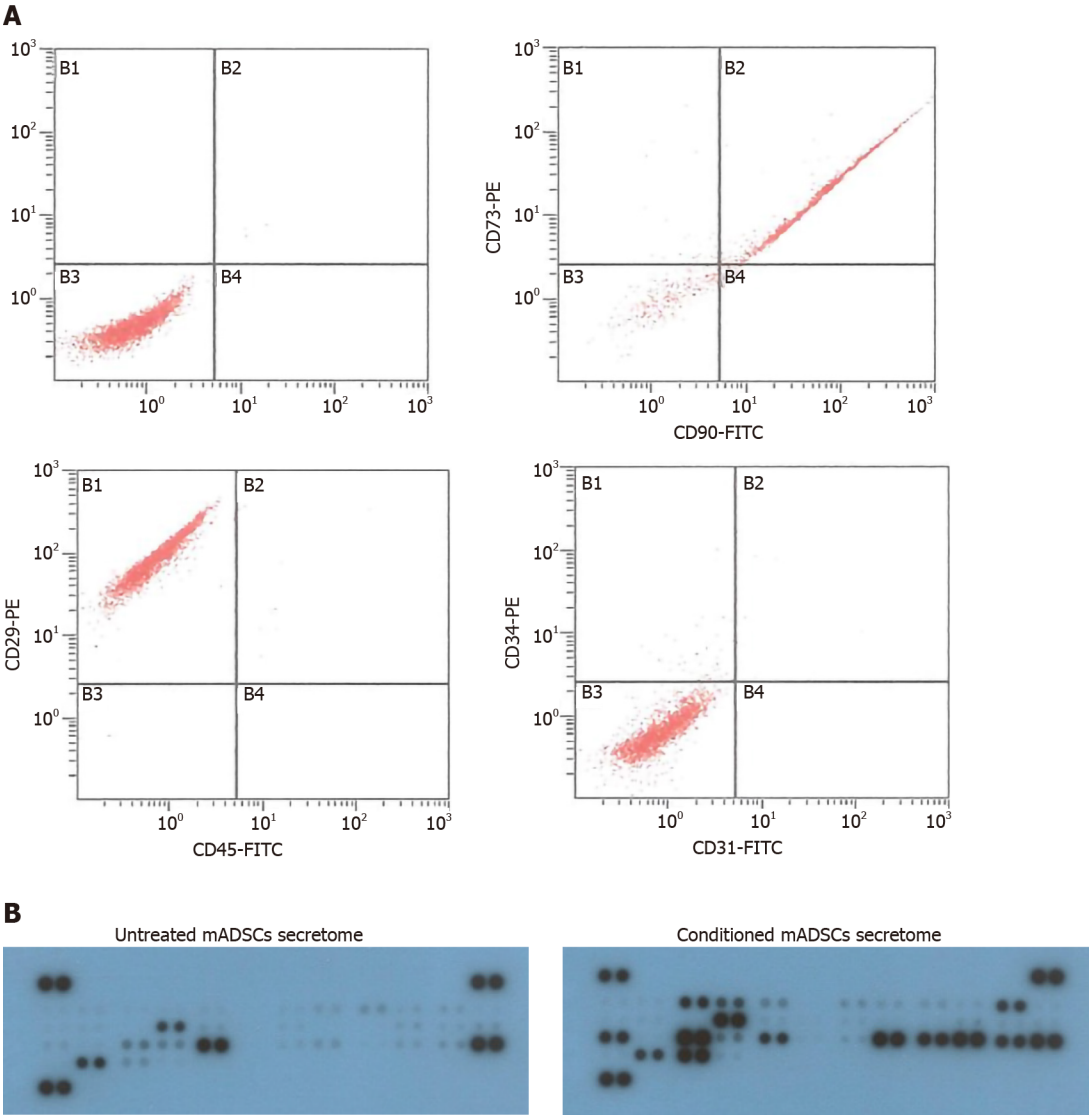


Figure 1 Characterization of mouse adipose-derived stem cells and secretome of mouse adipose-derived stem cells. A: Flow cytometric analysis of surface antigens of mouse adipose-derived stem cells (mADSCs) isolated from C57BL/6 mice. They were positive for Crohn's disease (CD)90, CD73 and CD29, and negative for CD45 and CD34, and CD31; B: Cytokine antibody arrays for secretome of mADSCs. Untreated mADSCs secretome was isolated from mADSCs without lipopolysaccharides (LPS) treatment, and conditioned mADSCs secretome was isolated from mADSCs cultured with LPS (1 μg/mL) for 24 h. Cell culture supernatants were subjected to cytokine antibody array. mADSCs: Mouse adipose-derived stem cells; CD: Crohn's disease.

suppressed the elevation of serum IL-6 concentration in DSS-induced mice.

DISCUSSION

ADSCs have emerged as a promising therapeutic tool for tissue inflammation and injury. The majority of clinical trials for MSC therapy have used ADSCs than other type of MSCs because of their availability, less invasiveness and cell yield from adipose tissue[34,35]. Although the regenerative effects of ADSCs may be the result of their ability to migrate and engraft into injured sites, their immune-modulatory activity is also mediated by soluble paracrine factors secreted from ADSCs. However, it is remains unclear whether the therapeutic efficacy of soluble paracrine factors is better than ADSCs themselves in inflammation-related disease. This study showed that the conditioned ADSCs secretome had more potent therapeutic effects than ADSCs themselves in the regeneration of damaged colon tissue in acute colitis through the suppression of IL-6 production

In this study, mADSCs isolated from abdominal adipose tissue of 7 w-old C57BL/6 mice were used for the transplantation and preparation of conditioned mADSCs secretome. The mADSCs expressed the stromal-associated markers (CD90, CD73 and

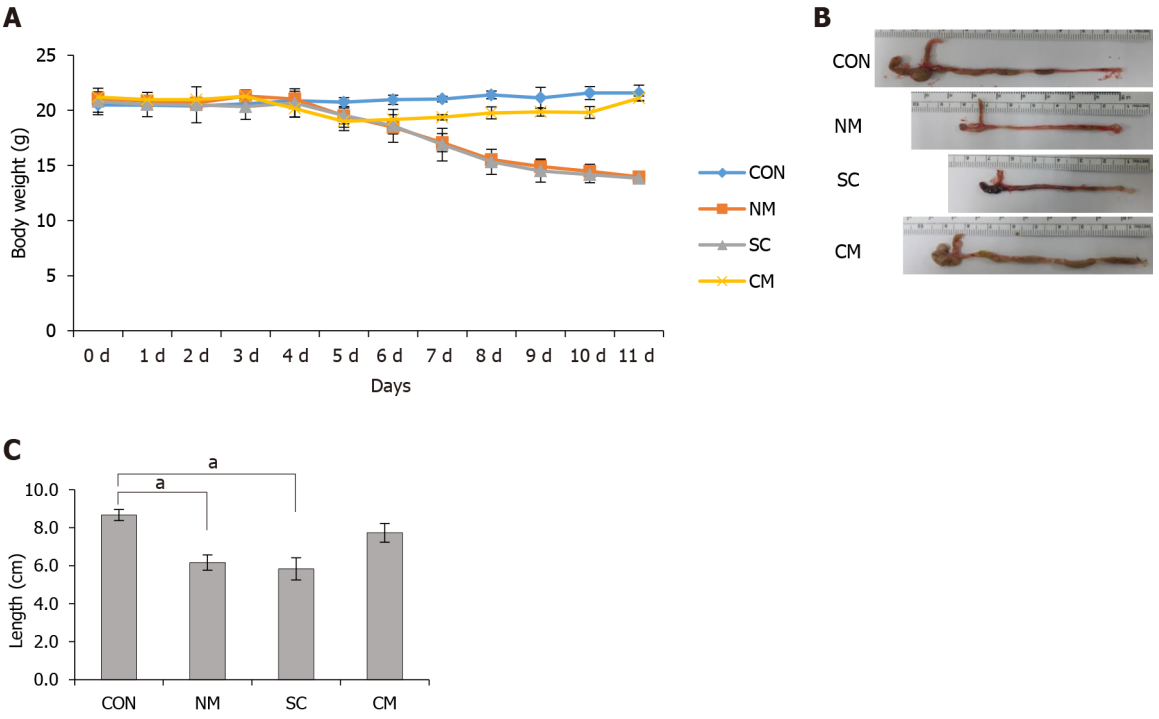


Figure 2 Effects of conditioned mouse adipose-derived stem cells secretome on body weight and colon length in dextran sulfate sodium-induced mice. A: The treatment of Conditioned mouse adipose-derived stem cells (mADSCs) secretom (CM) ameliorated the loss of body weight of dextran sulfate sodium-induced mice. Normal culture medium or CM was injected intraperitoneally into mice on days 4, 6 and 8. mADSCs was injected *via* tail vein on day 4. Mice were sacrificed on day 11; B and C: The treatment of CM ameliorated the shortening of colon length. Values are mean ± SEM (n = 4-6 mice *per* group). $^{a}P < 0.05$. CON: Control; NM: Normal culture medium; SC: Mouse adipose-derived stem cells (mADSCs); CM: Conditioned mADSCs secretome.

CD29) known as positive markers of mADSCs, and did not expressed hematopoiectic markers (CD34 and CD45) or endothelial cell marker (CD31), which are known as negative markers for mADSCs. The results of this study are consistent with the result of the previous study[36], which confirms the characteristics of the isolated mADSCs. It has been shown that ADSCs secretome consists of the growth factors for wound healing, cytokines for modulation of immune system, and chemokines for cell migration and engraftment[26,37]. A variety of culture conditions for ADSC culture affects the compositions of ADSCs secretome, which may influence the biological functions for tissue repair and regeneration[38]. It has been reported that the MSC secretome contains pro-inflammatory cytokines (IL-1b, IL-6, IL-8 and IL-9) as well as anti-inflammatory cytokines (IL-10, IL-13, IL-17 and IL-1ra), and the balance between these pro-inflammatory and anti-inflammatory cytokines may influence the final effect [26]. Moreover, the exposure of human ADSCs to LPS for 24 h increase the secretion of hematopoietic factors (G-CSF, GM-CSF and M-CSF) and the pro-inflammatory cytokines (IL-6, IL-8, IL-11 and TNF-α)[39]. In this study, we treated mADSCs with 1 μg/mL LPS to prepare the conditioned mADSCs secretome. The exposure of mADSCs to LPS for 24 h increased the secretion of pro-inflammatory cytokines (IL-6, IL-17 and TNF-α), anti-inflammatory cytokines (IL-1ra and IL-5), hematopoietic factors (G-CSF, GM-CSF and M-CSF), cell recruitment-related chemokines (CCL1, CCL3, CCL4 and CCL5) and inflammatory-related chemkines (CXCL1 and CXCL2), which is consistent with the previous studies[37,39]. Taken together, these results show that the conditioned mADSCs secretome used in this study contains various components involved in the immunomodulation and regeneration, which suggests that the conditioned mADSCs secretome may display more efficient regenerative effects in the recovery of damaged tissues.

Many studies have shown the therapeutic potent of ADSCs in several inflammatory diseases such as acute colitis, thyroiditis, and arthritis[36,40,41]. The injection of mADSCs reduced the histopathologic severity of colitis, body weight loss, diarrhea and inflammation in trinitrobenzene sulfonic acid (TNBS)- or DSS-induced colitis mouse models[42]. However, the clinical results of ADSCs therapy for inflammatory diseases are inconsistent, which may be due to the different cell sources and cell preparation methods[43,44]. Moreover, MSCs therapy requires a large number of cells and is influenced by uncertain factors such as administration routes and culture

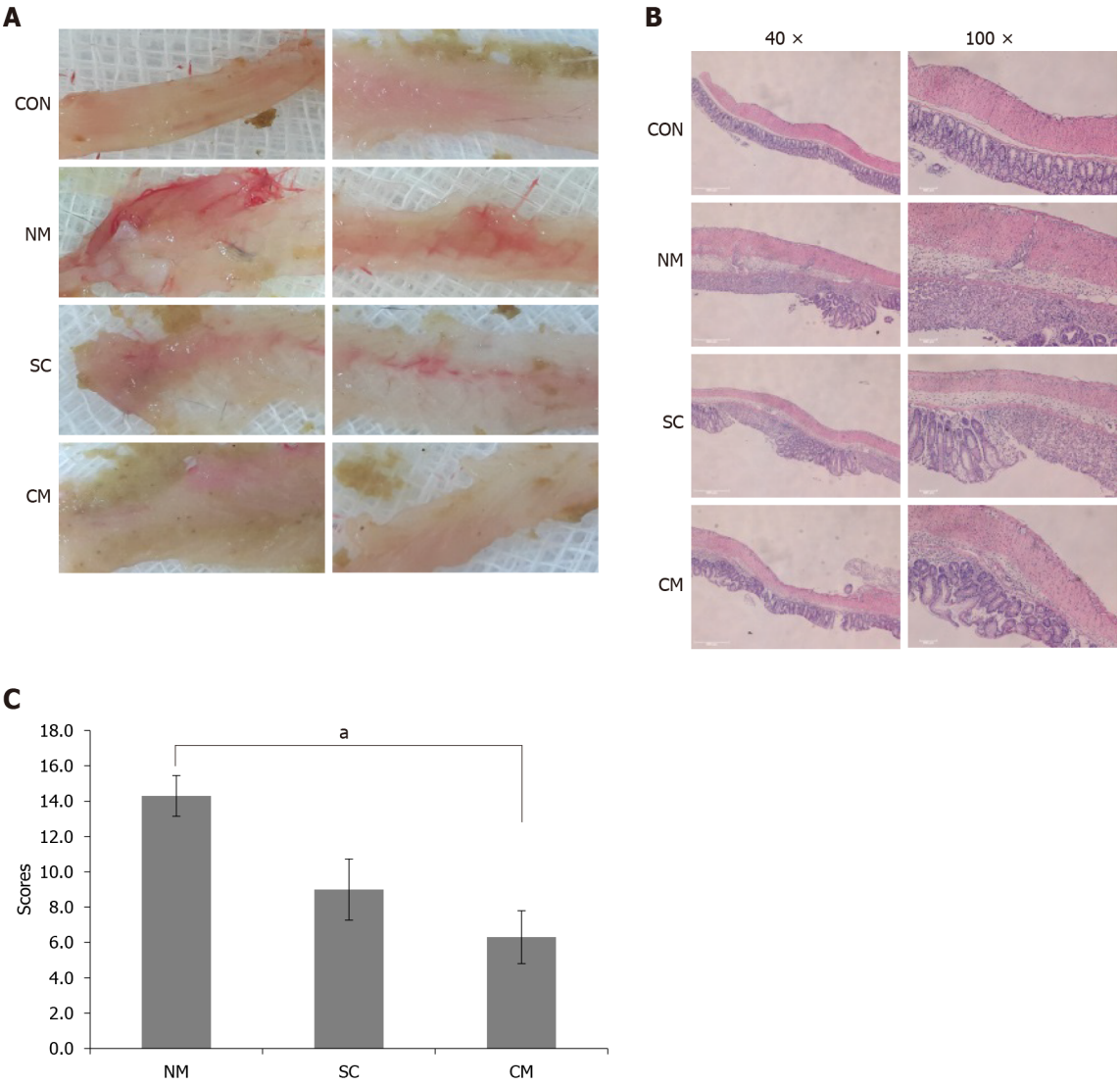


Figure 3 Effects of conditioned mouse adipose-derived stem cells secretome on damage of colon tissues in dextran sulfate sodium-induced mice. A: Macroscopic appearance of colon tissues; B: Representative hematoxylin and eosin stained sections of damaged colon tissues are shown on magnification, 40 × or 100 ×; C: The damage of colon tissues was assessed by histological score. Values are mean ± SEM (n = 4-6 mice *per* group). $^{a}P < 0.05$. CON: Control; NM: Normal culture medium; SC: Mouse adipose-derived stem cells (mADSCs); CM: Conditioned mADSCs secretome.

conditions, which indicates that there is a difficulty in standardizing MSCs therapy protocols. Therefore, recent studies have focused on the secretome from MSCS as an alternative therapeutic option for tissue regeneration[26]. In this study, we compared the therapeutic effects of conditioned mADSCs secretome with mADSCs in DSS-induced acute colitis mouse model. The injection of conditioned mADSCs secretome intraperitoneally into mice significantly improved the recovery of body weight, colon length and histopathological scores, but the intraperitoneal injection of mADSCs did not show any improvement in the recovery of mice. In a previous study, the intraperitoneal injection of mADSCs recovered body weight and significantly reduced histopathological signs in mice with TNBS-induced colitis[42]. Another study, however, showed that the intraperitoneal injection of mADSC did not improve the recovery of body weight, colon length and histological score in mice with DSS-induce colitis[45]. The inconsistent results in the effect of mADSCs on acute colitis may results from the different ADSCs injection protocols including variation in cell dose, injection timing and cell preparation method. However, our results showing the achievement of significant improvement by the treatment of mADSCs secretome in DSS-induced colitis is consistent with the results of a previous study reporting a reduction in DSS-induced colitis by the intraperitoneal injection of umbilical cord-derived MSC extract [46]. Therefore, the present study indicates that mADSCs secretome might be a more efficient and manageable therapeutic tool in regenerative medicine than the direct injection of mADSCs themselves.

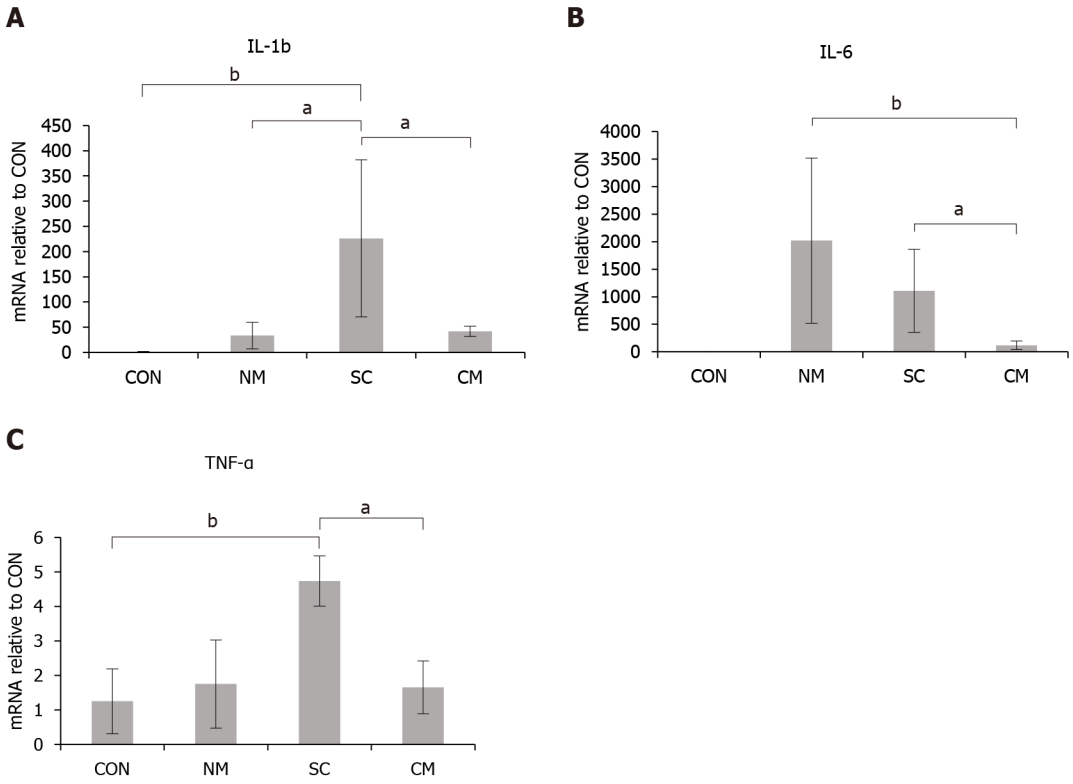


Figure 4 Effects of the conditioned mouse adipose-derived stem cells secretome on the expression of inflammatory cytokines in colon tissues of dextran sulfate sodium-induced mice on day 11. The mRNA expression levels of inflammatory cytokines interleukin (IL)-1b, IL-6 and tumor necrosis factor-α (TNF-α) were evaluated by quantitative real-time polymerase chain reaction analysis. The mRNA expression levels in normal culture medium, mouse adipose-derived stem cells (mADSCs) or conditioned mADSCs secretome were represented as the relative values to the expression levels in control. Values are mean ± SEM (n = 4-6 mice *per* group). A: IL-1b; B: IL-6; C: TNF-α. $^{a}P < 0.05$; $^{b}P < 0.005$. CON: Control; NM: Normal culture medium; SC: Mouse adipose-derived stem cells (mADSCs); CM: Conditioned mADSCs secretome; IL: Interleukin; TNF-α: Tumor necrosis factor-α.

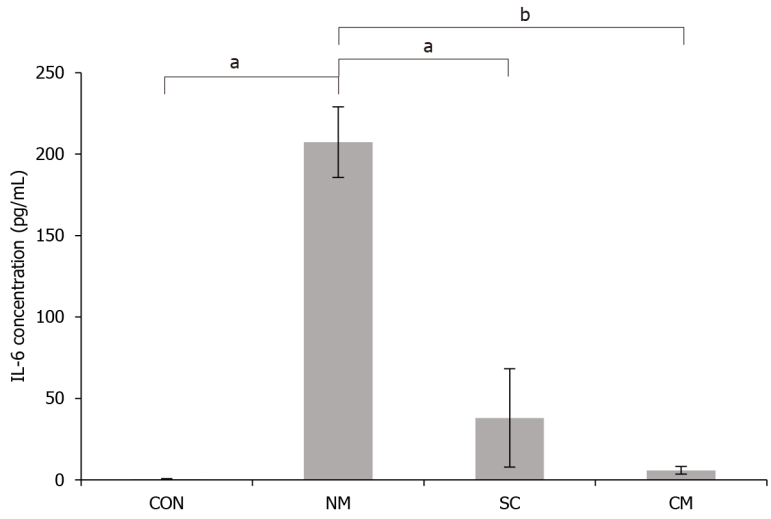


Figure 5 Effects of conditioned mouse adipose-derived stem cells secretome on the concentration of interleukin-6 in serum of dextran sulfate sodium-induced mice on day 11. The concentration of interleukin-6 in serum was evaluated by enzyme-linked immunosorbent assay. Values are mean ± SEM (n = 4-6 mice *per* group). $^{a}P < 0.05$; $^{b}P < 0.005$. CON: Control; NM: Normal culture medium; SC: Mouse adipose-derived stem cells (mADSCs); CM: Conditioned mADSCs secretome; IL-6: Interleukin-6.

Cytokines are key regulators in the intestinal immune response including inflammation. It has been known that IL-1b, IL-6 and TNF-α are important pro-inflammatory cytokines promoting inflammation in the innate immune response[47]. Previous studies have reported that ADSC or BMSC decreased the expression of IL-1b, IL-6 and TNF-α mRNA in colon tissues of mice treated with TNBS or DSS in comparison with

untreated mice[41,48]. In our results, mADSC treatment decreased the expression of IL-6 mRNA in colon tissue, but did not decrease the expression of IL-1b and TNF-α mRNA in colon tissue of DSS-treated mice. However, treatment with mADSC secretome decreased the expression of IL-1b, IL-6 and TNF-α mRNAs in colon tissue of DSS-treated mice. Moreover, the serum levels of IL-6 protein were much less with treatment of the conditioned mADSC secretome than with mADSCs themselves. Our results indicate that treatment using conditioned mADSCs secretome is more effective in suppressing inflammation than in treatment with mADSCs themselves, which is mediated through the inhibition of pro-inflammatory cytokine synthesis in colon tissue as well as the reduction of serum IL-6 levels.

CONCLUSION

In conclusion, conditioned mADSCs secretome could inhibit the synthesis of pro-inflammatory cytokines in colon tissue and reduce serum IL-6 levels more effectively than mADSCs themselves. This effective suppression of proinflammatory cytokines in colon tissue and serum might mainly contributes to the recovery of damaged colon tissue in a DSS-induced acute colitis model. Further studies are needed to identify the factors in conditioned mADSCs secretome involved in the suppression of pro-inflammatory cytokines in damaged colon tissue.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel diseases (IBD) causing chronic and destructive inflammation of the gastrointestinal tract is mainly related to uncontrolled immune response. Current treatment for IBD using immunosuppressive agents is not successful for significant improvement in remission rates. It is necessary to develop effective, feasible and safe therapeutic strategy for IBD. Stem cells having the ability to regulate immune response have emerged as attractive therapeutic tools for incurable IBD.

Research motivation

Mesenchymal stem cells (MSCs) including adipose-derived stem cells (ADSCs) has been known as a promising therapeutic for IBD. However, the transplantation of MACSs in IBD patients showed inconsistent results because of their distinct differentiation and regenerative potential, and the variety of protocols for treatment. In addition, the therapeutic ability of MSCs is mostly mediated by their secretome including cytokines, chemokines, growth factor, and hormones. Therefore, it has been suggested that the MSCs secretome may have therapeutic potential for IBD treatment.

Research objectives

Although the immune-modulatory activity of ADSC is mediated by soluble paracrine factors, it is still unclear whether the therapeutic efficacy of soluble factors secreted from ADSCs is better than ADSCs themselves in inflammation-related diseases. Therefore, the purpose of this study was to investigate the effects of conditioned mouse ADSCs (mADSCs) secretome on colitis-induced mice.

Research methods

mADSCs were isolated from C57BL/6 mice (female, 8-10 wk-old) and identified using fluorescence-activated cell sorting. The conditioned mADSCs secretome was obtained by culturing mADSCs in serum-free medium with lipopolysaccharide (1 μg/mL) for 24 h, and characterized using a Proteome Profiler mouse cytokine array kit. For induction of mouse acute colitis, mice (C57BL/6. female, 8-wk-old) were supplied with 2% dextran sodium sulfate for 7 d and then normal drinking water for 4 d. Animals were divided into 4 groups, control (CON) group receiving normal drinking water during the experimental period, normal culture medium (NM) group receiving 100 μL normal culture medium three times (on 4, 6 and 8 d), mADSCs (SC) group receiving 1 × 10^5 cells/100 μL ADSCs once (on 4 d) and conditioned mADSCs secretome (CM) group receiving 100 μL mADSCs secretome three times (on days 4, 6, and 8). The length of the colon and histopatholgy of colon tissues were evaluated after sacrificing animals on 11 d. The mRNA expression levels of inflammatory cytokines in colon

tissue were determined by quantitative real-time polymerase chain reaction assay, and the serum interleukin (IL)-6 levels were determined by enzyme-linked immunosorbent assay.

Research results

The isolated mADSCs maintained the mADSCs specific gene expression profiles during experiment. The conditioned mADSCs secretome obtained by culturing mADSCs with 1 μg/mL of lipopolysaccharides for 24 h contained high amounts of colony-stimulating factors, inflammatory chemokines and cytokines. The conditioned mADSCs secretome in dextran sulfate sodium (DSS)-induced mice ameliorated the loss of body weight and reduction of colon length, and improved the recovery of damaged colon tissue. The expression of IL-1b mRNA and IL-6 mRNA in colon tissues was significantly lower ($P < 0.05$) in the CM group than in the SC group ($P < 0.05$) and/or NM group ($P < 0.005$). However, the expression level of tumor necrosis factor-α mRNAs in the NM and CM groups did not differ from that in the CON group. The concentration of serum IL-6 in DSS-induced mice was significantly lower in the CM group (5.94 pg/mL, $P < 0.005$) and the SC group (38.01 pg/mL, $P < 0.05$) than in NM group (207.40 pg/mL), which indicates that the conditioned mADSCs secretome suppressed the elevation of serum IL-6 concentration in DSS-induced mice.

Research conclusions

This study suggests that conditioned mADSCs secretome may inhibit the synthesis of pro-inflammatory cytokines in the damaged colon tissue, and reduce serum IL-6 Levels more effectively than mADSCs themselves in DSS-induced mice. This effective suppression of proinflammatory cytokines in colon tissue and serum might mainly contributes to the recovery of damaged colon tissue in DSS-induced mice, which suggests the therapeutic potential of the conditioned mADSCs secretome for IBD treatment.

Research perspectives

The conditioned mADSCs secretome may serve as a novel therapeutic tool for IBD. Therefore, it is needed to investigate the factors in conditioned mADSCs secretome involved in the effective regeneration of damaged colon tissue.

REFERENCES

1 **Bouma G**, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003; **3**: 521-533 [PMID: 12876555 DOI: 10.1038/nri1132]

2 **Kaser A**, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol* 2010; **28**: 573-621 [PMID: 20192811 DOI: 10.1146/annurev-immunol-030409-101225]

3 **Pithadia AB**, Jain S. Treatment of inflammatory bowel disease (IBD). *Pharmacol Rep* 2011; **63**: 629-642 [PMID: 21857074 DOI: 10.1016/s1734-1140(11)70575-8]

4 **Sandborn WJ**, Feagan BG, Stoinov S, Honiball PJ, Rutgeerts P, Mason D, Bloomfield R, Schreiber S; PRECISE 1 Study Investigators. Certolizumab pegol for the treatment of Crohn's disease. *N Engl J Med* 2007; **357**: 228-238 [PMID: 17634458 DOI: 10.1056/NEJMoa067594]

5 **Watt FM**, Hogan BL. Out of Eden: stem cells and their niches. *Science* 2000; **287**: 1427-1430 [PMID: 10688781 DOI: 10.1126/science.287.5457.1427]

6 **Leiman DA**, Lichtenstein GR. Therapy of inflammatory bowel disease: what to expect in the next decade. *Curr Opin Gastroenterol* 2014; **30**: 385-390 [PMID: 24902037 DOI: 10.1097/MOG.0000000000000077]

7 **De Ugarte DA**, Alfonso Z, Zuk PA, Elbarbary A, Zhu M, Ashjian P, Benhaim P, Hedrick MH, Fraser JK. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunol Lett* 2003; **89**: 267-270 [PMID: 14556988 DOI: 10.1016/s0165-2478(03)00108-1]

8 **Orciani M**, Di Primio R. Skin-derived mesenchymal stem cells: isolation, culture, and characterization. *Methods Mol Biol* 2013; **989**: 275-283 [PMID: 23483402 DOI: 10.1007/978-1-62703-330-5_21]

9 **Zheng B**, Cao B, Crisan M, Sun B, Li G, Logar A, Yap S, Pollett JB, Drowley L, Cassino T, Gharaibeh B, Deasy BM, Huard J, Péault B. Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nat Biotechnol* 2007; **25**: 1025-1034 [PMID: 17767154 DOI: 10.1038/nbt1334]

10 **Villaron EM**, Almeida J, López-Holgado N, Alcoceba M, Sánchez-Abarca LI, Sanchez-Guijo FM, Alberca M, Pérez-Simon JA, San Miguel JF, Del Cañizo MC. Mesenchymal stem cells are present in peripheral blood and can engraft after allogeneic hematopoietic stem cell transplantation. *Haematologica* 2004; **89**: 1421-1427 [PMID: 15590390]

11 **Baksh D**, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med* 2004; **8**: 301-316 [PMID: 15491506 DOI: 10.1111/j.1582-4934.2004.tb00320.x]

12 **Kolf CM**, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther* 2007; **9**: 204 [PMID: 17316462 DOI: 10.1186/ar2116]

13 **Jiang XX**, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, Mao N. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005; **105**: 4120-4126 [PMID: 15692068 DOI: 10.1182/blood-2004-02-0586]

14 **Nauta AJ**, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 2007; **110**: 3499-3506 [PMID: 17664353 DOI: 10.1182/blood-2007-02-069716]

15 **Teo GS**, Ankrum JA, Martinelli R, Boetto SE, Simms K, Sciuto TE, Dvorak AM, Karp JM, Carman CV. Mesenchymal stem cells transmigrate between and directly through tumor necrosis factor-α-activated endothelial cells *via* both leukocyte-like and novel mechanisms. *Stem Cells* 2012; **30**: 2472-2486 [PMID: 22887987 DOI: 10.1002/stem.1198]

16 **Deans RJ**, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol* 2000; **28**: 875-884 [PMID: 10989188 DOI: 10.1016/s0301-472x(00)00482-3]

17 **Gnecchi M**, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* 2008; **103**: 1204-1219 [PMID: 19028920 DOI: 10.1161/CIRCRESAHA.108.176826]

18 **Parekkadan B**, Milwid JM. Mesenchymal stem cells as therapeutics. *Annu Rev Biomed Eng* 2010; **12**: 87-117 [PMID: 20415588 DOI: 10.1146/annurev-bioeng-070909-105309]

19 **Eggenhofer E**, Benseler V, Kroemer A, Popp FC, Geissler EK, Schlitt HJ, Baan CC, Dahlke MH, Hoogduijn MJ. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol* 2012; **3**: 297 [PMID: 23056000 DOI: 10.3389/fimmu.2012.00297]

20 **Griffin MD**, Elliman SJ, Cahill E, English K, Ceredig R, Ritter T. Concise review: adult mesenchymal stromal cell therapy for inflammatory diseases: how well are we joining the dots? *Stem Cells* 2013; **31**: 2033-2041 [PMID: 23766124 DOI: 10.1002/stem.1452]

21 **Flores AI**, Gómez-Gómez GJ, Masedo-González Á, Martínez-Montiel MP. Stem cell therapy in inflammatory bowel disease: A promising therapeutic strategy? *World J Stem Cells* 2015; **7**: 343-351 [PMID: 25815119 DOI: 10.4252/wjsc.v7.i2.343]

22 **L PK**, Kandoi S, Misra R, S V, K R, Verma RS. The mesenchymal stem cell secretome: A new paradigm towards cell-free therapeutic mode in regenerative medicine. *Cytokine Growth Factor Rev* 2019; **46**: 1-9 [PMID: 30954374 DOI: 10.1016/j.cytogfr.2019.04.002]

23 **Madrigal M**, Rao KS, Riordan NH. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J Transl Med* 2014; **12**: 260 [PMID: 25304688 DOI: 10.1186/s12967-014-0260-8]

24 **English K**. Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol Cell Biol* 2013; **91**: 19-26 [PMID: 23090487 DOI: 10.1038/icb.2012.56]

25 **González-González A**, García-Sánchez D, Dotta M, Rodríguez-Rey JC, Pérez-Campo FM. Mesenchymal stem cells secretome: The cornerstone of cell-free regenerative medicine. *World J Stem Cells* 2020; **12**: 1529-1552 [PMID: 33505599 DOI: 10.4252/wjsc.v12.i12.1529]

26 **Vizoso FJ**, Eiro N, Cid S, Schneider J, Perez-Fernandez R. Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. *Int J Mol Sci* 2017; **18** [PMID: 28841158 DOI: 10.3390/ijms18091852]

27 **Osugi M**, Katagiri W, Yoshimi R, Inukai T, Hibi H, Ueda M. Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. *Tissue Eng Part A* 2012; **18**: 1479-1489 [PMID: 22443121 DOI: 10.1089/ten.TEA.2011.0325]

28 **Pouya S**, Heidari M, Baghaei K, Asadzadeh Aghdaei H, Moradi A, Namaki S, Zali MR, Hashemi SM. Study the effects of mesenchymal stem cell conditioned medium injection in mouse model of acute colitis. *Int Immunopharmacol* 2018; **54**: 86-94 [PMID: 29112894 DOI: 10.1016/j.intimp.2017.11.001]

29 **Xia X**, Chiu PWY, Lam PK, Chin WC, Ng EKW, Lau JYW. Secretome from hypoxia-conditioned adipose-derived mesenchymal stem cells promotes the healing of gastric mucosal injury in a rodent model. *Biochim Biophys Acta Mol Basis Dis* 2018; **1864**: 178-188 [PMID: 28993190 DOI: 10.1016/j.bbadis.2017.10.009]

30 **van Buul GM**, Villafuertes E, Bos PK, Waarsing JH, Kops N, Narcisi R, Weinans H, Verhaar JA, Bernsen MR, van Osch GJ. Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. *Osteoarthritis Cartilage* 2012; **20**: 1186-1196 [PMID: 22771777 DOI: 10.1016/j.joca.2012.06.003]

31 **Ranganath SH**, Levy O, Inamdar MS, Karp JM. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell* 2012; **10**: 244-258 [PMID: 22385653 DOI: 10.1016/j.stem.2012.02.005]

32 **Kupcova Skalnikova H**. Proteomic techniques for characterisation of mesenchymal stem cell secretome. *Biochimie* 2013; **95**: 2196-2211 [PMID: 23880644 DOI: 10.1016/j.biochi.2013.07.015]

33 **Dieleman LA**, Palmen MJ, Akol H, Bloemena E, Peña AS, Meuwissen SG, Van Rees EP. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* 1998; **114**: 385-391 [PMID: 9844047 DOI: 10.1046/j.1365-2249.1998.00728.x]

34 **Strioga M**, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? *vs***21**: 2724-2752 [PMID: 22468918 DOI: 10.1089/scd.2011.0722]

35 **Fraser JK**, Wulur I, Alfonso Z, Hedrick MH. Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol* 2006; **24**: 150-154 [PMID: 16488036 DOI: 10.1016/j.tibtech.2006.01.010]

36 **Choi EW**, Shin IS, Park SY, Yoon EJ, Kang SK, Ra JC, Hong SH. Characteristics of mouse adipose tissue-derived stem cells and therapeutic comparisons between syngeneic and allogeneic adipose tissue-derived stem cell transplantation in experimental autoimmune thyroiditis. *Cell Transplant* 2014; **23**: 873-887 [PMID: 23485102 DOI: 10.3727/096368913X664586]

37 **Salgado AJ**, Reis RL, Sousa NJ, Gimble JM. Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine. *Curr Stem Cell Res Ther* 2010; **5**: 103-110 [PMID: 19941460 DOI: 10.2174/157488810791268564]

38 **Kapur SK**, Katz AJ. Review of the adipose derived stem cell secretome. *Biochimie* 2013; **95**: 2222-2228 [PMID: 23770442 DOI: 10.1016/j.biochi.2013.06.001]

39 **Kilroy GE**, Foster SJ, Wu X, Ruiz J, Sherwood S, Heifetz A, Ludlow JW, Stricker DM, Potiny S, Green P, Halvorsen YD, Cheatham B, Storms RW, Gimble JM. Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. *J Cell Physiol* 2007; **212**: 702-709 [PMID: 17477371 DOI: 10.1002/jcp.21068]

40 **Stavely R**, Robinson AM, Miller S, Boyd R, Sakkal S, Nurgali K. Human adult stem cells derived from adipose tissue and bone marrow attenuate enteric neuropathy in the guinea-pig model of acute colitis. *Stem Cell Res Ther* 2015; **6**: 244 [PMID: 26652292 DOI: 10.1186/s13287-015-0231-x]

41 **González MA**, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. *Arthritis Rheum* 2009; **60**: 1006-1019 [PMID: 19333946 DOI: 10.1002/art.24405]

42 **González MA**, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009; **136**: 978-989 [PMID: 19135996 DOI: 10.1053/j.gastro.2008.11.041]

43 **García-Olmo D**, García-Arranz M, Herreros D, Pascual I, Peiro C, Rodríguez-Montes JA. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. *Dis Colon Rectum* 2005; **48**: 1416-1423 [PMID: 15933795 DOI: 10.1007/s10350-005-0052-6]

44 **Liang J**, Zhang H, Wang D, Feng X, Wang H, Hua B, Liu B, Sun L. Allogeneic mesenchymal stem cell transplantation in seven patients with refractory inflammatory bowel disease. *Gut* 2012; **61**: 468-469 [PMID: 21617158 DOI: 10.1136/gutjnl-2011-300083]

45 **Gonçalves Fda C**, Schneider N, Pinto FO, Meyer FS, Visioli F, Pfaffenseller B, Lopez PL, Passos EP, Cirne-Lima EO, Meurer L, Paz AH. Intravenous *vs* intraperitoneal mesenchymal stem cells administration: what is the best route for treating experimental colitis? *World J Gastroenterol* 2014; **20**: 18228-18239 [PMID: 25561790 DOI: 10.3748/wjg.v20.i48.18228]

46 **Song JY**, Kang HJ, Hong JS, Kim CJ, Shim JY, Lee CW, Choi J. Umbilical cord-derived mesenchymal stem cell extracts reduce colitis in mice by re-polarizing intestinal macrophages. *Sci Rep* 2017; **7**: 9412 [PMID: 28842625 DOI: 10.1038/s41598-017-09827-5]

47 **Zhang JM**, An J. Cytokines, inflammation, and pain. *Int Anesthesiol Clin* 2007; **45**: 27-37 [PMID: 17426506 DOI: 10.1097/AIA.0b013e318034194e]

48 **Chen Z**, He X, Chen X, Lin X, Zou Y, Wu X, Lan P. Bone marrow mesenchymal stem cells ameliorate colitis-associated tumorigenesis in mice. *Biochem Biophys Res Commun* 2014; **450**: 1402-1408 [PMID: 25010644 DOI: 10.1016/j.bbrc.2014.07.002]

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ORIGINAL ARTICLE

Case Control Study

Pancreatic enzymes and abdominal adipose tissue distribution in new-onset prediabetes/diabetes after acute pancreatitis

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Abstract

BACKGROUND

New-onset prediabetes/diabetes after acute pancreatitis (NODAP) is the most common sequela of pancreatitis, and it differs from type 2 prediabetes/diabetes mellitus (T2DM).

AIM

To study the associations between circulating levels of pancreatic amylase, pancreatic lipase, chymotrypsin and fat phenotypes in NODAP, T2DM, and health.

METHODS

Individuals with NODAP (n = 30), T2DM (n = 30), and sex-matched healthy individuals (n = 30) were included. Five fat phenotypes (intra-pancreatic fat, liver fat, skeletal muscle fat, visceral fat, and subcutaneous fat) were determined using the same magnetic resonance imaging protocol and scanner magnet strength for all participants. One-way analysis of covariance, linear regression analysis, and relative importance analysis were conducted.

RESULTS

Intra-pancreatic fat deposition (IPFD) was higher in NODAP (9.4% ± 1.8%) and T2DM (9.8% ± 1.1%) compared with healthy controls (7.8% ± 1.9%) after adjusting for covariates (P = 0.003). Similar findings were observed in regards to visceral fat volume (P = 0.005), but not subcutaneous fat volume, liver fat, or skeletal muscle fat. Both IPFD (β = -2.201, P = 0.023) and visceral fat volume (β = -0.004, P = 0.028) were significantly associated with circulating levels of pancreatic amylase in NODAP, but not in T2DM or healthy individuals. Of the five fat phenotypes, IPFD explained the highest amount of variance in pancreatic amylase concentration (R^2 = 15.3% out of 41.2%). None of the phenotypes contributed meaningfully to the variance in pancreatic lipase or chymotrypsin.

CONCLUSION
Both NODAP and T2DM are characterized by increased IPFD and visceral fat volume. However, only NODAP is characterized by significant inverse associations between the two fat phenotypes and pancreatic amylase.

Key Words: Amylase; Lipase; Chymotrypsin; Pancreatitis; Diabetes; Intra-pancreatic fat; Visceral fat; Liver fat

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Core Tip: Intra-pancreatic fat deposition and visceral fat volume are significantly inversely associated with circulating levels of pancreatic amylase in individuals with new-onset prediabetes/diabetes after acute pancreatitis, but not in healthy individuals or those with type 2 prediabetes/diabetes mellitus.

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INTRODUCTION

Individuals after acute pancreatitis often develop metabolic sequelae such as post-pancreatitis diabetes, which accounts for 80% of cases of diabetes of the exocrine pancreas-the second most common type of adult-onset diabetes[1,2]. There is a circulating biomarker, involved in the regulation of exocrine pancreatic function, that distinguishes post-pancreatitis diabetes from type 2 diabetes[3]. Also, epidemiological data have shown that post-pancreatitis diabetes leads to worse clinical outcomes compared with type 2 diabetes. A population-based study found that individuals with post-pancreatitis diabetes were more likely to have poor glycemic control and to require more insulin than individuals with type 2 diabetes[4]. Another population-based study demonstrated that individuals with post-pancreatic diabetes (versus type 2 diabetes) were at a higher risk of mortality from cancer, gastrointestinal diseases, and infectious diseases, as well as hospitalization for chronic pulmonary disease, renal disease, and infectious disease[1]. The reasons for the above differences between post-pancreatic diabetes and type 2 diabetes are not fully understood but are worth investigating with a view to optimizing the management of both types of diabetes.

Excess deposition of body fat increases the risk of diabetes and has a deleterious effect on its clinical outcomes[5-9]. However, little evidence exists on the difference in excess body fat between post-pancreatitis diabetes and type 2 diabetes. A 2017 population-based study of people with new-onset diabetes showed that the proportion of individuals with obesity was higher in type 2 diabetes (48%) *vs* diabetes of the exocrine pancreas (35%)[4]. A 2020 population-based study of individuals with a history of clinically resolved acute pancreatitis demonstrated that the risk of new-onset diabetes was higher among individuals with normal body mass index (BMI) than those in the overall cohort (adjusted odds ratios of 3.1 and 2.1, correspondingly)[10]. BMI is a commonly used proxy for general adiposity but it may be suboptimal in quantifying excess abdominal fat[1,2]. Given that the effect of excess abdominal fat on metabolic functions depends on not only the degree of fat deposition but also its distribution[9,11-13], data on various abdominal fat phenotypes [determined with the use of magnetic resonance imaging (MRI)] are likely to provide useful insights[14-17]. For example, intra-pancreatic fat deposition (IPFD) and visceral fat volume were significantly higher in individuals with post-pancreatitis diabetes compared with healthy individuals[1]. Also, individuals with type 2 diabetes had a significantly higher IPFD compared with healthy individuals[18]. However, to date, no study has compared head-to-head abdominal fat phenotypes in post-pancreatitis diabetes *vs* type 2 diabetes. Also, individuals with metabolic disorders (including type 2 diabetes and obesity) not infrequently have within-normal but significantly lower circulating levels

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of pancreatic enzymes (amylase, lipase, and trypsin) compared with healthy individuals[19], and exocrine pancreatic dysfunction often develops after acute pancreatitis[2]. Hence, it is conceivable that the relationship between abdominal adipose tissue distribution and pancreatic enzymes may differ in post-pancreatitis diabetes *vs* type 2 diabetes.

The primary aim was to compare the differences in MRI-derived abdominal fat phenotypes between healthy individuals and the two types of diabetes. The secondary aim was to investigate the associations between abdominal fat phenotypes and circulating levels of pancreatic enzymes in the study groups.

MATERIALS AND METHODS

Study design

The present case-control study was nested into prospective cohort study of individuals after an attack of acute pancreatitis (ARIES project) and was approved by the Health and Disability Ethics Committee (13/STH/182) of New Zealand. Individuals with new-onset prediabetes or diabetes after acute pancreatitis (NODAP) were randomly selected and 1:1 matched on sex with individuals with type 2 prediabetes or diabetes mellitus (T2DM) from the same cohort. Individuals with fasting plasma glucose (FPG) ≥ 100 mg/dL (≥ 5.6 mmol/L) and/or glycated hemoglobin A1c (HbA1c) ≥ 5.7% (39 mmol/mol) beyond three months after an attack of acute pancreatitis constituted the NODAP group, in line with the published recommendations[1]. Individuals with HbA1c ≥ 5.7% (39 mmol/mol) before, during hospitalization for AP, or within three months after it constituted the T2DM group. FPG ≥ 100 mg/dL (≥ 5.6 mmol/L) during hospitalization was not considered as an eligibility criterion for the T2DM group due to the possibility of stress-induced hyperglycemia during acute illness[1]. All cases were at least 18 years old, provided informed consent, had a primary diagnosis of mild acute pancreatitis established prospectively at the time of hospitalization, and met the American Diabetes Association criteria for prediabetes or diabetes[6].

Individuals were excluded from the study if they had a recurrent attack of acute pancreatitis within three months of the enrollment date, chronic pancreatitis, post-endoscopic retrograde cholangiopancreatography pancreatitis, pancreatic cyst, pancreatic lipomatosis or lipomatous pseudohypertrophy, congenital anomalies of the pancreas, hereditary pancreatitis, cystic fibrosis, malignancy, cognitive disability, received surgical, endoscopic or radiological interventions involving the pancreas, had metallic foreign body implantations, heart pacemakers, or other electronic device implantations, received steroid therapy, or were pregnant.

The control group included healthy volunteers who were 1:1 matched on sex with the two case groups. These participants were at least 18 years old, provided informed consent, had no personal and family history of diseases of the exocrine pancreas and diabetes, had no family history of cystic fibrosis or coeliac diseases, had no upper abdominal symptoms in the 12 mo preceding the study, had no history or evaluation for infectious or inflammatory diseases in the 6 mo preceding the study, and had no history of cancer.

Quantification of fat phenotypes

Imaging protocol: Abdominal MRI for all participants was performed at the Centre of Advanced MRI (The University of Auckland) using 3.0 Tesla MAGNETOM Skyra scanner (Siemens, Erlangen, Germany). All participants underwent MRI wholly and exclusively for the purpose of the ARIES project. During the MRI, participants lied down in supine position and were asked to hold their breath during end-expiration. Axial T1-weighted volumetric interpolated breath-hold examination Dixon sequence was applied with the following parameters: true form abdomen shim mode; field of view, 420 mm; base resolution, 320; echo time, 1.27 ms, 2.5 ms; repetition time, 3.85 ms; flip angle, 9; pixel bandwidth, 920 Hz; slice thickness, 5 mm. All but liver fat phenotypes were quantified independently by two observers and average values of two independent MRI measurements were used for statistical analyses. The observers were blinded to the group allocation.

Intra-pancreatic fat: Intra-pancreatic fat was quantified using the 'MR-opsy' technique, as described in detail elsewhere[20]. In brief, two candidate slices with clear visualization of the pancreas were selected from a series of abdominal scans. Three regions of interest were placed in the head, body, and tail region of the pancreas for quantification of IPFD. Further, to prevent possible inclusion of non-parenchymal tissues

within the selected area of interest, a thresholding range of 1%-20% was applied, as recommended[21]. The intra-pancreatic fat percentage was calculated as the average pancreatic fat fraction of both slices.

Liver fat: Single-voxel spectroscopy was used to quantify liver fat. A voxel (20 mm × 20 mm × 20 mm) was placed in the right lobe of the liver, away from the blood vessels and bile ducts and at least 10 mm away from the edge. Automated shimming was performed prior to signal acquisition to improve B0 homogeneity. Spectra were acquired using a free-breathing navigator-triggered spin echo acquisition with repetition time 3000 ms, echo time 33 ms, 50 averages. Acquisition duration was 853 ms. Both water-suppressed and non-water-suppressed spectra were taken, with the non-water-suppressed spectrum acting as a reference for liver fat quantification. Spectra were processed and analyzed using SIVIC software (University of California-San Francisco, California, United States)[22]. The magnetic resonance spectroscopy fat fraction was defined as fat fraction = area under fat peak/area under fat and water peaks × 100%.

Skeletal muscle fat: Total muscle area and intra-muscular fat area of erector spinae muscles were measured using a single axial slice at the lower endplate of L3 vertebra, as it had been demonstrated that the L3 level is optimal for determination of skeletal muscle fat[23]. The free-hand tool of ImageJ software (National Institutes of Health, United States) was used to outline the left and right erector spinae muscles followed by measurement of total pixel content[23,24]. Further, to calculate the intra-muscular fat area, the threshold-function of ImageJ was used to convert grayscale pixels into binary images, using global histogram-derived method. Care was taken not to include extra-muscular fat (*i.e.*, beyond the fascial layer of the erector spinae muscles). Total muscle area and intra-muscular fat area were calculated by multiplying the selected total pixel content with pixel surface area. The ratio of fat-free cross-sectional muscle area to total cross-sectional muscle area was determined by subtracting intra-muscular fat area from the total muscle area and dividing this value by the total muscle area. Skeletal muscle fat percentage was defined as (1-fat-free cross-sectional muscle area to total cross-sectional muscle area ratio) × 100%.

Subcutaneous and visceral fat: Visceral fat volume and subcutaneous fat volume were quantified manually using ImageJ software. Identical fat-phase images (L2-L5) from the selected series were used for segmentation of visceral and subcutaneous fat compartments[25]. The threshold-function of ImageJ was used to convert grayscale pixels into binary images, using the global histogram-derived method[24]. Using the free-hand tool, visceral and subcutaneous fat regions were delineated from the abdominal musculature and measured separately. The non-adipose tissue, soft organs, and blood vessels were excluded from the measurement of visceral fat. The final step for all the above measurements involved summation of the pixel contents of all the slices in series and multiplied by the pixel area and slice thickness to obtain the total volume.

Measurement of pancreatic enzymes

Venous blood samples were obtained from each participant after at least 8 h of fasting to assess pancreatic enzymes. These blood samples were centrifuged 4000 *g* for 5.5 min and plasma was separated into aliquots and stored at -80 °C until further use. The active form of pancreatic amylase was measured in plasma using the Reflotron® Plus reflectance photometer (Roche®, Basel, Switzerland) and results were expressed in U/L. The active forms of pancreatic lipase and chymotrypsin were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits. Pancreatic lipase was measured using the Cloud-Clone Corporation ELISA kit (Houston, Texas, United States) and results were expressed in ng/mL. The intra- and inter-assay variations of the assay were < 10% and < 12%, respectively. Chymotrypsin concentration was measured using the Cusabio ELISA kit (Wuhan, Hubei Province, China) and results were expressed in ng/mL. The intra- and inter-assay variations of the assay were < 8% and < 10%, respectively. Absorbance was detected at 450 nm. Concentrations in each sample were estimated using a standard curve.

Covariates

Anthropometric data (height, weight, and waist circumference) of all study participants were recorded to calculate BMI and waist-height ratio. All measurements were taken over the light clothing of participants, and height and weight were measured in a standing position without shoes and headgear. Waist circumference

was measured at the level of the umbilicus. Blood samples for lipids (triglycerides, total cholesterol, high-density lipoproteins cholesterol, and low-density lipoprotein cholesterol) were measured at LabPlus-a tertiary referral medical laboratory at Auckland City Hospital. The same laboratory measured HbA1c, using the boronate affinity chromatography assay (Trinity Biotech, Wicklow, Ireland) that is certified by the National Glycohaemoglobin Standardisation Program and standardized to the Diabetes Control and Complications Trial reference assay. Fasting insulin was measured using chemiluminescence sandwich immunoassay (Roche Diagnostics, Auckland, New Zealand).

A standardized questionnaire was administered at the time of the study. For information on the use of antidiabetic medications, participants were asked, 'Are you currently on antidiabetic mediation?'. If the answer was 'yes', they were classified as antidiabetic medication user, otherwise they were classified as non-user. For information on smoking status, participants were asked, 'Have you ever smoked cigarettes?'. If the answer was 'yes', they were classified as ever-smokers, otherwise, they were classified as never-smokers[26]. For information on alcohol consumption, participants were asked, 'On average, how much alcohol do you consume in a week?'. A reference diagram for drink volumes *per* unit was provided. The response to this question was presented as grams *per* week and used to determine the average amount of alcohol consumption[26].

Statistical analysis

All analyses were performed using SPSS for Windows Version 25 (SPSS Inc., Illinois, United States). A two-sided $P < 0.05$ was deemed to be statistically significant. The mean and standard deviation of the five studied abdominal fat phenotypes (*i.e.*, intra-pancreatic fat, liver fat, skeletal muscle fat, visceral fat, and subcutaneous fat) in the three groups (NODAP, T2DM, and healthy controls) were compared using one-way analysis of variance (ANOVA).

To examine the differences in the five studied abdominal fat phenotypes between the three groups, one-way ANOVA and one-way analysis of covariance (ANCOVA) were conducted. ANCOVA enabled the reduction of within-group variance while adjusting for covariates. The following four models were constructed: (1) Unadjusted; (2) Adjusted for age and sex; (3) Adjusted for age, sex, triglycerides, and HbA1c; and (4) Adjusted for age, sex, triglycerides, HbA1c, BMI, use of antidiabetic medications, alcohol consumption, and smoking status. The Fisher's least significant difference method was used for post-hoc pair-wise comparisons.

To investigate the associations between the five fat phenotypes and pancreatic enzymes (pancreatic amylase, pancreatic lipase, and chymotrypsin) in each study group, linear regression analyses were conducted. In these analyses, each abdominal fat phenotype was entered as an independent variable and concentrations of pancreatic enzymes were treated as the dependent variable. In addition, relative importance of each abdominal fat phenotype in explaining the variance of pancreatic enzymes concentrations was determined in each study group. Using the 'relaimpo' package in R Studio Version3.6.1 (RStudio Inc., Massachusetts, United States), a multivariable linear regression model was constructed in each study group, including the five abdominal fat phenotypes as independent variables and pancreatic enzymes concentrations as the dependent variable[27]. The resulting individual R^2 values of all the independent variables were obtained and plotted.

RESULTS

Characteristics of study participants

A total of 90 individuals were included (30 NODAP, 30 T2DM, and 30 healthy controls). The median time since the last attack of pancreatitis was 29 mo (interquartile range, 15.7-42.8 mo) and 29 mo (interquartile range, 21.1-36.2 mo) in the NODAP group and T2DM group, respectively. Other characteristics are presented in Table 1.

Abdominal fat phenotypes in the study groups

The intra-pancreatic fat percentage was 9.4 ± 1.8%, 9.8 ± 1.1%, and 7.8 ± 1.9% in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was statistically significant in both the unadjusted ($P < 0.001$) and all the adjusted models ($P = 0.002$ in model 2; $P = 0.001$ in model 3; $P = 0.003$ in model 4).

Table 1 Characteristics of the study groups at the time of magnetic resonance imaging

Characteristic	Healthy controls (*n* = 30)	T2DM (*n* = 30)	NODAP (*n* = 30)	*P* value[1]
Age (yr)	50.0 (36.5-68.8)	55.5 (41.8-66.3)	58.5 (48.5-67.3)	0.213
Men, *n* (%)	21 (70.0)	21 (70.0)	21 (70.0)	1.000
Body mass index (kg/m^2)	24.0 (21.8-28.1)	30.3 (26.4-35.4)	27.5 (24.1-32.7)	< 0.001
Waist-height ratio	0.5 (0.5-0.5)	0.6 (0.5-0.6)	0.6 (0.5-0.6)	< 0.001
Triglycerides (mmol/L)	1.0 (0.6-1.2)	1.7 (1.3-3.7)	2.2 (1.3-3.6)	0.035
Total cholesterol (mmol/L)	4.5 (3.4-5.6)	5.1 (3.9-5.8)	5.1 (4.1-5.7)	0.388
HDL cholesterol (mmol/L)	1.2 (0.8-1.8)	1.2 (0.9-1.4)	1.2 (1.1-1.4)	0.680
LDL cholesterol (mmol/L)	2.7 (2.0-3.4)	2.7 (1.6-3.3)	2.6 (2.1-3.5)	0.542
Fasting insulin (mIU/L)	11.8 (4.8-16.6)	12.2 (8.2-17.2)	13.3 (7.4-18.3)	0.523
HOMA-IR (mIU/L·mmol/L)	2.8 (1.2-3.3)	3.3 (2.1-5.1)	3.3 (1.9-5.3)	0.244
Smoking status[2]	0 (0-1)	0 (0-1)	1 (0-1)	0.016
Alcohol consumption (g/wk)	39 (4.5-96)	12 (6-144)	12 (0-108)	0.279
Amylase (U/L)	29.0 (20.2-33.9)	20.2 (14.0-31.5)	28.7 (19.4-33.3)	0.158
Lipase (pg/mL)	7.2 (5.5-8.7)	7.6 (5.9-10.5)	6.5 (5.5-8.6)	0.340
Chymotrypsin (U/L)	6.2 (5.2-7.1)	4.6 (2.9-6.7)	5.9 (4.9-6.6)	0.314

Data are presented as median and interquartile range or percentage.
[1]*P* values were from one-way analysis of variance.
[2]Smoking status was classified as either ever-smokers or never-smokers.
T2DM: Type 2 prediabetes/diabetes mellitus; NODAP: New-onset prediabetes/diabetes after acute pancreatitis; HDL: High-density lipoproteins; LDL: Low-density lipoprotein; HOMA-IR: Homeostasis model assessment of insulin resistance.

The liver fat percentage was 12.0 ± 9.7%, 11.3 ± 11.1%, and 9.3 ± 7.7% in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was not statistically significant in all the models.

The skeletal muscle fat percentage was 14.9 ± 6.1%, 15.5 ± 6.0%, and 14.1 ± 7.0% in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was not statistically significant in all the models.

The visceral fat volume was 2205.0 ± 1098.1 cm^3, 2622.5 ± 1172.2 cm^3, and 1208.8 ± 808.1cm^3 in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was statistically significant in both the unadjusted (P < 0.001) and all the adjusted models (P = 0.010 in model 2; P = 0.001 in model 3; P = 0.005 in model 4).

The subcutaneous fat volume was 3011.4 ± 1432.2 cm^3, 3463.0 ± 1323.7 cm^3, and 2523.8 ± 1437.7 cm^3 in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was statistically significant in the unadjusted (P = 0.013) and two adjusted models (P = 0.038 in model 3; P = 0.034 in model 4).

Results of all the pair-wise comparisons between the study groups are presented in Table 2.

Associations between abdominal fat phenotypes and pancreatic enzymes in the study groups

In the NODAP group, the five abdominal fat phenotypes altogether explained 41.2% of the variance in pancreatic amylase, 4.5% of the variance in pancreatic lipase, and 11.1% of the variance in chymotrypsin. Of the fat phenotypes studied, the variance in pancreatic amylase concentration was explained the most by IPFD (R^2 = 15.3%) (Figure 1A); the variance in pancreatic lipase concentration was explained the most by IPFD (R^2 = 3.0%) (Figure 2A); the variance in chymotrypsin concentration was explained the most by liver fat (R^2 = 6.5%) (Figure 3A). IPFD and visceral fat volumes were significantly associated with pancreatic amylase concentration (β = -2.201, P = 0.023; and β = -0.004, P = 0.028, correspondingly). The other abdominal fat phenotypes were not significantly associated with the studied pancreatic enzymes (Table 3).

Table 2 Differences in abdominal fat phenotypes between the study groups

Fat phenotype	Overall[1]	T2DM *vs* NODAP	T2DM *vs* healthy controls	NODAP *vs* healthy controls
Intra-pancreatic fat (%)				
Model 1	< 0.001	0.290	< 0.001	0.001
Model 2	0.002	0.555	0.001	0.004
Model 3	0.001	0.440	0.001	0.001
Model 4	0.003	0.187	0.002	0.012
Liver fat (%)				
Model 1	0.416	0.806	0.322	0.211
Model 2	0.681	0.457	0.983	0.465
Model 3	0.556	0.998	0.393	0.312
Model 4	0.612	0.428	0.329	0.801
Skeletal muscle fat (%)				
Model 1	0.348	0.148	0.516	0.420
Model 2	0.329	0.137	0.455	0.489
Model 3	0.585	0.319	0.692	0.544
Model 4	0.477	0.243	0.551	0.460
Visceral fat (cm^3)				
Model 1	< 0.001	0.093	< 0.001	0.001
Model 2	0.010	0.596	0.005	0.012
Model 3	0.001	0.249	< 0.001	0.003
Model 4	0.005	0.787	0.012	0.003
Subcutaneous fat (cm^3)				
Model 1	0.013	0.214	0.003	0.082
Model 2	0.740	0.488	0.508	0.987
Model 3	0.038	0.245	0.012	0.081
Model 4	0.034	0.063	0.009	0.301

[1]Overall *P* values were from one-way analysis of variance in model 1 (unadjusted) and one-way analysis of covariance in models 2-4 (adjusted as described below). Data are presented as *P* values. Fisher's least significant difference method was used in pair-wise comparison. Model 2: Adjusted for age and sex; Model 3: Adjusted for age, sex, triglycerides, and glycated hemoglobin A1c; Model 4: Adjusted for age, sex, triglycerides, glycated hemoglobin A1c, use of antidiabetic medications, average weekly alcohol consumption, and smoking status. T2DM: Type 2 prediabetes/diabetes mellitus; NODAP: New-onset prediabetes/diabetes after acute pancreatitis.

In the T2DM group, the five abdominal fat phenotypes altogether explained 4.0% of the variance in pancreatic amylase, 14.7% of the variance in pancreatic lipase, and 29.9% of the variance in chymotrypsin. Of the fat phenotypes studied, the variance in pancreatic amylase concentration was explained the most by skeletal muscle fat (R^2 = 1.5%) (Figure 1B); the variance in pancreatic lipase concentration was explained the most by skeletal muscle fat (R^2 = 10.7%) (Figure 2B); the variance in chymotrypsin concentration was explained the most by IPFD (R^2 = 8.9%) (Figure 3B). None of the abdominal fat phenotypes were significantly associated with the studied pancreatic enzymes (Table 3).

In the healthy controls group, the five abdominal fat phenotypes altogether explained 17.8% of the variance in pancreatic amylase, 31.3% of the variance in pancreatic lipase, and 7.5% of the variance in chymotrypsin. Of the abdominal fat phenotypes studied, the variance in pancreatic amylase concentration was explained the most by liver fat (R^2 = 7.1%) (Figure 1C); the variance in pancreatic lipase concentration was explained the most by skeletal muscle fat (R^2 = 13.0%) (Figure 2C); the variance in chymotrypsin concentration was explained the most by visceral fat (R^2 = 4.0%) (Figure 3C). Skeletal muscle fat percentage was significantly associated with

Table 3 Associations between abdominal fat phenotypes and pancreatic enzymes

Fat phenotype	Healthy			T2DM			NODAP		
	β	S.E.	*P* value	β	S.E.	*P* value	β	S.E.	*P* value
Intra-pancreatic fat (%)									
Pancreatic amylase	-0.832	2.220	0.712	0.573	1.709	0.741	-2.201	0.899	0.023
Pancreatic lipase	0.258	0.421	0.546	-0.631	0.397	0.876	1.343	1.462	0.367
Chymotrypsin	-0.137	0.179	0.452	-0.579	0.610	0.343	0.164	0.270	0.550
Liver fat (%)									
Pancreatic amylase	0.685	0.444	0.140	-0.022	0.157	0.890	-0.493	0.331	0.151
Pancreatic lipase	-0.136	0.089	0.141	-0.134	0.351	0.709	-0.098	0.280	0.730
Chymotrypsin	0.002	0.040	0.967	-0.071	0.069	0.314	0.059	0.051	0.259
Skeletal muscle fat (%)									
Pancreatic amylase	-0.570	0.595	0.351	-0.241	0.474	0.619	-0.185	0.125	0.151
Pancreatic lipase	0.195	0.093	0.047	-1.345	0.912	0.161	-0.071	0.446	0.875
Chymotrypsin	-0.023	0.044	0.609	-0.129	0.105	0.238	-0.014	0.086	0.868
Visceral fat (cm^3)									
Pancreatic amylase	0.002	0.005	0.664	0.000	0.002	0.841	-0.004	0.002	0.028
Pancreatic lipase	-0.588	0.839	0.490	-3.247	0.462	0.493	0.533	0.250	0.833
Chymotrypsin	-0.314	0.362	0.394	0.287	0.566	0.619	0.240	0.458	0.606
Subcutaneous fat (cm^3)									
Pancreatic amylase	0.003	0.002	0.224	0.000	0.002	0.821	-0.002	0.001	0.232
Pancreatic lipase	-0.573	0.482	0.246	-2.032	3.444	0.564	-0.036	1.931	0.985
Chymotrypsin	-0.030	0.215	0.890	-0.527	0.534	0.338	-0.091	0.350	0.797

Data are presented as beta coefficients, standard errors, and *P* values (from linear regression). T2DM: Type 2 prediabetes/diabetes mellitus; NODAP: New-onset prediabetes/diabetes after acute pancreatitis.

pancreatic lipase concentration (β = 0.195, *P* = 0.047). The other abdominal fat phenotypes were not significantly associated with the studied pancreatic enzymes (Table 3).

DISCUSSION

In the present study, a uniformed MRI protocol on a single 3T scanner was used to comprehensively compare, for the first time, abdominal fat phenotypes in 90 matched individuals with NODAP, T2DM, and healthy controls. An important finding of the study was that both NODAP and T2DM were characterized by a significantly larger amount of intra-pancreatic fat and visceral fat (but not liver fat, skeletal muscle fat, or subcutaneous fat) compared with healthy controls, consistently in both unadjusted and all the adjusted analyses. In addition, IPFD and visceral fat volume were significantly inversely associated with circulating levels of pancreatic amylase in the NODAP group (but not the other two groups). The relative importance analyses revealed that the five studied abdominal fat phenotypes altogether explained 41% of the variance in pancreatic amylase concentration in individuals with NODAP. By contrast, only 4% and 13% of the variance in this pancreatic enzyme was explained by the five abdominal phenotypes in individuals with T2DM and healthy controls, respectively.

Abdominal fat phenotypes are often used to differentiate between type 1 diabetes and the much more common type 2 diabetes. Several studies have compared head-to-head these two types of diabetes and have agreed that individuals with type 1 diabetes typically have lower levels of adiposity (as evidenced by both BMI and visceral

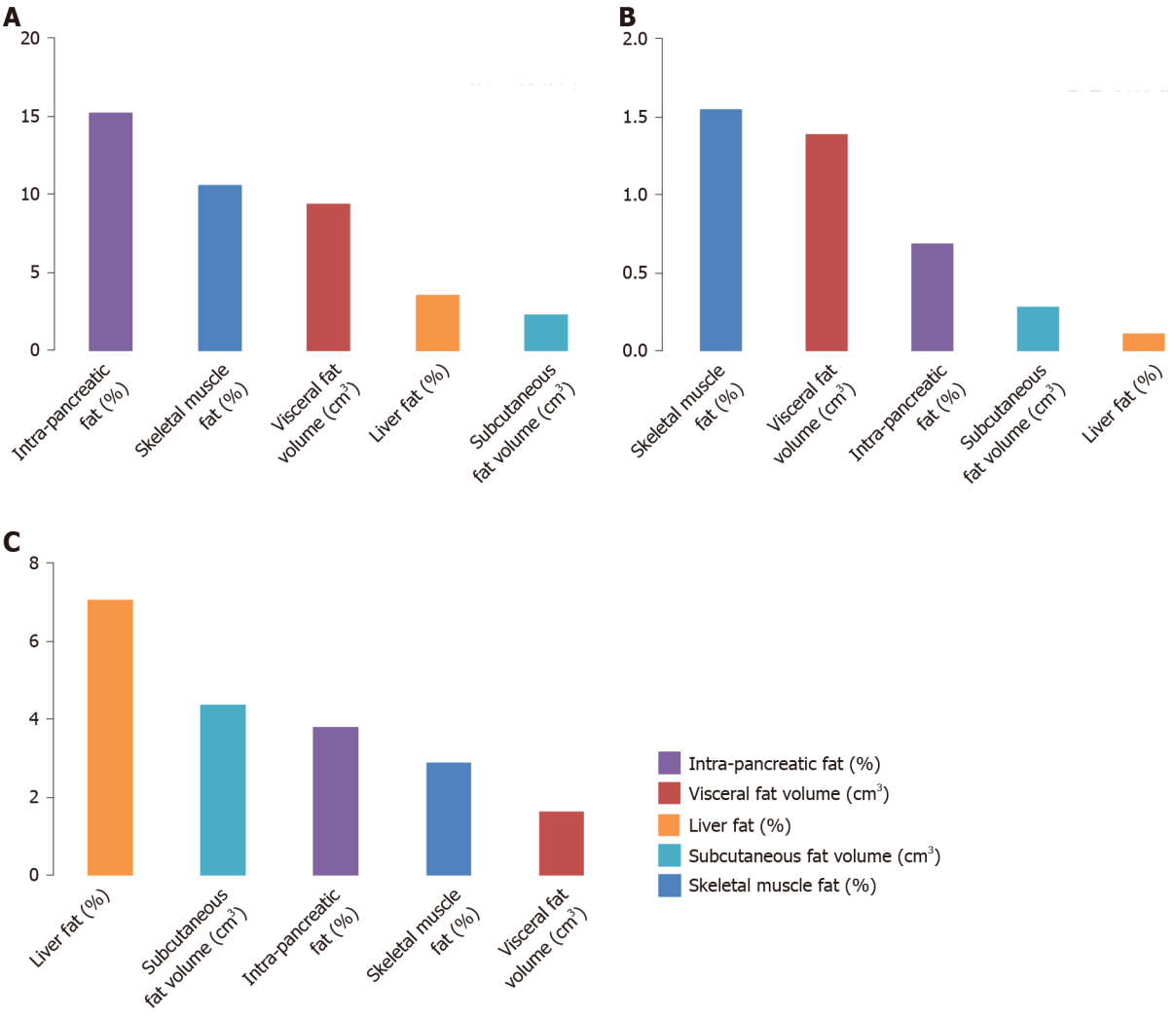


Figure 1 Contributions of the studied fat phenotypes to the variance in circulating levels of pancreatic amylase in the healthy controls, type 2 prediabetes/diabetes mellitus, and new-onset prediabetes/diabetes after acute pancreatitis groups. Footnote: Data are presented as a percentage of the corresponding abdominal fat phenotype that explains the variance in circulating levels of pancreatic amylase. A: New-onset prediabetes/diabetes after acute pancreatitis; B: Type 2 prediabetes/diabetes mellitus; C: Healthy controls.

adiposity) than those with type 2 diabetes[28-30]. Moreover, a large study from the United Kingdom demonstrated that the prevalence of type 2 diabetes, but not type 1 diabetes, was significantly associated with BMI[29]. NODAP, another type of diabetes that is much less common than type 2 diabetes, is often misdiagnosed as type 2 diabetes[4]. Specifically, a 2017 population-based study of new-onset diabetes demonstrated that 93% cases of new-onset diabetes after pancreatitis were misclassified as type 2 diabetes (and 4% as type 1 diabetes)[4]. In the present study, the NODAP group did not significantly differ from the T2DM group in terms of visceral adiposity, which was significantly higher in the two case groups in comparison with the healthy control group. Although we did not specifically measure insulin sensitivity in the present study, our earlier study showed that individuals with NODAP were characterized by decreased insulin sensitivity compared with healthy controls[31]. The findings of the two studies are complementary as increased visceral adiposity is known to be more strongly linked with decreased insulin sensitivity than subcutaneous adiposity.

IPFD has recently emerged as another fat phenotype strongly linked with insulin sensitivity[14,31,32]. This is epitomized in the 'twin cycle hypothesis' that posits that type 2 diabetes is caused by excess fat deposition in the pancreas (and liver). During chronic positive caloric balance, β-cells enter a 'survival mode' and fail to function adequately in the pancreas because of the fat-induced metabolic stress[33]. Studies from a primary care-based weight management program in the United Kingdom found that the reduction in fat depositions in the pancreas (and liver) in the first few years after diabetes onset can normalize hepatic insulin responsiveness and possibly

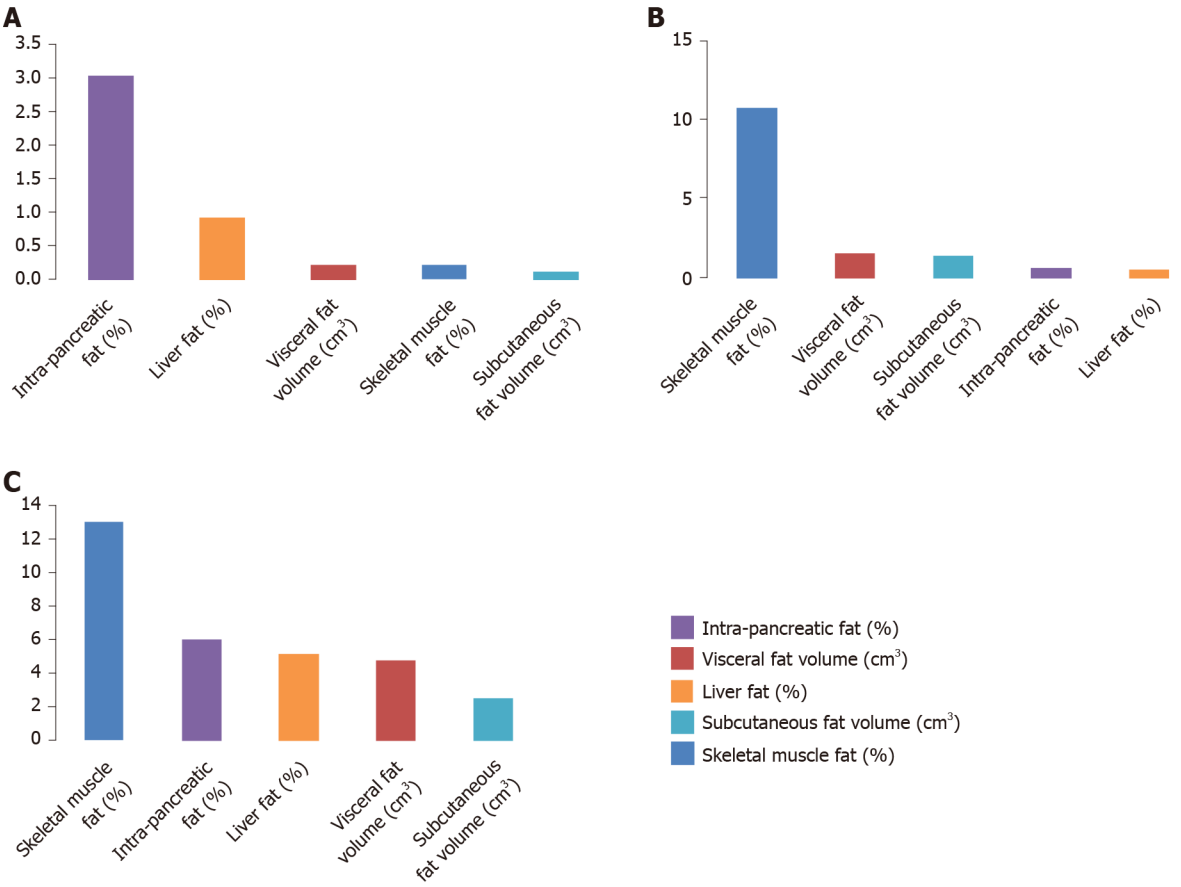


Figure 2 Contributions of the studied fat phenotypes to the variance in circulating levels of pancreatic lipase in the healthy controls, type 2 prediabetes/diabetes mellitus, and new-onset prediabetes/diabetes after acute pancreatitis groups. Data are presented as a percentage of the corresponding abdominal fat phenotype that explains the variance in circulating levels of pancreatic lipase. A: New-onset prediabetes/diabetes after acute pancreatitis; B: Type 2 prediabetes/diabetes mellitus; C: Healthy controls.

trigger β-cell re-differentiation[34]. Collectively, these changes can lead to normalization of blood glucose levels and reversal of biochemical diabetes status[33-35]. Further, the link between IPFD and insulin traits, specifically in NODAP, was investigated in a 2019 study. It showed that a fasted state index of insulin sensitivity (specifically, Raynaud index) was significantly inversely associated with IPFD in individuals with NODAP and it explained 20% of the variance in IPFD, which was the highest among the nearly 30 body composition variables and insulin traits investigated [31]. The present study bridges the gap in the literature by showing, for the first time, that IPFD is similarly increased in NODAP and T2DM as compared with healthy controls.

The other novel finding in the present study was that, although high IPFD and visceral fat volume characterized both NODAP and T2DM, the two fat phenotypes were significantly inversely associated with circulating levels of pancreatic amylase in the NODAP group only. Further, IPFD contributed the most to the variance in circulating levels of pancreatic amylase (R^2 = 15.0%). By contrast, there was only a small contribution of IPFD to the variance in circulating levels of pancreatic amylase in the healthy controls group (R^2 = 3.8%) and the T2DM group (R^2 = 0.7%). The exact mechanism underlying the above findings is yet to be elucidated but we believe it may relate to a more prominent role of exocrine pancreatic dysfunction in NODAP *vs* T2DM. A 2015 study demonstrated that MRI-derived IPFD was inversely associated with serum lipase activity (pancreatic amylase and other pancreatic enzymes were not studied) in the general population, suggesting that increased IPFD is associated with reduced pancreatic acinar cell mass[36]. A subsequent add-on study of 1458 participants with available fecal elastase measurements showed that MRI-derived IPFD was significantly inversely associated with exocrine pancreatic function (defined based on fecal elastase levels), in both crude analysis and after adjustment for age, sex, and BMI[37]. Interestingly, other fat phenotypes (subcutaneous fat, visceral fat, and liver fat) were not significantly associated with exocrine pancreatic function in that

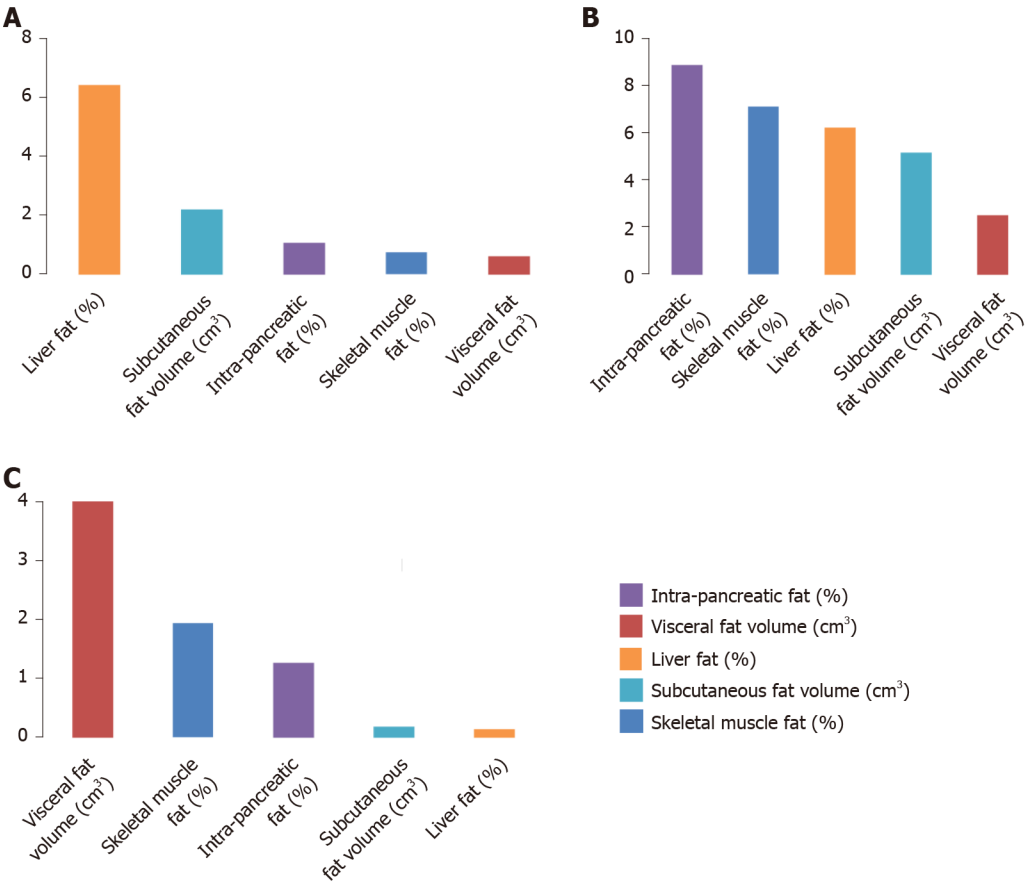


Figure 3 Contributions of the studied fat phenotypes to the variance in circulating levels of chymotrypsin in the healthy controls, type 2 prediabetes/diabetes mellitus, and new-onset prediabetes/diabetes after acute pancreatitis groups. Data are presented as a percentage of the corresponding abdominal fat phenotype that explains the variance in circulating levels of chymotrypsin. A: New-onset prediabetes/diabetes after acute pancreatitis; B: Type 2 prediabetes/diabetes mellitus; C: Healthy controls.

study. Another study of fecal elastase and MRI-derived IPFD included 56 individuals without diabetes or pancreatitis and showed that individuals with excess IPFD had a significantly higher frequency of exocrine pancreatic dysfunction than controls[38].

Amylase is the main enzyme responsible for the hydrolysis of carbohydrates[39] and, hence, its observed involvement in the pathogenesis of NODAP may have practical implications for the dietary management of this disorder. In most human populations, starch is the primary source of carbohydrates[40-42]. A 2012 study found that individuals with low amylase activity had higher postprandial plasma glucose concentrations after starch ingestion than individuals with high amylase activity[43]. A 2016 metabolomic study showed that utilization of glucose in the body for energy was attenuated in individuals with low serum amylase who have an energy dependence on fats rather than carbohydrates[44]. Based on the above findings, it is likely that individuals with NODAP have a different glycemic response in comparison with individuals with T2DM. Glycemic load reflects the quantity and quality of carbohydrates in the diet[40,45]. Because individuals with NODAP may not be fully adapted to a diet rich in carbohydrates, they may benefit from the determination of glycemic load for foods that are high in starch. The differential association between amylase and fat phenotypes in NODAP *vs* T2DM may also influence response to weight-loss dietary interventions and this needs to be taken into account in the design of future randomized controlled trials[41].

Several limitations have to be acknowledged. First, genetic factors were not analyzed in the present study. Some genes (*e.g.*, the α-amylase gene cluster) are highly relevant to the present research. The α-amylase cluster comprises 2 pancreatic amylase genes (*AMY2A* and *AMY2B*), 3 salivary amylase genes (*AMY1A*, *AMY1B*, and *AMY1C*), and a related pseudogene[46]. Several studies showed that genetic factors (such as copy number variants in amylase genes) may account for as much as 11% of the population variance in body fat[47]. The present study opens up a potential avenue for future research into genetic factors that predispose to IPFD and visceral fat deposition.

Second, levels of amylase, lipase, and chymotrypsin in the gastrointestinal tract were not measured. Hence, it is unknown whether their circulating and intraluminal levels correlate in our study population. However, a 2020 systematic review identified circulating levels of amylase and lipase as biomarkers of diabetes[19]. Third, we did not analyze lifestyle factors (*e.g.*, diet, physical activity)[48,49]. It is well established that obesogenic risk factors are positively associated with ectopic fat deposition in several organs[2,50]. However, there is no evidence that these factors would differentially affect ectopic fat deposition in the liver, pancreas, and skeletal muscle. Third, the study sample size was rather limited. However, this was a pilot study that will inform the design and sample size calculation of future studies. Last, given the cross-sectional nature of the study, inference of causality between IPFD and circulating levels of pancreatic enzymes cannot be drawn. To the best of our knowledge, no longitudinal study has explored the association between IPFD (or the other phenotypes investigated in the present study) and circulating levels of pancreatic enzymes. Their temporal relationship is warranted to be on the research agenda.

CONCLUSION

IPFD and visceral fat were significantly increased in individuals with NODAP and T2DM (in comparison with healthy controls). However, only individuals with NODAP were characterized by significant inverse associations between the two abdominal fat phenotypes and circulating levels of pancreatic amylase. Pancreatic amylase may have implications for the pathogenesis and management of NODAP and, hence, the role of this pancreatic enzyme in NODAP warrants purposely designed investigations in the future.

ARTICLE HIGHLIGHTS

Research background

Abdominal adipose tissue distribution is an important factor in the pathogenesis of diabetes in general and new-onset diabetes after acute pancreatitis in particular.

Research motivation

The role of pancreatic enzymes in the pathogenesis of new-onset diabetes after acute pancreatitis is unknown.

Research objectives

The objective was to compare head-to-head abdominal adipose tissue distribution in new-onset prediabetes or diabetes after acute pancreatitis (NODAP), type 2 prediabetes or diabetes, and healthy controls.

Research methods

The design was a case-control study. Intra-pancreatic fat, liver fat, skeletal muscle fat, visceral fat, and subcutaneous fat were quantified in a blinded fashion with the use of magnetic resonance imaging. Circulating levels of pancreatic amylase, pancreatic lipase, and chymotrypsin were determined.

Research results

The intra-pancreatic fat percentage was 9.4 ± 1.8%, 9.8 ± 1.1%, and 7.8 ± 1.9% in NODAP, type 2 prediabetes or diabetes, and healthy controls, respectively ($P < 0.001$). The visceral fat volume was 2205 ± 1098 cm^3, 2622 ± 1172 cm^3, and 1209 ± 808 cm^3 in NODAP, type 2 prediabetes or diabetes, and healthy controls, respectively ($P < 0.001$). The other fat phenotypes did not differ between the groups. The amount of intra-pancreatic fat and visceral fat was significantly associated with circulating levels of pancreatic amylase in NODAP (but not type 2 prediabetes or diabetes or healthy controls).

Research conclusions

Excess intra-pancreatic fat deposition is a key factor in the pathogenesis of new-onset diabetes after acute pancreatitis. There is a significant inverse relationship between circulating levels of pancreatic amylase and intra-pancreatic fat.

Research perspectives

Human studies on the role of pancreatic amylase in new-onset diabetes after acute pancreatitis are warranted.

REFERENCES

1 **Petrov MS**. Post-pancreatitis diabetes mellitus: prime time for secondary disease. *Eur J Endocrinol* 2021; **184**: R137-R149 [PMID: 33460393 DOI: 10.1530/EJE-20-0468]

2 **Petrov MS**, Yadav D. Global epidemiology and holistic prevention of pancreatitis. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 175-184 [PMID: 30482911 DOI: 10.1038/s41575-018-0087-5]

3 **Bharmal SH**, Cho J, Stuart CE, Alarcon Ramos GC, Ko J, Petrov MS. Oxyntomodulin may distinguish new-onset diabetes after acute pancreatitis from type 2 diabetes. *Clin Transl Gastroenterol* 2020; **11**: e00132 [PMID: 32463621 DOI: 10.14309/ctg.0000000000000132]

4 **Woodmansey C**, McGovern AP, McCullough KA, Whyte MB, Munro NM, Correa AC, Gatenby PAC, Jones SA, de Lusignan S. Incidence, demographics, and clinical characteristics of diabetes of the exocrine pancreas (type 3c): a retrospective cohort study. *Diabetes Care* 2017; **40**: 1486-1493 [PMID: 28860126 DOI: 10.2337/dc17-0542]

5 **Han TS**, Al-Gindan YY, Govan L, Hankey CR, Lean MEJ. Associations of BMI, waist circumference, body fat, and skeletal muscle with type 2 diabetes in adults. *Acta Diabetol* 2019; **56**: 947-954 [PMID: 30927105 DOI: 10.1007/s00592-019-01328-3]

6 **American Diabetes Association**. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes-2020. *Diabetes Care* 2020; **43**: S14-S31 [PMID: 31862745 DOI: 10.2337/dc20-S002]

7 **Gudipaty L**, Rosenfeld NK, Fuller CS, Cuchel M, Rickels MR. Different β-cell secretory phenotype in non-obese compared to obese early type 2 diabetes. *Diabetes Metab Res Rev* 2020; **36**: e3295 [PMID: 32017362 DOI: 10.1002/dmrr.3295]

8 **Chait A**, den Hartigh LJ. Adipose tissue distribution, inflammation and its metabolic consequences, including diabetes and cardiovascular disease. *Front Cardiovasc Med* 2020; **7**: 22 [PMID: 32158768 DOI: 10.3389/fcvm.2020.00022]

9 **Wu WC**, Wang CY. Association between non-alcoholic fatty pancreatic disease (NAFPD) and the metabolic syndrome: case-control retrospective study. *Cardiovasc Diabetol* 2013; **12**: 77 [PMID: 23688357 DOI: 10.1186/1475-2840-12-77]

10 **Bendor CD**, Bardugo A, Zucker I, Cukierman-Yaffe T, Lutski M, Derazne E, Shohat T, Mosenzon O, Tzur D, Sapir A, Pinhas-Hamiel O, Kibbey RG, Raz I, Afek A, Gerstein HC, Tirosh A, Twig G. Childhood pancreatitis and risk for incident diabetes in adulthood. *Diabetes Care* 2020; **43**: 145-151 [PMID: 31694859 DOI: 10.2337/dc19-1562]

11 **Roh E**, Kim KM, Park KS, Kim YJ, Chun EJ, Choi SH, Jang HC, Lim S. Comparison of pancreatic volume and fat amount linked with glucose homeostasis between healthy Caucasians and Koreans. *Diabetes Obes Metab* 2018; **20**: 2642-2652 [PMID: 29934972 DOI: 10.1111/dom.13447]

12 **Wang CY**, Ou HY, Chen MF, Chang TC, Chang CJ. Enigmatic ectopic fat: prevalence of nonalcoholic fatty pancreas disease and its associated factors in a Chinese population. *J Am Heart Assoc* 2014; **3**: e000297 [PMID: 24572250 DOI: 10.1161/JAHA.113.000297]

13 **Shulman GI**. Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. *N Engl J Med* 2014; **371**: 1131-1141 [PMID: 25229917 DOI: 10.1056/NEJMra1011035]

14 **Sakai NS**, Taylor SA, Chouhan MD. Obesity, metabolic disease and the pancreas-quantitative imaging of pancreatic fat. *Br J Radiol* 2018; **91**: 20180267 [PMID: 29869917 DOI: 10.1259/bjr.20180267]

15 **Acharya C**, Navina S, Singh VP. Role of pancreatic fat in the outcomes of pancreatitis. *Pancreatology* 2014; **14**: 403-408 [PMID: 25278311 DOI: 10.1016/j.pan.2014.06.004]

16 **Komada H**, Sakaguchi K, Hirota Y, Sou A, Nakamura T, Kyotani K, Kawamitsu H, Sugimura K, Okuno Y, Ogawa W. Pancreatic fat content assessed by 1H magnetic resonance spectroscopy is correlated with insulin resistance, but not with insulin secretion, in Japanese individuals with normal glucose tolerance. *J Diabetes Investig* 2018; **9**: 505-511 [DOI: 10.1111/jdi.12720]

17 **Wong VW**, Wong GL, Yeung DK, Abrigo JM, Kong AP, Chan RS, Chim AM, Shen J, Ho CS, Woo J, Chu WC, Chan HL. Fatty pancreas, insulin resistance, and β-cell function: a population study using fat-water magnetic resonance imaging. *Am J Gastroenterol* 2014; **109**: 589-597 [PMID: 24492753 DOI: 10.1038/ajg.2014.1]

18 **Nadarajah C**, Fananapazir G, Cui E, Gichoya J, Thayalan N, Asare-Sawiri M, Menias CO, Sandrasegaran K. Association of pancreatic fat content with type II diabetes mellitus. *Clin Radiol* 2020; **75**: 51-56 [PMID: 31711639 DOI: 10.1016/j.crad.2019.05.027]

19 **Ko J**, Cho J, Petrov MS. Low serum amylase, lipase, and trypsin as biomarkers of metabolic disorders: a systematic review and meta-analysis. *Diabetes Res Clin Pract* 2020; **159**: 107974 [PMID: 31811884 DOI: 10.1016/j.diabres.2019.107974]

20 **Al-Mrabeh A**, Hollingsworth KG, Steven S, Taylor R. Morphology of the pancreas in type 2 diabetes: effect of weight loss with or without normalisation of insulin secretory capacity. *Diabetologia* 2016; **59**: 1753-1759 [PMID: 27179658 DOI: 10.1007/s00125-016-3984-6]

21 **Al-Mrabeh A**, Hollingsworth KG, Steven S, Tiniakos D, Taylor R. Quantification of intrapancreatic

fat in type 2 diabetes by MRI. *PLoS One* 2017; **12**: e0174660 [PMID: 28369092 DOI: 10.1371/journal.pone.0174660]

22 **Crane JC**, Olson MP, Nelson SJ. SIVIC: open-source, standards-based software for DICOM MR spectroscopy workflows. *Int J Biomed Imaging* 2013; **2013**: 169526 [PMID: 23970895 DOI: 10.1155/2013/169526]

23 **Kiefer LS**, Fabian J, Lorbeer R, Machann J, Storz C, Kraus MS, Wintermeyer E, Schlett C, Roemer F, Nikolaou K, Peters A, Bamberg F. Inter- and intra-observer variability of an anatomical landmark-based, manual segmentation method by MRI for the assessment of skeletal muscle fat content and area in subjects from the general population. *Br J Radiol* 2018; **91**: 20180019 [PMID: 29658780 DOI: 10.1259/bjr.20180019]

24 **Schneider CA**, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012; **9**: 671-675 [PMID: 22930834 DOI: 10.1038/nmeth.2089]

25 **Irlbeck T**, Massaro JM, Bamberg F, O'Donnell CJ, Hoffmann U, Fox CS. Association between single-slice measurements of visceral and abdominal subcutaneous adipose tissue with volumetric measurements: the Framingham Heart Study. *Int J Obes (Lond)* 2010; **34**: 781-787 [PMID: 20065971 DOI: 10.1038/ijo.2009.279]

26 **Whitcomb DC**, Yadav D, Adam S, Hawes RH, Brand RE, Anderson MA, Money ME, Banks PA, Bishop MD, Baillie J, Sherman S, DiSario J, Burton FR, Gardner TB, Amann ST, Gelrud A, Lo SK, DeMeo MT, Steinberg WM, Kochman ML, Etemad B, Forsmark CE, Elinoff B, Greer JB, O'Connell M, Lamb J, Barmada MM; North American Pancreatic Study Group. Multicenter approach to recurrent acute and chronic pancreatitis in the United States: the North American Pancreatitis Study 2 (NAPS2). *Pancreatology* 2008; **8**: 520-531 [PMID: 18765957 DOI: 10.1159/000152001]

27 **Grömping U**. Relative importance for linear regression in R: The package relaimpo. *J Stat Softw* 2006; **17**: 1-27

28 **Dubé MC**, Joanisse DR, Prud'homme D, Lemieux S, Bouchard C, Pérusse L, Lavoie C, Weisnagel SJ. Muscle adiposity and body fat distribution in type 1 and type 2 diabetes: varying relationships according to diabetes type. *Int J Obes (Lond)* 2006; **30**: 1721-1728 [PMID: 16652137 DOI: 10.1038/sj.ijo.0803337]

29 **Song SH**. Complication characteristics between young-onset type 2 vs type 1 diabetes in a UK population. *BMJ Open Diabetes Res Care* 2015; **3**: e000044 [PMID: 25713725 DOI: 10.1136/bmjdrc-2014-000044]

30 **Zhu A**, Cui B, Dang H, Yao D, Yu H, Jia H, Hu Z, Zhang X. Correlation of abdominal fat distribution with different types of diabetes in a Chinese population. *J Diabetes Res* 2013; **2013**: 651462 [PMID: 24350301 DOI: 10.1155/2013/651462]

31 **Singh RG**, Nguyen NN, DeSouza SV, Pendharkar SA, Petrov MS. Comprehensive analysis of body composition and insulin traits associated with intra-pancreatic fat deposition in healthy individuals and people with new-onset prediabetes/diabetes after acute pancreatitis. *Diabetes Obes Metab* 2019; **21**: 417-423 [PMID: 30187644 DOI: 10.1111/dom.13523]

32 **Rattarasarn C**. Dysregulated lipid storage and its relationship with insulin resistance and cardiovascular risk factors in non-obese Asian patients with type 2 diabetes. *Adipocyte* 2018; **7**: 71-80 [PMID: 29411678 DOI: 10.1080/21623945.2018.1429784]

33 **Taylor R**, Al-Mrabeh A, Sattar N. Understanding the mechanisms of reversal of type 2 diabetes. *Lancet Diabetes Endocrinol* 2019; **7**: 726-736 [PMID: 31097391 DOI: 10.1016/S2213-8587(19)30076-2]

34 **Lean ME**, Leslie WS, Barnes AC, Brosnahan N, Thom G, McCombie L, Peters C, Zhyzhneuskaya S, Al-Mrabeh A, Hollingsworth KG, Rodrigues AM, Rehackova L, Adamson AJ, Sniehotta FF, Mathers JC, Ross HM, McIlvenna Y, Stefanetti R, Trenell M, Welsh P, Kean S, Ford I, McConnachie A, Sattar N, Taylor R. Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial. *Lancet* 2018; **391**: 541-551 [PMID: 29221645 DOI: 10.1016/S0140-6736(17)33102-1]

35 **Lean ME**, Leslie WS, Barnes AC, Brosnahan N, Thom G, McCombie L, Peters C, Zhyzhneuskaya S, Al-Mrabeh A, Hollingsworth KG, Rodrigues AM, Rehackova L, Adamson AJ, Sniehotta FF, Mathers JC, Ross HM, McIlvenna Y, Welsh P, Kean S, Ford I, McConnachie A, Messow CM, Sattar N, Taylor R. Durability of a primary care-led weight-management intervention for remission of type 2 diabetes: 2-year results of the DiRECT open-label, cluster-randomised trial. *Lancet Diabetes Endocrinol* 2019; **7**: 344-355 [PMID: 30852132 DOI: 10.1016/S2213-8587(19)30068-3]

36 **Kühn JP**, Berthold F, Mayerle J, Völzke H, Reeder SB, Rathmann W, Lerch MM, Hosten N, Hegenscheid K, Meffert PJ. Pancreatic steatosis demonstrated at MR imaging in the general population: clinical relevance. *Radiology* 2015; **276**: 129-136 [PMID: 25658037 DOI: 10.1148/radiol.15140446]

37 **Kromrey ML**, Friedrich N, Hoffmann RT, Bülow R, Völzke H, Weiss FU, Lerch MM, Motosugi U, Kühn JP. Pancreatic steatosis is associated with impaired exocrine pancreatic function. *Invest Radiol* 2019; **54**: 403-408 [PMID: 30817380 DOI: 10.1097/RLI.0000000000000554]

38 **Tahtacı M**, Algın O, Karakan T, Yürekli ÖT, Alışık M, Köseoğlu H, Metin MR, Bolat AD, Erel Ö, Ersoy O. Can pancreatic steatosis affect exocrine functions of pancreas? *Turk J Gastroenterol* 2018; **29**: 588-594 [PMID: 30260782 DOI: 10.5152/tjg.2018.17696]

39 **Cummings JH**, Stephen AM. Carbohydrate terminology and classification. *Eur J Clin Nutr* 2007; **61** Suppl 1: S5-18 [PMID: 17992187 DOI: 10.1038/sj.ejcn.1602936]

40 **Shahdadian F**, Saneei P, Milajerdi A, Esmaillzadeh A. Dietary glycemic index, glycemic load, and

risk of mortality from all causes and cardiovascular diseases: a systematic review and dose-response meta-analysis of prospective cohort studies. *Am J Clin Nutr* 2019; **110**: 921-937 [PMID: 31187856 DOI: 10.1093/ajcn/nqz061]

41 **Heianza Y**, Sun D, Wang T, Huang T, Bray GA, Sacks FM, Qi L. Starch digestion-related amylase genetic variant affects 2-year changes in adiposity in response to weight-loss diets: the POUNDS Lost Trial. *Diabetes* 2017; **66**: 2416-2423 [PMID: 28659346 DOI: 10.2337/db16-1482]

42 **Reynolds A**, Mann J, Cummings J, Winter N, Mete E, Te Morenga L. Carbohydrate quality and human health: a series of systematic reviews and meta-analyses. *Lancet* 2019; **393**: 434-445 [PMID: 30638909 DOI: 10.1016/S0140-6736(18)31809-9]

43 **Mandel AL**, Breslin PA. High endogeneous salivary amylase activity is associated with improved glycemic homeostasis following starch ingestion in adults. *J Nutr* 2012; **142**: 853-858 [PMID: 22492122 DOI: 10.3945/jn.111.156984]

44 **Arredouani A**, Stocchero M, Culeddu N, Moustafa JE, Tichet J, Balkau B, Brousseau T, Manca M, Falchi M. Metabolomic profile of low-copy number carriers at the salivary α-amylase gene suggests a metabolic shift toward lipid-based energy production. *Diabetes* 2016; **65**: 3362-3368 [PMID: 27436124 DOI: 10.2337/db16-0315]

45 **Jenkins DJ**, Dehghan M, Mente A, Bangdiwala SI, Rangarajan S, Srichaikul K, Mohan V, Avezum A, Díaz R, Rosengren A, Lanas F, Lopez-Jaramillo P, Li W, Oguz A, Khatib R, Poirier P, Mohammadifard N, Pepe A, Alhabib KF, Chifamba J, Yusufali AH, Iqbal R, Yeates K, Yusoff K, Ismail N, Teo K, Swaminathan S, Liu X, Zatońska K, Yusuf R, Yusuf S; PURE Study Investigators. Glycemic index, glycemic load, and cardiovascular disease and mortality. *N Engl J Med* 2021; **384**: 1312-1322 [PMID: 33626252 DOI: 10.1056/NEJMoa2007123]

46 **Santos JL**, Saus E, Smalley SV, Cataldo LR, Alberti G, Parada J, Gratacòs M, Estivill X. Copy number polymorphism of the salivary amylase gene: implications in human nutrition research. *J Nutrigenet Nutrigenomics* 2012; **5**: 117-131 [PMID: 22965187 DOI: 10.1159/000339951]

47 **Falchi M**, El-Sayed Moustafa JS, Takousis P, Pesce F, Bonnefond A, Andersson-Assarsson JC, Sudmant PH, Dorajoo R, Al-Shafai MN, Bottolo L, Ozdemir E, So HC, Davies RW, Patrice A, Dent R, Mangino M, Hysi PG, Dechaume A, Huyvaert M, Skinner J, Pigeyre M, Caiazzo R, Raverdy V, Vaillant E, Field S, Balkau B, Marre M, Visvikis-Siest S, Weill J, Poulain-Godefroy O, Jacobson P, Sjostrom L, Hammond CJ, Deloukas P, Sham PC, McPherson R, Lee J, Tai ES, Sladek R, Carlsson LM, Walley A, Eichler EE, Pattou F, Spector TD, Froguel P. Low copy number of the salivary amylase gene predisposes to obesity. *Nat Genet* 2014; **46**: 492-497 [PMID: 24686848 DOI: 10.1038/ng.2939]

48 **Neuenschwander M**, Ballon A, Weber KS, Norat T, Aune D, Schwingshackl L, Schlesinger S. Role of diet in type 2 diabetes incidence: umbrella review of meta-analyses of prospective observational studies. *BMJ* 2019; **366**: l2368 [PMID: 31270064 DOI: 10.1136/bmj.l2368]

49 **Galani C**, Schneider H. Prevention and treatment of obesity with lifestyle interventions: review and meta-analysis. *Int J Public Health* 2007; **52**: 348-359 [PMID: 18368998 DOI: 10.1007/s00038-007-7015-8]

50 **Tamura Y**. Ectopic fat, insulin resistance and metabolic disease in non-obese Asians: investigating metabolic gradation. *Endocr J* 2019; **66**: 1-9 [PMID: 30518722 DOI: 10.1507/endocrj.EJ18-0435]

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ORIGINAL ARTICLE

Retrospective Cohort Study

Effect of type 2 diabetic mellitus in the prognosis of acute-on-chronic liver failure patients in China

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Abstract

BACKGROUND

Acute-on-chronic liver failure (ACLF) patients have a high short-term mortality rate, and the severity evaluation of ACLF is necessary for prognostication. Therefore, it was meaningful to evaluate the association between type 2 diabetic mellitus (DM) and ACLF and further explore the feasibility of using DM as a prognostic indicator in ACLF patients. The association between type 2 DM and the prognosis of patients with severe liver disease remains unclear.

AIM

To examine the effect of type 2 DM on the prognosis of patients with ACLF.

METHODS

Clinical data from 222 ACLF patients were collected and analyzed. The patients were categorized into two groups depending on whether they had DM or not, and the clinical data of ACLF patients were measured within 48 h after admission. Complications of ACLF were documented during treatment, such as hepatic encephalopathy, hepatorenal syndrome, acute upper gastrointestinal hemorrhage, and spontaneous peritonitis (SBP). Values of laboratory parameters, complication rates, and hospital mortality rates were compared between two groups.

RESULTS

Among 222 ACLF patients, 38 cases were categorized into DM groups, the mean age was 56.32 years and 73.68% were male. The prognosis of ACLF patients was

significantly correlated with DM in univariate [hazard ratio (HR) = 2.4, 95% confidence interval (CI) =1.5-3.7, $P < 0.001$] and multivariable analysis (HR = 3.17, 95%CI =1.82-5.523, $P < 0.001$). The incident of SBP (34.21% *vs* 13.59%, $P = 0.038$) and other infections like lung, urinary, blood, and cholecyst (44.74% *vs* 28.26%, $P = 0.046$) were higher in DM patients than non-DM counterparts. In addition, the ACLF patients with DM tended to have a high mortality rate ($P < 0.001$). Cumulative survival time was also significantly shorter in the ACLF patients with DM than non-DM.

CONCLUSION

A significant association between DM and the prognosis of ACLF patients was found in China. The ACLF patients with DM had higher incidence of hospital mortality and infection than those without DM.

Key Words: Acute-on-chronic liver failure; Diabetic mellitus; Prognosis; Infection; Type 2 diabetic mellitus

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Core Tip: This study evaluated the association between type 2 diabetic mellitus (DM) and the prognosis of acute-on-chronic liver failure (ACLF) patients. The 222 ACLF patients were categorized into two groups depending on whether they had DM or not. Values of laboratory parameters, complication rates, and hospital mortality rates were compared between two groups. We observed a significant association between DM and the prognosis of ACLF patients in the study. The ACLF patients with DM had higher incidence of hospital mortality and infection than those without DM.

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INTRODUCTION

Liver failure is usually manifested as coagulation dysfunction, jaundice, ascites, and hepatic encephalopathy (HE). Acute-on-chronic liver failure (ACLF) is a clinical type of liver failure, and the term was firstly raised in order to describe a condition with two insults operating on the liver simultaneously[1]. ACLF has been featured as a clinical syndrome in which an acute injury factor in a patient with chronic liver disease caused rapid deterioration of liver function, resulting in one or more organ failure. Causes of ACLF could be attributed to hepatitis B virus (HBV), autoimmune hepatitis, severe nonalcoholic fatty liver disease (NAFLD), and other sources. According to Asian Pacific Association for the Study of the Liver (APASL) ACLF Research Consortium consensus, which was published in 2014 and subsequently updated in 2019, ACLF was defined as acute hepatic insult manifested as jaundice and coagulopathy, complicated within 4 wk by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease/cirrhosis, and associated with a high 28-d mortality[1,2]. ACLF diagnostic criteria is uniform in the world, and the Asia-Pacific consensus emphasizes the emergence of liver failure based on chronic liver disease, mainly focusing on the performance of liver failure in order to get early diagnosis and intervention of disease[3]. It was found that ACLF patients had a high short-term mortality rate (> 15% at 28 d)[4] and the severity evaluation of ACLF was necessary for prognostication. Hence well-established prognostic indicators are extremely important, which are helpful for early intervention and reversibility of ACLF as well as improving survival rate[5,6].

Diabetic mellitus (DM) is one of the leading causes of morbidity and mortality across the globe[7]. Previous studies showed that DM was associated with higher incidence of encephalopathy, spontaneous bacterial peritonitis, and hepatocellular

obtained after each patient agreed to treatment by written consent.

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carcinoma in chronic liver disease[8-10]. With the improvement of people's living standards, the prevalence of fatty liver disease and DM has increased significantly in China. NAFLD is the most common chronic liver disease, and the risk of progressed liver fibrosis in NAFLD patients is significantly associated with DM[11,12]. The prevalence of DM has increased significantly in recent decades, results in the coexistence of DM and chronic liver disease being common[13]. DM, NAFLD, and obesity are manifestations of symptoms known as metabolic syndrome, and these symptoms are all related to each other. A recent study showed that morbid obesity is an important risk factor for the development of ACLF in liver cirrhosis patients[14]. However, there are very few studies that have evaluated the association between DM and ACLF. Therefore, it may be meaningful to investigate the association between DM and ACLF and further explore the feasibility of using DM as a prognostic indicator in ACLF patients.

MATERIALS AND METHODS

Research population

In this retrospective single-center study, all patients diagnosed with liver failure who were hospitalized at the Department of Hepatology Research Institute of the First Affiliated Hospital, Fujian Medical University, China from July 2013 to July 2020 were recruited in this study. ACLF was defined by APASL definition in 2019, an acute hepatic insult manifesting as jaundice [serum bilirubin ≥ 5 mg/dL (85 mmol/L) and coagulopathy (international normalized ratio (INR) ≥ 1.5 or prothrombin activity < 40%)] complicated within 4 wk by clinical ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease/cirrhosis and was associated with a high 28-d mortality[1]. The diagnosed complications of ACLF patients also were in accord with this definition. Type 2 DM was defined using any of the following criteria: (1) The World Health Organization 2013 criteria; and (2) documented history of diabetes. All ACLF patients were included as follow criteria: (1) patients aged 18 years and 85 years; (2) ACLF diagnostic criteria accord with APASL definition in 2019; and (3) sufficient data for the study. Patients were excluded if they had the following conditions: (1) *de novo* tumors; (2) liver transplantation or liver operation; (3) current use of hormone medication; and (4) other fatal disease, or gestation. After exclusion, a total of 222 patients with basis for chronic liver disease were recruited, including 190 HBV infection patients, 17 alcoholic liver disease patients, 15 other disease patients, among which 94 deaths were observed. This prospective study was approved by the ethics committee at the First Affiliated Hospital of Fujian Medical University, China.

Clinical and laboratory parameters

All parameters were collected from the electronic medical record system. The demographic parameters included age, gender, and body mass index (BMI). The clinical laboratory parameters collected within 48 h after admission, included prothrombin time (PT), INR, total bilirubin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, blood urea nitrogen (BUN), serum creatinine, albumin (ALB), fasting blood-glucose (FBG), total cholesterol (TCHO), triglyceride, K^+, Na^+, leukocyte count (white blood cell), erythrocyte count [red blood cell (RBC)], hemoglobin, platelet count (PLT), alpha fetoprotein, and ammonia. The BMI was calculated as weight (kg)/height^2 (m^2). This study used the West Haven criteria for grading HE. This grading system differentiated four grades of clinically manifest HE[15]. Ascites can be divided into 3 grades based on the volume of fluid by ultrasound examination[16]. The Child-Turcotte-Pugh (CTP) score, Model for End-Stage Liver Disease (MELD) score, and MELD with serum sodium (MELD-Na) score were calculated using the latest published criteria[5,17,18]. All prognostic scores were calculated according to all parameters at 48 h of admission.

Statistical analysis

Statistical analyses were performed using SPSS 24.0 (Armonk, NY, United States). The normally distributed continuous variables are presented as mean ± SD, which were further evaluated by Student's *t*-test between those with and without DM, whereas variables showing skewed distributions were evaluated by the Mann-Whitney *U* test, which are presented as median (interquartile range). Categorical variables were described using frequencies and proportions, and Pearson's chi-squared test was used to compare categorical variables.

The Cox proportional hazard regression analysis was used to estimate the hazard ratio (HR) for the association between DM and all-cause mortality. Participants were followed up from date of hospital admission to date of death or date of discharge. Proportional hazard assumption was tested using Kaplan-Meier survival curves method. A multivariable Cox model was chosen by considering all variables with $P < 0.1$ in univariate analysis, meanwhile DM was considered as the main variable in statistical analysis model. Further, the nomogram and forest plot were built in terms of results of Cox regression analyses. A two sided P value less than 0.05 were considered significant.

RESULTS

Demographic and clinical characteristics

The baseline demographics characteristics and clinical laboratory parameters of the patients are shown in Table 1, and HBV was the most common etiology of ACLF in both groups (68.42% and 75.54%, respectively). The study included 38 DM patients, the mean age was 56.32 years, and 73.68% were male. As expected, BMI and fasting glucose were higher in DMs than their non-DM counterparts. In contrast, the level of ALB was higher in non-DM ($P = 0.045$). The scoring systems included CTP, MELD, and MELD-Na, and there were no statistically significant differences between the two groups. The mortality rate in DM patients was significantly higher than non-DM patients (65.79% *vs* 37.5%, $P = 0.001$).

The incidence of complications between DM and non-DM patients with ACLF

The incidence of common complications in ACLF patients with or without DM is shown in Table 2, and ascites was the most frequent complication in both groups (89.47% and 75.54%, respectively), most of whom was mild. Compared to non-DM participants, DMs had a higher incidence of HE complication ($P = 0.026$). There was no statistical difference in other clinical complications including ascites, hepatorenal syndrome (HRS), and acute upper gastrointestinal bleeding (AUGIB) between the two groups. Spontaneous peritonitis (SBP) was the common infection complication of ACLF patients, and the incidence of SBP was higher in DMs than their non-DM counterparts ($P = 0.038$). Other infections, including lung infections, urinary infections, blood infections, and cholecyst infections, were statistically different ($P = 0.046$), though the total incidence rates were low. Furthermore, lung infections were the main infection (34.21% and 23.37%, respectively).

Independent risk factor to predict mortality in ACLF patients

As shown in Table 3, univariate and multivariable analysis were used to evaluate the predictors of prognosis for ACLF patients. Among demographic risk factors, gender and BMI were associated with the prognosis of ACLF patients in univariate analysis. With regard to clinical laboratory parameters, PT, INR, Na^+, FBG, BUN, PLT, and RBC were significantly associated with the prognosis of ACLF patients in univariate analysis, moreover, INR, FBG, and BUN also had significant association in multivariable analysis. However, TCHO was not significantly associated with the prognosis of ACLF patients in univariate analysis, while it was significantly in multivariable analysis. Among scoring systems reflecting severity of liver dysfunction, CTP, MELD, and MELD-Na were associated with the prognosis of ACLF patients in univariate analysis, while they had no significant association in multivariable analysis. All four risk factors identified in the univariate analysis (DM, HRS, AUGIB, and SBP) remained significant in the multivariable analysis. Further, nomogram and forest plots that incorporate parameters previously shown to be associated with the prognosis of ACLF patients in the multivariable analysis, including gender, DM, INR, BUN, TCHO, HRS, AUGIB, and SBP, were then constructed and are presented in Figures 1 and 2. As shown by nomogram plot in Figure 1, the total points accumulated by the various variables correspond to the predicted probability of survival for individual patient, and the predicted probability of 1- or 2-mo survival ranged from 0.1 to 0.9. The forest plot in Figure 2 illustrated the intuitive correlation between each risk variable, and the prognosis of ACLF and DM was the most important risk factor (HR = 3.257) influencing the prognosis of ACLF patients.

DM was independent risk factor to predict mortality in ACLF patients

Survival curves were used to describe the survival status of DM and non-DM patients

Table 1 Demographic characteristics and clinical features of the patients between diabetic mellitus and non-diabetic mellitus

	DM (yes) (*n* = 38)	DM (no) (*n* = 184)	*P* value
Age (yr)	56.32 ± 14.23	49.16 ± 12.84	0.002
Gender, *n* (%)			0.309
Male	28 (73.68)	149 (80.98)	
Female	10 (26.32)	35 (19.02)	
Cause of disease, *n* (%)			0.201
Hepatitis B virus	26 (68.42)	139 (75.54)	
Hepatitis B virus + other	5 (13.16)	20 (10.87)	
Alcohol	2 (5.26)	15 (8.15)	
Others	5 (13.16)	10 (5.44)	
WBC (10^9/L)	6.17 ± 4.03	7.35 ± 3.58	0.07
RBC (10^{12}/L)	3.68 ± 0.87	3.94 ± 0.84	0.084
Hb (g/L)	117.21 ± 24.71	121.95 ± 23.13	0.257
PLT (10^9/L)	100.34 ± 42.20	118.79 ± 59.09	0.069
PT (s)	23.01 ± 5.38	24.45 ± 6.95	0.229
INR	1.97 ± 0.45	2.10 ± 0.59	0.229
ALT (U/L)	396.08 ± 448.56	560.36 ± 693.06	0.163
AST (U/L)	365.95 ± 391.18	419.99 ± 513.42	0.541
γ-GGT (U/L)	174.16 ± 305.61	137.57 ± 127.33	0.231
TBIL (μmol/L)	320.71 ± 141.31	309.56 ± 134.00	0.644
ALB (g/L)	29.25 ± 4.51	30.73 ± 4.03	0.045
Scr (μmol/L)	56.37 ± 22.00	63.45 ± 27.28	0.134
BUN (mmol/L)	3.94 ± 2.65	4.25 ± 2.98	0.56
TCHO (mmol/L)	2.67 ± 0.81	2.65 ± 1.05	0.919
TG (mmol/L)	1.45 ± 0.67	1.26 ± 0.70	0.124
Na^+ (mmol/L)	136.98 ± 3.97	136.86 ± 4.43	0.878
K^+ (mmol/L)	3.90 ± 0.46	4.08 ± 0.58	0.072
AMON (μmol/L)	64.40 ± 40.39	71.60 ± 41.85	0.332
AFP (ng/mL)	118.63 ± 202.80	128.19 ± 192.02	0.784
BMI ($kg/m^{2)}$	24.99 ± 3.32	22.78 ± 3.03	< 0.001
FBG (mmol/L)	5.34 ± 1.87	3.83 ± 1.07	< 0.001
Scoring systems			
CTP	10.79 ± 1.49	10.40 ± 1.35	0.115
MELD	19.38 ± 4.52	20.74 ± 5.06	0.128
MELD-Na	20.89 ± 5.00	22.27 ± 6.84	0.239
Death, *n* (%)	25 (65.79)	69 (37.5)	0.001

γ-GGT: Gamma-glutamyl transpeptidase; AFP: Alpha fetal protein; ALB: Albumin; ALT: Alanine aminotransferase; AMMON: Ammonia; AST: Aspartate aminotransferase; BMI: Body mass index; BUN: Blood urea nitrogen; CTP: Child-Turcotte-Pugh; DM: Diabetic mellitus; FBG: Fasting blood-glucose; HB: Hemoglobin; INR: International normalized ratio; K^+: Kalium; MELD: Model for End-Stage Liver Disease; MELD-Na: Model for End-Stage Liver Disease with serum sodium; Na^+: Natriumion; PLT: Platelet count; PT: Prothrombin time; Scr: Serum creatinine; TBIL: Total bilirubin; TCHO: Total cholesterol; TG: Triglyceride; RBC: Red blood cell; WBC: White blood cell.

with ACLF. Figure 3 showed that the cumulative survival rate of ACLF patients was significantly distinguished between DM and non-DM (P = 0.00019). The survival time

Table 2 The incidence of complications between diabetic mellitus and non-diabetic mellitus patients with acute-on-chronic liver failure

	DM (yes) (*n* = 38)	DM (no) (*n* = 184)	*P* value
Ascites, *n* (%)	34 (89.47)	139 (75.54)	0.271
Mild	21 (55.26)	80 (43.48)	
Moderate	5 (13.16)	26 (14.13)	
Severe	8 (21.05)	33 (17.93)	
HE, *n* (%)	4 (10.53)	5 (2.72)	0.026
Grade (1-2)	4 (10.53)	3 (1.63)	
Grade (3-4)	0 (0)	2 (1.09)	
HRS, *n* (%)	4 (10.53)	12 (6.52)	0.385
AUGIB, *n* (%)	3 (7.89)	4 (2.17)	0.066
SBP, *n* (%)	13 (34.21)	25 (13.59)	0.038
Other infections, *n* (%)	17 (44.74)	52 (28.26)	0.046
Lung	13 (34.21)	43 (23.37)	
Urinary	1 (2.63)	5 (2.72)	
Blood	2 (5.26)	2 (1.09)	
Cholecyst	1 (2.63)	2 (1.09)	

Ascites mild: The depth of abdomen was < 3 cm; Ascites moderate: The depth of the middle of the abdomen was 3-10 cm; Ascites severe: The depth of the middle of the abdomen was > 10 cm; HE: Hepatic encephalopathy; HE grade 1: Patients showed a lack of attention and some subtle personality changes; HE grade 2: The most intriguing finding was disorientation for time combined; HE grade 3: Patients were stuporous but responded to stimuli; HE grade 4: Patients were in coma. AUGIB: Acute upper gastrointestinal bleeding; AUGIH: Acute upper gastrointestinal hemorrhage; HRS: Hepatorenal syndrome; SBP: Spontaneous peritonitis.

of ACLF patients with non-DM was longer than that in the patients with DM. With the follow-up time increased, the survival time of patients with DM decreased significantly. Therefore, DM was associated with a higher death risk in ACLF patients.

DISCUSSION

ACLF was characterized by acute decompensation of chronic liver disease, and the course of ACLF was complex and associated with high short-term mortality, complicated with multiple organ failure[19]. The effective prognostic predictors of ACLF would help in identifying assessment of patients at specific time points either requiring early organ support treatment or urgent liver transplantation for ACLF patients, thereafter, doctors may provide a rational therapy for the patients in time[20, 21]. Previous study demonstrated that patients who survived for the first 3 mo usually had a good prognosis[22]. Therefore, the exploration of ACLF related prognostic indicators was helpful to reduce the mortality by predicting the outcome of patients and providing early clinical intervention.

It has been reported that there is a strong association between DM and liver disease. During a 12-year follow-up cohort study, compared to non-DM patients with chronic hepatitis B (CHB), DM patients with CHB developed more frequently to cirrhosis and cirrhosis decompensation[23]. Similarity, it has been shown that the presence of DM was an independent predictor of mortality in CHB patients[24]. Therefore, it is necessary to pay attention to the impact of DM on the clinical outcomes with ACLF. In this study, it was found that DM was as an independent prognostic factor for ACLF patients, and the incidence of infection and mortality were higher in DMs than that in non-DM patients. In addition to DM, gender, INR, BUN, TCHO, HRS, AUGIB, SBP, and FBG were independent risk factors for the prognosis of ACLF patients in the multivariate analysis.

Previous studies had shown that nomogram enabled more-accurate individualized prediction of survival than MELD, MELD-Na, CTP, sequential organ failure assessment, or CLIF-C scores for HBV patients and demonstrated superior net benefits

Table 3 Factors associated with the survival time of acute-on-chronic liver failure patients based on Cox proportional hazards regression

	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gender	0.58 (0.37-0.91)	0.019	0.535 (0.332-0.861)	0.01
Age (yr)	1 (0.99-1)	0.23		
WBC (10^9/L)	1 (0.99-1.1)	0.12		
RBC (10^{12}/L)	0.73 (0.57-0.92)	0.009		
Hb (g/L)	0.99 (0.98-1)	0.01		
PLT (10^9/L)	0.99 (0.99-1)	0.021		
PT (s)	1.1 (1-1.1)	< 0.001		
INR	1.9 (1.4-2.5)	< 0.001	2.725 (1.949-3.811)	< 0.001
ALT (U/L)	1 (1-1)	0.53		
AST (U/L)	1 (1-1)	0.8		
γ-GGT (U/L)	1 (1-1)	0.93		
TBIL (μmol/L)	1 (1-1)	0.77		
ALB (g/L)	0.95 (0.9-1)	0.079		
Scr (μmol/L)	1 (1-1)	0.15		
BUN (mmol/L)	1.1 (1-1.2)	0.021	1.095 (1.025-1.170)	0.0069
TCHO (mmol/L)	1 (0.85-1.3)	0.76	1.220 (1.006-1.170)	0.0436
TG (mmol/L)	0.78 (0.55-1.1)	0.18		
Na^+ (mmol/L)	0.94 (0.9-0.98)	0.005		
K^+ (mmol/L)	0.83 (0.58-1.2)	0.34		
AMON (μmol/L)	1 (0.99-1)	0.64		
AFP (ng/ml)	1 (1-1)	0.075		
BMI (kg/$m^{2)}$	0.99 (0.93-1.1)	0.7		
FBG (mmol/L)	0.58 (0.39-0.87)	0.009	0.842 (0.719-0.986)	0.0325
CTP	1.7 (1-2.9)	0.042		
MELD	1.1 (1-1.1)	0.02		
MELD-Na	1 (1-1.1)	0.002		
DM	2.4 (1.5-3.7)	< 0.001	3.17 (1.82-5.523)	< 0.001
Ascites	1.2 (1-1.5)	0.036		
HE	1.3 (0.65-2.5)	0.48		
HRS	3.3 (1.9-5.8)	< 0.001	2.71 (1.513-4.857)	0.001
AUGIB	2.6 (1.1-5.9)	0.027	2.444 (1.037-5.76)	0.041
SBP	2.5 (1.6-3.9)	< 0.001	2.262 (1.438-3.558)	< 0.001
Other infections	1.2 (0.82-1.9)	0.32		

γ-GGT: Gamma-glutamyl transpeptidase; AFP: Alpha fetal protein; ALB: Albumin; ALT: Alanine aminotransferase; AMMON: Ammonia; AST: Aspartate aminotransferase; AUGIB: Acute upper gastrointestinal bleeding; AUGIH: Acute upper gastrointestinal hemorrhage; BMI: Body mass index; BUN: Blood urea nitrogen; CI: Confidence interval; CTP: Child-Turcotte-Pugh; DM: Diabetic mellitus; FBG: Fasting blood glucose; HB: Hemoglobin; HE: Hepatic encephalopathy; HR: Hazard ratio; HRS: Hepatorenal syndrome; INR: International normalized ratio; Na^+: Natriumion; K^+: Kalium; MELD: Model for End-Stage Liver Disease; MELD-Na: Model for End-Stage Liver Disease with serum sodium; PLT: Platelet count; PT: Prothrombin time; RBC: Red blood cell; SBP: Spontaneous peritonitis; Scr: Serum creatinine; TBIL: Total bilirubin; TCHO: Total cholesterol; TG: Triglyceride; WBC: White blood cell.

over other score models[25,26]. Therefore, an effective prognostic nomogram for ACLF patients was established, as shown in Figure 1; the nomogram of our study could

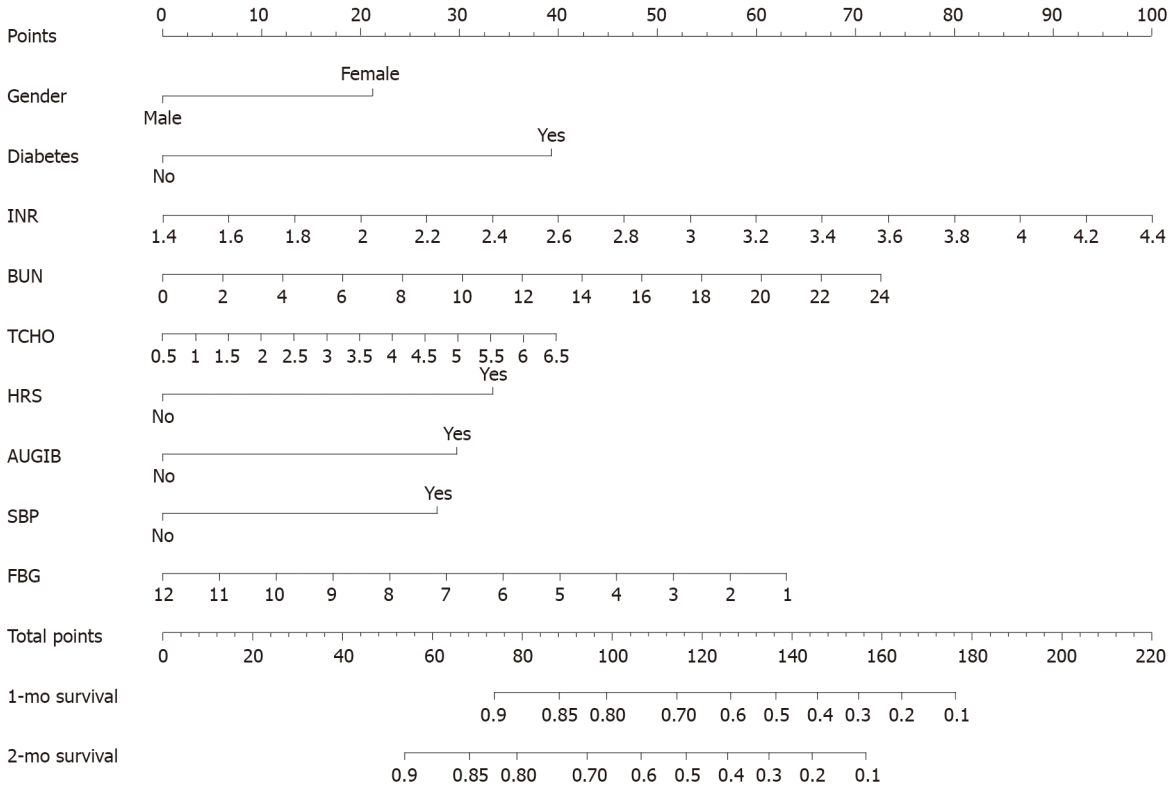


Figure 1 Acute-on-chronic liver failure patients' survival nomogram predicted the probability of 1- and 2-mo survival. AUGIH: Acute upper gastrointestinal hemorrhage; BUN: Blood urea nitrogen; FBG: Fasting blood-glucose; HRS: Hepatorenal syndrome; INR: International normalized ratio; SBP: Spontaneous peritonitis; TCHO: Total cholesterol.

accurately predict 1- and 2-mo survival in patients with ACLF.

Liver plays an important role in glucose homeostasis, and it is the major storage site of glycogen. Massive damage and necrosis of liver cells can cause serious deficiency of liver glycogen synthesis and storage, which are the main features of ACLF disease. Meanwhile, gastrointestinal dysfunction and gastrointestinal mucous edema could lead to impaired nutrient absorption in ACLF patients. Therefore, the mechanism of blood glucose regulation may be abnormal when liver function was severely damaged due to ACLF. In addition, the influence of DM on chronic liver related clinical outcomes had become a focus of research in recent years. It was proved that DM emerged as a risk factor of experiencing liver related death in the chronic liver patients [27]. Furthermore, a meta-analysis showed that DM was independently associated with poor survival in hepatocellular carcinoma patients[28]. Thus, the presence of DM looked as an "at risk" sub-population of liver-related patients. In a conclusion, DM had a tremendous impact on liver disease, which should be fully considered by clinicians. Our study was consistent with previous studies, as shown in Figures 2 and 3, which implied that the level of fast blood glucose and DM were important prognostic indicators for ACLF patients. The cumulative survival time of ACLF patients with non-DM was significantly longer than the patients with DM (P = 0.00019). DM was identified as a risk factor in the univariate analysis (P = 0.009) and remained significant in the multivariable analysis (P = 0.038) in ACLF patients.

DM is a disorder of glucose metabolism; in order to maintain body composition and nitrogen balance, metabolic control and sufficient protein and energy intake are required[29]. ACLF is in a hyper-metabolism state, and upregulated inflammatory response may lead to accelerated proteolysis and increased body energy expenditure, which contributes to negative nitrogen balance[30]. Our study showed that increased level of BUN, which may due to negative nitrogen balance, was associated with the prognosis of ACLF patients in Cox regression analysis. In ACLF patients, the function of protein synthesis was significantly reduced, and ALB had become an effective indicator for ACLF nutritional surveillance[31,32]. A retrospective study had shown that ALB was associated with prognosis in ACLF patients; the patients with hypoproteinemia tended to have a higher mortality rate[33]. However, in this study, ALB was not associated with the prognosis of liver failure, the result may be interfered by exogenous ALB infusion during treatment. Therefore, ACLF patients with DM should

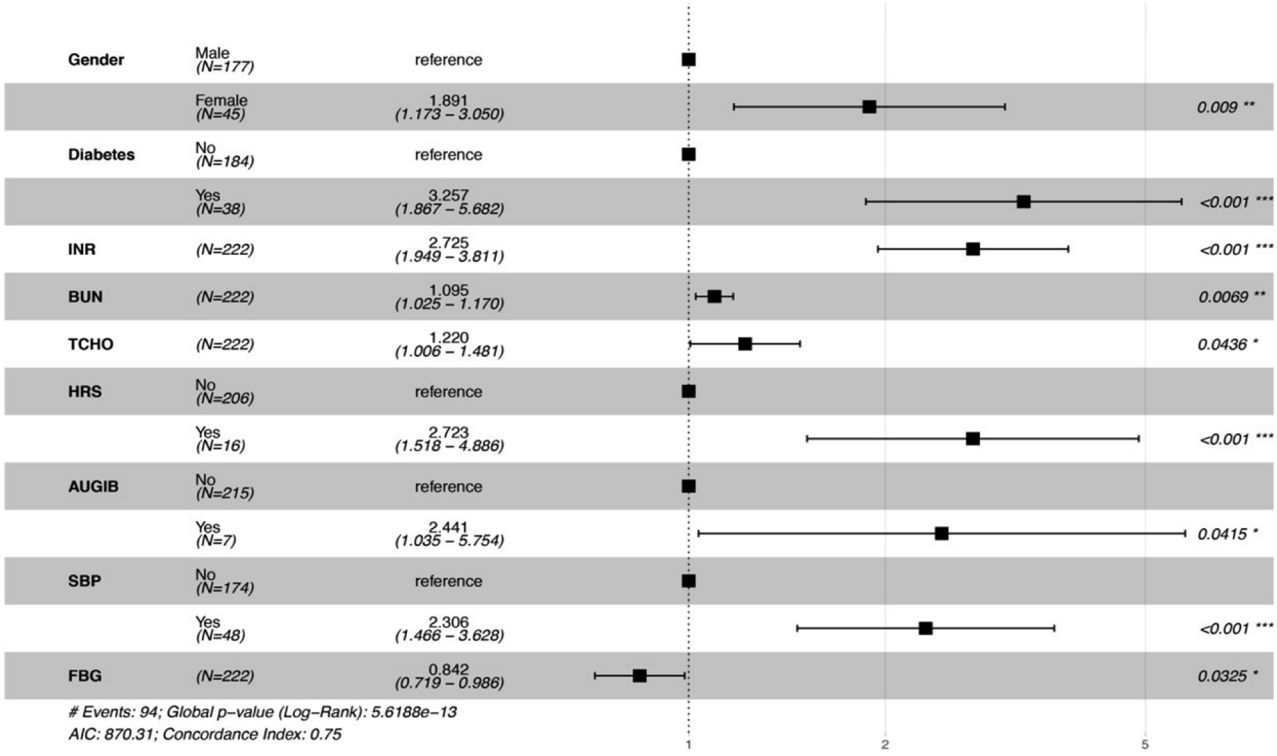


Figure 2 Forest plot of prognostic factors for acute-on-chronic liver failure patients based on multivariate Cox regression analysis.
AUGIH: Acute upper gastrointestinal hemorrhage; BUN: Blood urea nitrogen; FBG: Fasting blood-glucose; HRS: Hepatorenal syndrome; INR: International normalized ratio; SBP: Spontaneous peritonitis; TCHO: Total cholesterol.

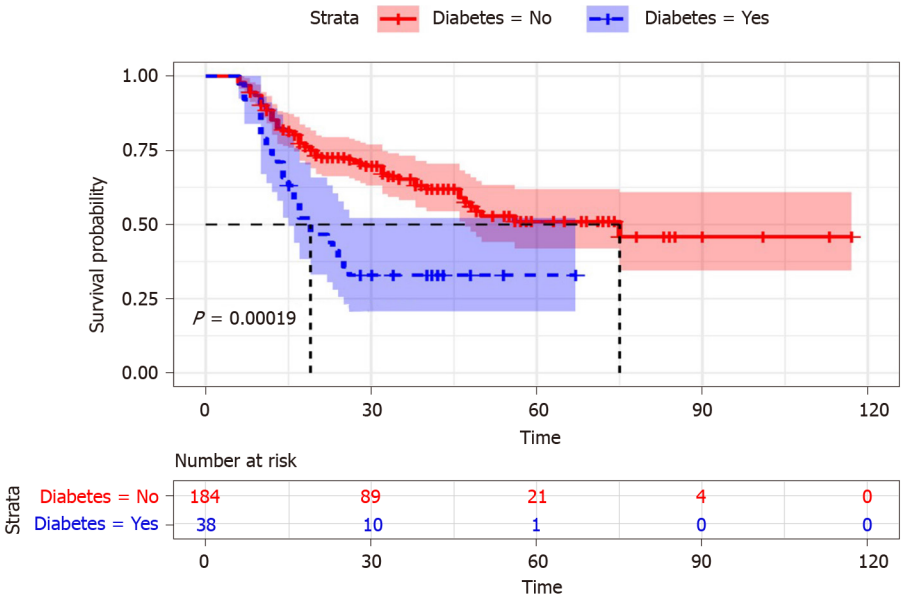


Figure 3 Cumulative survival time in acute-on-chronic liver failure patients with diabetic mellitus and non-diabetic mellitus.

be paid more attention to ensure sufficient protein and energy intake.

Malnutrition, protein consumption, and chronic inflammation could lead to sarcopenia in chronic liver disease, which is related with adverse clinical outcomes and the increased risk of mortality[34,35]. Further research found that sarcopenia was strongly associated with ACLF development and impacted the poor prognosis of ACLF[36]. Previous study revealed that DM was significantly associated with sarcopenia[37]. DM increased the risk of sarcopenia, which may be a risk factor to induce the poor prognosis in ACLF patients. Therefore, it was important to give adequate nutritional support for the patients with liver failure in order to reduce the

incidence of sarcopenia.

DM, BMI, and NALFD as metabolic risk factors were strongly related with the pathogenesis of metabolic syndrome. This research showed that BMI in DM patients were higher than that in non-DM counterparts. Our previous study showed BMI was associated with hospital mortality in ACLF patients[33], however, this study showed that BMI was not related with ACLF, which may do with the lack of subgroup analysis of BMI. At the same time, our study found that ACLF patients with DM had a higher BMI than the patients with non-DM, which may be a risk factor for the high mortality in ACLF patients with DM.

DM was a significant risk factor for progression of the chronic liver disease[38]. In fact, several previous surveys indicated that survival was significantly lower in DM than in non-DM cirrhotic[10,39]. DM patients were accompanied by high occurrence of NAFLD, and the prevalence of NAFLD in DM varied from 40% to 70%[40,41]. A recent study implied that NAFLD emerged as the rapidly growing etiology of chronic liver disease associated with ACLF[42]. DM was a significant risk factor for progression of the chronic liver disease. In fact, several previous surveys indicated that survival was significantly lower in DM than in non-DM cirrhotic. Our research further confirmed this finding that DM could increase mortality rate in ACLF patients. DM is an important metabolic risk factor that leads to increased mortality in ACLF patients (HR = 3.257, P = 0.001). For all of the above reasons, DM may increase the incidence of NAFLD and affect the prognosis of ACLF patients.

Presence of DM was also shown to be related with the high incidence of HE in patients with liver cirrhosis[43,44]. DM increased the risk of HE in cirrhosis patients, the previous studies showed that insulin resistance, muscle breakdown, and glutaminase activity may be mechanistic[44,45]. In terms of complications of liver failure, DM patients had a higher incidence of HE complication than non-DM participants (P = 0.026); our study produced exactly the same result as above studies. Compared with the patients without DM, it was known that the patients with DM had higher rates for all infections[46]. Previous studies had shown that DM was associated with high incidence of infection in cirrhotic individuals[10]. Our study showed the incidence of infection was higher in ACLF patients with DM than in counterparts without DM (P = 0.038). SBP and pneumonia were common infectious complication of ACLF patients; especially SBP was significantly related with the prognosis of ACLF patients in Cox regression analysis. Therefore, it was extremely important to prevent and control infection in patients with ACLF, especially in the patients with concurrent DM. Among all possible infections, SBP and pulmonary infection should be considered in priority.

Limits of the study

There are several limitations in our research. Firstly, this was not a multicenter study and the number of DM patients with ACLF was relatively small. Therefore, the magnitude of association between DM and the prognosis of ACLF could have been less precise. Secondly, because of the retrospective design of the study, not all relevant variables were obtained continuously; glycosylated hemoglobin (HBA1c) parameter and blood sugar control level were available sporadically in ACLF patients with DM. Therefore, these variables were not taken into account in the statistical analysis. Finally, Because of the drawback of this retrospective research, to assess further the impact of DM on the prognosis in ACLF patients, a larger cohort study should be conducted.

CONCLUSION

DM and chronic liver disease in terms of prevalence and mortality are on the rise worldwide. Because of the liver's important role in glucose metabolism, the correlation between liver disease and DM was close. Through this study, we found that DM could predict the prognosis of ACLF patients, and the ACLF patients with DM had higher incidence of mortality and infection, especially in abdominal and pulmonary infections. Consequently, the clinician should pay attention to the result that DM was a major predictor of short-term mortality in ACLF.

ARTICLE HIGHLIGHTS

Research background

The acute-on-chronic liver failure (ACLF) patients have a high short-term mortality rate, and the severity evaluation of ACLF is necessary for prognostication. The prevalence of diabetic mellitus (DM) has increased significantly in recent decades, resulting in coexistence of DM and chronic liver disease be common. There were very few studies that have evaluated the association between DM and ACLF. Therefore, the association between type 2 DM and the prognosis of patients with ACLF remained unclear.

Research motivation

Previous studies suggested that DM increased higher incidences of complications in chronic liver disease, included encephalopathy, spontaneous bacterial peritonitis, and hepatocellular carcinoma, but it was not clear whether DM would similarly affect ACLF patients. We needed further to evaluate the association between DM and the prognosis of ACLF patients in order to explore the feasibility of using DM as a prognostic indicator in ACLF patients.

Research objectives

We aimed to examine the effect of DM on the prognosis of patients with ACLF and established a predictive model to predict the risk of mortality for ACLF patients. A well-established prognostic predictive model was extremely important, which was helpful for early intervention of ACLF patients as well as improved survival rate.

Research methods

Clinical data from 222 ACLF patients were retrospectively collected and analyzed between July 2013 and July 2020 from the Department of Hepatology Research Institute of the First Affiliated Hospital, Fujian Medical University. The patients were categorized into two groups depending on whether they had DM or not, and complications of ACLF were documented during treatment. Values of laboratory parameters, complication rates, and hospital mortality rates were compared between two groups. The Cox proportional hazard regression analysis was used to estimate hazard ratio (HR) for the association between DM and all-cause mortality. DM was independent risk factor to predict mortality in ACLF patients. Further, the nomogram and forest plot were built in terms of results of Cox regression analyses. A survival nomogram model was constructed to predict the probability of 1- and 2-mo survival for ACLF patients.

Research results

The prognosis of ACLF patients was significantly correlated with DM in univariate (HR = 2.4, $P < 0.001$) and multivariable analysis (HR = 3.17, $P < 0.001$). The ACLF patients with DM tended to have a high mortality rate ($P < 0.001$). Survival curves showed that the cumulative survival rate of ACLF patients was significantly distinguished between DM and non-DM ($P = 0.00019$). The survival time of ACLF patients with non-DM was longer than the patients with DM. As a good prognostic indicator, DM was helpful to predict the outcome of ACLF patients. The incidence of infection was higher in DM patients than non-DM counterparts ($P = 0.038$). Among all possible infections, spontaneous peritonitis and pulmonary infection should be considered a priority for ACLF patients. ACLF patients' survival nomogram could predict the probability of 1- and 2-mo survival, which would be helpful to provide early clinical intervention. Because of the retrospective design of the study, we needed to assess further the impact of DM on the prognosis in ACLF patients by a prospective cohort study.

Research conclusions

Our study found a significant association between DM and the prognosis of ACLF patients in China. The survival time of ACLF patients with non-DM was longer than that of patients with DM. The ACLF patients with DM had higher incidence of hospital mortality and infection than those without DM. DM was an independent risk factor affecting the prognosis of ACLF patients.

Research perspectives

This was not a multicenter study and the number of DM patients with ACLF was

relatively small. Therefore, a multi-center prospective cohort study would evaluate further the impact of DM on the prognosis in ACLF patients. Meanwhile, a noninvasive model should be established based on an extensive clinical database to predict effectively the survival time of ACLF patients.

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REFERENCES

1 **Sarin SK**, Choudhury A, Sharma MK, Maiwall R, Al Mahtab M, Rahman S, Saigal S, Saraf N, Soin AS, Devarbhavi H, Kim DJ, Dhiman RK, Duseja A, Taneja S, Eapen CE, Goel A, Ning Q, Chen T, Ma K, Duan Z, Yu C, Treeprasertsuk S, Hamid SS, Butt AS, Jafri W, Shukla A, Saraswat V, Tan SS, Sood A, Midha V, Goyal O, Ghazinyan H, Arora A, Hu J, Sahu M, Rao PN, Lee GH, Lim SG, Lesmana LA, Lesmana CR, Shah S, Prasad VGM, Payawal DA, Abbas Z, Dokmeci AK, Sollano JD, Carpio G, Shresta A, Lau GK, Fazal Karim M, Shiha G, Gani R, Kalista KF, Yuen MF, Alam S, Khanna R, Sood V, Lal BB, Pamecha V, Jindal A, Rajan V, Arora V, Yokosuka O, Niriella MA, Li H, Qi X, Tanaka A, Mochida S, Chaudhuri DR, Gane E, Win KM, Chen WT, Rela M, Kapoor D, Rastogi A, Kale P, Sharma CB, Bajpai M, Singh V, Premkumar M, Maharashi S, Olithselvan A, Philips CA, Srivastava A, Yachha SK, Wani ZA, Thapa BR, Saraya A, Shalimar, Kumar A, Wadhawan M, Gupta S, Madan K, Sakhuja P, Vij V, Sharma BC, Garg H, Garg V, Kalal C, Anand L, Vyas T, Mathur RP, Kumar G, Jain P, Pasupuleti SSR, Chawla YK, Chowdhury A, Song DS, Yang JM, Yoon EL; APASL ACLF Research Consortium (AARC) for APASL ACLF working Party. Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific association for the study of the liver (APASL): an update. *Hepatol Int* 2019; **13**: 353-390 [PMID: 31172417 DOI: 10.1007/s12072-019-09946-3]

2 **Sarin SK**, Kedarisetty CK, Abbas Z, Amarapurkar D, Bihari C, Chan AC, Chawla YK, Dokmeci AK, Garg H, Ghazinyan H, Hamid S, Kim DJ, Komolmit P, Lata S, Lee GH, Lesmana LA, Mahtab M, Maiwall R, Moreau R, Ning Q, Pamecha V, Payawal DA, Rastogi A, Rahman S, Rela M, Saraya A, Samuel D, Saraswat V, Shah S, Shiha G, Sharma BC, Sharma MK, Sharma K, Butt AS, Tan SS, Vashishtha C, Wani ZA, Yuen MF, Yokosuka O; APASL ACLF Working Party. Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the Study of the Liver (APASL) 2014. *Hepatol Int* 2014; **8**: 453-471 [PMID: 26202751 DOI: 10.1007/s12072-014-9580-2]

3 **Olson JC**, Wendon JA, Kramer DJ, Arroyo V, Jalan R, Garcia-Tsao G, Kamath PS. Intensive care of the patient with cirrhosis. *Hepatology* 2011; **54**: 1864-1872 [PMID: 21898477 DOI: 10.1002/hep.24622]

4 **Dhiman RK**, Agrawal S, Gupta T, Duseja A, Chawla Y. Chronic Liver Failure-Sequential Organ Failure Assessment is better than the Asia-Pacific Association for the Study of Liver criteria for defining acute-on-chronic liver failure and predicting outcome. *World J Gastroenterol* 2014; **20**: 14934-14941 [PMID: 25356054 DOI: 10.3748/wjg.v20.i40.14934]

5 **Jalan R**, Saliba F, Pavesi M, Amoros A, Moreau R, Ginès P, Levesque E, Durand F, Angeli P, Caraceni P, Hopf C, Alessandria C, Rodriguez E, Solis-Muñoz P, Laleman W, Trebicka J, Zeuzem S, Gustot T, Mookerjee R, Elkrief L, Soriano G, Cordoba J, Morando F, Gerbes A, Agarwal B, Samuel D, Bernardi M, Arroyo V; CANONIC study investigators of the EASL-CLIF Consortium. Development and validation of a prognostic score to predict mortality in patients with acute-on-chronic liver failure. *J Hepatol* 2014; **61**: 1038-1047 [PMID: 24950482 DOI: 10.1016/j.jhep.2014.06.012]

6 **Xue R**, Duan Z, Liu H, Chen L, Yu H, Ren M, Zhu Y, Jin C, Han T, Gao Z, Meng Q. A novel dynamic model for predicting outcome in patients with hepatitis B virus related acute-on-chronic liver failure. *Oncotarget* 2017; **8**: 108970-108980 [PMID: 29312583 DOI: 10.18632/oncotarget.22447]

7 **Carracher AM**, Marathe PH, Close KL. International Diabetes Federation 2017. *J Diabetes* 2018; **10**: 353-356 [PMID: 29345068 DOI: 10.1111/1753-0407.12644]

8 **Wlazlo N**, van Greevenbroek MM, Curvers J, Schoon EJ, Friederich P, Twisk JW, Bravenboer B, Stehouwer CD. Diabetes mellitus at the time of diagnosis of cirrhosis is associated with higher incidence of spontaneous bacterial peritonitis, but not with increased mortality. *Clin Sci (Lond)* 2013; **125**: 341-348 [PMID: 23566037 DOI: 10.1042/CS20120596]

9 **Yang JD**, Ahmed F, Mara KC, Addissie BD, Allen AM, Gores GJ, Roberts LR. Diabetes Is Associated With Increased Risk of Hepatocellular Carcinoma in Patients With Cirrhosis From Nonalcoholic Fatty Liver Disease. *Hepatology* 2020; **71**: 907-916 [PMID: 31309602 DOI: 10.1002/hep.30858]

10 **Elkrief L**, Chouinard P, Bendersky N, Hajage D, Larroque B, Babany G, Kutala B, Francoz C, Boyer N, Moreau R, Durand F, Marcellin P, Rautou PE, Valla D. Diabetes mellitus is an independent

prognostic factor for major liver-related outcomes in patients with cirrhosis and chronic hepatitis C. *Hepatology* 2014; **60**: 823-831 [PMID: 24841704 DOI: 10.1002/hep.27228]

11 **Khan R**, Foster GR, Chowdhury TA. Managing diabetes in patients with chronic liver disease. *Postgrad Med* 2012; **124**: 130-137 [PMID: 22913901 DOI: 10.3810/pgm.2012.07.2574]

12 **Younossi Z**, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 11-20 [PMID: 28930295 DOI: 10.1038/nrgastro.2017.109]

13 **Silva TE**, Ronsoni MF, Schiavon LL. Challenges in diagnosing and monitoring diabetes in patients with chronic liver diseases. *Diabetes Metab Syndr* 2018; **12**: 431-440 [PMID: 29279271 DOI: 10.1016/j.dsx.2017.12.013]

14 **Sundaram V**, Jalan R, Ahn JC, Charlton MR, Goldberg DS, Karvellas CJ, Noureddin M, Wong RJ. Class III obesity is a risk factor for the development of acute-on-chronic liver failure in patients with decompensated cirrhosis. *J Hepatol* 2018; **69**: 617-625 [PMID: 29709681 DOI: 10.1016/j.jhep.2018.04.016]

15 **Vilstrup H**, Amodio P, Bajaj J, Cordoba J, Ferenci P, Mullen KD, Weissenborn K, Wong P. Hepatic encephalopathy in chronic liver disease: 2014 Practice Guideline by the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver. *Hepatology* 2014; **60**: 715-735 [PMID: 25042402 DOI: 10.1002/hep.27210]

16 **Chinese Society of Hepatology**; Chinese Medical Association, Xu X, Duan Z, Ding H, Li W, Jia J, Wei L, Linghu E, Zhuang H. Chinese guidelines on the management of ascites and its related complications in cirrhosis. *Hepatol Int* 2019; **13**: 1-21 [PMID: 30656520 DOI: 10.1007/s12072-018-09923-2]

17 **Perdigoto DN**, Figueiredo P, Tomé L. The Role of the CLIF-C OF and the 2016 MELD in Prognosis of Cirrhosis with and without Acute-on-Chronic Liver Failure. *Ann Hepatol* 2019; **18**: 48-57 [PMID: 31113608 DOI: 10.5604/01.3001.0012.7862]

18 **Song DS**, Kim TY, Kim DJ, Kim HY, Sinn DH, Yoon EL, Kim CW, Jung YK, Suk KT, Lee SS, Lee CH, Kim TH, Choe WH, Yim HJ, Kim SE, Baik SK, Jang JY, Kim HS, Kim SG, Yang JM, Sohn JH, Choi EH, Cho HC, Jeong SW, Kim MY; Korean Acute-on-Chronic Liver Failure (KACLiF) Study Group. Validation of prognostic scores to predict short-term mortality in patients with acute-on-chronic liver failure. *J Gastroenterol Hepatol* 2018; **33**: 900-909 [PMID: 28921629 DOI: 10.1111/jgh.13991]

19 **Hernaez R**, Solà E, Moreau R, Ginès P. Acute-on-chronic liver failure: an update. *Gut* 2017; **66**: 541-553 [PMID: 28053053 DOI: 10.1136/gutjnl-2016-312670]

20 **Gustot T**, Fernandez J, Garcia E, Morando F, Caraceni P, Alessandria C, Laleman W, Trebicka J, Elkrief L, Hopf C, Solís-Munoz P, Saliba F, Zeuzem S, Albillos A, Benten D, Montero-Alvarez JL, Chivas MT, Concepción M, Córdoba J, McCormick A, Stauber R, Vogel W, de Gottardi A, Welzel TM, Domenicali M, Risso A, Wendon J, Deulofeu C, Angeli P, Durand F, Pavesi M, Gerbes A, Jalan R, Moreau R, Ginés P, Bernardi M, Arroyo V; CANONIC Study Investigators of the EASL-CLIF Consortium. Clinical Course of acute-on-chronic liver failure syndrome and effects on prognosis. *Hepatology* 2015; **62**: 243-252 [PMID: 25877702 DOI: 10.1002/hep.27849]

21 **Wu SJ**, Yan HD, Zheng ZX, Shi KQ, Wu FL, Xie YY, Fan YC, Ye BZ, Huang WJ, Chen YP, Zheng MH. Establishment and validation of ALPH-Q score to predict mortality risk in patients with acute-on-chronic hepatitis B liver failure: a prospective cohort study. *Medicine (Baltimore)* 2015; **94**: e403 [PMID: 25590846 DOI: 10.1097/MD.0000000000000403]

22 **Lin S**, Zhang K, Zhang J, Wang M, Velani B, Zhu Y. Long-term outcomes of patients with hepatitis B virus-related acute on chronic liver failure: An observational cohort study. *Liver Int* 2019; **39**: 854-860 [PMID: 30753752 DOI: 10.1111/liv.14072]

23 **Huang YW**, Wang TC, Lin SC, Chang HY, Chen DS, Hu JT, Yang SS, Kao JH. Increased risk of cirrhosis and its decompensation in chronic hepatitis B patients with newly diagnosed diabetes: a nationwide cohort study. *Clin Infect Dis* 2013; **57**: 1695-1702 [PMID: 24051864 DOI: 10.1093/cid/cit603]

24 **Stepanova M**, Rafiq N, Younossi ZM. Components of metabolic syndrome are independent predictors of mortality in patients with chronic liver disease: a population-based study. *Gut* 2010; **59**: 1410-1415 [PMID: 20660697 DOI: 10.1136/gut.2010.213553]

25 **Lin S**, Chen J, Wang M, Han L, Zhang H, Dong J, Zeng D, Jiang J, Zhu Y. Prognostic nomogram for acute-on-chronic hepatitis B liver failure. *Oncotarget* 2017; **8**: 109772-109782 [PMID: 29312647 DOI: 10.18632/oncotarget.21012]

26 **Gao F**, Li X, Wan G, Li Y, Zhang Q, Liu Y, Liu H, Li H, Wang X. Development and external validation of a prognostic nomogram for acute decompensation of chronic hepatitis B cirrhosis. *BMC Gastroenterol* 2018; **18**: 179 [PMID: 30509201 DOI: 10.1186/s12876-018-0911-y]

27 **Bertot LC**, Jeffrey GP, de Boer B, MacQuillan G, Garas G, Chin J, Huang Y, Adams LA. Diabetes impacts prediction of cirrhosis and prognosis by non-invasive fibrosis models in non-alcoholic fatty liver disease. *Liver Int* 2018; **38**: 1793-1802 [PMID: 29575516 DOI: 10.1111/liv.13739]

28 **Wang YG**, Wang P, Wang B, Fu ZJ, Zhao WJ, Yan SL. Diabetes mellitus and poorer prognosis in hepatocellular carcinoma: a systematic review and meta-analysis. *PLoS One* 2014; **9**: e95485 [PMID: 24830459 DOI: 10.1371/journal.pone.0095485]

29 **Franz MJ**. Protein and diabetes: much advice, little research. *Curr Diab Rep* 2002; **2**: 457-464 [PMID: 12643172 DOI: 10.1007/s11892-002-0111-5]

30 **Patel JJ**, McClain CJ, Sarav M, Hamilton-Reeves J, Hurt RT. Protein Requirements for Critically Ill

Patients With Renal and Liver Failure. *Nutr Clin Pract* 2017; **32**: 101S-111S [PMID: 28208022 DOI: 10.1177/0884533616687501]

31 **Bémeur C**, Desjardins P, Butterworth RF. Role of nutrition in the management of hepatic encephalopathy in end-stage liver failure. *J Nutr Metab* 2010; **2010**: 489823 [PMID: 21234351 DOI: 10.1155/2010/489823]

32 **Tsiaousi ET**, Hatzitolios AI, Trygonis SK, Savopoulos CG. Malnutrition in end stage liver disease: recommendations and nutritional support. *J Gastroenterol Hepatol* 2008; **23**: 527-533 [PMID: 18397483 DOI: 10.1111/j.1440-1746.2008.05369.x]

33 **Lai R**, Chen T, Wu Z, Lin S, Zhu Y. Associations between body mass index and mortality in acute-on-chronic liver failure patients. *Ann Hepatol* 2019; **18**: 893-897 [PMID: 31506215 DOI: 10.1016/j.aohep.2019.07.008]

34 **Ooi PH**, Hager A, Mazurak VC, Dajani K, Bhargava R, Gilmour SM, Mager DR. Sarcopenia in Chronic Liver Disease: Impact on Outcomes. *Liver Transpl* 2019; **25**: 1422-1438 [PMID: 31242345 DOI: 10.1002/lt.25591]

35 **Carey EJ**, Lai JC, Wang CW, Dasarathy S, Lobach I, Montano-Loza AJ, Dunn MA; Fitness, Life Enhancement; and Exercise in Liver Transplantation Consortium. A multicenter study to define sarcopenia in patients with end-stage liver disease. *Liver Transpl* 2017; **23**: 625-633 [PMID: 28240805 DOI: 10.1002/lt.24750]

36 **Mauro E**, Crespo G, Martinez-Garmendia A, Gutierrez-Acevedo MN, Diaz JM, Saidman J, Bermudez C, Ortiz-Patron J, Garcia-Olveira L, Zalazar F, Narvaez A, Spina JC, Orta R, Savluk L, Piano S, Marciano S, Gadano A. Cystatin C and Sarcopenia Predict Acute on Chronic Liver Failure Development and Mortality in Patients on the Liver Transplant Waiting List. *Transplantation* 2020; **104**: e188-e198 [PMID: 32150034 DOI: 10.1097/TP.0000000000003222]

37 **Tanimoto Y**, Watanabe M, Sun W, Sugiura Y, Hayashida I, Kusabiraki T, Tamaki J. Sarcopenia and falls in community-dwelling elderly subjects in Japan: Defining sarcopenia according to criteria of the European Working Group on Sarcopenia in Older People. *Arch Gerontol Geriatr* 2014; **59**: 295-299 [PMID: 24852668 DOI: 10.1016/j.archger.2014.04.016]

38 **Hamed AE**, Elsahar M, Elwan NM, El-Nakeep S, Naguib M, Soliman HH, Ahmed Aboubakr A, AbdelMaqsod A, Sedrak H, Assaad SN, Elwakil R, Esmat G, Salh S, Mostafa T, Mogawer S, Sadek SE, Saber MM, Ezelarab H, Mahmoud AA, Sultan S, El Kassas M, Kamal E, ElSayed NM, Moussa S. Managing diabetes and liver disease association. *Arab J Gastroenterol* 2018; **19**: 166-179 [PMID: 30420265 DOI: 10.1016/j.ajg.2018.08.003]

39 **Elkrief L**, Rautou PE, Sarin S, Valla D, Paradis V, Moreau R. Diabetes mellitus in patients with cirrhosis: clinical implications and management. *Liver Int* 2016; **36**: 936-948 [PMID: 26972930 DOI: 10.1111/liv.13115]

40 **Chan WK**, Tan AT, Vethakkan SR, Tah PC, Vijayananthan A, Goh KL. Non-alcoholic fatty liver disease in diabetics--prevalence and predictive factors in a multiracial hospital clinic population in Malaysia. *J Gastroenterol Hepatol* 2013; **28**: 1375-1383 [PMID: 23517307 DOI: 10.1111/jgh.12204]

41 **Kabir MA**, Uddin MZ, Siddiqui NI, Robi IH, Malek MS, Islam MS, Rahman S, Hossain MS, Mahapatra SK, Alam MJ, Ahmad F, Alam MS, Islam MA. Prevalence of Non-Alcoholic Fatty Liver Disease and Its Biochemical Predictors in Patients with Type-2 Diabetes Mellitus. *Mymensingh Med J* 2018; **27**: 237-244 [PMID: 29769484]

42 **Axley P**, Ahmed Z, Arora S, Haas A, Kuo YF, Kamath PS, Singal AK. NASH Is the Most Rapidly Growing Etiology for Acute-on-Chronic Liver Failure-Related Hospitalization and Disease Burden in the United States: A Population-Based Study. *Liver Transpl* 2019; **25**: 695-705 [PMID: 30861321 DOI: 10.1002/lt.25443]

43 **Jepsen P**, Watson H, Andersen PK, Vilstrup H. Diabetes as a risk factor for hepatic encephalopathy in cirrhosis patients. *J Hepatol* 2015; **63**: 1133-1138 [PMID: 26206073 DOI: 10.1016/j.jhep.2015.07.007]

44 **Kalaitzakis E**, Olsson R, Henfridsson P, Hugosson I, Bengtsson M, Jalan R, Björnsson E. Malnutrition and diabetes mellitus are related to hepatic encephalopathy in patients with liver cirrhosis. *Liver Int* 2007; **27**: 1194-1201 [PMID: 17919230 DOI: 10.1111/j.1478-3231.2007.01562.x]

45 **Ampuero J**, Ranchal I, del Mar Díaz-Herrero M, del Campo JA, Bautista JD, Romero-Gómez M. Role of diabetes mellitus on hepatic encephalopathy. *Metab Brain Dis* 2013; **28**: 277-279 [PMID: 23180316 DOI: 10.1007/s11011-012-9354-2]

46 **Critchley JA**, Carey IM, Harris T, DeWilde S, Hosking FJ, Cook DG. Glycemic Control and Risk of Infections Among People With Type 1 or Type 2 Diabetes in a Large Primary Care Cohort Study. *Diabetes Care* 2018; **41**: 2127-2135 [PMID: 30104296 DOI: 10.2337/dc18-0287]

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ORIGINAL ARTICLE

Observational Study

Preliminary prospective study of real-time post-gastrectomy glycemic fluctuations during dumping symptoms using continuous glucose monitoring

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Author contributions: Ri M, Nunobe S, Ida S, Kumagai K, Ohashi M and Sano T designed this study; Ri M, Ida S and Atsumi S acquired data; Ri M prepared the final database; Ri M, Nunobe S and Ida S undertook the statistical analysis; Ishizuka N supervised the statistical analysis; Ri M wrote the draft paper; all authors critically reviewed and gave final approval of the version to be published.

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Abstract

BACKGROUND

Although dumping symptoms constitute the most common post-gastrectomy syndromes impairing patient quality of life, the causes, including blood sugar fluctuations, are difficult to elucidate due to limitations in examining dumping symptoms as they occur.

AIM

To investigate relationships between glucose fluctuations and the occurrence of dumping symptoms in patients undergoing gastrectomy for gastric cancer.

METHODS

Patients receiving distal gastrectomy with Billroth-I (DG-BI) or Roux-en-Y reconstruction (DG-RY) and total gastrectomy with RY (TG-RY) for gastric cancer (March 2018-January 2020) were prospectively enrolled. Interstitial tissue glycemic profiles were measured every 15 min, up to 14 d, by continuous glucose monitoring. Dumping episodes were recorded on 5 patient-selected days by diary. Within 3 h postprandially, dumping-associated glycemic changes were defined as a dumping profile, those without symptoms as a control profile. These profiles were compared.

RESULTS

Thirty patients were enrolled (10 DG-BI, 10 DG-RY, 10 TG-RY). The 47 early dumping profiles of DG-BI showed immediately sharp rises after a meal, which 47

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control profiles did not ($P < 0.05$). Curves of the 15 late dumping profiles of DG-BI were similar to those of early dumping profiles, with lower glycemic levels. DG-RY and TG-RY late dumping profiles (7 and 13, respectively) showed rapid glycemic decreases from a high glycemic state postprandially to hypoglycemia, with a steeper drop in TG-RY than in DG-RY.

CONCLUSION

Postprandial glycemic changes suggest dumping symptoms after standard gastrectomy for gastric cancer. Furthermore, glycemic profiles during dumping may differ depending on reconstruction methods after gastrectomy.

Key Words: Gastric cancer; Gastrectomy; Billroth-I reconstruction; Roux-en-Y reconstruction; Dumping syndrome; Continuous glucose monitoring

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Core Tip: Glucose variability at dumping onset was investigated using continuous glucose monitoring and subject diaries after standard gastrectomy for gastric cancer. Postprandial glycemic changes suggest both early and late dumping symptoms. Glycemic profiles during dumping may differ depending on reconstruction methods after gastrectomy, considering the similar glucose fluctuation curves with both early and late dumping after distal gastrectomy with Billroth I reconstruction and rapidly decreasing glucose profiles with late dumping after distal and total gastrectomy, both with Roux-en-Y reconstruction.

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INTRODUCTION

Globally, gastric cancer (GC) is among the most life-threatening malignancies[1,2]. Gastric resection with lymph node dissection is still the only curative treatment option for resectable GC, though it is noteworthy that early lesions can be resected endoscopically[3]. However, gastrectomy can result in various gastrointestinal symptoms known as post-gastrectomy syndrome, which is characterized by functional deficits and disorders due to loss of some or all of the stomach, often giving rise to clinical issues reflecting deterioration of quality of life for patients[4,5].

Dumping symptoms constitute the most common post-gastrectomy syndrome adversely affecting quality of life[6-8]. According to the time of onset, dumping syndrome is classified into early and late symptoms[9,10] but cannot always be clearly separated into these two categories. Therefore, while some patients develop either early or late dumping symptoms, others may have both. The mechanisms underlying late dumping symptoms especially are thought to involve hypoglycemia in response to hyperinsulinemia after carbohydrate ingestion[10,11]. However, blood glucose changes appearing while patients experience dumping symptoms are still not fully understood because a method allowing blood glucose to be measured easily and continuously has been lacking.

However, the continuous glucose monitoring (CGM) system developed for the management of diabetes allows interstitial glucose levels, which are closely related to blood glucose levels, to be tracked continuously[12,13]. Therefore, details of the 24 h glycemic profile, which includes both postprandial and nocturnal trends not measurable with a simple conventional glucometer, can be obtained using CGM. This also means that CGM has the potential to provide essential information about the glucose profiles of patients suffering from dumping symptoms after gastrectomy.

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Herein, we designed a prospective exploratory pilot study to investigate relationships between glucose fluctuations and the occurrence of dumping symptoms in patients who had undergone gastrectomy for GC, with various reconstructions. To our knowledge, this is the first examination of real-time glucose variability during the onset of dumping symptoms.

MATERIALS AND METHODS

Patients

During the period from March 2018 to January 2020, patients who underwent distal or total gastrectomy for GC at the Department of Gastroenterological Surgery, Cancer Institute Hospital, Tokyo, Japan, were prospectively enrolled in this study. The inclusion criteria were as follows: Diagnosed as having pathological stage I or II gastric adenocarcinoma, underwent R0 resection, age 20 to 75 years, 3 mo to 3 years after the operation and Eastern Cooperative Oncology Group Performance Status score 0 or 1. Patients with simultaneous resection of other organs (other than cholecystectomy or splenectomy), diabetes under treatment, receiving adjuvant chemotherapy, and/or taking supplements including enteral nutrition were excluded. Pathological stages were determined according the 14th edition of the Japanese classification of gastric carcinoma[14]. This study was approved by the institutional review board of the Cancer Institute Hospital (No. 2017-1110). All participants signed a written informed consent for the present study. All protocols are carried out in accordance with relevant guidelines.

Continuous glucose monitoring

FreeStyle Libre Pro (Abbot Diabetes Care Inc., Alameda, CA, United States), a CGM device, was used to continuously measure glucose concentrations. The sensor attached to the posterior surface of the patient's upper arm continuously measured and recorded the glucose concentration in the subcutaneous tissue interstitial fluid every 15 min for up to 14 d. Measurement results automatically saved on the sensor were transferred wirelessly to the reader and then analyzed using FreeStyle Libre Pro Software *via* the reader.

Assessment of dumping syndrome

Dumping symptoms were evaluated using a diary recording diet and symptoms. Diary entries were made every 15 min and listed the 15 typical symptoms related to dumping, as previously reported[15,16]. The patient filled in the times of starting and completing meals, whether symptoms appeared, allowing the times and corresponding symptoms to be checked. Considering that patient dietary records after gastrectomy often document three or more meals, snacks described by the patient as being about the same amount as an ordinary meal were regarded as meals and were recorded as such in the diary. The diary entries were made for 5 patient-selected days within the 14 d period with the sensor attached.

Definition of dumping and control

Our strategy for defining the dumping and control profiles is presented in Figure 1. Dumping syndrome is defined as the development of one or more of the 15 symptoms listed in the diary within 3 h of eating a meal. Furthermore, symptoms that occurred within 1 h after the start of a meal were regarded as early dumping and those occurring within 1 h to 3 h after starting a meal were regarded as late dumping, as previously reported[10,17]. To avoid effects of the intakes of other foods, we excluded cases in which another meal was consumed from two hours before to three hours after the baseline meal. The total number of symptoms was described as N-symp. In addition, within 3 h after the start of a meal, glycemic changes associated with early or late dumping symptoms were defined as a dumping profile, while those with no symptoms were defined as a control profile. The control profiles consisted of up to one series per patient per day. The total number with each profile was designated the N-profile. If multiple symptoms appeared simultaneously, the symptoms were counted accordingly, and the profile was only counted once.

Statistical analysis

The patient background characteristics, surgical details and postoperative findings were collected from our database and information contained in electronic medical

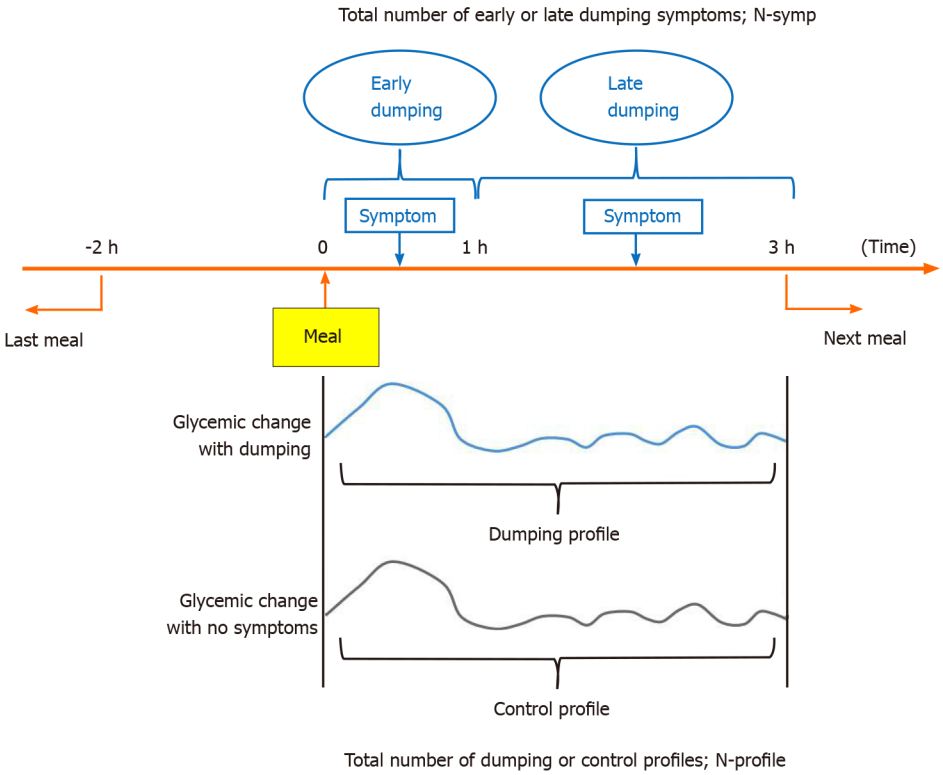


Figure 1 How dumping and control profiles were defined.

records. Based on the glucose concentration values measured every 15 min by CGM and the details of the dumping symptoms described in the diary, dumping and control profiles were compared. All missing values in the data obtained employing CGM were replaced by linear interpolation as single imputation method[18]. All continuous variables were expressed as median values. Although we used *P* value of Mann-Whitney *U* test and the χ^2 test in addition to basic statistics, statistical analysis is solely exploratory and descriptive without any formal general linear models. The statistical methods of this study were reviewed by Naoki Ishizuka, a biostatistician. A *P* value less than 0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed with JMP Pro 13 (SAS Institute Japan Ltd, Japan) for Windows.

RESULTS

Patient characteristics

Patient background data are presented in Table 1. In total, 30 patients were enrolled prospectively: 10 patients each underwent distal gastrectomy with Billroth I reconstruction (DG-BI), distal gastrectomy with Roux-en-Y reconstruction (DG-RY) and total gastrectomy with Roux-en-Y reconstruction (TG-RY). The DG-BI group had a significantly shorter period since surgery than the other two surgical groups ($P = 0.01$). Surgical approach, degree of lymph node dissection and pathological stage differed significantly among the three groups. There were no significant differences in nutritional status or HbA1c levels among the three groups.

Details of dumping symptoms

The details of early and late dumping symptoms are shown in Tables 2 and 3. In all patients, early dumping symptoms consisted mainly of abdominal symptoms such as borborygmi, bloating and abdominal pain (Table 2). The rates of borborygmi in TG-RY, bloating in DG-BI and drowsiness in DG-RY were significantly higher than those associated with other procedures. On the other hand, late dumping exhibited a wide range of symptoms in all three patient groups, and hypoglycemic symptoms such as cold sweat and drowsiness were relatively common among the features of late dumping syndrome (Table 3).

Dumping and control profiles

Dumping and control profiles obtained by CGM and the diary results are shown in Figure 2. During the 5 d of diary recording, the respective N-profiles of early dumping were 47, 25 and 54, those of late dumping were 15, 7 and 13, in the order of DG-BI, DG-RY and TG-RY.

The early dumping profiles in DG-BI showed a sharp and immediate rise when starting a meal and then dropped, with significant increases up to 60 min postprandially as compared with the control group ($P < 0.05$, Figure 2A). The curves of late dumping profiles in DG-BI were similar to those of early dumping profiles, with generally lower glucose levels (Figure 2A). The early dumping profiles in TG-RY increased significantly from the start of a meal up to 60 min postprandially as compared to the control profiles ($P < 0.05$, Figure 2C).

When late dumping developed in DG-RY and TG-RY (Figure 2B and 2C), the dumping profiles in the former showed a sharp decrease from the peak glycemic value at 75 min after starting a meal. A similar but more rapid drop was observed in the dumping profiles in TG-RY from the hyperglycemic state at 45 min after starting a meal. Most notably, glucose levels in TG-RY ultimately decreased to 69 mg/dL at 90 min postprandially.

DISCUSSION

Dumping syndrome was first reported by Mix in 1922 as a serious complication of gastrectomy[19]. Several mechanisms have been speculated to underlie the development of dumping symptoms[9,10,20]. In early dumping, because of the rapid flow of hyperosmolar food into the jejunum, the plasma fluid rapidly moves into the intestinal lumen and the plasma volume decreases. Furthermore, the release of gastrointestinal hormones, including vasoactive agents, incretins and glucose modulators, is also increased[21]. Consequently, vasomotor symptoms such as palpitations, weakness and faintness and gastrointestinal symptoms such as abdominal pain, bloating and borborygmi develop[10]. On the other hand, hypoglycemia in response to hyperinsulinemia after carbohydrate ingestion has been said to cause hypoglycemic symptoms including the drowsiness and cold sweat characteristic of late dumping[9,10,20]. In fact, early and late dumping symptoms similar to those previously reported were also observed in the present study. However, the causes of the onset of dumping symptoms have not yet been sufficiently elucidated because, as mentioned above, it is difficult to measure changes in plasma volume, various hormones and blood glucose levels when a dumping symptom is actually occurring.

To our knowledge, this is the first exploratory study of real-time changes in glucose profiles when a dumping symptom actually occurs after gastrectomy using CGM and daily recording of diet and symptoms. Although a few studies have demonstrated continuous glucose profiles using CGM after GC surgery, with most simply measuring the glucose fluctuations, the occurrence of dumping symptoms over time has not previously been examined[22-24]. Results of the present study indicated glucose fluctuations to be involved in the onset of late dumping as well as early dumping symptoms after standard gastrectomy for GC.

The early dumping profiles in DG-BI showed rises immediately after the start of meals and subsequently dropped in contrast to the control profiles. Postprandial hyperglycemia is usually observed in patients with impaired glucose tolerance, which is representative of diabetes. However, it was recently found that healthy subjects without diabetes show a similar phenomenon called a blood glucose spike[25]. Although the main mechanisms have been considered to differ between early and late dumping symptoms, a series of similar mechanisms might underlie the development of both early and late dumping symptoms, considering that similar curves of glycemic changes were observed in both symptomatic groups. Mine *et al*[8], who demonstrated a strong correlation between early and late dumping, suggested that a faster or greater flow of food into the small intestine may be the cause of both of early and late dumping symptoms.

On the other hand, in the late dumping profiles in DG-RY and TG-RY, a marked decrease in glucose levels was observed from a high glycemic state around 1 h after starting a meal to a hypoglycemic level around 2 h postprandially. Although hypoglycemia has been regarded as a cause of late dumping symptoms as described above, many hypoglycemic episodes are reportedly asymptomatic, and the appearance of hypoglycemia-like symptoms does not consistently correlate with biochemically verified hypoglycemia after gastrectomy with RY reconstruction for obese patients[26,

Table 1 Patient demographic and clinical features

	DG-BI, *n* = 10	DG-RY, *n* = 10	TG-RY, *n* = 10	*P* value
Sex, *n* (%)				0.30
Male	3 (30)	6 (60)	6 (60)	
Female	7 (70)	4 (40)	4 (40)	
Age, yr (IQR)	60 (48-70)	63 (55-68)	62 (46-70)	0.97
Period from operation, mo (IQR)	7.1 (6.7-19.2)	23.5 (19.5-26.6)	20.3 (14.9-26.1)	0.01
Body mass index, kg/m^2 (IQR)	19.0 (16.1-23.1)	21.3 (18.4-23.5)	20.5 (19.4-23.1)	0.32
Serum total protein, g/dL (IQR)	7.1 (6.8-7.4)	7.0 (6.7-7.3)	6.8 (6.5-7.3)	0.57
Serum prealbumin, mg/dL (IQR)	23.4 (18.6-28.7)	23.0 (21.8-29.3)	22.0 (18.4-26.5)	0.62
Serum albumin, g/dL (IQR)	4.3 (4.2-4.6)	4.2 (4.0-4.5)	4.1 (4.1-4.3)	0.45
Serum hemoglobin, g/dL (IQR)	12.9 (12.2-14.3)	13.6 (12.2-15.0)	12.1 (11.3-13.2)	0.21
Blood glucose level, mg/dL (IQR)	96 (95-99)	96 (89-114)	93 (88-100)	0.73
HbA1c, % (IQR)	5.7 (5.3-5.9)	5.7 (5.6-5.9)	5.6 (5.5-5.8)	0.73
Approach				< 0.01
Open	0 (0)	2 (20)	6 (60)	
Laparoscopic	10 (100)	8 (80)	4 (40)	
Lymph node dissection, *n* (%)				< 0.01
D1+	10 (100)	5 (50)	2 (20)	
D2	0 (0)	5 (50)	8 (80)	
pStage, *n* (%)				< 0.01
I	10 (100)	9 (90)	3 (30)	
II	0 (0)	1 (10)	7 (70)	

DG-BI: Distal gastrectomy with Billroth I reconstruction; DG-RY: Distal gastrectomy with Roux-en-Y reconstruction; HbA1c: Hemoglobin A1c; IQR: Interquartile range; TG-RY: Total gastrectomy with Roux-en-Y reconstruction.

Table 2 Details of early dumping symptoms in all patients, *n* (%)

	Early dumping symptoms				
	All, N-symp = 185	DG-BI, N-symp = 80	DG-RY, N-symp = 35	TG-RY, N-symp = 70	*P* value
Borborygmi	49 (26.5)	14 (17.5)	9 (25.7)	26 (37.1)	0.02
Bloating	47 (25.4)	28 (35.0)	4 (11.4)	15 (21.4)	0.01
Abdominal pain	21 (11.4)	9 (11.3)	2 (5.7)	10 (14.3)	0.42
Drowsiness	21 (11.4)	9 (11.3)	8 (22.9)	4 (5.7)	0.03
Palpitation	15 (8.1)	3 (3.8)	2 (5.7)	10 (14.3)	0.05
Weakness	11 (5.9)	6 (7.5)	3 (8.6)	2 (2.9)	0.37
Others	21 (11.4)	11 (13.8)	7 (20.0)	3 (4.3)	0.03

DG-BI: Distal gastrectomy with Billroth I reconstruction; DG-RY: Distal gastrectomy with Roux-en-Y reconstruction; N-symp: Total number of dumping symptoms; TG-RY: Total gastrectomy with Roux-en-Y reconstruction.

27]. Therefore, the results of the present study raise the possibility that the development of late dumping after RY reconstruction may be related not to hypoglycemia but rather to rapid drops in glucose profiles following meals. A possible mechanism would be that postprandial hyperglycemia, resulting from increased carbohydrate absorption from the upper jejunum, further promotes the secretion of

Table 3 Details of late dumping symptoms in all patients, *n* (%)

	Late dumping symptoms				
	All, N-symp = 62	DG-BI, N-symp = 28	DG-RY, N-symp = 12	TG-RY, N-symp = 22	*P* value
Cold sweat	9 (14.5)	3 (10.7)	2 (16.7)	4 (18.2)	0.73
Drowsiness	8 (12.9)	3 (10.7)	1 (8.3)	4 (18.2)	0.64
Diarrhea	8 (12.9)	5 (17.9)	3 (25.0)	0 (0)	0.06
Weakness	6 (9.7)	3 (10.7)	0 (0)	3 (13.6)	0.42
Bloating	6 (9.7)	3 (10.7)	1 (8.3)	2 (9.1)	0.96
Abdominal pain	6 (9.7)	1 (3.6)	3 (25.0)	2 (9.1)	0.10
Borborygmi	5 (8.1)	3 (10.7)	2 (16.7)	0 (0)	0.18
Palpitations	4 (6.5)	1 (3.6)	0 (0)	3 (13.6)	0.21
Tremulousness	4 (6.5)	2 (7.1)	0 (0)	2 (9.1)	0.57
Others	6 (9.7)	4 (14.3)	0 (0)	2 (9.1)	0.37

DG-BI: Distal gastrectomy with Billroth I reconstruction; DG-RY: Distal gastrectomy with Roux-en-Y reconstruction; N-symp: Total number of dumping symptoms; TG-RY: Total gastrectomy with Roux-en-Y reconstruction.

incretins such as GLP-1, one of the gastrointestinal hormones, resulting in hyperinsulinemia and subsequently a rapid decrease in glucose levels[28].

Although a similar glycemic variability was observed in the late dumping groups that had undergone DG-RY and TG-RY, the curve of glucose profiles was steeper in the TG-RY than in the DG-RY dumping group. Due to the lack of storage capacity, that is the absence of part or all of the stomach, there might be a faster flow of food into the jejunum in TG-RY than in DG-RY, resulting in a more rapid glucose level change and higher number of dumping symptoms. In contrast, some of the observed variation in glycemic profiles might have been due to differences in the size of the remnant stomach in DG-RY, resulting in a lack of statistical significance. In addition, considering that the control profiles in DG-RY and TG-RY were more remarkable than those in DG-BI, RY reconstruction, a non-physiological reconstruction method in which food does not pass through the duodenum, may impact glucose fluctuations after a meal more adversely than the other procedures.

The present study has potential limitations. First, although prospective, this was a preliminary study with a small sample size conducted at a single institution. It is possible that statistical differences could not be demonstrated due to the small number of events. In addition, variation in the frequency of dumping among patients might have produced statistical bias. Further accumulation of cases, allowing a study with a larger sample size, is needed. Second, postoperative periods were not similar among the three groups. Although we aimed herein to enroll patients in the mid to long term after surgery, differences in postoperative periods may have influenced the onset of dumping symptoms and glycemic change. Third, dietary details were not documented in this study. Caloric intake has a significant effect on blood glucose levels, and the manner in which a meal is consumed may affect the onset of dumping symptoms. Finally, although glucose profiles and dumping symptoms were sequentially investigated, other factors possibly involved in the onset of dumping were not examined. The evaluation of changes in hemodynamics and various hormones, which are considered to be factors causing dumping, is another topic for future research.

CONCLUSION

Postprandial rapid glycemic changes appear to be involved in the onset of early and late dumping symptoms after standard gastrectomy for GC. Given the similar glucose fluctuation curves with early and late dumping in DG-BI and the rapid decrease in glucose profiles with late dumping in DG-RY and TG-RY, the glycemic profiles associated with dumping symptoms may differ depending on the reconstruction methods employed after gastrectomy.

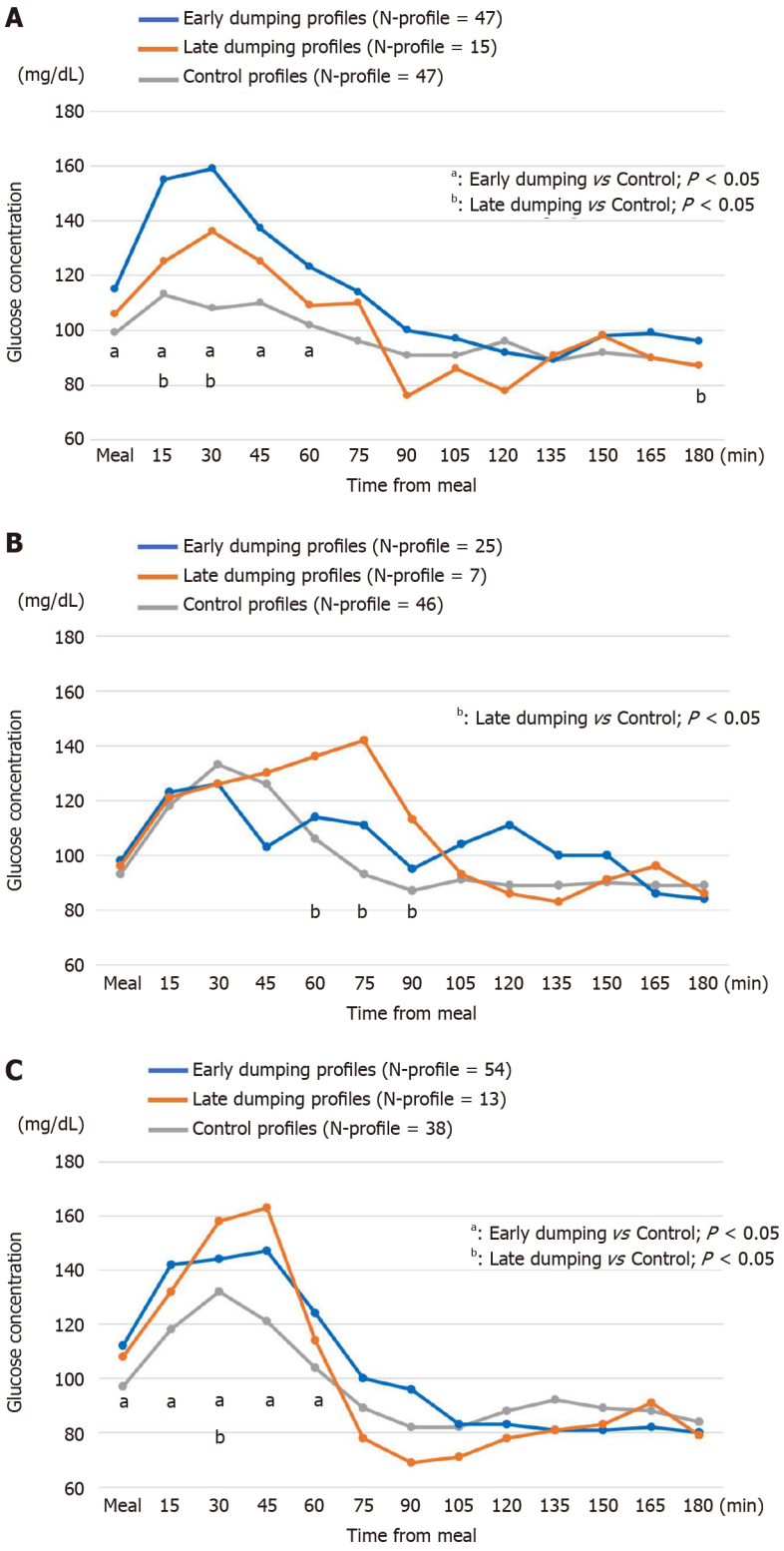


Figure 2 Glycemic changes with dumping symptoms (dumping profiles) and without symptoms (control profiles) after gastrectomy. A: Dumping and control profiles after distal gastrectomy with Billroth I reconstruction; B: Dumping and control profiles after distal gastrectomy with Roux-en-Y reconstruction; C: Dumping and control profiles after total gastrectomy with Roux-en-Y reconstruction.

ARTICLE HIGHLIGHTS

Research background

Dumping symptoms constitute the most common post-gastrectomy syndrome adversely affecting quality of life. However, the causes of dumping symptoms, including blood glucose changes, remain poorly understood due to limitations in examining dumping symptoms as they occur.

Research motivation

The continuous glucose monitoring (CGM) system, which continuously measures interstitial glucose levels to reflect blood glucose levels, was developed for the management of diabetes. CGM also has the potential to provide long awaited essential information about the glucose profiles of patients suffering from dumping symptoms after gastrectomy.

Research objectives

We designed a prospective pilot study to investigate relationships between glucose fluctuations and the occurrence of dumping symptoms in patients undergoing gastrectomy for gastric cancer (GC). Our results may contribute to devising future treatments for dumping syndrome.

Research methods

During the period from March 2018 to January 2020, GC patients who underwent distal gastrectomy with Billroth I reconstruction (DG-BI), distal gastrectomy with Roux-en-Y reconstruction (DG-RY) or total gastrectomy with Roux-en-Y reconstruction (TG-RY) were prospectively enrolled in this study. Based on the glucose concentration values measured every 15 min by CGM and the details of the dumping symptoms (early dumping within 1 h postprandially, late dumping within 1 h to 3 h postprandially) described in diaries, patients with dumping-associated glycemic changes (dumping profiles) were compared to those without symptoms (control profiles). This is the first examination of real-time glucose variability during the onset of dumping symptoms using CGM.

Research results

Thirty patients were enrolled (10 DG-BI, 10 DG-RY, 10 TG-RY). The early dumping profiles of DG-BI (47 profiles) showed a sharp and immediate rise after a meal, with significant increases up to 60 min postprandially as compared with the control group (47 profiles) ($P < 0.05$). The curves of late dumping profiles in DG-BI were similar to those of early dumping profiles, with generally lower glucose levels. DG-RY and TG-RY late dumping profiles (7 and 13, respectively) showed rapid glycemic decreases from a high glycemic state postprandially to hypoglycemia, with the drop being steeper in TG-RY than in DG-RY.

Research conclusions

Postprandial rapid glycemic changes appear to be involved in the onset of early and late dumping symptoms after standard gastrectomy for GC. In addition, the glycemic profiles associated with dumping symptoms may differ depending on the reconstruction methods employed after gastrectomy, considering the similar glucose fluctuation curves with both early and late dumping after DG-BI and rapidly decreasing glucose profiles with late dumping after DG-RY and TG-RY.

Research perspectives

We will conduct a prospective interventional study with the aim of developing new treatments ameliorating dumping symptoms associated with GC surgery.

REFERENCES

1 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

2 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

3 **Bonenkamp JJ**, Hermans J, Sasako M, van de Velde CJ, Welvaart K, Songun I, Meyer S, Plukker JT, Van Elk P, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H; Dutch Gastric Cancer Group. Extended lymph-node dissection for gastric cancer. *N Engl J Med* 1999; **340**: 908-914 [PMID: 10089184 DOI: 10.1056/NEJM199903253401202]

4 **Bolton JS**, Conway WC 2nd. Postgastrectomy syndromes. *Surg Clin North Am* 2011; **91**: 1105-1122 [PMID: 21889032 DOI: 10.1016/j.suc.2011.07.001]

5 **Davis JL**, Ripley RT. Postgastrectomy Syndromes and Nutritional Considerations Following Gastric Surgery. *Surg Clin North Am* 2017; **97**: 277-293 [PMID: 28325187 DOI: 10.1016/j.suc.2016.11.005]

6 **Bender MA**, Moore GE, Webber BM. Dumping syndrome; an evaluation of some current etiologic concepts. *N Engl J Med* 1957; **256**: 285-289 [PMID: 13400253 DOI:

10.1056/NEJM195702142560702]
7 **Abbott WE**, Krieger H, Levey S, Bradshaw J. The etiology and management of the dumping syndrome following a gastroenterostomy or subtotal gastrectomy. *Gastroenterology* 1960; **39**: 12-27 [PMID: 13791387]
8 **Mine S**, Sano T, Tsutsumi K, Murakami Y, Ehara K, Saka M, Hara K, Fukagawa T, Udagawa H, Katai H. Large-scale investigation into dumping syndrome after gastrectomy for gastric cancer. *J Am Coll Surg* 2010; **211**: 628-636 [PMID: 20829078 DOI: 10.1016/j.jamcollsurg.2010.07.003]
9 **Tack J**, Arts J, Caenepeel P, De Wulf D, Bisschops R. Pathophysiology, diagnosis and management of postoperative dumping syndrome. *Nat Rev Gastroenterol Hepatol* 2009; **6**: 583-590 [PMID: 19724252 DOI: 10.1038/nrgastro.2009.148]
10 **van Beek AP**, Emous M, Laville M, Tack J. Dumping syndrome after esophageal, gastric or bariatric surgery: pathophysiology, diagnosis, and management. *Obes Rev* 2017; **18**: 68-85 [PMID: 27749997 DOI: 10.1111/obr.12467]
11 **Machella TE**. The Mechanism of the Post-gastrectomy "Dumping" Syndrome. *Ann Surg* 1949; **130**: 145-159 [PMID: 17859417]
12 **Bolinder J**, Antuna R, Geelhoed-Duijvestijn P, Kröger J, Weitgasser R. Novel glucose-sensing technology and hypoglycaemia in type 1 diabetes: a multicentre, non-masked, randomised controlled trial. *Lancet* 2016; **388**: 2254-2263 [PMID: 27634581 DOI: 10.1016/S0140-6736(16)31535-5]
13 **Wood A**, O'Neal D, Furler J, Ekinci EI. Continuous glucose monitoring: a review of the evidence, opportunities for future use and ongoing challenges. *Intern Med J* 2018; **48**: 499-508 [PMID: 29464891 DOI: 10.1111/imj.13770]
14 **Japanese Gastric Cancer Association**. Japanese classification of gastric carcinoma: 3rd English edition. *Gastric Cancer* 2011; **14**: 101-112 [PMID: 21573743 DOI: 10.1007/s10120-011-0041-5]
15 **Linehan IP**, Weiman J, Hobsley M. The 15-minute dumping provocation test. *Br J Surg* 1986; **73**: 810-812 [PMID: 3768652 DOI: 10.1002/bjs.1800731017]
16 **Vecht J**, Masclee AA, Lamers CB. The dumping syndrome. Current insights into pathophysiology, diagnosis and treatment. *Scand J Gastroenterol Suppl* 1997; **223**: 21-27 [PMID: 9200302]
17 **Arts J**, Caenepeel P, Bisschops R, Dewulf D, Holvoet L, Piessevaux H, Bourgeois S, Sifrim D, Janssens J, Tack J. Efficacy of the long-acting repeatable formulation of the somatostatin analogue octreotide in postoperative dumping. *Clin Gastroenterol Hepatol* 2009; **7**: 432-437 [PMID: 19264574 DOI: 10.1016/j.cgh.2008.11.025]
18 **Millard LAC**, Patel N, Tilling K, Lewcock M, Flach PA, Lawlor DA. GLU: a software package for analysing continuously measured glucose levels in epidemiology. *Int J Epidemiol* 2020; **49**: 744-757 [PMID: 32737505 DOI: 10.1093/ije/dyaa004]
19 **Mix C**. Dumping stomach following gastrojejunostomy. *Surg Clin North Am* 1922; **2**: 617-622
20 **Tack J**. Gastric motor disorders. *Best Pract Res Clin Gastroenterol* 2007; **21**: 633-644 [PMID: 17643905 DOI: 10.1016/j.bpg.2007.04.001]
21 **Øhrstrøm CC**, Worm D, Kielgast UL, Holst JJ, Hansen DL. Evidence for Relationship Between Early Dumping and Postprandial Hypoglycemia After Roux-en-Y Gastric Bypass. *Obes Surg* 2020; **30**: 1038-1045 [PMID: 31907828 DOI: 10.1007/s11695-020-04387-6]
22 **Buscemi S**, Mattina A, Genova G, Genova P, Nardi E, Costanzo M. Seven-day subcutaneous continuous glucose monitoring demonstrates that treatment with acarbose attenuates late dumping syndrome in a woman with gastrectomy for gastric cancer. *Diabetes Res Clin Pract* 2013; **99**: e1-e2 [PMID: 23146372 DOI: 10.1016/j.diabres.2012.10.021]
23 **Kurihara K**, Tamai A, Yoshida Y, Yakushiji Y, Ueno H, Fukumoto M, Hosoi M. Effectiveness of sitagliptin in a patient with late dumping syndrome after total gastrectomy. *Diabetes Metab Syndr* 2018; **12**: 203-206 [PMID: 29208515 DOI: 10.1016/j.dsx.2017.11.001]
24 **Kubota T**, Shoda K, Ushigome E, Kosuga T, Konishi H, Shiozaki A, Kudo M, Arita T, Murayama Y, Morimura R, Ikoma H, Kuriu Y, Nakanishi M, Fujiwara H, Okamoto K, Fukui M, Otsuji E. Utility of continuous glucose monitoring following gastrectomy. *Gastric Cancer* 2020; **23**: 699-706 [PMID: 31916026 DOI: 10.1007/s10120-019-01036-5]
25 **Hall H**, Perelman D, Breschi A, Limcaoco P, Kellogg R, McLaughlin T, Snyder M. Glucotypes reveal new patterns of glucose dysregulation. *PLoS Biol* 2018; **16**: e2005143 [PMID: 30040822 DOI: 10.1371/journal.pbio.2005143]
26 **Abrahamsson N**, Edén Engström B, Sundbom M, Karlsson FA. Hypoglycemia in everyday life after gastric bypass and duodenal switch. *Eur J Endocrinol* 2015; **173**: 91-100 [PMID: 25899582 DOI: 10.1530/EJE-14-0821]
27 **Laurenius A**, Werling M, Le Roux CW, Fändriks L, Olbers T. More symptoms but similar blood glucose curve after oral carbohydrate provocation in patients with a history of hypoglycemia-like symptoms compared to asymptomatic patients after Roux-en-Y gastric bypass. *Surg Obes Relat Dis* 2014; **10**: 1047-1054 [PMID: 25205571 DOI: 10.1016/j.soard.2014.04.007]
28 **Salehi M**, Gastaldelli A, D'Alessio DA. Blockade of glucagon-like peptide 1 receptor corrects postprandial hypoglycemia after gastric bypass. *Gastroenterology* 2014; **146**: 669-680. e2 [PMID: 24315990 DOI: 10.1053/j.gastro.2013.11.044]

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ORIGINAL ARTICLE

Prospective Study

Real-world treatment patterns and disease control over one year in patients with inflammatory bowel disease in Brazil

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Abstract

BACKGROUND

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBDs) with a remission-relapsing presentation and symptomatic exacerbations that have detrimental impacts on patient quality of life and are associated with a high cost burden, especially in patients with moderate-to-severe disease. The Real-world Data of Moderate-to-Severe Inflammatory Bowel Disease in Brazil (RISE BR) study was a noninterventional study designed to evaluate disease control, treatment patterns, disease burden and health-related quality of life in patients with moderate-to-severe active IBD. We report findings from the prospective follow-up phase of the RISE BR study in patients with active UC or CD.

AIM

To describe the 12-mo disease evolution and treatment patterns among patients with active moderate-to-severe IBD in Brazil.

METHODS

This was a prospective, noninterventional study of adult patients with active Crohn's disease (CD: Harvey-Bradshaw Index ≥ 8, CD Activity Index ≥ 220), inadequate CD control (*i.e.*, calprotectin > 200 μg/g or colonoscopy previous results), or active ulcerative colitis (UC: Partial Mayo score ≥ 5). Enrollment occurred in 14 centers from October 2016 to February 2017. The proportion of active IBD patients after 9-12 mo of follow-up, Kaplan-Meier estimates of the time to mild or no activity and a summary of treatment initiation, discontinuation and dose changes were examined.

RESULTS

The study included 118 CD and 36 UC patients, with mean ± SD ages of 43.3 ± 12.6 and 44.9 ± 16.5 years, respectively. The most frequent drug classes at index were biologics for CD (62.7%) and 5-aminosalicylate derivates for UC patients

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(91.7%). During follow-up, 65.3% of CD and 86.1% of UC patients initiated a new treatment at least once. Discontinuations/dose changes occurred in 68.1% of CD patients [median 2.0 (IQR: 2-5)] and 94.3% of UC patients [median 4.0 (IQR: 3-7)]. On average, CD and UC patients had 4.4 ± 2.6 and 5.0 ± 3.3 outpatient visits, respectively. The median time to first mild or no activity was 319 (IQR: 239-358) d for CD and 320 (IQR: 288-358) d for UC patients. At 9-12 mo, 22.0% of CD and 20.0% of UC patients had active disease.

CONCLUSION

Although a marked proportion of active IBD patients achieved disease control within one year, the considerable time to achieve this outcome represents an unmet medical need of the current standard of care in a Brazilian real-world setting.

Key Words: Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Prospective study

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Core Tip: This was a prospective, noninterventional study of 118 adult patients with active Crohn's disease (CD) and 36 with active ulcerative colitis (UC). The aim was to describe the 12-mo disease evolution and treatment patterns of active moderate-to-severe inflammatory bowel disease (IBD) patients in Brazil. The median time to first mild or no activity was 319 (IQR: 239-358) d for CD and 320 (IQR: 288-358) d for UC patients. A marked proportion of active IBD patients achieved disease control within one year, which is considered a long time to achieve the goal of reducing inflammation.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBDs) with a remission-relapsing presentation and symptomatic exacerbations that have a detrimental impact on patient quality of life and are associated with a high cost burden, especially in the case of moderate to severe disease[1,2]. The pharmacological treatment of IBD aims to achieve and maintain long-term remission and heal the gut mucosa, thus delaying the progression of disease and associated complications as well as the need for surgery[3]. For CD patients with moderate-to-severe disease activity, guidelines usually recommend the use of antitumor necrosis factor-α (anti-TNFα) either as a monotherapy or combined with an immunosuppressant to induce remission for steroid-refractory disease or steroid-intolerant patients[4]. With regard to moderate-to-severe active UC, an immunosuppressant and/or a biologic drug are recommended[5,6]. Systemic 5-aminosalicylate (5-ASA) agents are also indicated for maintenance treatment of UC[1]. Corticosteroids are recommended for short-term use (≤ 3 mo) during relapses of both CD and UC and are not recommended as maintenance therapy[1,4,6].

In clinical practice, physicians often try several options and treatment sequences for IBD management, both in monotherapy or combined regimens[3]. Primary nonresponse, secondary loss of response and development of adverse reactions to anti-TNFα drugs may occur and lead to discontinuation, with the subsequent prescription of a second advanced therapy with a different mechanism of action (*e.g.*, anti-integrins

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or JAK inhibitors)[7-11]. However, some studies have reported that up to half of IBD patients who discontinue their first-line biologic (infliximab or adalimumab) do not restart or switch to another therapy[3,12]. In Brazil, vedolizumab, ustekinumab and tofacitinib are available at private health services, the latter being approved only for UC treatment since 2018. Moreover, the VARSITY study recently demonstrated that the gut-selective anti-integrin vedolizumab was superior to the anti-TNFα adalimumab in achieving clinical remission in patients with moderate-to-severe active UC after one year[13].

Real-world data on IBD management could unveil unmet medical needs and treatment gaps[3]. This is of utmost relevance in developing countries such as Brazil, where the prevalence of IBD is increasing but access to biologic treatment may be restricted, and information on IBD treatment, in general, and associated outcomes is scarce[14,15]. The Real-world Data of Moderate-to-Severe Inflammatory Bowel Disease in Brazil (RISE BR) study was a noninterventional study designed to evaluate disease control, treatment patterns, burden of disease and health-related quality of life in patients with a previous diagnosis of moderate-to-severe active IBD[16,17]. Here, we report findings from the prospective follow-up phase of the RISE BR study in patients with active UC or CD and aim to describe the real-world treatment patterns and outcomes of IBD-related therapies during a 12-mo follow-up period.

MATERIALS AND METHODS

Study design

The RISE BR study (ClinicalTrials.gov Identifier: NCT02822235) was a national, multicenter, noninterventional study designed to evaluate disease control, treatment patterns, burden of disease and health-related quality of life among IBD patients in Brazil. It involved a cross-sectional evaluation of IBD patients (index date) with a retrospective data collection component and a 12-mo prospective follow-up period of IBD patients who had active disease (at the index date). The cross-sectional evaluation was conducted at routine IBD outpatient visits between October 2016 and February 2017, and patients were consecutively recruited from 14 study sites (nine clinics from the public health system and five private clinics) covering Brazil's most populated regions. In this article, we present data from the follow-up period. All participants provided written informed consent prior to the study procedures. The study was approved by the local ethics committees and was conducted according to the principles of the Helsinki Declaration.

Study population

At the index date, patients were included if they were aged 18 years or older and had a confirmed diagnosis of moderate-to-severe CD or UC at least 6 mo prior to index. Patients were excluded if they presented indeterminate/unclassified colitis, mental incapacity, unwillingness, or language barriers precluding adequate understanding or cooperation with the study, were hospitalized, participated in a clinical trial within the last 3 years, or received off-label treatment with vedolizumab. All patients presenting active disease at the index date were invited to participate in the prospective follow-up phase of the study. Active CD was defined as a Harvey-Bradshaw Index (HBI) ≥ 8 points or a Crohn's Disease Activity Index (CDAI) ≥ 220 points or fecal calprotectin levels > 200 μg/g or colonoscopy results in the previous year suggestive of inadequate control (as per investigator criteria)[18]. Active UC was defined as a partial Mayo (pMayo) score ≥ 5 points.

Prospective follow-up period and study variables

The prospective observational period was defined as a 12-mo follow-up since the index date. Data were collected from patients' medical records by staff personnel of the participating site. Sociodemographic characteristics (age, sex, educational level, and professional status), weight and body mass index (BMI), smoking habits, and family history of IBD were collected at the study appointment. Other clinical variables included the time since first IBD diagnosis, extension/severity and location of CD or UC (Montreal classification), corticosteroid dependency/intolerance status, and presence of extraintestinal manifestations. In addition, IBD treatment patterns and changes during the follow-up period (at the index date and the following medical appointments) were collected.

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CD activity during follow-up was evaluated with the HBI[18] and/or CDAI[19], depending on local practice. Moderate-to-severe active CD was defined as patients with an HBI ≥ 8 or a CDAI ≥ 220, while CD disease control (*i.e.*, mild or no disease activity) was defined as an HBI < 8 or a CDAI < 220[18-20]. UC disease activity during follow-up was assessed with the 9-item pMayo score; moderate-to-severe active UC was defined as pMayo ≥ 5, and UC disease control (*i.e.*, mild or no UC activity) was defined as pMayo < 5[21]. Given the noninterventional design of the study, there were no predefined time points of IBD activity assessment, and measures were collected in the course of typical disease treatment.

Statistical analysis

Descriptive statistics [mean, median, standard deviation (SD) and minimum-maximum] were used to analyze sociodemographic, clinical, and treatment-related variables. Student's *t*-test for independent samples or the Mann-Whitney test were used to compare CD and UC patients regarding quantitative variables. Fisher's exact test and the chi-square test were used for qualitative variables.

Overall, 407 patients (264 CD and 143 UC) were enrolled in the cross-sectional assessment. All patients with active IBD at the cross-sectional appointment were eligible for the prospective phase of the study, including 118 CD patients and 36 UC patients. These sample sizes allowed the use of a desirable central limit theorem in outcome inference, even accounting for sample losses [11/118 (9.3%) in CD; 2/36 (5.6%) in UC] after 12 mo of follow-up.

The time to disease control was calculated using the Kaplan-Meier approach, defined as the time (in days) from index date until the date of the first documentation of disease control [*i.e.*, achieved disease control, mild or no active disease (defined as event)]. Patients who did not achieve disease control, died (without the event), discontinued the study or were lost to follow-up had their time censored at death or at the date of the last visit.

The proportion of patients with moderate-to-severe active IBD at 12 mo was calculated by IBD type at the last visit between 9 and 12 mo of follow-up.

Treatment changes were defined as drug discontinuations or dose changes (*i.e.*, changes in frequency and/or dosage administered) during follow-up for ongoing medications or initiated at the index date and any new medication initiated after the index date. Patients who did not have any visits during follow-up or patients who had no ongoing treatments were considered missing in the survival analysis.

For each IBD type, bivariate analyses were conducted to evaluate sociodemographic, clinical and treatment-related variables between subjects who achieved disease control during the 12-mo follow-up period *vs* those who did not. Chi-square or Fisher's exact tests were used to compare categorical variables, *t*-tests were used for independent samples, and the nonparametric Mann-Whitney test was used to compare quantitative variables. The incidence rate of treatment changes by person-years was also determined.

All statistical tests were two-tailed, with a significance level of 0.05, using SAS® (version 9.4, SAS Institute Inc., Cary).

RESULTS

Patient disposition and characteristics

Of 407 subjects (75.4%, n = 307, from public clinics) evaluated at the index date, 37.8% (n = 118 CD and 36 UC) were included in the prospective study phase (Figure 1). The majority of included patients were from public clinics: 74.6% (n = 88) CD and 72.2% (n = 26) UC patients. During the 12-mo follow-up, 11 (9.3%) CD patients and 2 (5.6%) UC patients discontinued the study, and one CD patient died. Overall, the mean ± SD follow-up times were 340.4 ± 59.2 d and 339.2 ± 64.8 d for CD and UC patients, respectively. A total of 107 CD patients and 34 UC patients completed the study and had a medical appointment between months 9 and 12, of which 82 CD patients and 25 UC patients had at least one evaluation of disease activity during this time window.

Most patients were female (58.5% and 78.8% of CD and UC patients, respectively), and the mean ± SD age at baseline was 43.3 ± 12.6 years for CD patients and 44.9 ± 16.5 years for UC patients (Table 1). A total of 45 (38.1%) CD patients and 22 (61.1%) UC patients had their first diagnosis of moderate-to-severe IBD within the past 5 years. The median CD disease duration was similar between patients receiving biologics and those not receiving biologics at the index date [11.0 (IQR: 0.5-11.0) *vs* 11.5 (IQR: 0.5-29.1) years]. UC patients on biologics at index had a shorter disease duration than

Table 1 Sociodemographic and disease characteristics of patients at the index date

	CD patients (*n* = 118)	UC patients (*n* = 36)
Age (yr), mean ± SD	43.3 ± 12.6	44.9 ± 16.5
Female, *n* (%)	69 (58.5)	26 (72.2)
Employed, *n* (%)	36 (36.7)	5 (16.1)
Attended higher education, *n* (%)	32 (34.4)	5 (23.8)
Body mass index (kg/m^2), mean ± SD	24.9 ± 4.7	24.9 ± 5.1
Current smokers, *n* (%)[1]	7 (5.9)	0 (0.0)
Family history of IBD, *n* (%)	15 (12.7)	2 (5.6)
IBD duration (yr), median (IQR)	11.0 (0.5-11.0)	6.5 (0.5-29.1)
Biologic group	11.0 (5.0-16.0)	6.0 (3.0-13.0)
Nonbiologic group	11.5 (4.5-18.5)	10.0 (4.0-17.0)
5-ASA derivates group	9.0 (4.0-13.0)	5.5 (4.0-14.0)
Non-5-ASA derivates group	11.5 (5.0-17.5)	13.0 (4.0-15.0)
Corticosteroid status, *n* (%)[2]		
Steroid-dependent	17 (14.4)	10 (27.8)
Steroid-refractory disease	5 (4.2)	3 (8.3)
Not applicable (no previous use)	33 (28.0)	3 (8.3)
Unknown	37 (31.4)	15 (41.7)
Location of CD, *n* (%)		
L1 ileal	26 (22.0)	-
L2 colonic	21 (17.8)	-
L3 ileocolonic	71 (60.2)	-
CD Behavior, *n* (%)		
B1 nonstricturing/penetrating	31 (26.3)	-
B2 stricturing	52 (44.1)	-
B3 penetrating	35 (29.7)	-
Symptomatic perianal disease, *n* (%)	37 (31.6)	-
HBI result (available for 101 CD patients), median (IQR)	5.0 (1.0-9.0)	-
CDAI result (available for 43 CD patients), median (IQR)	200.0 (114.0-274.0)	-
Fecal calprotectin level > 200 μg/g during previous year, *n* (%)	40 (33.9)	-
Colonoscopy with CD activity [3] during previous year, *n* (%)	69 (58.5)	-
Extent of UC inflammation - Montreal classification, *n* (%)		
E1 ulcerative proctitis	-	10 (27.8)
E2 left-sided UC	-	8 (22.2)
E3 pancolitis	-	18 (50.0)
Partial Mayo score, median (IQR)	-	6.0 (5.0-6.0)
Total Mayo score (available for 13 UC patients)	-	8.0 (7.0-8.0)
Extra intestinal manifestations[4], *n* (%)	29 (24.6)	8 (22.2)
Patients with at least one IBD surgery during previous 3 yr, *n* (%)	32 (27.1)	1 (2.8)
Total colectomy	0 (0.0)	1 (2.8)
Fistulectomy	7 (5.9)	0 (0.0)
Enterostomy	6 (5.1)	0 (0.0)

Partial colectomy	5 (4.2)	0 (0.0)
Closure of enterostomy	5 (4.2)	0 (0.0)
Drainage of anorectal abscess	3 (2.5)	0 (0.0)
Jejunostomy/Ileostomy	1 (0.8)	0 (0.0)
Other	15 (12.7)	1 (2.8)
Patients with at least one IBD-related hospitalizations during previous 3 yr, *n* (%)	51 (43.1)	11 (30.6)

[1]Smoking at Day 1 and has smoked 100 cigarettes in his/her lifetime.
[2]Steroid-dependent disease - defined as: (1) Being unable to reduce steroids below the equivalent of prednisolone at 10 mg/day within 3 mo of starting steroids, without recurrent disease; or (2) having a relapse within 3 mo of stopping glucocorticoids. Steroid-refractory disease - defined as having active disease despite prednisolone of up to 0.75 mg/kg/d over a period of 4 wk.
[3]As per investigator criteria.
[4]During the entire study period (3 retrospective years and 1 year of prospective follow-up), extraintestinal manifestations included arthralgia, arthritis, sacroiliitis, ankylosing spondylitis, erythema nodosum, pyoderma gangrenosum, pustular dermatitis, psoriasis, uveitis, sclerosing cholangitis, cholelithiasis, nephrolithiasis, autoimmune disease, hypertension, and dyslipidemia. SD: Standard deviation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; HBI: Harvey-Bradshaw index; CDAI: Crohn's disease activity index; pMayo: Partial Mayo score; 5-ASA: 5-aminosalicylate; IQR: Interquartile.

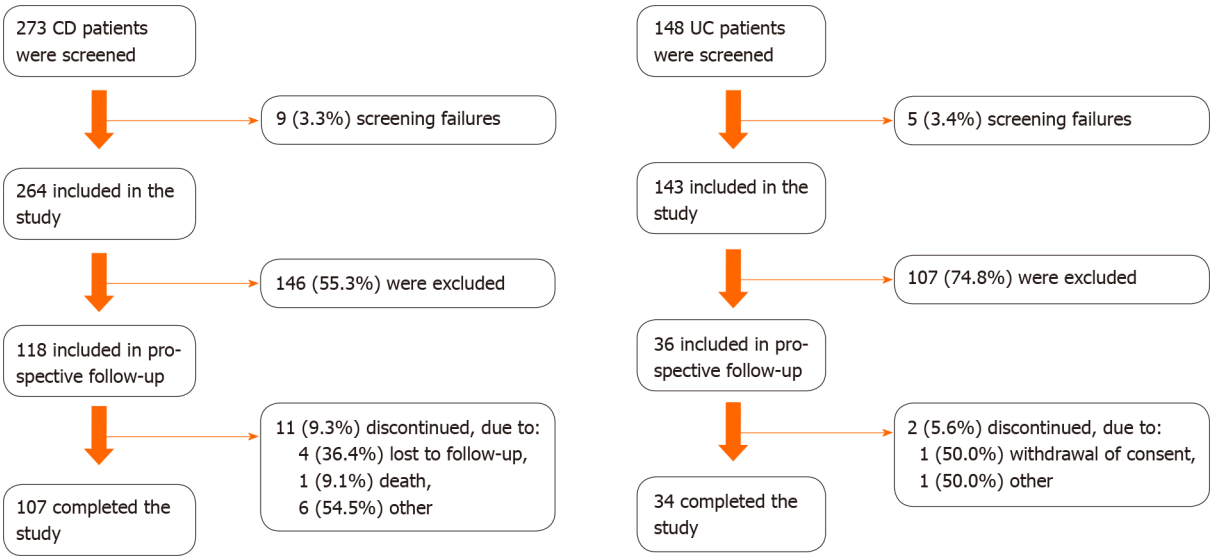


Figure 1 Patient disposition for Crohn's disease patients and ulcerative colitis patients. UC: Ulcerative colitis; CD: Crohn's disease.

patients not receiving biologics (6.0 years *vs* 10.0 years).

At the index date, 46 (39.0%) CD patients had active disease according to the CDAI or HBI, of whom 24 were identified based on these indexes only, with six presenting severely active CD (HBI > 16 or CDAI > 450). Regarding disease behavior, 52 (44.1%) and 35 (29.7%) CD patients had B2 stricturing and B3 penetrating disease, respectively. In addition, 40 (33.9%) patients had fecal calprotectin levels > 200 μg/g (22 patients identified only by calprotectin levels), and 69 (58.5%) had a colonoscopy results suggestive of disease activity during the previous year (35 patients identified by this criterion only). UC patients with active disease had a median pMayo of 6.0 (IQR: 5.0-6.0) at baseline, four (11.1%) had severe UC based on pMayo > 7, and 50.0% of patients presented with pancolitis.

IBD treatment patterns and changes during follow-up

Biologics were the most frequent drug class that CD patients were receiving at the index date appointment (62.7%), and 5-ASA derivates (91.7%) were the most common for UC patients; however, it is important to highlight that biologics were not available as a UC treatment strategy in Brazil at the time of the study (Table 2). Approximately 48% of CD patients and 58.3% of UC patients were receiving corticosteroids at the index date. Half (50.0%) of UC patients had received at least one corticosteroid for a period of 3 mo or longer. At the start of the 12-mo follow-up period, 58.5% of CD

Table 2 Treatment patterns and medical appointments during the follow-up period (Crohn's disease and ulcerative colitis patients)

	CD patients (*n* = 118)	UC patients (*n* = 36)
IBD treatment at index date appointment, *n* (%)[1]	111 (94.1)	35 (97.2)
5-ASA derivates	36 (30.5)	33 (91.7)
Corticosteroids	56 (47.5)	21 (58.3)
Immunosuppressants	65 (55.1)	21 (58.3)
Biologics	74 (62.7)	14 (38.9)
Patients who received at least one corticosteroid for ≥ 3 mo, *n* (%)[2]	31 (26.3)	18 (50.0)
Treatments at the start of 12-mo follow-up (index date), *n* (%)		
IBD treatment initiated or maintained	112 (94.9)	35 (97.2)
Biologic therapy		
Maintained or initiated at index date	75 (63.6)	14 (38.9)
Maintained	69 (58.5)	11 (30.6)
Initiated new	6 (5.1)	3 (8.3)
Naïve to biologics[3]	1 (0.8)	1 (2.8)
Discontinued at index date	0 (0.0)	0 (0.0)
Immunosuppressants		
Maintained or initiated at index date	73 (61.9)	19 (52.8)
Maintained	71 (60.2)	14 (38.9)
Initiated new	2 (1.7)	5 (13.9)
Discontinued at index date	1 (0.8)	0 (0 .0)
5-ASA compounds		
Maintained or initiated at index date	19 (16.1)	27 (75.0)
Maintained	17 (14.4)	20 (55.6)
Initiated new	2 (1.7)	5 (13.9)
Naïve to 5-ASA compounds	2 (1.7)	5 (13.9)
Discontinued at index date	1 (0.8)	0 (0.0)
Corticosteroids		
Maintained or initiated at index date	16 (13.6)	15 (41.7)
Maintained	9 (7.6)	6 (16.7)
Initiated new	6 (5.1)	8 (22.2)
Naïve to corticosteroids[3]	2 (1.7)	1 (2.8)
Discontinued at index date	2 (1.7)	4 (11.1)
Any antibiotic initiated at index date	12 (10.2)	5 (13.9)
Treatment changes during 12-mo follow-up period		
New treatment initiated during follow-up	77 (65.3)	31 (86.1)
Discontinuation or dose change during follow-up[2]	77 (68.1)	33 (94.3)
Median (IQR) number of treatment changes by patient	2 (2-5)	4 (3-7)
Biologic therapy		
Initiated	45 (38.1)	6 (16.7)
Naïve to biologics[4]	13 (11.0)	1 (2.8)
Discontinued	23 (19.5)	5 (13.9)
Dose change	14 (11.9)	4 (11.1)

Immunosuppressants		
Initiated	24 (20.3)	14 (38.9)
Naïve to immunosuppressants[4]	4 (3.4)	2 (5.6)
Discontinued	20 (16.9)	11 (9.3)
Dose change	7 (5.9)	7 (19.4)
5-ASA derivates		
Initiated	10 (8.5)	21 (58.3)
Naïve to 5-ASA compounds[4]	1 (0.8)	2 (5.6)
Discontinued	7 (5.9)	8 (22.2)
Dose change	4 (3.4)	14 (38.8)
Corticosteroids		
Initiated	24 (20.3)	16 (44.4)
Naïve to corticosteroids[4]	5 (4.2)	2 (5.6)
Discontinued	23 (19.5)	17 (47.2)
Any antibiotic initiated after index date	23 (19.5)	5 (13.9)

[1]Inflammatory bowel disease (IBD) treatment patients were receiving at the time of enrollment, irrespective of the physician's decision to continue or discontinue it at the end of the medical appointment.
[2]Percentages calculated from 113 Crohn's disease and 35 ulcerative colitis patients, since two patients had no IBD medication and four had no medical appointments during follow-up.
[3]Assuming the information from the previous 3 year.
[4]Assuming the information from the previous 3 years and index date. Values are *n* (%), and percentages calculated within the total of the study population in each group, except if otherwise mentioned. SD: standard deviation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; 5-ASA: 5-aminosalicylate.

patients maintained their current biologic therapy, while 5.1% initiated a new biologic. Regarding UC patients, 30.6% maintained their biologic therapy, while 8.3% initiated a new biologic treatment. Current 5-ASA agents were maintained by 14.4% of CD patients, while 55.6% of UC patients maintained the same therapy.

During follow-up, 65.3% of CD and 86.1% of UC patients switched to a new treatment at least once. Discontinuation or dose changes (excluding antibiotics) of IBD-related treatments occurred in 68.1% of CD and 94.3% of UC patients.

The incidence rates of drug discontinuations and dose changes were 9.2 and 6.8 changes/person-years for CD and UC patients, respectively (data not shown). During follow-up, the median number of treatment changes among CD patients was 2.0 (IQR 2-5), with 26 (33.8%) patients having more than four changes. Regarding drug classes, 19.5% of CD patients discontinued biologic therapy, 16.9% discontinued immunosuppressants, and 5.9% discontinued 5-ASA derivates.

In UC patients, the median number of treatment changes was 4.0 (IQR 3-7), with 18 (54.5%) patients having more than four treatment changes. Regarding drug classes, 22.2% of UC patients discontinued 5-ASA derivates, 13.9% discontinued biologics, and 9.3% discontinued immunosuppressants.

Azathioprine was the most frequently maintained or newly initiated agent in CD patients (61.9%), while mesalazine was the agent most maintained or newly initiated treatment by UC patients (72.2%), whether as monotherapy or combination therapy (Figure 2).

Treatment patterns at the medical appointments during follow-up

The vast majority (96.6%) of CD patients had at least one outpatient visit during the follow-up period, with a mean ± SD number of 4.4 ± 2.6 visits per patient and a total of 518 visits. Treatment changes were observed in 25.3% (n = 131) of CD outpatient visits, and disease activity was assessed by the HBI and/or CDAI scores in 12.6% (n = 51) and 6.4% (n = 26) of visits, respectively.

Biologics were the most frequent treatments used during follow-up (76.8% in the first quarter of the follow-up), followed by immunosuppressants (65.2%) and 5-ASA compounds (16.1%). The use of corticosteroids started to decrease from the third quarter of follow-up (Figure 3).

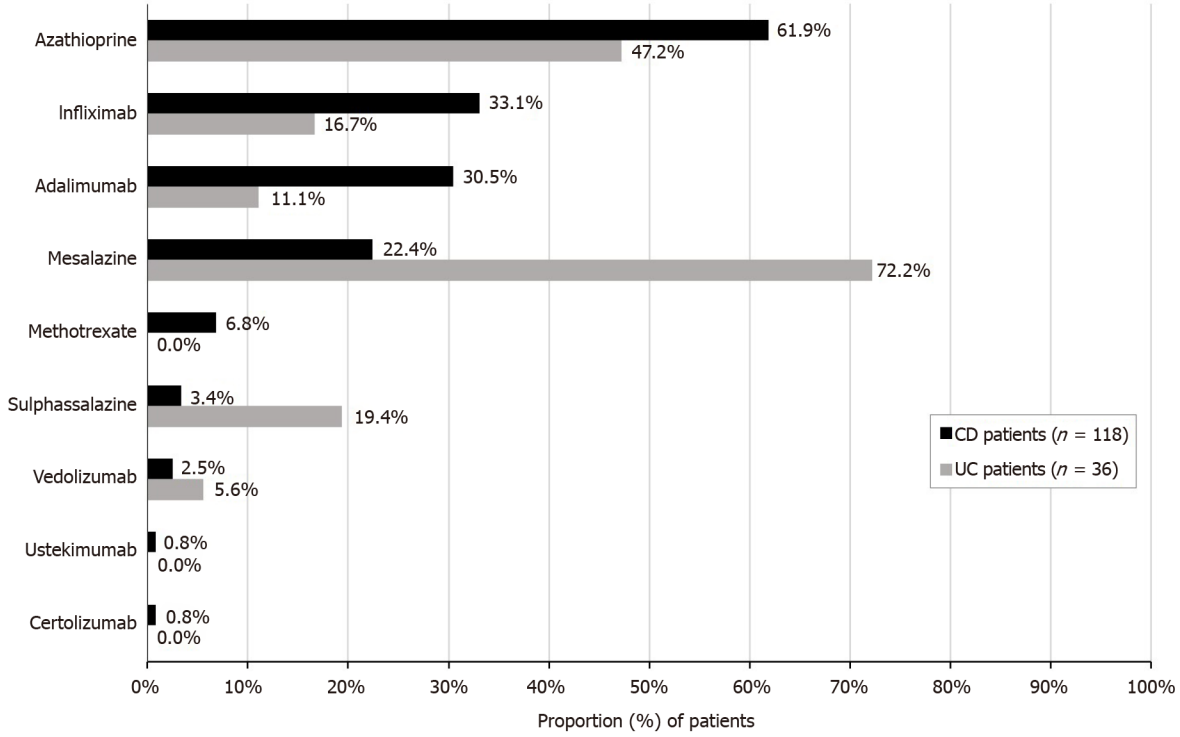


Figure 2 Proportion (%) of Crohn's disease and ulcerative colitis patients who maintained or initiated new inflammatory bowel disease drugs at the index date. UC: Ulcerative colitis; CD: Crohn's disease.

All UC patients had at least one visit during the follow-up period, with a mean ± SD number of 5.0 ± 3.3 visits per patient and a total of 179 visits. Treatment changes were observed among 40.2% (n = 72) of UC outpatient visits, and the assessment of UC activity with the Mayo score was performed in 11.2% (n = 16) of visits. The most prescribed agent during the first quarter of follow-up was 5-ASAs (83.3%), followed by immunosuppressants (58.3%) and biologics (38.9%). Corticosteroid use decreased from 50.0% in the first trimester to 26.5% in the last trimester of follow-up (Figure 3).

Time to IBD disease control during follow-up

During the 12-mo follow-up period, 86 (72.9%) CD patients achieved disease control. The median time to first disease control for CD from the index date was 319 d (IQR 239-358) [95%CI: 295-330], *i.e.*, approximately 10.6 mo (Figure 4A). There were five switches from "mild or no disease activity" to "moderate or severe disease" among the patients who had the event (control disease, n = 86).

Overall, 25 (69.4%) UC patients achieved disease control at least once, with 30.6% (n = 11) of patients remaining with "moderate or severe" disease at the end of the 12-mo period (censored observations). The median time for achieving the first episode of disease control from the index date was 320 d (IQR: 288-358) [95%CI: 302-358], *i.e.*, approximately 10.7 mo (Figure 4B), and there were two changes from "mild or no disease activity" to "moderate or severe disease".

Of the 107 patients who had a final evaluation during the remaining 90 d of the follow-up period, 23 (21.5%) showed moderate or severe disease activity. The proportions of CD and UC patients with active disease at the last follow-up visit were 22.0% (n = 15/82, 95%CI: 13.6%-32.5%) and 20.0% (n = 5/25, 95%CI: 6.8%-40.7%), respectively.

CD patients with active disease at the end of follow-up had a higher mean BMI at index than patients with 'mild or no activity' (27.5 ± 3.7 *vs* 24.2 ± 4.4; P = 0.007) (Table 3). Higher education was also more frequent among CD patients with mild or no disease activity at the end of follow-up than among patients with moderate-to-severe activity (42.6% *vs* 12.5%; P = 0.029).

Despite a higher proportion of CD patients with symptomatic perianal disease among patients with moderate to severe disease activity, the results were not statistically significant (55.6% *vs* 30.2% among patients with mild or no disease activity; P = 0.066).

Table 3 Sociodemographic, clinical and treatment characteristics of ulcerative colitis and Crohn's disease patients at the index date according to the final disease activity assessment

Characteristics at index date	CD patients (*n* = 82)			UC patients (*n* = 25)		
	Moderate-to-severe activity (*n* = 18)	No or mildactivity (*n* = 64)	*P* value	Moderate-to-severe activity (*n* = 5)	No or mild activity (*n* = 20)	*P* value
Age (yr), mean ± SD	42.0 ± 13.0	43.1 ± 12.7	0.732[3]	38.0 ± 14.4	46.9 ± 17.5	0.310[4]
Female, *n* (%)	12 (66.7)	39 (60.9)	0.786[5]	4 (80.0)	15 (75.0)	> 0.999[5]
Employed, *n* (%)	4 (23.5)	22 (45.8)	0.328[6]	1 (20.0)	2 (10.5)	0.977[5]
Attended higher education, *n* (%)	2 (12.5)	20 (42.6)	0.029[6]	1 (20.0)	2 (18.2)	0.462[5]
Body Mass Index (kg/m^2), mean ± SD	27.5 ± 3.7	24.2 ± 4.4	0.007[4]	25.4 ± 7.5	24.8 ± 5.5	0.838[3]
Current smokers, *n* (%)[1]	2 (12.5)	4 (7.0)	0.607[5]	0 (0.0)	0 (0.0)	NA
IBD duration (yr), median (IQR)	6.0 (1-27)	13.0 (0-45)	0.0433[3]	5 (1-23)	6.5 (1-23)	0.7032[4]
Time since moderate to severe IBD (yr), median (IQR)	5.5 (1-25)	7.0 (0-26)	0.8839[3]	2 (0-18)	3.5 (1-16)	0.8101[3]
Extra intestinal manifestation, *n* (%)	3 (50.0)	17 (51.5)	0.946[6]	1 (33.3)	4 (40.0)	NA
HBI result, median (IQR)	9.0 (7-13)	4.0 (1-8)	NA	-	-	-
CDAI result, median (IQR)	200.0 (114-311)	224.0 (113-281)	NA	-	-	-
Previous calprotectin level > 200 μg/g, *n* (%)	3 (16.7)	24 (37.5)	0.097[6]	-	-	-
Previous colonoscopy with CD activity, *n* (%)	12 (66.7)	33 (51.6)	0.256[6]	-	-	-
Ileocolonic disease, *n* (%)	13 (72.2)	36 (56.3)	0.282[5]	-	-	-
Stricturing or penetrating CD behavior, *n* (%)	11 (61.1)	48 (75.0)	0.247[6]	-	-	-
Symptomatic perianal disease, *n* (%)	10 (55.6)	19 (30.2)	0.066[6]	-	-	-
Partial Mayo score, median (IQR)	-	-	-	7.0 (5-8)	6.0 (5-6)	NA
Left-sided or pancolitis UC, *n* (%)	-	-	-	3 (60.0)	15 (75.0)	
Current IBD treatment at baseline[2], *n* (%)	15 (83.3)	61 (95.3)	0.233[5]	5 (100.0)	19 (95.0)	0.871[5]
Treatment ongoing or initiated at index, *n* (%)	16 (88.9)	62 (96.9)	0.416[5]	5 (100.0)	20 (100.0)	NA
5-ASA compounds	2 (12.5)	10 (16.1)	3 (60.0)		16 (80.0)	
Biologic therapy	13 (81.3)	41 (66.1)	3 (60.0)		4 (20.0)	
Corticosteroids	3 (18.8)	9 (14.5)	3 (60.0)		9 (45.0)	
Immunosuppressants	5 (31.3)	44 (71.0)	3 (60.0)		10 (50.0)	

[1]Smoking at Day 1 and has smoked 100 cigarettes in his/her lifetime.
[2]Ongoing or discontinued at baseline.
[3]*P* values from theMann-Whitney test.
[4]*t*-test.
[5]Fisher's exact test.
[6]Chi-square test. SD: Standard deviation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; HBI: Harvey-Bradshaw index; CDAI: Crohn's disease activity index; pMayo: Partial Mayo score; 5-ASA: 5-aminosalicylate; NA: Not applicable.

No statistically significant differences were observed when comparing sociodemographic, clinical and treatment characteristics between UC patients with *vs* without disease activity at the end of follow-up.

DISCUSSION

This study aimed to provide a real-world perspective on the management of moderate-to-severe active IBD patients in Brazil, with a focus on treatment patterns

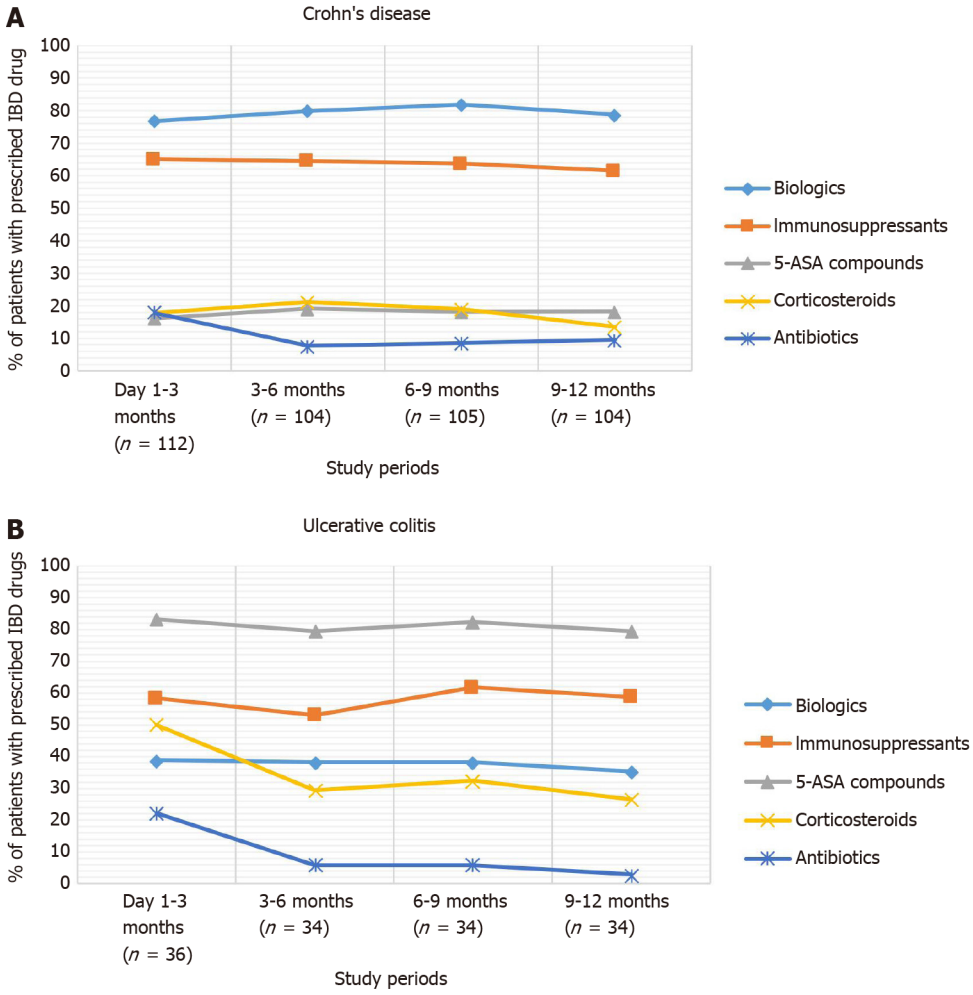


Figure 3 Proportion of patients by prescribed inflammatory bowel disease treatment classes during the prospective phase and grouped by trimester for Crohn's disease (A) and ulcerative colitis patients (B) with at least one treatment. 5-ASA: 5-aminosalicylate.

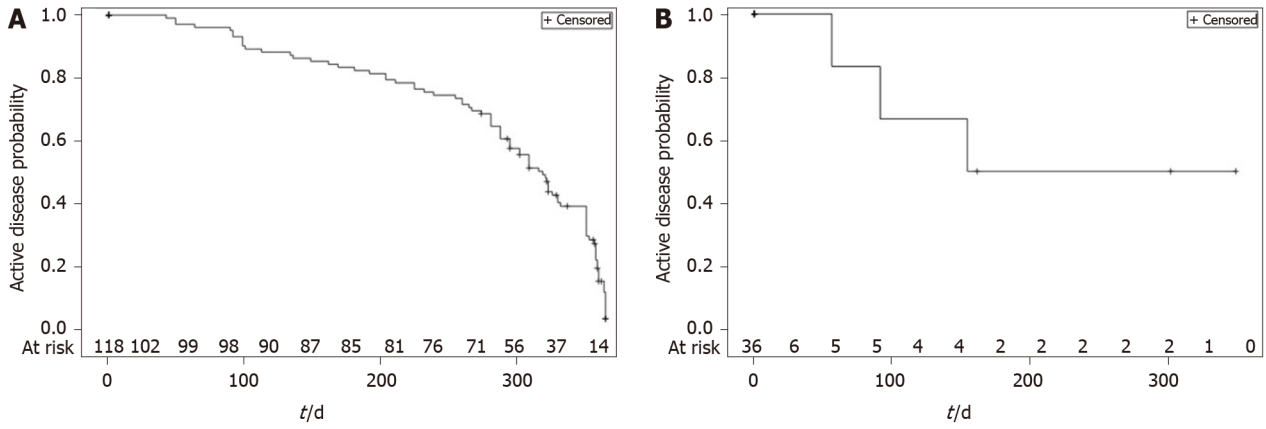


Figure 4 Probability of staying in active disease over 12 mo in Crohn's disease (A) and ulcerative colitis (B) patients.

and outcomes.

We found that more than two-thirds of IBD patients (73% and 69% for CD and UC patients) with active disease at the index date achieved disease control at least once during the 12-mo period. In addition, at the end of follow-up, approximately 20% of IBD patients had moderate-to-severe activity.

On average, almost 11 mo were required for half of UC and CD patients to achieve disease control. Considering that this is an observational study with no predefined visits, some patients did not undergo the 12-mo visit. Ideally, the control of disease

activity should be achieved within 4-6 mo[22,23]. One possible explanation for this long period could be attributed to the difficulty in the public setting in Brazil to access fecal biomarkers (such as calprotectin) or routinely perform colonoscopy, which are essential assessments to evaluate the response to treatment[2,6,24]. These barriers can delay the assessment of treatment response and thus the optimization of or change in medications.

The lack of public access to serum and anti-drug antibody level testing (unless solicited and justified) could also contribute to this delay. Evidence shows the usefulness of these tests in the decision to proactively or reactively change or optimize biologic therapies[25,26]. During the follow-up period, a mean of 4.4 medical appointments were required for CD patients and 5.0 for UC patients. We observed that clinical scores to assess disease activity were not collected at the majority of appointments (the HBI was assessed only in 12.6%, the CDAI in 6.4% and the Mayo score in 11.2% of outpatient visits).

Since the assessment of symptoms is part of several disease activity scores and relevant for both patients and physicians[27-30], these data suggest that assessment of IBD activity can be improved.

For the purpose of this study, we grouped "no activity" and "mildly active" disease in the same category. The ultimate treatment goal in IBD consists of achieving mucosal healing; however, the definition for this outcome can vary. Some studies in UC consider an endoscopic response as a Mayo endoscopic score ≤ 1[6].

Biological agents were the most frequent treatment among CD patients, while 5-ASA compounds were largely the most common therapy among UC patients. Despite 5-ASA derivates not being recommended for routine use in CD patients for induction or maintenance therapy, 14.4% of CD patients received this treatment strategy. This highlights the need for continuing education of gastroenterologists in Brazil. In addition, the lack of other therapies in some regions of the country may have led to the prescription of salicylic derivatives as the only CD treatment option. In a systematic literature review conducted in 2019, the lack of evidence for conventional treatment effectiveness, especially for remission maintenance, mucosal healing and fecal calprotectin, was highlighted[31].

We observed that the majority (65.5%) of CD patients started a new therapy after the study appointment, and the most common new treatment was biologics. New treatments were found for 86.1% of UC patients, with 5-ASA compounds being the most frequent. The more recent treatment guidelines, which are also followed in Brazil, recommend 5-ASA compounds in UC treatment irrespective of disease activity, while CD patients with moderate-to-severe disease activity would benefit more from the early use of biologics and immunosuppressants[2,6]. However, we cannot exclude differences in accessibility to new and more effective drugs, as clinics from the public health system in Brazil have no reimbursement when prescribing biologics to UC patients. Furthermore, the use of immunosuppressants and biologics increased among UC patients during follow-up, suggesting that 5-ASA compounds may not be enough for achieving disease control in many patients.

A relevant proportion of CD and UC patients were receiving biologic therapy, not only at the time of the study appointment but also during the follow-up period. This finding suggests that despite difficulties in accessing biologic therapies in hospitals in Brazil, especially in UC, physicians prescribe these agents for patients with moderate-to-severe disease activity. At the time of data collection, biologic therapy was available for use but not reimbursed by public or private healthcare systems in Brazil (which would mean medication without cost for patient); however, patients with UC had access to biologics *via* other strategies such as judicial petition and out-of-pocket. Only from March 2020, was infliximab available for patients diagnosed with UC in the public healthcare system; infliximab, vedolizumab and golimumab were only available in the private setting in 2021. We observed a decrease in the use of corticosteroids during follow-up for both CD and UC, suggesting an adequate control of disease overtime. Nevertheless, approximately one-quarter of CD patients and half of UC patients required corticosteroids for a period longer than three months.

One of the strengths of this study was the inclusion of treated IBD patients from both public and private settings, allowing a broad characterization of this population.

Some study limitations should be recognized. The inclusion of prevalent IBD cases, with baseline variability in terms of time since diagnosis and previous treatments, was a barrier to the assessment of the association between treatment options and disease activity. Even though we found no statistically significant association between clinical and treatment variables and presenting disease activity at the end of follow-up, we cannot exclude that treatment at the index date and during follow-up may have contributed to this outcome, as we did not adjust for combined therapy, dosing

information or duration of treatment (due to missing information). Moreover, the disease activity outcome was assessed at one time point only during the prospective phase (end of follow-up or month 12), making it difficult to determine whether it was a sustained control.

The inclusion of patients who had a previous event of moderate-to-severe IBD (irrespective of disease activity at index date) may affect the generalization of results for the whole IBD population in Brazil, even though these patients were selected for their higher burden of disease and thus needed closer medical follow-up. Disease activity was not assessed with the proposed scores in all outpatient medical appointments. Nevertheless, concerning disease activity, one limitation was the subjective assessment of colonoscopy, which was based on investigator criteria, which also impacted the selection of UC patients. The prospective design aimed to reduce missing data, but due to the observational character of the study, we did not proactively interfere with the usual practices for IBD activity evaluation. Finally, the sample size affected the group comparison regarding the main baseline and follow-up variables, especially for the UC patients. Furthermore, due to the small sample, one should be cautious when extrapolating the findings to a nationwide population. Additional studies are required to further understand the management of IBD in Brazil and understand the gaps and barriers in this setting.

CONCLUSION

In conclusion, to our knowledge, this was the first study aiming to characterize IBD evolution in a real-world setting in Brazil. Although a marked proportion of patients with active IBD achieved disease control within one year, the prolonged time to achieve this outcome suggests an area of unmet need with the current standard of care.

ARTICLE HIGHLIGHTS

Research background

The Real-world Data of Moderate-to-Severe Inflammatory Bowel Disease in Brazil (RISE BR) study was a noninterventional study designed to evaluate disease control, treatment patterns, disease burden and health-related quality of life in patients with moderate-to-severe active inflammatory bowel diseases (IBD).

Research motivation

Real-world data on IBD management could unveil unmet medical needs and treatment gaps. This is of utmost relevance in developing countries such as Brazil, where the prevalence of IBD is increasing, but access to biologic treatment may be restricted, and information on IBD treatment, in general, and associated outcomes is scarce.

Research objectives

The aim was to describe the 12-mo disease evolution and treatment patterns among patients with active moderate-to-severe IBD in Brazil.

Research methods

We report findings from the prospective follow-up phase of the RISE BR study in patients with active ulcerative colitis (UC) or Crohn's disease (CD). This was a prospective, noninterventional study of adult patients with active CD, inadequate CD control or active UC. The proportion of active IBD patients after 9-12 mo of follow-up, the time to mild or no activity and a summary of treatment initiation, discontinuation and dose changes were evaluated.

Research results

The study included 118 CD and 36 UC patients. The most frequent drug classes at index were biologics for CD (62.7%) and 5-aminosalicylate derivates for UC patients (91.7%). During follow-up, 65.3% of CD and 86.1% of UC patients initiated a new treatment at least once. Considering the prospective follow-up period, discontinuations/dose changes occurred in 68.1% of CD patients [median 2.0 (IQR: 2-5)] and

94.3% of UC patients [median 4.0 (IQR: 3-7)]. On average, CD and UC patients had 4.4 ± 2.6 and 5.0 ± 3.3 outpatient visits, respectively. The median time to first mild or no activity was 319 (IQR: 239-358) d for CD and 320 (IQR: 288-358) d for UC patients. At 9-12 mo, 22.0% of CD and 20.0% of UC patients had active disease.

Research conclusions

Although a marked proportion of active IBD patients achieved disease control within one year, the considerable time to achieve this outcome represents an unmet medical need of the current standard of care in a Brazilian real-world setting.

Research perspectives

This was the first study aiming to characterize IBD evolution in a real-world setting in Brazil. Additional studies are required to further understand the management of IBD in Brazil and understand the gaps and barriers in this setting.

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REFERENCES

1 **Mowat C**, Cole A, Windsor A, Ahmad T, Arnott I, Driscoll R, Mitton S, Orchard T, Rutter M, Younge L, Lees C, Ho GT, Satsangi J, Bloom S; IBD Section of the British Society of Gastroenterology. Guidelines for the management of inflammatory bowel disease in adults. *Gut* 2011; **60**: 571-607 [PMID: 21464096 DOI: 10.1136/gut.2010.224154]

2 **Gomollón F**, Dignass A, Annese V, Tilg H, Van Assche G, Lindsay JO, Peyrin-Biroulet L, Cullen GJ, Daperno M, Kucharzik T, Rieder F, Almer S, Armuzzi A, Harbord M, Langhorst J, Sans M, Chowers Y, Fiorino G, Juillerat P, Mantzaris GJ, Rizzello F, Vavricka S, Gionchetti P; ECCO. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. *J Crohns Colitis* 2017; **11**: 3-25 [PMID: 27660341 DOI: 10.1093/ecco-jcc/jjw168]

3 **Brady JE**, Stott-Miller M, Mu G, Perera S. Treatment Patterns and Sequencing in Patients With Inflammatory Bowel Disease. *Clin Ther* 2018; **40**: 1509-1521. e5 [PMID: 30126706 DOI: 10.1016/j.clinthera.2018.07.013]

4 **Lichtenstein GR**, Loftus EV, Isaacs KL, Regueiro MD, Gerson LB, Sands BE. ACG Clinical Guideline: Management of Crohn's Disease in Adults. *Am J Gastroenterol* 2018; **113**: 481-517 [PMID: 29610508 DOI: 10.1038/ajg.2018.27]

5 **Kornbluth A**, Sachar DB; Practice Parameters Committee of the American College of Gastroenterology. Ulcerative colitis practice guidelines in adults: American College Of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol* 2010; **105**: 501-23; quiz 524 [PMID: 20068560 DOI: 10.1038/ajg.2009.727]

6 **Harbord M**, Eliakim R, Bettenworth D, Karmiris K, Katsanos K, Kopylov U, Kucharzik T, Molnár T, Raine T, Sebastian S, de Sousa HT, Dignass A, Carbonnel F; European Crohn's and Colitis Organisation [ECCO]. Third European Evidence-based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 2: Current Management. *J Crohns Colitis* 2017; **11**: 769-784 [PMID: 28513805 DOI: 10.1093/ecco-jcc/jjx009]

7 **Lindsay JO**, Armuzzi A, Gisbert JP, Bokemeyer B, Peyrin-Biroulet L, Nguyen GC, Smyth M, Patel H. Indicators of suboptimal tumor necrosis factor antagonist therapy in inflammatory bowel disease. *Dig Liver Dis* 2017; **49**: 1086-1091 [PMID: 28826571 DOI: 10.1016/j.dld.2017.07.010]

8 **Gisbert JP**, Marín AC, McNicholl AG, Chaparro M. Systematic review with meta-analysis: the efficacy of a second anti-TNF in patients with inflammatory bowel disease whose previous anti-TNF treatment has failed. *Aliment Pharmacol Ther* 2015; **41**: 613-623 [PMID: 25652884 DOI: 10.1111/apt.13083]

9 **Sandborn WJ**, Feagan BG, Rutgeerts P, Hanauer S, Colombel JF, Sands BE, Lukas M, Fedorak RN, Lee S, Bressler B, Fox I, Rosario M, Sankoh S, Xu J, Stephens K, Milch C, Parikh A; GEMINI 2 Study Group. Vedolizumab as induction and maintenance therapy for Crohn's disease. *N Engl J Med* 2013; **369**: 711-721 [PMID: 23964933 DOI: 10.1056/NEJMoa1215739]

10 **Sandborn WJ**, Gasink C, Gao LL, Blank MA, Johanns J, Guzzo C, Sands BE, Hanauer SB, Targan S, Rutgeerts P, Ghosh S, de Villiers WJ, Panaccione R, Greenberg G, Schreiber S, Lichtiger S, Feagan BG; CERTIFI Study Group. Ustekinumab induction and maintenance therapy in refractory

Crohn's disease. *N Engl J Med* 2012; **367**: 1519-1528 [PMID: 23075178 DOI: 10.1056/NEJMoa1203572]

11 **Kirchgesner J**, Lemaitre M, Rudnichi A, Racine A, Zureik M, Carbonnel F, Dray-Spira R. Therapeutic management of inflammatory bowel disease in real-life practice in the current era of anti-TNF agents: analysis of the French administrative health databases 2009-2014. *Aliment Pharmacol Ther* 2017; **45**: 37-49 [PMID: 27781286 DOI: 10.1111/apt.13835]

12 **Null KD**, Xu Y, Pasquale MK, Su C, Marren A, Harnett J, Mardekian J, Manuchehri A, Healey P. Ulcerative Colitis Treatment Patterns and Cost of Care. *Value Health* 2017; **20**: 752-761 [PMID: 28577692 DOI: 10.1016/j.jval.2017.02.005]

13 **Schreiber S**, Peyrin-Biroulet L, Loftus EV, Danese S, Colombel JF, Abhyankar B, Chen J, Rogers R, Lirio RA, Bornstein JD, Sands BE. OP34 VARSITY: A double-blind, double-dummy, randomised, controlled trial of vedolizumab *vs* adalimumab in patients with active ulcerative colitis. *J Crohn's Colitis* 2019; **13** Suppl 1: S612-S613 [DOI: 10.1093/ecco-jcc/jjz046.001]

14 **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 2017; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]

15 **Lima Martins A**, Volpato RA, Zago-Gomes MDP. The prevalence and phenotype in Brazilian patients with inflammatory bowel disease. *BMC Gastroenterol* 2018; **18**: 87 [PMID: 29914399 DOI: 10.1186/s12876-018-0822-y]

16 **Parra RS**, Chebli JMF, Amarante HMBS, Flores C, Parente JML, Ramos O, Fernandes M, Rocha JJR, Feitosa MR, Feres O, Scotton AS, Nones RB, Lima MM, Zaltman C, Goncalves CD, Guimaraes IM, Santana GO, Sassaki LY, Hossne RS, Bafutto M, Junior RLK, Faria MAG, Miszputen SJ, Gomes TNF, Catapani WR, Faria AA, Souza SCS, Caratin RF, Senra JT, Ferrari MLA. Quality of life, work productivity impairment and healthcare resources in inflammatory bowel diseases in Brazil. *World J Gastroenterol* 2019; **25**: 5862-5882 [PMID: 31636478 DOI: 10.3748/wjg.v25.i38.5862]

17 **Zaltman C**, Parra RS, Sassaki LY, Santana GO, Ferrari MLA, Miszputen SJ, Amarante HMBS, Kaiser Junior RL, Flores C, Catapani WR, Parente JML, Bafutto M, Ramos O, Gonçalves CD, Guimaraes IM, da Rocha JJR, Feitosa MR, Feres O, Saad-Hossne R, Penna FGC, Cunha PFS, Gomes TN, Nones RB, Faria MAG, Parente MPPD, Scotton AS, Caratin RF, Senra J, Chebli JM. Real-world disease activity and sociodemographic, clinical and treatment characteristics of moderate-to-severe inflammatory bowel disease in Brazil. *World J Gastroenterol* 2021; **27**: 208-223 [PMID: 33510560 DOI: 10.3748/wjg.v27.i2.208]

18 **Harvey RF**, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980; **1**: 514 [PMID: 6102236 DOI: 10.1016/s0140-6736(80)92767-1]

19 **Vilela EG**, Torres HO, Martins FP, Ferrari Mde L, Andrade MM, Cunha AS. Evaluation of inflammatory activity in Crohn's disease and ulcerative colitis. *World J Gastroenterol* 2012; **18**: 872-881 [PMID: 22408345 DOI: 10.3748/wjg.v18.i9.872]

20 **Lahiff C**, Safaie P, Awais A, Akbari M, Gashin L, Sheth S, Lembo A, Leffler D, Moss AC, Cheifetz AS. The Crohn's disease activity index (CDAI) is similarly elevated in patients with Crohn's disease and in patients with irritable bowel syndrome. *Aliment Pharmacol Ther* 2013; **37**: 786-794 [PMID: 23432394 DOI: 10.1111/apt.12262]

21 **Lewis JD**, Chuai S, Nessel L, Lichtenstein GR, Aberra FN, Ellenberg JH. Use of the noninvasive components of the Mayo score to assess clinical response in ulcerative colitis. *Inflamm Bowel Dis* 2008; **14**: 1660-1666 [PMID: 18623174 DOI: 10.1002/ibd.20520]

22 **Kuenzig ME**, Benchimol EI, Lee L, Targownik LE, Singh H, Kaplan GG, Bernstein CN, Bitton A, Nguyen GC, Lee K, Cooke-Lauder J, Murthy SK. The Impact of Inflammatory Bowel Disease in Canada 2018: Direct Costs and Health Services Utilization. *J Can Assoc Gastroenterol* 2019; **2**: S17-S33 [PMID: 31294382 DOI: 10.1093/jcag/gwy055]

23 **Sales-Campos H**, Basso PJ, Alves VB, Fonseca MT, Bonfá G, Nardini V, Cardoso CR. Classical and recent advances in the treatment of inflammatory bowel diseases. *Braz J Med Biol Res* 2015; **48**: 96-107 [PMID: 25466162 DOI: 10.1590/1414-431X20143774]

24 **Colombel JF**, Narula N, Peyrin-Biroulet L. Management Strategies to Improve Outcomes of Patients With Inflammatory Bowel Diseases. *Gastroenterology* 2017; **152**: 351-361. e5 [PMID: 27720840 DOI: 10.1053/j.gastro.2016.09.046]

25 **Kopylov U**, Ben-Horin S, Seidman E. Therapeutic drug monitoring in inflammatory bowel disease. *Ann Gastroenterol* 2014; **27**: 304-312 [PMID: 25331715]

26 **López-Ibáñez M**, Marín-Jiménez I. [Drugs and anti-drug antibody levels in the management of patients with inflammatory bowel disease]. *Gastroenterol Hepatol* 2016; **39**: 265-272 [PMID: 26601991 DOI: 10.1016/j.gastrohep.2015.09.012]

27 **Levesque BG**, Sandborn WJ, Ruel J, Feagan BG, Sands BE, Colombel JF. Converging goals of treatment of inflammatory bowel disease from clinical trials and practice. *Gastroenterology* 2015; **148**: 37-51. e1 [PMID: 25127678 DOI: 10.1053/j.gastro.2014.08.003]

28 **Peyrin-Biroulet L**, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant RV, D'Haens G, Dotan I, Dubinsky M, Feagan B, Fiorino G, Gearry R, Krishnareddy S, Lakatos PL, Loftus EV Jr, Marteau P, Munkholm P, Murdoch TB, Ordás I, Panaccione R, Riddell RH, Ruel J, Rubin DT, Samaan M, Siegel CA, Silverberg MS, Stoker J, Schreiber S, Travis S, Van Assche G, Danese S, Panes J, Bouguen G, O'Donnell S, Pariente B, Winer S, Hanauer S, Colombel JF. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for

Treat-to-Target. *Am J Gastroenterol* 2015; **110**: 1324-1338 [PMID: 26303131 DOI: 10.1038/ajg.2015.233]

29 **Kim AH**, Roberts C, Feagan BG, Banerjee R, Bemelman W, Bodger K, Derieppe M, Dignass A, Driscoll R, Fitzpatrick R, Gaarentstroom-Lunt J, Higgins PD, Kotze PG, Meissner J, O'Connor M, Ran ZH, Siegel CA, Terry H, van Deen WK, van der Woude CJ, Weaver A, Yang SK, Sands BE, Vermeire S, Travis SP. Developing a Standard Set of Patient-Centred Outcomes for Inflammatory Bowel Disease-an International, Cross-disciplinary Consensus. *J Crohns Colitis* 2018; **12**: 408-418 [PMID: 29216349 DOI: 10.1093/ecco-jcc/jjx161]

30 **Papay P**, Ignjatovic A, Karmiris K, Amarante H, Milheller P, Feagan B, D'Haens G, Marteau P, Reinisch W, Sturm A, Steinwurz F, Egan L, Panés J, Louis E, Colombel JF, Panaccione R. Optimising monitoring in the management of Crohn's disease: a physician's perspective. *J Crohns Colitis* 2013; **7**: 653-669 [PMID: 23562672 DOI: 10.1016/j.crohns.2013.02.005]

31 **Damião AOMC**, de Azevedo MFC, Carlos AS, Wada MY, Silva TVM, Feitosa FC. Conventional therapy for moderate to severe inflammatory bowel disease: A systematic literature review. *World J Gastroenterol* 2019; **25**: 1142-1157 [PMID: 30863001 DOI: 10.3748/wjg.v25.i9.1142]

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SYSTEMATIC REVIEWS

Local ablation of pancreatic tumors: State of the art and future perspectives

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Abstract

BACKGROUND

Currently, the technologies most commonly used to treat locally advanced pancreatic cancer are radiofrequency ablation (RFA), microwave ablation, and irreversible (IRE) or reversible electroporation combined with low doses of chemotherapeutic drugs.

AIM

To report an overview and updates on ablative techniques in pancreatic cancer.

METHODS

Several electronic databases were searched. The search covered the years from January 2000 to January 2021. Moreover, the reference lists of the found papers were analysed for papers not indexed in the electronic databases. All titles and abstracts were analysed.

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RESULTS

We found 30 studies (14 studies for RFA, 3 for microwave therapy, 10 for IRE, and 3 for electrochemotherapy), comprising 1047 patients, which were analysed further. Two randomized trials were found for IRE. Percutaneous and laparotomy approaches were performed. In the assessed patients, the median maximal diameter of the lesions was in the range of 2.8 to 4.5 cm. All series included patients unfit for surgical treatment, but Martin *et al* assessed a subgroup of patients with borderline resectable tumours who underwent resection with margin attenuation with IRE. Most studies administered chemotherapy prior to ablative therapies. However, several studies suggest that the key determinant of improved survival is attributable to ablative treatment alone. Nevertheless, the authors suggested chemotherapy before local therapies for several reasons. This strategy may not only downstage a subgroup of patients to curative-intent surgery but also support to recognize patients with biologically unfavourable tumours who would likely not benefit from ablation treatments. Ablation therapies seem safe based on the 1047 patients assessed in this review. The mortality rate ranged from 1.8% to 2%. However, despite the low mortality, the reported rates of severe post procedural complications ranged from 0%-42%. Most reported complications have been self-limiting and manageable. Median overall survival varied between 6.0 and 33 mo. Regarding the technical success rate, assessed papers reported an estimated rate in the range of 85% to 100%. However, the authors reported early recurrence after treatment. A distinct consideration should be made on whether local treatments induce an immune response in the ablated area. Preclinical and clinical studies have shown that RFA is a promising mechanism for inducing antigen-presenting cell infiltration and enhancing the systemic antitumour T-cell immune response and tumour regression.

CONCLUSION

In the management of patients with pancreatic cancer, the possibility of a multimodal approach should be considered, and conceptually, the combination of RFA with immunotherapy represents a novel angle of attack against this tumour.

Key Words: Pancreatic cancer; Ablation treatment; Radiofrequency ablation; Microwave ablation; Irreversible; Electrochemotherapy

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Core Tip: In the current state of knowledge, the most commonly used technologies in locally advanced pancreatic cancer are radiofrequency ablation, microwave ablation, and irreversible or reversible electroporation combined with low doses of chemotherapeutic drugs. Our purpose is to report an updated overview of these techniques, highlighting the advantages and limitations of each technology.

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INTRODUCTION

Worldwide, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020[1]. Pancreatic cancer accounts for almost as many deaths (466000) as cases (496000) because of its poor prognosis, and it is the seventh leading cause of cancer death in both sexes. Both incidence and mortality rates have been stable or slightly increased in many countries, likely reflecting the increasing prevalence of obesity, diabetes, and alcohol consumption, although improvements in diagnostic and cancer registration practices may also be factors in some countries.

Given that the rates of this disease are rather stable relative to the declining rates of breast cancer, it has been projected in a study of 28 European countries that pancreatic cancer will surpass breast cancer as the third leading cause of cancer death by 2025[1].

The only curative treatment is surgery; however, many patients have locally advanced or metastatic disease at diagnosis, and systemic chemotherapy is usually the main treatment[2-6]. The median survival of patients with metastatic disease treated with FOLFIRINOX therapy is only 3 mo[2,5]. FOLFIRINOX or modified FOLFIRINOX and gemcitabine/albumin-bound nab-paclitaxel remain the first-line treatment regimens, and for patients with *BRCA1/2* and *PALB2* mutations, FOLFIRINOX or modified FOLFIRINOX and gemcitabine/cisplatin are a second option[2,3]. Despite the recent introduction of novel chemotherapeutic schemes, these treatments still correlate with inadequate survival and significant systemic complications. Additionally, only one-third of patients are responsive to chemotherapy[6,7].

Local ablation treatment is considered in some centres for patients with persistent locally advanced disease after chemotherapy. Although randomized trials to establish the role of ablation treatments in addition to chemotherapy alone are absent and there are no concluded trials that have compared various ablative modalities[8], patients with persistent locally advanced disease who are in good clinical condition (World Health Organization performance status 0-1) and Response Evaluation Criteria in Solid Tumours (RECIST) stable disease after 2-4 mo of chemotherapy can be treated by local ablation therapies. Moreover, there is a growing interest in these techniques related to the fact that they can encourage a systemic antitumour response. Therefore, it is proposed to combine ablative treatments with immunotherapy to improve disease control[8]. Nonetheless, ablative treatments should be employed in pancreatic cancers that show a local growth pattern without systemic involvement and should be chosen as consolidative treatments in a multimodal approach. The superior technique between the two remains unknown; therefore, the choice to employ one or the other should be reserved for a multidisciplinary team, considering the patients' comorbidities, the tumour characteristics and, particularly, the response to medical therapies[9-19].

In the current state of knowledge, the most commonly used technologies in locally advanced pancreatic cancer (LAPC) are radiofrequency ablation (RFA), microwave ablation (MWA), and irreversible electroporation (IRE) or reversible electroporation combined with low-dose chemotherapeutic drugs (ECT).

We report an overview and an update of these procedures, highlighting the advantages and limitations of each technology.

MATERIALS AND METHODS

This study is an autonomous study with no protocol or registration number.

Search criteria

The following electronic databases were used for search: PubMed (United States National Library of Medicine, http://www.ncbi.nlm.nih.gov/pubmed), Scopus (Elsevier, http://www.scopus.com/), Web of Science (Thomson Reuters, http://apps.webofknowledge.com/), and Google Scholar (https://scholar.google.it/). The following search criteria were used: "Pancreatic Cancer" AND "Ablative Therapies"; "Pancreatic Cancer" AND "RFA"; "Pancreatic Cancer" AND "MWA"; "Pancreatic Cancer" AND "IRE"; "Pancreatic Cancer" AND "ECT".

The search covered the years from January 2000 to January 2021. Reference lists of the found papers were analysed for papers not indexed in the electronic databases. The included papers were required to be clinical studies (*e.g.*, retrospective analyses, case series, and prospective cohort studies) evaluating the safety and efficacy of ablative therapies in pancreatic adenocarcinoma. Articles published in the English language from January 2000 to January 2021 were included. The exclusion criteria were: Different topics, unavailability of full text, insufficient data, and case reports, reviews, or letters to editors.

RESULTS

The search strategy resulted in 30 studies [14 studies for RFA, 3 for MWA, 10 for IRE, and 3 for electrochemotherapy (ECT)] (Figure 1), including 1047 patients, which were



Figure 1 Included and excluded studies in systematic review.

further assessed. We found two randomized trials for IRE. Percutaneous and laparotomy approaches were performed. In the assessed patients, the median maximal diameter of the lesions was in the range of 2.8-4.5 cm, including patients unfit for surgical treatment. Additionally, Martin *et al*[19] evaluated patients with borderline resectable tumours who underwent resection with margin attenuation with IRE. Most series administered chemotherapy prior to IRE. The specific types of drugs varied between series, but gemcitabine- or 5-FU-based regimens were common.

In Table 1, we report the sample size, overall survival (OS), major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with RFA according to the studies assessed.

In Table 2, we report the sample size, OS, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with MWA according to the studies assessed.

In Table 3, we report the sample size, OS, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with IRE according to the studies assessed.

In Table 4, we report the sample size, OS, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with ECT according to the studies assessed.

DISCUSSION

Ablation techniques-physical principles

RFA and MWA are hyper-thermic techniques that utilize energy to heat the lesions to at least 60°C[16].

RFA produces necrosis due to thermocoagulation. With this treatment, the area of active tissue heating is limited to a few millimetres near the electrode[16]. Consequently, the efficacy is closely correlated to the lesion size, and the maximum result is obtained for targets less than 3.5 cm[16]. Additionally, some tissue features, such as electrical conductivity, thermal conductivity, dielectric permittivity, and blood perfusion rate, have an effect on the efficacy of the RFA procedure. In particular, RFA treatment should be avoided when the target is near large vessels because of the heat sink effect[19]. However, the bipolar system of RFA can reduce the heat sink effect and lower pancreatic injury[10].

MWA uses the dielectric effect, which occurs when an imperfect dielectric material is subjected to an alternating electromagnetic field, to generate a larger area of active heating (up to 2 cm close to the antenna), allowing more homogeneous necrosis in the target zone compared to RFA[16]. Additionally, MWA has several supposed improvements with respect to RFA: The target can be greater given that it generates a larger area of necrosis; the treatment time is quicker; and it is less influenced by the defence of the neighbouring tissues, which is due to vaporization and charring, so the heat-sink effect impacts the efficacy of MWA[16].

In contrast to RFA and MWA, IRE and ECT are non-thermal techniques that cause ablation, changing cell membrane permeability through an induced electric field

Table 1 Sample size, overall survival, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with radiofrequency ablation

Ref.	Sample size	Overall survival	Major complication rate	Minor complication rate	Mortality rate
D'Onofrio *et al*[28], 2016	51	-	-	-	-
D'Onofrio *et al*[29], 2017	18	Median, 185 d (range, 62-398 d)	0%	0%	0%
Giardino *et al*[30], 2013	168	34.0 mo	3.70%	17.70%	1.80%
Hadjicostas *et al*[31], 2006	4	6 mo	0%	25%	0%
Kallis *et al*[33], 2015	23	226 d (range, 140-526 d)	0%	4.30%	0%
Song *et al*[37], 2016	6	NR	0%	33.30%	0%
Spiliotis *et al*[38], 2007	25	33 mo	0%	-	0%
Varshney *et al*[39], 2006	3	-	0%	66.70%	0%
Zou *et al*[41], 2010	32	17.5 mo	3.10%	0%	0%
Giardino *et al*[44], 2017	10	NR	30%	0%	0%
D'Onofrio *et al*[46], 2020	35	310 (65–718) d	0%	0%	0%
Wang *et al*[48], 2020	11	12 mo	0%	0%	0%
He *et al*[50], 2020	18	1-yr 40.5%; 2-yr 27.0%	22.20%	50%	0%
Fegrachi *et al*[52], 2019	17	9 mo (range, 5-11 mo)	6%	24%	0%

NR: Not reported.

Table 2 Sample size, overall survival, major complication rate, minor complication rates, and mortality rate in pancreatic cancer treated with microwave ablation

Ref.	Sample size	Overall survival	Major complication rate	Minor complication rate	Mortality rate
Carrafiello *et al*[59], 2013	10	80% at 1 yr	10%	20%	0%
Ierardi *et al*[60], 2018	5	-	0%	20%	0%
Vogl *et al*[61], 2017	20	-	0%	9.10%	0%

(electroporation). IRE is considered a direct ablation tool since electroporation is used in an irreversible manner[17-26]. The use of short high-voltage electric current fields (up to 3000 V and 50 A for milliseconds) cause irreversible permeabilization of the lipid bilayer, disruption of cellular homeostasis, and stimulation of apoptotic pathways, causing death of neoplastic cells[9-17,26]. Taking into account its mechanism of action, IRE can protect surrounding structures, such as vessels, and it is a central element if the tumour encases the peripancreatic vessels in which the employment of RFA could be unsafe[22]. ECT conceives of the electroporation of cells and the associated administration of low doses of non-permanent or poorly permanent chemotherapeutic agents[11,13-27]. The application of an electrical field to a cell causes a transient and reversible orientation of its polar membrane molecules, with increased permeability[11-13]. This transient permeability allows the cell to receive a higher dose of chemotherapeutic drugs than would occur otherwise, increasing the cytotoxic effects of the agents. This local potentiation of chemotherapy allows reducing the doses of the drugs, lowering the side effects and increasing the chemotherapy efficacy [11-13].

Clinical study

RFA: RFA is the typical treatment worldwide for the treatment of LAPC if further benefit from chemotherapy is expected. Several studies have evaluated the role of RFA in metastatic pancreatic cancer[9]. To the best of our knowledge, 14 studies assessed the safety and efficacy of RFA in pancreatic cancer[28-52]. In several studies, the use of RFA was limited to patients with locally advanced cancer and/or metastatic cancer. Only in cases where the patients were unfit for surgery was RFA used in resectable

Table 3 Sample size, overall survival, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with irreversible electroporation

Ref.	Sample size	Overall survival	Major complication rate	Minor complication rate	Mortality rate
Vroomen *et al*[14], 2017	25	-	8%	20%	0%
Martin *et al*[19], 2015	200	24.9 mo (range, 4.9–85 mo)	18.50%	50,5%	2%
Martin *et al*[20], 2013	54	20 mo	24%	55,5%	2%
Lambert *et al*[23], 2016	21	10.2 mo	23.80%	-	0%
Yan *et al*[24], 2016	25	-	36%	16%	0%
Scheffer *et al*[26], 2017	25	11 mo	40%	40%	0%
Ruarus *et al*[65], 2020	50	11.6 mo (no induction chemotherapy or gemcitabine-based induction chemotherapy) and 14.9 mo (FOLFIRINOX)	42%	28%	2%
van Veldhuisen *et al* [66], 2020	30	17.0 (range, 5-35 mo)	20%	23%	0%
Narayananet al[67], 2017	50	27 mo	20%	-	0%
Liu *et al*[68], 2019	54	16.2 and 20.3 mo in the IRE and IRE + chemo groups	7.40%	81%	0%

IRE: Irreversible electroporation.

Table 4 Sample size, overall survival, major complication rates, minor complication rate, and mortality rate in pancreatic cancer treated with Electrochemotherapy

Ref.	Sample size	Overall survival	Major complication rate	Minor complication rate	Mortality rate
Granata *et al* [11], 2015	13	-	0%	23%	0%
Granata *et al* [12], 2017	19	-	-	-	-
Granata *et al* [71], 2020	25	In fixed geometry, treated patients 6 mo (range, 1-74 mo); in variable geometry treated patients 12 mo (range, 2-50 mo)	0%	23%	0%

cancer[25-52]. Recently, RFA has been used as an upfront option at the time of diagnosis[9], justified based on immunological antitumoral stimulation[43,44].

The results of the assessed studies in terms of OS, major and minor complication rates, and mortality rates are reported in Table 1.

In the treatment of pancreatic adenocarcinoma, RFA has been mainly applied during laparotomy or with an endoscopic approach. The percutaneous approach has rarely been described in the literature, and worldwide experience is still limited[46]. However, the percutaneous approach should be favoured to avoid the invasiveness of the intraoperative approach. If percutaneous RFA is feasible, it could avoid unnecessary laparotomy, thus reducing the risk of surgical complications as well as the time and costs of the treatment[46]. Moreover, while surgery involves an impaired immune response, enhanced immune system stimulation and immune response against the tumour have been described in a percutaneously treated patient[43,47].

Nonrandomized studies showed a promising OS up to 25.6 mo after RFA for LAPC [53]. However, no randomized controlled trials have been performed, so the true effectiveness of RFA combined with systemic chemotherapy regimens remains unknown. Several studies[9] reported an excellent outcome with a median OS of 30 mo for patients subjected to RFA and a median OS of 25.6 mo in the patients subjected to primary treatments plus RFA plus further systemic treatments. Few studies have assessed the efficacy of RFA compared to other treatments[50]. He *et al*[50] compared

the efficacy of IRE with RFA in patients with LAPC, showing that IRE after induction chemotherapy is superior to RFA after induction chemotherapy for treating LAPC, while these two therapies have comparable efficacy for tumours that were larger than 4 cm. However, the study was not a randomized controlled trial but a retrospective study.

Morbidity rates range from 14% to 28% and seem to depend on RFA temperature settings, preventive duodenal cooling, and safety margins from vital structures[52]. Since pancreatic tissue is sensitive to heat and rich in blood vessels and since the anatomical position is close to arteries and bile ducts, the application of thermotherapy techniques carries a high risk. However, as RFA application becomes increasingly mature, the incidence of postoperative complications has decreased significantly[52]. Complications related to RFA included pancreatic fistulae, portal vein thrombosis, gastrointestinal bleeding, and acute pancreatitis. The rates of RFA-related mortality ranged from 0% to 19%[51]. The RFA-related complications that resulted in patient deaths included gastrointestinal bleeding and sepsis. The rates of overall complications ranged from 10% to 43%[51]. The types of complications reported varied widely and included pneumonia, peritoneal cavity abscess, acute renal failure, transient ascites, hepatic insufficiency, pseudomembrane colitis, hemoperitoneum, abdominal fluid collection, gastric bypass fistula, gastric ulcer, and choledocholithiasis.

Computed tomography (CT) is the diagnostic tool most often employed to evaluate treatment in terms of efficacy and safety. Although CT is best known for its role in the evaluation of abdominal emergencies, it is also an excellent tool in the evaluation of posttreatment complications[54-58]. However, CT has significant limitations in assessing treatment effectiveness[6]. RFA produces side effects such as interstitial oedema, haemorrhage, carbonization, necrosis, and fibrosis. These are responsible for heterogeneous appearances on imaging. The assessment of the treatment response in terms of dimensional criteria, according to RECIST 1.1[15] criteria, is not applicable because effectiveness is not always correlated to a size decrease[6]. Nevertheless, the assessed studies evaluated short- and long-term RFA efficacy according to dimensional criteria[15]. The assessment time was between 7 and 34 mo considering only dimensional criteria. According to Paiella *et al*[9] for RFA the technique to choose is CT, and the effectiveness is related to a post treatment hypodense zone. However, pancreatic tumours are also hypodense, so a "qualitative evaluation" based on a visual assessment could cause misdiagnosis. A quantitative evaluation founded on functional analysis allows a more objective assessment and a more correct diagnosis[6].

Distinct consideration should be made regarding whether RFA induces an immune response in the ablated area. Preclinical and clinical studies have shown that RFA is a favourable tool to induce antigen-presenting cell infiltration and to enhance the systemic antitumour T-cell immune response and tumour regression. The treatment is followed by a significant inflammatory response with intense T-cell infiltration[43,45].

Therefore, in the management of patients with pancreatic cancer, the possibility of a multimodal approach should be considered, and theoretically, the association of RFA with immunotherapy is a novel strategy against this tumour.

Microwave ablation

The results of the assessed studies in terms of OS, major and minor complication rates, and mortality rates are reported in Table 2.

Carrafiello *et al*[59] evaluated the efficacy of MWA in ten unresectable pancreatic head adenocarcinomas. The mean follow-up was 9.2 mo (range, 3 to 16 mo). The rate of MWA-related morbidity was 30% (3 patients). The authors found pancreatitis in two patients and gastroduodenal artery pseudoaneurysm in one patient. CT was executed up to 15 mo after the procedure. At the first follow-up, the researchers found one case with partial response (PR), eight with stable disease (SD), and one with progressive disease.

Ierardi *et al*[60] evaluated the feasibility and safety of MWA in five head pancreatic locally advanced cancer patients using a new technology with a high power and frequency of 2450 MH. CT was performed after 1, 3, 6, and 12 mo. No major complications were reported with a safe treatment in all patients (100%). Minor complications resolved during hospitalization (median, 4 d)[60].

Vogl *et al*[61] cured 22 lesions: In 17 (77.3%) patients, the tumour was in the pancreatic head and in 5 (22.7%) in the pancreatic tail. The rate of MWA efficacy was 100%. No major complications were reported; however, in two (9.1%) cases, minor complications were found because of severe local pain post-MWA treatment. Only ten patients underwent follow-up magnetic resonance imaging (MRI) examinations (median, 3 mo); local tumour progression was reported in one (10%) case.

Unlike RFA, the percutaneous approach was the most commonly used during MWA treatment, probably explaining the lower complication and death rates. However, the complication rates of MWA varied among the assessed studies. This finding might be due to the heterogeneity and the sample size of the studies assessed. In fact, Carrafiello *et al*[59] treated ten unresectable head pancreatic adenocarcinomas, Ierardi *et al*[60] treated five locally advanced head pancreatic cancers, and Vogl *et al* [61] treated 22 patients: In 17 (77.3%) patients, the tumour was in the pancreatic head and in five (22.7%) in the pancreatic tail.

CT is the diagnostic tool most often employed to evaluate the treatment, for either efficacy and/or safety. However, an assessment centred only on dimensional criteria is inappropriate to evaluate this procedure.

No data on MWA and immune response in the ablated area are described in the literature.

Although MWA is a promising treatment for LAPC, further studies are needed to increase the data about its safety and efficacy as well as the oncological outcome.

Irreversible electroporation

Currently, IRE is applied in stage III LAPC[17,26], even if several studies have reported three cases of IRE in stage IV with liver metastases[62] and the option to employ IRE as a technique to reduce the rate of R1 resections[19,21,63]. According to the reported data[17-26,62,64], IRE works better on target areas not larger than 3-3.5 cm; in addition, IRE should be more suitable than thermal tools when the tumour encapsulates the superior mesenteric artery. However, IRE has the disadvantage of necessitating general anaesthesia.

The results of the assessed studies in terms of OS, major and minor complication rates, and mortality rates are reported in Table 3.

Rombouts *et al*[53] described an IRE-related complication rate of 13%, with a mortality of 2%. The complication rate increases with the percutaneous treatment (29% *vs* 13%)[53]. Martin *et al*[19], evaluating 200 cured lesions, reported an overall rate of adverse events of 37% and a mortality rate of 2%. The most common complications reported are abdominal pain as a minor complication and pancreatitis, bile leakage, pancreatic leakage, duodenal leakage, duodenal ulcer, pneumothorax, haematoma, and deep vein thrombosis as major complications[6]. Several studies have confirmed the safety profile of IRE, with encouraging survival outcomes. Most studies, however, were retrospective, had limited sample sizes, and had a relatively short follow-up time. The aim of the PANFIRE study was to evaluate the efficacy and safety of percutaneous IRE for LAPC and isolated local recurrence following surgical resection of pancreatic cancer[65]. In this prospective single-arm phase II study, 40 patients with LAPC and 10 with local recurrence after resection were treated. The primary endpoint was the median survival times with primary LAPC (median OS, 17 mo) and with local recurrence (median OS, 16 mo), which exceeded the target median survival times based on chemotherapy alone. These results show a survival benefit compared with the current standard of care[65]. The reported overall complication rate was of 58%, including 21 major adverse events and two deaths within 90 d of the treatment. In addition, 13 (33%) patients were treated upfront with IRE. No survival benefit was demonstrated for patients receiving a 5-FU-based regimen. This finding suggests that the key determinant of improved survival is attributable to IRE treatment alone. Nevertheless, the authors recommend at least four cycles of a 5-FU-based regimen before IRE for several reasons. First, a 5-FU-based regimen enables the identification of patients with aggressive tumour biologic features, allowing the exclusion of those who would not benefit from IRE. Second, a 5-FU-based regimen can result in downstaging, potentially rendering 15%–25% of patients with resectable disease. Last, an upfront 5-FU-based regimen has the potential to result in a longer OS[65]. The combination of systemic chemotherapy and cytoreductive ablation using IRE may prove synergistic for several reasons[66]. Induction chemotherapy may not only downstage a subgroup of lesions to curative-intent surgery but also identify biologically unfavourable tumours with rapid progression that would likely not benefit from ablative treatment [66]. As systemic chemotherapy remains the only treatment for LAPC proven to be beneficial, patients should first receive systemic chemotherapy followed by experimental treatment in the setting of a clinical trial[66-69].

IRE can be implemented successfully as an adjunctive measure for attempting to achieve negative microscopic operative margins in selected patients[21]. This treatment is limited to patients who have generally stable disease at the time of resection. To date, there are few options for effective therapy to facilitate microscopically negative margin resections outside of patient selection and meticulous operative dissection. Accepting that true margin-positive rates are significantly high (> 75%) in

resected pancreatic cancers, intraoperative IRE could accentuate negative-margin dissection of the retroperitoneal margin and its surrounding perivascular soft tissue, primarily the perineural and mesenteric tissue adjacent to critical vascular structures [21].

MRI and CT are the diagnostic tools mostly used to assess IRE.

IRE produces the formation of nanoscale pores within the cell membrane, changing the transmembrane potential and causing cell death. Experimental models showed that diffusion-weighted imaging (DWI) could be used to assess therapeutic effects[14,70]. Vroomen *et al*[14] evaluated specific imaging parameters with contrast-enhanced (ce) MRI and ce-CT. The authors evaluated pre- and post-treatment, for MRI, the signal intensity (SI) on T2-weighted (W) images, on T1-W images before and after contrast medium, on DWI, and on apparent coefficient of diffusion (ADC) maps and for CT attenuation in the arterial and portal-venous phases. These authors showed that the most significant features to evaluate efficacy and outcome were SI on images with b = 800s/mm^2 and contrast-enhanced MRI.

Only two studies[19,67] reported an outstanding median OS of 24.9 and 27 mo, respectively. Consequently, there is a need for a greater number of studies that assess efficacy in terms of oncological outcomes.

Electrochemotherapy

Today, few studies have evaluated the role of ECT in LAPC[11-13,71]. In our previous study, we assessed 13 patients with LAPC. In seven (53.8%) patients, the tumour was localized in the head, and in six (46.2%), it was localized in the body tail (Figure 2). The treatment was safe in all patients without major complications. The types of minor complications reported varied widely and included transient ascites, transient pleural effusion, and gastric emptying documented by radiological studies, without clinically significant signs. The mean duration of hospitalization was 12 d. CT and MR were utilized for the follow-up[11]. In an ongoing study, we found that the median OS was 11.5 mo with a range in values of 73 mo. At 1 mo after ECT, 76.0% of patients were in PR, and 20.0% were in SD. Moreover, we found that the use of pre-treatment planning (Figure 3), which optimizes the multiple insertions of single electrodes, increases the local disease control rate (LDCR) and the OS compared with the use of fixed-geometry electrodes (hexagonal or linear). The patients treated with fixed geometry had an LDCR of 46.1%, whereas the group treated with variable geometry (Figure 4) had an LDCR of 66.7%. For the 13 patients treated with fixed geometry, the median OS was 6 mo (range, 1–74 mo), whereas for the 12 patients treated with variable geometry, the median OS was 12 mo (range, 2 to 50 mo)[71].

Although ECT is a promising tool for cancer treatment, how to assess tumour treatment response is still a problem. In fact, as highlighted in our preliminary experience, the RECIST 1.1 criterion, using the variation of the largest diameter on both CT and MRI images, does not provide appropriate patient stratification in responders or non-responders[12,13]. It is clear that when considering therapeutic effects on tumours, imaging observations are sometimes difficult to interpret, so functional imaging should resolve this problem. We evaluated several functional features as follows: for MRI, wash-in slope and wash-out slope by dynamic contrast-enhanced MRI, pseudo-diffusivity, perfusion fraction, and tissue diffusivity by the intravoxel incoherent motion model, ADC by the conventional mono-exponential approach, and the mean of the diffusion coefficient and the mean of diffusional kurtosis by diffusion kurtosis imaging. In addition, for positron emission tomography, maximum standardized uptake value was assessed and for CT, lesion density was evaluated. We found that conventional morphologic criteria were not able to differentiate partial, complete, or incomplete responses after ECT, while changes in functional parameters could be more suitable to assess ECT responses[11,13].

Today, ECT is recommended during clinical trials in dedicated centres[11-13].

Comments and future perspectives

Several studies have suggested that the key determinant of improved survival is attributable to ablative treatment alone[72-75]. Nevertheless, the authors recommend at least four cycles of a 5-FU-based regimen before local therapy for several reasons. Induction chemotherapy may not only downstage a subgroup of lesions to curative-intent surgery but also identify biologically unfavourable tumours with rapid progression that would likely suffer from ablation treatment[66,76-80]. As systemic chemotherapy remains the only treatment for LAPC proven to be beneficial, patients should first receive systemic chemotherapy followed by experimental treatment in the setting of a clinical trial[66].

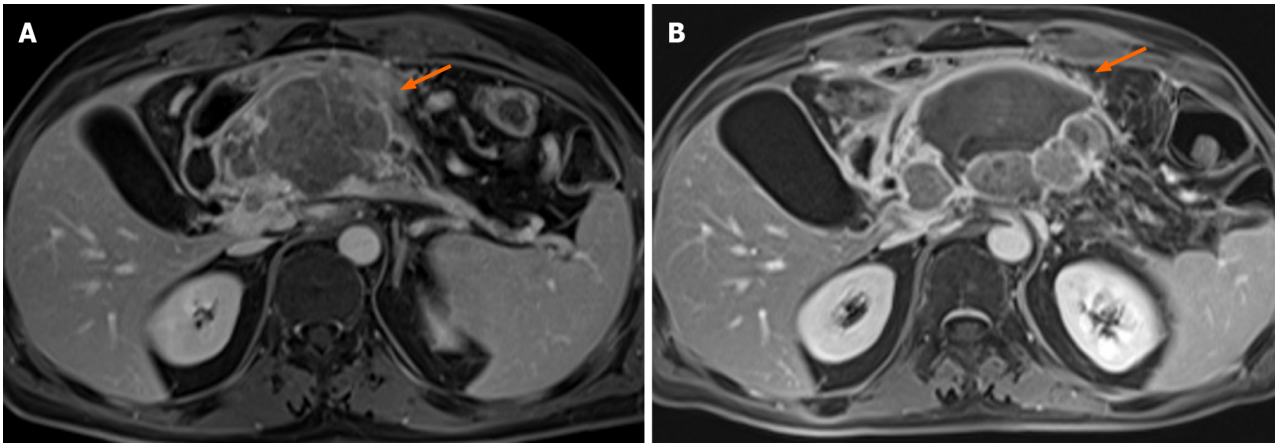


Figure 2 A patient with body pancreatic cancer. A: Volume-interpolated breath-hold examination (VIBE) T1-W post contrast sequence during the portal phase in axial plane for pretreatment evaluation of the lesion (arrow); B: VIBE T1-W post contrast sequence during the portal phase in axial plane showing the ablated area (arrow). According to qualitative assessment (significant differences in signal intensity in pre and post treatment sequences), the lesion was in partial response.

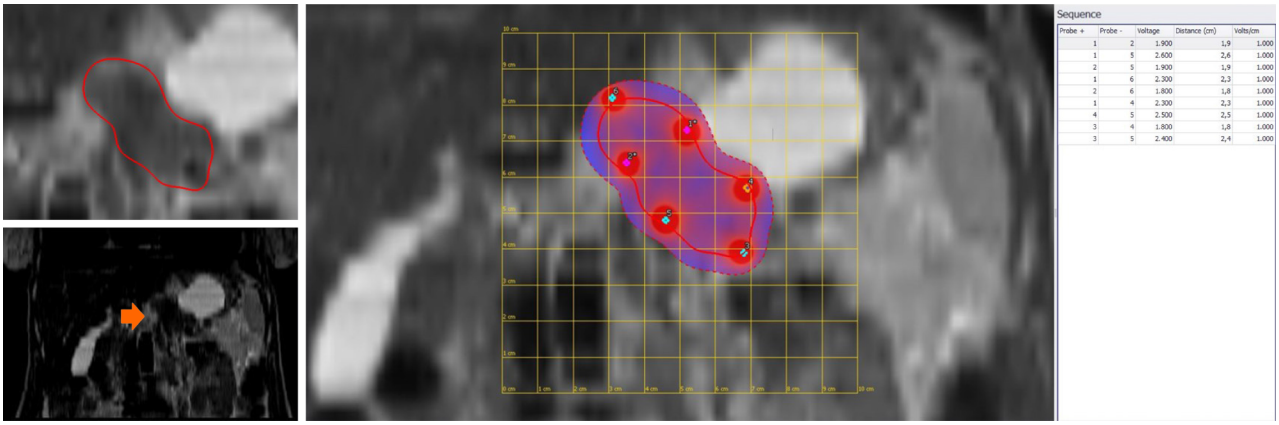


Figure 3 Preoperative planning for electrochemotherapy treatment with multiple single needles in a variable geometry for locally advanced pancreatic cancer.

Ablation therapies seem to be safe in 1047 patients assessed in this study. The mortality rate ranged from 1.8% to 2%. However, despite the low mortality, the reported rates of severe post procedural complications ranged from 0%–42%. Additionally, for laparotomy, the series reported by Martin *et al*[19] had more severe complications, including procedure-related deaths. The major drawback inherent to all thermal ablation techniques is the fact that these therapies comprise the risk of the heat-sink effect[69]. This issue is particularly important, as the pancreas is an organ with a peculiar position that is closely related to the duodenum, bile duct, and major vessels. This feature turned IRE into an attractive tool for LAPC ablation[69]. Taken together, pathological studies revealed that one-third of patients died of PC as a result of local tumour infiltration, without evidence of metastatic disease. This population appears to be ideal for IRE to increase patient survival and, importantly, quality of life [69]. A registry-based study showed a high rate of complications (42%) post-IRE. An important point demonstrated in this study is the correlation of the learning curve to the rate of complications, which seems to drop after a cumulative experience of a minimal of five IRE cases in PC[69].

Median OS varied between 6.0 and 33 mo. However, these data are very problematic to understand because of the heterogeneity between the series.

Regarding the technical success rate, several studies reported an estimated technical success rate in the range of 85%–100%. However, the authors reported early recurrence after treatment, indicating the limitations of the radiological assessment post-treatment [16]. In addition, none of these studies assessed the relationship between the technical success rate and tumour size.

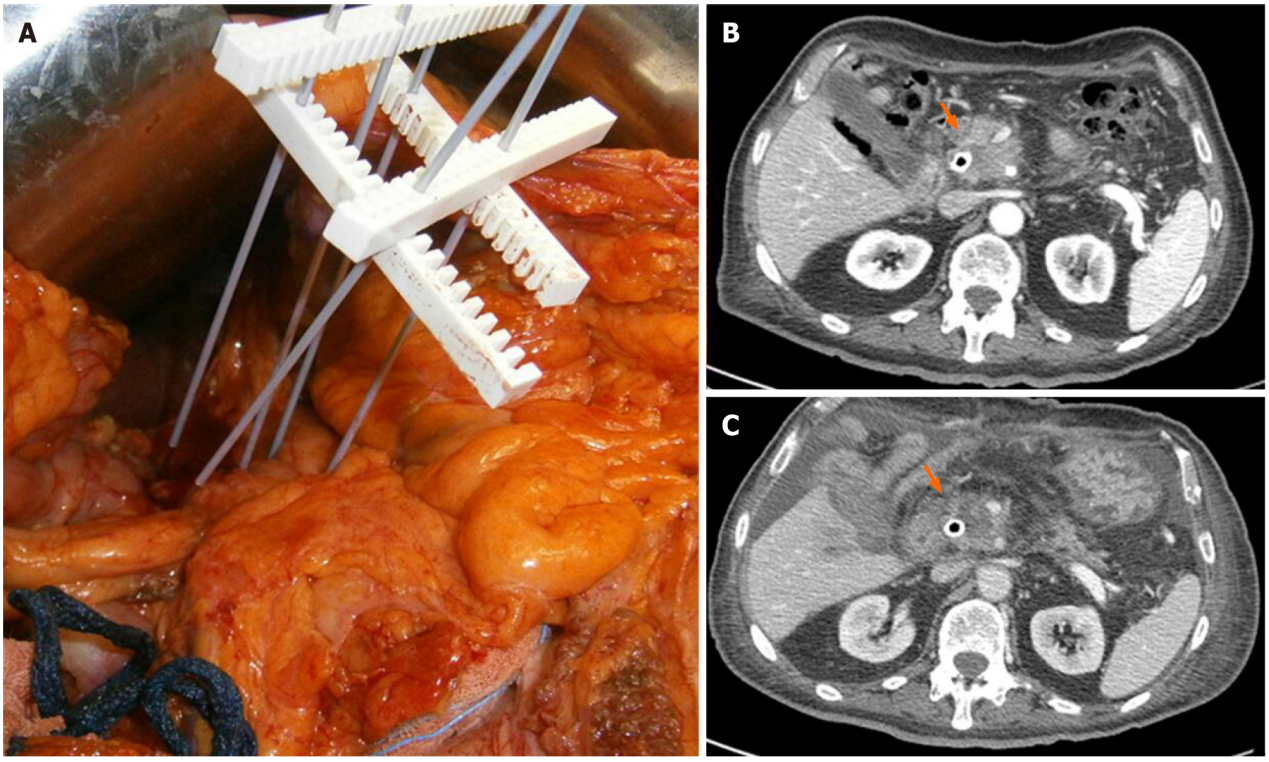


Figure 4 Electrochemotherapy with variable geometry and computed tomography pre-treatment and 6 mo post-treatment. A: Electrochemotherapy treatment with variable geometry; B: Computed tomography pre-treatment; C: Computed tomography 6 mo post-treatment. Computed tomography density showed a reduction as a positive response to treatment.

A distinct consideration should be made on whether local treatments induce an immune response in the ablated area. Preclinical and clinical studies have shown that RFA is an interesting tool to induce antigen-presenting cell infiltration and to enhance the systemic antitumour response. To the best of our knowledge, no data on other local treatments are available; therefore, studies that also evaluate this aspect for the other methods would be interesting.

Therefore, in the management of patients with pancreatic cancer, the possibility of a multimodal approach should be considered, and theoretically, the association of RFA with immunotherapy is a novel strategy against this tumour.

CONCLUSION

In conclusion, ablation therapies seem effective and safe with low post-treatment mortality. Although complications are mostly self-limiting, severe complications do occur. The technical success rate is high at 85%–100%, but this feature may be an over-estimation. Further efforts are also needed to address patient selection, as well as the use of IRE for "margin accentuation" during surgical resection, so the combination of RFA with immunotherapy represents a novel strategy against this tumour.

ARTICLE HIGHLIGHTS

Research background

In the current state of knowledge, the most commonly used technologies in locally advanced pancreatic cancer (LAPC) are radiofrequency ablation (RFA), microwave ablation, and irreversible electroporation (IRE) or reversible electroporation combined with low doses of chemotherapeutic drugs.

Research motivation

In the management of patients with pancreatic cancer, the possibility of a multimodal approach should be considered.

Research objectives

The research purpose was to report an overview and an update on ablation techniques, highlighting the advantages and limitations of each technology.

Research methods

The search covered the years from January 2000 to January 2021 and was performed using data from several electronic databases.

Research results

Ablation therapies seem effective and safe with low post-treatment mortality. Although complications are mostly self-limiting, severe complications do occur.

Research conclusions

Overall survival varies widely between different studies, and the additional value of ablation treatments for LAPC needs to be further explored.

Research perspectives

Further efforts are also needed to address patient selection, as well as the use of IRE for "margin accentuation" during surgical resection, so the combination of RFA with immunotherapy represents a novel angle of attack against this tumour type.

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REFERENCES

1 **Sung H**, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021 [PMID: 33538338 DOI: 10.3322/caac.21660]

2 NCCN Guidelines Updates: Pancreatic Cancer. [cited 28 March 2021]. Available from: https://www.nccn.org/patients/guidelines/content/PDF/pancreatic-patient.pdf

3 **Tempero MA**. NCCN Guidelines Updates: Pancreatic Cancer. *J Natl Compr Canc Netw* 2019; **17**: 603-605 [PMID: 31117041 DOI: 10.6004/jnccn.2019.5007]

4 **Alistar A**, Morris BB, Desnoyer R, Klepin HD, Hosseinzadeh K, Clark C, Cameron A, Leyendecker J, D'Agostino R Jr, Topaloglu U, Boteju LW, Boteju AR, Shorr R, Zachar Z, Bingham PM, Ahmed T, Crane S, Shah R, Migliano JJ, Pardee TS, Miller L, Hawkins G, Jin G, Zhang W, Pasche B. Safety and tolerability of the first-in-class agent CPI-613 in combination with modified FOLFIRINOX in patients with metastatic pancreatic cancer: a single-centre, open-label, dose-escalation, phase 1 trial. *Lancet Oncol* 2017; **18**: 770-778 [PMID: 28495639 DOI: 10.1016/S1470-2045(17)30314-5]

5 **Usón Junior PLS**, Rother ET, Maluf FC, Bugano DDG. Meta-analysis of Modified FOLFIRINOX Regimens for Patients With Metastatic Pancreatic Cancer. *Clin Colorectal Cancer* 2018; **17**: 187-197 [PMID: 29615310 DOI: 10.1016/j.clcc.2018.03.007]

6 **Granata V**, Grassi R, Fusco R, Setola SV, Palaia R, Belli A, Miele V, Brunese L, Petrillo A, Izzo F. Assessment of Ablation Therapy in Pancreatic Cancer: The Radiologist's Challenge. *Front Oncol* 2020; **10**: 560952 [PMID: 33330028 DOI: 10.3389/fonc.2020.560952]

7 **Chin V**, Nagrial A, Sjoquist K, O'Connor CA, Chantrill L, Biankin AV, Scholten RJ, Yip D. Chemotherapy and radiotherapy for advanced pancreatic cancer. *Cochrane Database Syst Rev* 2018; **3**: CD011044 [PMID: 29557103 DOI: 10.1002/14651858.CD011044.pub2]

8 **van Veldhuisen E**, van den Oord C, Brada LJ, Walma MS, Vogel JA, Wilmink JW, Del Chiaro M, van Lienden KP, Meijerink MR, van Tienhoven G, Hackert T, Wolfgang CL, van Santvoort H, Groot Koerkamp B, Busch OR, Molenaar IQ, van Eijck CH, Besselink MG; Dutch Pancreatic Cancer Group and International Collaborative Group on Locally Advanced Pancreatic Cancer. Locally Advanced Pancreatic Cancer: Work-Up, Staging, and Local Intervention Strategies. *Cancers (Basel)* 2019; **11** [PMID: 31336859 DOI: 10.3390/cancers11070976]

9 **Paiella S**, Salvia R, Ramera M, Girelli R, Frigerio I, Giardino A, Allegrini V, Bassi C. Local Ablative Strategies for Ductal Pancreatic Cancer (Radiofrequency Ablation, Irreversible Electroporation): A Review. *Gastroenterol Res Pract* 2016; **2016**: 4508376 [PMID: 26981115 DOI: 10.1155/2016/4508376]

10 **Testoni SGG**, Healey AJ, Dietrich CF, Arcidiacono PG. Systematic review of endoscopy ultrasound-guided thermal ablation treatment for pancreatic cancer. *Endosc Ultrasound* 2020; **9**: 83-100 [PMID:

32295966 DOI: 10.4103/eus.eus_74_19]
11 **Granata V**, Fusco R, Piccirillo M, Palaia R, Petrillo A, Lastoria S, Izzo F. Electrochemotherapy in locally advanced pancreatic cancer: Preliminary results. *Int J Surg* 2015; **18**: 230-236 [PMID: 25917204 DOI: 10.1016/j.ijsu.2015.04.055]
12 **Granata V**, Fusco R, Setola SV, Piccirillo M, Leongito M, Palaia R, Granata F, Lastoria S, Izzo F, Petrillo A. Early radiological assessment of locally advanced pancreatic cancer treated with electrochemotherapy. *World J Gastroenterol* 2017; **23**: 4767-4778 [PMID: 28765698 DOI: 10.3748/wjg.v23.i26.4767]
13 **Granata V**, Fusco R, Setola SV, Palaia R, Albino V, Piccirillo M, Grimm R, Petrillo A, Izzo F. Diffusion kurtosis imaging and conventional diffusion weighted imaging to assess electrochemotherapy response in locally advanced pancreatic cancer. *Radiol Oncol* 2019; **53**: 15-24 [PMID: 30681974 DOI: 10.2478/raon-2019-0004]
14 **Vroomen LGPH**, Scheffer HJ, Melenhorst MCAM, de Jong MC, van den Bergh JE, van Kuijk C, van Delft F, Kazemier G, Meijerink MR. MR and CT imaging characteristics and ablation zone volumetry of locally advanced pancreatic cancer treated with irreversible electroporation. *Eur Radiol* 2017; **27**: 2521-2531 [PMID: 27659702 DOI: 10.1007/s00330-016-4581-2]
15 **Pelle E**, Al-Toubah T, Morse B, El-Haddad G, Strosberg J. Desmoplastic mesenteric lesions do not respond radiographically to peptide receptor radionuclide therapy. *J Neuroendocrinol* 2021; **33**: e12936 [PMID: 33474756 DOI: 10.1111/jne.12936]
16 **Izzo F**, Granata V, Grassi R, Fusco R, Palaia R, Delrio P, Carrafiello G, Azoulay D, Petrillo A, Curley SA. Radiofrequency Ablation and Microwave Ablation in Liver Tumors: An Update. *Oncologist* 2019; **24**: e990-e1005 [PMID: 31217342 DOI: 10.1634/theoncologist.2018-0337]
17 **Granata V**, de Lutio di Castelguidone E, Fusco R, Catalano O, Piccirillo M, Palaia R, Izzo F, Gallipoli AD, Petrillo A. Irreversible electroporation of hepatocellular carcinoma: preliminary report on the diagnostic accuracy of magnetic resonance, computer tomography, and contrast-enhanced ultrasound in evaluation of the ablated area. *Radiol Med* 2016; **121**: 122-131 [PMID: 26345332 DOI: 10.1007/s11547-015-0582-5]
18 **Ierardi AM**, Lucchina N, Petrillo M, Floridi C, Piacentino F, Bacuzzi A, Fonio P, Fontana F, Fugazzola C, Brunese L, Carrafiello G. Systematic review of minimally invasive ablation treatment for locally advanced pancreatic cancer. *Radiol Med* 2014; **119**: 483-498 [PMID: 24981482 DOI: 10.1007/s11547-014-0417-9]
19 **Martin RC 2nd**, Kwon D, Chalikonda S, Sellers M, Kotz E, Scoggins C, McMasters KM, Watkins K. Treatment of 200 locally advanced (stage III) pancreatic adenocarcinoma patients with irreversible electroporation: safety and efficacy. *Ann Surg* 2015; **262**: 486-494; discussion 492 [PMID: 26258317 DOI: 10.1097/SLA.0000000000001441]
20 **Martin RC 2nd**, McFarland K, Ellis S, Velanovich V. Irreversible electroporation in locally advanced pancreatic cancer: potential improved overall survival. *Ann Surg Oncol* 2013; **20** Suppl 3: S443-S449 [PMID: 23128941 DOI: 10.1245/s10434-012-2736-1]
21 **Marsanic P**, Mellano A, Sottile A, De Simone M. Irreversible electroporation as treatment of locally advanced and as margin accentuation in borderline resectable pancreatic adenocarcinoma. *Med Biol Eng Comput* 2017; **55**: 1123-1127 [PMID: 28078482 DOI: 10.1007/s11517-016-1603-9]
22 **Ansari D**, Kristoffersson S, Andersson R, Bergenfeldt M. The role of irreversible electroporation (IRE) for locally advanced pancreatic cancer: a systematic review of safety and efficacy. *Scand J Gastroenterol* 2017; **52**: 1165-1171 [PMID: 28687047 DOI: 10.1080/00365521.2017.1346705]
23 **Lambert L**, Horejs J, Krska Z, Hoskovec D, Petruzelka L, Krechler T, Kriz P, Briza J. Treatment of locally advanced pancreatic cancer by percutaneous and intraoperative irreversible electroporation: general hospital cancer center experience. *Neoplasma* 2016; **63**: 269-273 [PMID: 26774149 DOI: 10.4149/213_150611N326]
24 **Yan L**, Chen YL, Su M, Liu T, Xu K, Liang F, Gu WQ, Lu SC. A Single-institution Experience with Open Irreversible Electroporation for Locally Advanced Pancreatic Carcinoma. *Chin Med J (Engl)* 2016; **129**: 2920-2925 [PMID: 27958223 DOI: 10.4103/0366-6999.195476]
25 **Zhang Y**, Shi J, Zeng J, Alnagger M, Zhou L, Fang G, Long X, Pan Z, Li Y, Chen J, Xu K, Qian W, Niu L. Percutaneous Irreversible Electroporation for Ablation of Locally Advanced Pancreatic Cancer: Experience From a Chinese Institution. *Pancreas* 2017; **46**: e12-e14 [PMID: 28085755 DOI: 10.1097/MPA.0000000000000703]
26 **Scheffer HJ**, Vroomen LG, de Jong MC, Melenhorst MC, Zonderhuis BM, Daams F, Vogel JA, Besselink MG, van Kuijk C, Witvliet J, de van der Schueren MA, de Gruijl TD, Stam AG, van den Tol PM, van Delft F, Kazemier G, Meijerink MR. Ablation of Locally Advanced Pancreatic Cancer with Percutaneous Irreversible Electroporation: Results of the Phase I/II PANFIRE Study. *Radiology* 2017; **282**: 585-597 [PMID: 27604035 DOI: 10.1148/radiol.2016152835]
27 **Izzo F**, Ionna F, Granata V, Albino V, Patrone R, Longo F, Guida A, Delrio P, Rega D, Scala D, Pezzuto R, Fusco R, Di Bernardo E, D'Alessio V, Grassi R, Contartese D, Palaia R. New Deployable Expandable Electrodes in the Electroporation Treatment in a Pig Model: A Feasibility and Usability Preliminary Study. *Cancers (Basel)* 2020; **12** [PMID: 32102182 DOI: 10.3390/cancers12020515]
28 **D'Onofrio M**, Barbi E, Girelli R, Tinazzi Martini P, De Robertis R, Ciaravino V, Salvia R, Butturini G, Frigerio I, Milazzo T, Crosara S, Paiella S, Pederzoli P, Bassi C. Variation of tumoral marker after radiofrequency ablation of pancreatic adenocarcinoma. *J Gastrointest Oncol* 2016; **7**: 213-220 [PMID: 27034788 DOI: 10.3978/j.issn.2078-6891.2015.085]
29 **D'Onofrio M**, Crosara S, De Robertis R, Butturini G, Salvia R, Paiella S, Bassi C, Mucelli RP.

Percutaneous Radiofrequency Ablation of Unresectable Locally Advanced Pancreatic Cancer: Preliminary Results. *Technol Cancer Res Treat* 2017; **16**: 285-294 [PMID: 27193941 DOI: 10.1177/1533034616649292]

30 **Giardino A**, Girelli R, Frigerio I, Regi P, Cantore M, Alessandra A, Lusenti A, Salvia R, Bassi C, Pederzoli P. Triple approach strategy for patients with locally advanced pancreatic carcinoma. *HPB (Oxford)* 2013; **15**: 623-627 [PMID: 23458679 DOI: 10.1111/hpb.12027]

31 **Hadjicostas P**, Malakounides N, Varianos C, Kitiris E, Lerni F, Symeonides P. Radiofrequency ablation in pancreatic cancer. *HPB (Oxford)* 2006; **8**: 61-64 [PMID: 18333241 DOI: 10.1080/13651820500466673]

32 **Ikuta S**, Kurimoto A, Iida H, Aihara T, Takechi M, Kamikonya N, Yamanaka N. Optimal combination of radiofrequency ablation with chemoradiotherapy for locally advanced pancreatic cancer. *World J Clin Oncol* 2012; **3**: 12-14 [PMID: 22247824 DOI: 10.5306/wjco.v3.i1.12]

33 **Kallis Y**, Phillips N, Steel A, Kaltsidis H, Vlavianos P, Habib N, Westaby D. Analysis of Endoscopic Radiofrequency Ablation of Biliary Malignant Strictures in Pancreatic Cancer Suggests Potential Survival Benefit. *Dig Dis Sci* 2015; **60**: 3449-3455 [PMID: 26038094 DOI: 10.1007/s10620-015-3731-8]

34 **Lakhtakia S**, Ramchandani M, Galasso D, Gupta R, Venugopal S, Kalpala R, Reddy DN. EUS-guided radiofrequency ablation for management of pancreatic insulinoma by using a novel needle electrode (with videos). *Gastrointest Endosc* 2016; **83**: 234-239 [PMID: 26394384 DOI: 10.1016/j.gie.2015.08.085]

35 **Pai M**, Habib N, Senturk H, Lakhtakia S, Reddy N, Cicinnati VR, Kaba I, Beckebaum S, Drymousis P, Kahaleh M, Brugge W. Endoscopic ultrasound guided radiofrequency ablation, for pancreatic cystic neoplasms and neuroendocrine tumors. *World J Gastrointest Surg* 2015; **7**: 52-59 [PMID: 25914783 DOI: 10.4240/wjgs.v7.i4.52]

36 **Rossi S**, Viera FT, Ghittoni G, Cobianchi L, Rosa LL, Siciliani L, Bortolotto C, Veronese L, Vercelli A, Gallotti A, Ravetta V. Radiofrequency ablation of pancreatic neuroendocrine tumors: a pilot study of feasibility, efficacy, and safety. *Pancreas* 2014; **43**: 938-945 [PMID: 24717825 DOI: 10.1097/MPA.0000000000000133]

37 **Song TJ**, Seo DW, Lakhtakia S, Reddy N, Oh DW, Park DH, Lee SS, Lee SK, Kim MH. Initial experience of EUS-guided radiofrequency ablation of unresectable pancreatic cancer. *Gastrointest Endosc* 2016; **83**: 440-443 [PMID: 26344883 DOI: 10.1016/j.gie.2015.08.048]

38 **Spiliotis JD**, Datsis AC, Michalopoulos NV, Kekelos SP, Vaxevanidou A, Rogdakis AG, Christopoulou AN. Radiofrequency ablation combined with palliative surgery may prolong survival of patients with advanced cancer of the pancreas. *Langenbecks Arch Surg* 2007; **392**: 55-60 [PMID: 17089173 DOI: 10.1007/s00423-006-0098-5]

39 **Varshney S**, Sewkani A, Sharma S, Kapoor S, Naik S, Sharma A, Patel K. Radiofrequency ablation of unresectable pancreatic carcinoma: feasibility, efficacy and safety. *JOP* 2006; **7**: 74-78 [PMID: 16407624]

40 **Waung JA**, Todd JF, Keane MG, Pereira SP. Successful management of a sporadic pancreatic insulinoma by endoscopic ultrasound-guided radiofrequency ablation. *Endoscopy* 2016; **48** Suppl 1: E144-E145 [PMID: 27081874 DOI: 10.1055/s-0042-104650]

41 **Zou YP**, Li WM, Zheng F, Li FC, Huang H, Du JD, Liu HR. Intraoperative radiofrequency ablation combined with 125 iodine seed implantation for unresectable pancreatic cancer. *World J Gastroenterol* 2010; **16**: 5104-5110 [PMID: 20976848 DOI: 10.3748/wjg.v16.i40.5104]

42 **Saccomandi P**, Lapergola A, Longo F, Schena E, Quero G. Thermal ablation of pancreatic cancer: A systematic literature review of clinical practice and pre-clinical studies. *Int J Hyperthermia* 2018; **35**: 398-418 [PMID: 30428728 DOI: 10.1080/02656736.2018.1506165]

43 **Waitz R**, Solomon SB. Can local radiofrequency ablation of tumors generate systemic immunity against metastatic disease? *Radiology* 2009; **251**: 1-2 [PMID: 19332838 DOI: 10.1148/radiol.2511082215]

44 **Giardino A**, Innamorati G, Ugel S, Perbellini O, Girelli R, Frigerio I, Regi P, Scopelliti F, Butturini G, Paiella S, Bacchion M, Bassi C. Immunomodulation after radiofrequency ablation of locally advanced pancreatic cancer by monitoring the immune response in 10 patients. *Pancreatology* 2017; **17**: 962-966 [PMID: 29037917 DOI: 10.1016/j.pan.2017.09.008]

45 **Fei Q**, Pan Y, Lin W, Zhou Y, Yu X, Hou Z, Lin X, Lin R, Lu F, Guan H, Huang H. High-dimensional single-cell analysis delineates radiofrequency ablation induced immune microenvironmental remodeling in pancreatic cancer. *Cell Death Dis* 2020; **11**: 589 [PMID: 32719347 DOI: 10.1038/s41419-020-02787-1]

46 **D'Onofrio M**, Beleù A, Sarno A, De Robertis R, Paiella S, Viviani E, Frigerio I, Girelli R, Salvia R, Bassi C. US-Guided Percutaneous Radiofrequency Ablation of Locally Advanced Pancreatic Adenocarcinoma: A 5-Year High-Volume Center Experience. *Ultraschall Med* 2020 [PMID: 32797463 DOI: 10.1055/a-1178-0474]

47 **Gao S**, Pu N, Yin H, Li J, Chen Q, Yang M, Lou W, Chen Y, Zhou G, Li C, Li G, Yan Z, Liu L, Yu J, Wang X. Radiofrequency ablation in combination with an mTOR inhibitor restrains pancreatic cancer growth induced by intrinsic HSP70. *Ther Adv Med Oncol* 2020; **12**: 1758835920953728 [PMID: 32973929 DOI: 10.1177/1758835920953728]

48 **Wang J**, Wang Y, Zhao Y, Wu X, Zhang M, Hou W, Chen Q, Cheng B. Endoscopic ultrasound-guided radiofrequency ablation of unresectable pancreatic cancer with low ablation power and multiple applications: a preliminary study of 11 patients. *Ann Palliat Med* 2021; **10**: 1842-1850

[PMID: 33440967 DOI: 10.21037/apm-20-1468]
49 **Al-Jumah R**, Urits I, Viswanath O, Kaye AD, Hasoon J. Radiofrequency Ablation and Alcohol Neurolysis of the Splanchnic Nerves for a Patient With Abdominal Pain From Pancreatic Cancer. *Cureus* 2020; **12**: e10758 [PMID: 33150109 DOI: 10.7759/cureus.10758]
50 **He C**, Wang J, Zhang Y, Cai Z, Lin X, Li S. Comparison of combination therapies in the management of locally advanced pancreatic cancer: Induction chemotherapy followed by irreversible electroporation vs radiofrequency ablation. *Cancer Med* 2020; **9**: 4699-4710 [PMID: 32410380 DOI: 10.1002/cam4.3119]
51 **Fegrachi S**, Besselink MG, van Santvoort HC, van Hillegersberg R, Molenaar IQ. Radiofrequency ablation for unresectable locally advanced pancreatic cancer: a systematic review. *HPB (Oxford)* 2014; **16**: 119-123 [PMID: 23600801 DOI: 10.1111/hpb.12097]
52 **Fegrachi S**, Walma MS, de Vries JJJ, van Santvoort HC, Besselink MG, von Asmuth EG, van Leeuwen MS, Borel Rinkes IH, Bruijnen RC, de Hingh IH, Klaase JM, Molenaar IQ, van Hillegersberg R. Safety of radiofrequency ablation in patients with locally advanced, unresectable pancreatic cancer: A phase II study. *Eur J Surg Oncol* 2019; **45**: 2166-2172 [PMID: 31227340 DOI: 10.1016/j.ejso.2019.06.008]
53 **Rombouts SJ**, Vogel JA, van Santvoort HC, van Lienden KP, van Hillegersberg R, Busch OR, Besselink MG, Molenaar IQ. Systematic review of innovative ablative therapies for the treatment of locally advanced pancreatic cancer. *Br J Surg* 2015; **102**: 182-193 [PMID: 25524417 DOI: 10.1002/bjs.9716]
54 **Trinci M**, Cirimele V, Cozzi D, Galluzzo M, Miele V. Diagnostic accuracy of pneumo-CT-cystography in the detection of bladder rupture in patients with blunt pelvic trauma. *Radiol Med* 2020; **125**: 907-917 [PMID: 32274620 DOI: 10.1007/s11547-020-01190-2]
55 **Giannitto C**, Campoleoni M, Maccagnoni S, Angileri AS, Grimaldi MC, Giannitto N, De Piano F, Ancona E, Biondetti PR, Esposito AA. Unindicated multiphase CT scans in non-traumatic abdominal emergencies for women of reproductive age: a significant source of unnecessary exposure. *Radiol Med* 2018; **123**: 185-190 [PMID: 29086381 DOI: 10.1007/s11547-017-0819-6]
56 **Miele V**, Piccolo CL, Trinci M, Galluzzo M, Ianniello S, Brunese L. Diagnostic imaging of blunt abdominal trauma in pediatric patients. *Radiol Med* 2016; **121**: 409-430 [PMID: 27075018 DOI: 10.1007/s11547-016-0637-2]
57 **Schueller G**, Scaglione M, Linsenmaier U, Schueller-Weidekamm C, Andreoli C, De Vargas Macciucca M, Gualdi G. The key role of the radiologist in the management of polytrauma patients: indications for MDCT imaging in emergency radiology. *Radiol Med* 2015; **120**: 641-654 [PMID: 25634793 DOI: 10.1007/s11547-015-0500-x]
58 **Sessa B**, Trinci M, Ianniello S, Menichini G, Galluzzo M, Miele V. Blunt abdominal trauma: role of contrast-enhanced ultrasound (CEUS) in the detection and staging of abdominal traumatic lesions compared to US and CE-MDCT. *Radiol Med* 2015; **120**: 180-189 [PMID: 24961341 DOI: 10.1007/s11547-014-0425-9]
59 **Carrafiello G**, Ierardi AM, Fontana F, Petrillo M, Floridi C, Lucchina N, Cuffari S, Dionigi G, Rotondo A, Fugazzola C. Microwave ablation of pancreatic head cancer: safety and efficacy. *J Vasc Interv Radiol* 2013; **24**: 1513-1520 [PMID: 24070507 DOI: 10.1016/j.jvir.2013.07.005]
60 **Ierardi AM**, Biondetti P, Coppola A, Fumarola EM, Biasina AM, Alessio Angileri S, Carrafiello G. Percutaneous microwave thermosphere ablation of pancreatic tumours. *Gland Surg* 2018; **7**: 59-66 [PMID: 29770302 DOI: 10.21037/gs.2017.11.05]
61 **Vogl TJ**, Panahi B, Albrecht MH, Naguib NNN, Nour-Eldin NA, Gruber-Rouh T, Thompson ZM, Basten LM. Microwave ablation of pancreatic tumors. *Minim Invasive Ther Allied Technol* 2018; **27**: 33-40 [PMID: 29278340 DOI: 10.1080/13645706.2017.1420664]
62 **Narayanan G**, Hosein PJ, Arora G, Barbery KJ, Froud T, Livingstone AS, Franceschi D, Rocha Lima CM, Yrizarry J. Percutaneous irreversible electroporation for downstaging and control of unresectable pancreatic adenocarcinoma. *J Vasc Interv Radiol* 2012; **23**: 1613-1621 [PMID: 23177107 DOI: 10.1016/j.jvir.2012.09.012]
63 **Weiss MJ**, Wolfgang CL. Irreversible electroporation: a novel pancreatic cancer therapy. *Curr Probl Cancer* 2013; **37**: 262-265 [PMID: 24331180 DOI: 10.1016/j.currproblcancer.2013.10.002]
64 **Scheffer HJ**, Nielsen K, de Jong MC, van Tilborg AA, Vieveen JM, Bouwman AR, Meijer S, van Kuijk C, van den Tol PM, Meijerink MR. Irreversible electroporation for nonthermal tumor ablation in the clinical setting: a systematic review of safety and efficacy. *J Vasc Interv Radiol* 2014; **25**: 997-1011; quiz 1011 [PMID: 24656178 DOI: 10.1016/j.jvir.2014.01.028]
65 **Ruarus AH**, Vroomen LGPH, Geboers B, van Veldhuisen E, Puijk RS, Nieuwenhuizen S, Besselink MG, Zonderhuis BM, Kazemier G, de Gruijl TD, van Lienden KP, de Vries JJJ, Scheffer HJ, Meijerink MR. Percutaneous Irreversible Electroporation in Locally Advanced and Recurrent Pancreatic Cancer (PANFIRE-2): A Multicenter, Prospective, Single-Arm, Phase II Study. *Radiology* 2020; **294**: 212-220 [PMID: 31687922 DOI: 10.1148/radiol.2019191109]
66 **van Veldhuisen E**, Vroomen LG, Ruarus AH, Derksen TC, Busch OR, de Jong MC, Kazemier G, Puijk RS, Sorgedrager NS, Vogel JA, Scheffer HJ, van Lienden KP, Wilmink JW, Besselink MG, Meijerink MR. Value of CT-Guided Percutaneous Irreversible Electroporation Added to FOLFIRINOX Chemotherapy in Locally Advanced Pancreatic Cancer: A Post Hoc Comparison. *J Vasc Interv Radiol* 2020; **31**: 1600-1608 [PMID: 32861569 DOI: 10.1016/j.jvir.2020.02.024]
67 **Narayanan G**, Hosein PJ, Beulaygue IC, Froud T, Scheffer HJ, Venkat SR, Echenique AM, Hevert EC, Livingstone AS, Rocha-Lima CM, Merchan JR, Levi JU, Yrizarry JM, Lencioni R. Percutaneous

Image-Guided Irreversible Electroporation for the Treatment of Unresectable, Locally Advanced Pancreatic Adenocarcinoma. *J Vasc Interv Radiol* 2017; **28**: 342-348 [PMID: 27993507 DOI: 10.1016/j.jvir.2016.10.023]

68 **Liu S**, Qin Z, Xu J, Zeng J, Chen J, Niu L, Xu M. Irreversible electroporation combined with chemotherapy for unresectable pancreatic carcinoma: a prospective cohort study. *Onco Targets Ther* 2019; **12**: 1341-1350 [PMID: 30863100 DOI: 10.2147/OTT.S186721]

69 **Linecker M**, Pfammatter T, Kambakamba P, DeOliveira ML. Ablation Strategies for Locally Advanced Pancreatic Cancer. *Dig Surg* 2016; **33**: 351-359 [PMID: 27216160 DOI: 10.1159/000445021]

70 **Zhang Z**, Li W, Procissi D, Tyler P, Omary RA, Larson AC. Rapid dramatic alterations to the tumor microstructure in pancreatic cancer following irreversible electroporation ablation. *Nanomedicine (Lond)* 2014; **9**: 1181-1192 [PMID: 24024571 DOI: 10.2217/nnm.13.72]

71 **Granata V**, Fusco R, Palaia R, Belli A, Petrillo A, Izzo F. Comments on "Electrochemotherapy with Irreversible Electroporation and FOLFIRINOX Improves Survival in Murine Models of Pancreatic Adenocarcinoma". *Ann Surg Oncol* 2020; **27**: 954-955 [PMID: 33011913 DOI: 10.1245/s10434-020-09183-1]

72 **De Filippo M**, Bozzetti F, Martora R, Zagaria R, Ferretti S, Macarini L, Brunese L, Rotondo A, Rossi C. Radiofrequency thermal ablation of renal tumors. *Radiol Med* 2014; **119**: 499-511 [PMID: 25024061 DOI: 10.1007/s11547-014-0412-1]

73 **Vogl TJ**, Farshid P, Naguib NN, Darvishi A, Bazrafshan B, Mbalisike E, Burkhard T, Zangos S. Thermal ablation of liver metastases from colorectal cancer: radiofrequency, microwave and laser ablation therapies. *Radiol Med* 2014; **119**: 451-461 [PMID: 24894923 DOI: 10.1007/s11547-014-0415-y]

74 **Cornalba G**, Melchiorre F. Interventional oncology: state of the art. *Radiol Med* 2014; **119**: 449-450 [PMID: 25001586 DOI: 10.1007/s11547-014-0428-6]

75 **Garetto I**, Busso M, Sardo D, Filippini C, Solitro F, Grognardi ML, Veltri A. Radiofrequency ablation of thoracic tumours: lessons learned with ablation of 100 lesions. *Radiol Med* 2014; **119**: 33-40 [PMID: 24234185 DOI: 10.1007/s11547-013-0308-5]

76 **Kaufman JD**, Fesmire CC, Petrella RA, Fogle CA, Xing L, Gerber D, Sano MB. High-Frequency Irreversible Electroporation Using 5,000-V Waveforms to Create Reproducible 2- and 4-cm Ablation Zones-A Laboratory Investigation Using Mechanically Perfused Liver. *J Vasc Interv Radiol* 2020; **31**: 162-168. e7 [PMID: 31530492 DOI: 10.1016/j.jvir.2019.05.009]

77 **Timmer FEF**, Geboers B, Ruarus AH, Schouten EAC, Nieuwenhuizen S, Puijk RS, de Vries JJJ, Meijerink MR, Scheffer HJ. Irreversible Electroporation for Locally Advanced Pancreatic Cancer. *Tech Vasc Interv Radiol* 2020; **23**: 100675 [PMID: 32591191 DOI: 10.1016/j.tvir.2020.100675]

78 **Reames BN**, Blair AB, Krell RW, Groot VP, Gemenetzis G, Padussis JC, Thayer SP, Falconi M, Wolfgang CL, Weiss MJ, Are C, He J. Management of Locally Advanced Pancreatic Cancer: Results of an International Survey of Current Practice. *Ann Surg* 2019 [PMID: 31449138 DOI: 10.1097/SLA.0000000000003568]

79 **Tartaglia E**, Fabozzi M, Rizzuto A, Settembre A, Abete R, Guerriero L, Favoriti P, Cuccurullo D, Corcione F. Irreversible electroporation for locally advanced pancreatic cancer through a minimally invasive surgery supported by laparoscopic ultrasound. *Int J Surg Case Rep* 2018; **42**: 290-294 [PMID: 29335228 DOI: 10.1016/j.ijscr.2017.12.036]

80 **Latouche EL**, Sano MB, Lorenzo MF, Davalos RV, Martin RCG 2nd. Irreversible electroporation for the ablation of pancreatic malignancies: A patient-specific methodology. *J Surg Oncol* 2017; **115**: 711-717 [PMID: 28185295 DOI: 10.1002/jso.24566]

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OPINION REVIEW

Trial eligibility in advanced hepatocellular carcinoma: Does it support clinical practice in underrepresented subgroups?

Federico Piñero, Leonardo Gomes da Fonseca

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Abstract

Although hepatocellular carcinoma is considered a highly lethal malignancy, recent therapeutic advances have been achieved during the last 10 years. This scenario resulted in an unprecedented improvement in survival for patients with advanced hepatocellular carcinoma, almost reaching 20-26 mo of overall survival after first-second line sequential treatment. The advent of the combination of atezolizumab with bevacizumab showed, for the first time, superiority over sorafenib with improvement in overall survival. However, first and second-line trials were correctly based on the premise that a strict selection of patients enhances the power to capture the positive effect of treatment by excluding competing risks for mortality such as liver failure, decompensated cirrhosis or other underlying medical conditions. As a result, the inclusion criteria used in clinical trials do not support the use of novel therapies in several real-world scenarios involving underrepresented subgroups, such as patients with unpreserved liver function, other comorbid conditions, a history of solid-organ transplantation, autoimmune disorders and those with a high risk of bleeding. The present text aims at discussing treatment strategies in these subgroups.

Key Words: Eligibility; Systemic therapies; End-stage; Hepatocellular carcinoma

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Core Tip: The strict criteria used in clinical trials in advanced hepatocellular carcinoma have led to a scarcity of available data in a considerable proportion of patients in the real-world practice. The daily challenge of treating these underrepresented subgroups

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can be overcome by future clinical trials addressing special situations, collaborative studies and real-world data.

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INTRODUCTION

Although hepatocellular carcinoma (HCC) is considered a highly lethal malignancy, recent therapeutic advances have been achieved during the last 10 years. These achievements were unthinkable 20 years before. Historically, patients with HCC at advanced stages or refractory to locoregional therapies (such as surgery, ablation or intra-arterial treatments) were associated with a dismal prognosis[1]. This scenario has fortunately changed.

In 2008, the first positive phase III trial (SHARP trial) using a systemic agent for HCC was published, showing that sorafenib improved overall survival over placebo in a selected population[2]. This result was observed in the Asia-Pacific trial, repeating similar observations yet in another population[3]. Sorafenib has succeeded due to its activity against different tumor pathways, particularly angiogenesis and proliferation signaling activation even in the absence of significant tumor shrinkage. It showed a favorable safety profile, particularly in patients with a well-preserved liver function (Child-Pugh A), a performance status of 2 or less and no other organ failure.

Following sorafenib, several drugs with similar or different targets were tested with disappointing results in phase III trials[4]. On the other hand, lenvatinib, shown to be non-inferior to sorafenib in the phase III REFLECT trial in patients without main portal trunk tumor invasion or without more than 50% of liver involvement[5], became an alternative in the first-line setting. Other agents such as regorafenib[6], cabozantinib[7] and ramucirumab[8] were incorporated as second-line options after sorafenib failure. This scenario resulted in an unprecedented improvement in survival for patients with advanced HCC, almost reaching 20-26 mo of overall survival after first-second line sequential treatment[9,10].

The advent of immune checkpoint inhibitors (ICI) with impressive results in solid tumors underpinned trials in advanced HCC. ICIs were rapidly incorporated after encouraging results with nivolumab and pembrolizumab in phase II trials with HCC patients, with durable objective response rates in 15%-20% of the patients[11,12]. In 2020, the combination of atezolizumab (a programmed death ligand 1 inhibitor) with bevacizumab [an anti-vascular endothelial growth factor-vascular endothelial growth factor (VEGF)-antibody] showed for the first time superiority over sorafenib in the phase III IMBRAVE150 trial[13]. This result was followed by approval of this combination as the standard first-line treatment for advanced HCC in different countries.

However, first and second-line trials were correctly based on the premise that a strict selection of patients enhances the power to capture the positive effect of treatment by excluding competing risks of mortality such as liver failure, decompensated cirrhosis or other underlying medical conditions (Figure 1). As a result, the inclusion criteria used in clinical trials do not support novel therapies in several real-world scenarios involving underrepresented subgroups. Moreover, due to the mechanism of action of ICIs and the risk of immune-related adverse events, the IMBRAVE trial did not enroll specific subgroups, such as patients with a history of solid-organ transplantation, auto-immune disorders, and a high risk of bleeding. The present text aims at discussing treatment strategies in these subgroups.

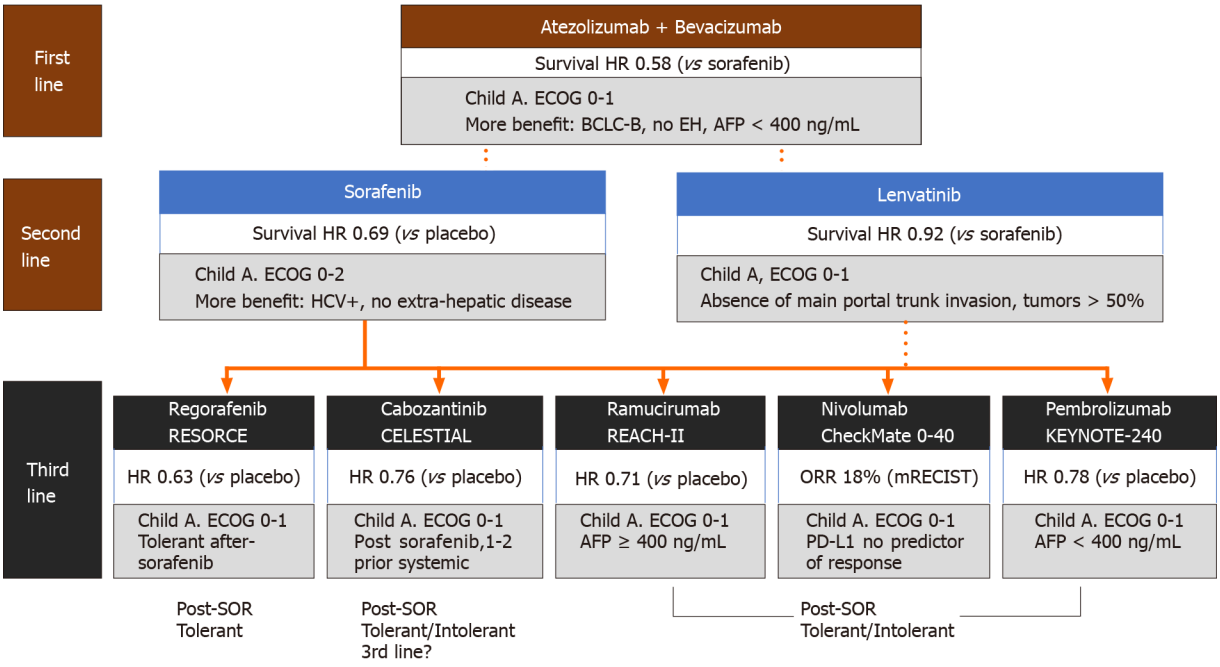


Figure 1 Systemic therapy for advanced hepatocellular carcinoma. Note: First and second-line options may be presented as first-line options in parallel. Exclusion criteria in the REFLECT trial shown for lenvatinib[5]. AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; ECOG: Eastern Cooperative Oncology Group; EH: Extrahepatic; HCV: Hepatitis C virus; HR: Hazard ratio; ORR: Overall response rate; PD-L1: Programmed death ligand 1; SOR: Sorafenib.

CHALLENGES IN REAL-WORLD SCENARIOS

Etiology of underlying liver disease: does it really matter for decision making?

In Western countries (mainly Europe and the United States), the leading risk factor for HCC is chronic hepatitis C virus (HCV) infection. In contrast, hepatitis B virus (HBV) chronic infection is predominant in China, Asia and sub-Saharan Africa, where a higher burden of HCC is found compared to the rest of the world[14]. In Latin America, HCV represents the most prevalent risk factor for HCC[15,16], but other etiologies, such as nonalcoholic fatty liver disease, are steadily increasing[17,18].

This geographic heterogeneity directly impacts the recruitment of patients. Trials that restrict enrollment to a specific region are likely to be enriched with the predominant local etiology. Clinical trials that recruit globally tend to have HBV as the most frequent etiology when the Asian population predominates. A noticeable transition in risk factors has been observed in Western countries, with growing evidence that metabolic-associated fatty liver disease is an increasing cause of HCC, often associated with other comorbidities such as obesity, hypertension and diabetes [19].

This geographical eligibility is exemplified by the pivotal trials exploring sorafenib. In the SHARP trial, only 18.4% of the enrolled population had HBV-related HCC[2], while 73% of the patients enrolled in the Asia-Pacific trial had HBV-related HCC[3]. Although both trials showed a benefit in overall survival irrespective from underlying liver disease, a combined analysis of these two trials demonstrated a significant benefit in patients with HCV[20].

Nonviral etiologies represent only 30% to 45% of the included population in recent immunotherapy trials (Table 1). Whether immunotherapy is equally effective across all etiologies is still uncertain[13], shown through subgroup analysis in the IMBRAVE150 study suggesting that atezolizumab plus bevacizumab may have a lower benefit over sorafenib in nonviral etiologies [hazard ratio (HR): 0.91, 95% confidence interval (CI): 0.51-1.60], when compared to HBV (HR: 0.51; 95%CI: 0.32-0.81) or HCV-associated HCC (HR: 0.43; 95%CI: 0.22-0.87). This was also shown in the first-line trial comparing nivolumab *vs* sorafenib (Checkmate-459 trial, NCT02576509), in which nivolumab did not reach superiority over sorafenib in the overall population. Stratified subgroup analysis showed an HR of 0.91 (95%CI: 0.72-1.16) in nonviral etiologies when compared to HBV (HR: 0.79; 95%CI: 0.59-1.07) or HCV-associated HCC (HR: 0.72; 95%CI: 0.51-1.02). More recently, it has been shown that chronic inflammation in nonalcoholic fatty liver leads to liver injury and promotes liver cancer, impairing tumor surveillance. A meta-analysis of three randomized-control trials (IMbrave150,

Table 1 Studies reporting the effect and safety of first-line therapies in advanced hepatocellular carcinoma

	IMbrave: Phase III, open-label		REFLECT: Phase III, open-label		CheckMate-459: Phase III, open-label	
	Atezo + Bev	Sorafenib	Lenvatinib	Sorafenib	Nivolumab	Sorafenib
n	336	165	478	476	371	372
Age, median	64	66	63	62	65	65
≥ 65 yr, %	48	55	44	41	NR	NR
Male, %	82	83	85	84	85	85
Asia, %	56	58	70	68	NR	NR
ECOG 1, %	38	38	36	37	27	30
AFP ≥ 200 ng/mL, %	43	45	46	39	39	43
HBV, %	49	46	53	48	31	31
HCV, %	21	22	19	26	23	23
Nonviral, %	30	32	28	26	45	45
MVI trunk			Excluded		Excluded	
MVI, %	38	43	23	19	NR	NR
EH, %	63	56	61	62	NR	NR

AFP: Alpha-fetoprotein; Atezo + Bev: Atezolizumab + bevacizumab; ECOG: Eastern Cooperative Oncology Group; EH: Extrahepatic tumor disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MVI: Macrovascular invasion or neoplastic thrombosis of the main portal trunk; NR: Not reported.

Checkmate-459 and KEYNOTE-240), showed that treatment with ICIs in these patients is associated with reduced survival compared to other etiologies[21].

Although not conclusive, subgroup analysis might offer partial information and generate a hypothesis for future trials. In regard with etiology, there is some molecular background that supports the existence of different molecular activated pathways according to molecular and transcriptomic-based features, which may lead to distinct activation of antitumor immunity or even to ICI resistance[22].

Elderly patients: discrepancy between trials and real-world scenario?

Approximately 70% of patients with cancer are aged 65 or older. The number of patients with cancer in this age group is projected to increase over the next decades significantly[23]. The aging process has been associated with changes in antineoplastic agents' pharmacokinetics due to a number of age-related changes, including modifications in renal and liver function, leading to altered drug absorption, metabolism and distribution.

The mean age of patients with HCC included in clinical trials is around 65-years-old. However, a substantial proportion of HCC patients are older. In HCC and other malignancies, elderly patients are underrepresented in clinical trials. In HCC, exceptionally, the concomitance of advanced age with chronic liver disease raises concerns about toxicity and clinical benefit.

A pooled analysis of both sorafenib pivotal trials (SHARP and Asia-Pacific) did not demonstrate prognostic differences between patients < or ≥ 75 years[20], suggesting that well-selected individuals could derive benefit from systemic treatment irrespective of age.

A concern that elderlies may present a poor tolerance to systemic treatment primes a trend for early discontinuation of sorafenib in field-practice studies[24]. However, in this cohort study (SOFIA Italian study), patients with half dosing sorafenib were associated with improved overall survival and discontinuation with worse outcomes. Consequently, early reduction avoiding definitive treatment discontinuation should be mandatory[24]. On the other hand, other studies did not show significant differences in overall survival and class-specific adverse events with lenvatinib in older patients [25]. A subanalysis of the IMBRAVE150 trial evaluating patients aged < or ≥ 65 years showed a similar toxicity profile, patient-reported outcomes and survival outcomes [26].

Most available data come from retrospective studies and subanalysis of prospective trials, mainly with sorafenib. Most of these data support that age alone should not

restrict treatment in advanced HCC, but a multidisciplinary approach and frailty metrics, apart from Eastern Cooperative Oncology Group grades, can be helpful in managing this group.

The limits of liver function: Is it unquestionable?

Cirrhosis and its complications (ascites among others) are the most significant competing risk for mortality in patients with HCC. In fact, prior evaluation of liver function, liver decompensation (prior history of ascites and its complications) or clinically significant portal hypertension are mandatory before systemic therapy initiation or selection (*e.g.*, presence of gastric or esophageal varices, other abdominal collaterals, enlarged spleen more than 120 mm, low platelet count < 150000 mm^3, among others). Due to this fact, clinical trials specifically selected those populations in which HCC determines the risk of mortality so that the antitumor treatment effect is more likely to be captured without distortion by cirrhosis-imposed threats. The majority of trials strictly included patients with preserved liver function, Child-Pugh A, or without liver decompensation events. It results in the lack of robust data showing how to manage patients with advanced HCC and impaired liver function. On the other hand, liver decompensation during systemic therapy leads to a significant impact on overall survival and an exclusion of sequencing systemic options[27,28].

The GIDEON study[29], the most extensive real-world data including patients treated with sorafenib, demonstrated that the median survival of patients with unpreserved liver function or Child-Pugh B and C was 5.2 mo and 2.6 mo, respectively. On the one hand, this result shows almost futility and discourages systemic agent use in patients with very poor liver function (Child-Pugh C) due to lack of treatment benefit. On the other hand, Child-Pugh B patient data suggests that well-selected patients can be considered for treatment, although more robust data is lacking. It seems that the presence of clinically significant ascites is a mandatory exclusion criterion. For example, in patients treated with sorafenib, those with a Child-Pugh B7 score without ascites presented similar outcomes than Child-Pugh A6[30]. Another retrospective study showed poor survival in patients with Child-Pugh B treated with lenvatinib[31].

Some authors recommend against grading ascites due to its subjective assessment, showing that the albumin-bilirubin score may be an alternative tool to evaluate prognosis in candidates for systemic treatment[32]. However, events of liver decompensation, such as ascites, jaundice or encephalopathy, have been associated with a significant worse prognosis and should always be part of eligibility criteria in trials and in the real-world setting.

Safety and efficacy in patients with liver dysfunction should not be extrapolated to all tyrosine kinase inhibitors (TKIs). The GIDEON cohort study showed an increasing incidence rate of serious adverse events from Child Pugh A to B or C, with a rising rate of sorafenib discontinuation[29]. Moreover, almost 20% of the patients may experience clinical deterioration due to liver impairment with the treatment with TKIs, particularly during the first 4 wk of therapy[6,24,33]. Nevertheless, in the second-line setting, patients allocated to cabozantinib in the CELESTIAL trial who presented worsening in liver function by week 8 had a manageable safety profile and maintained treatment benefit compared to the total cohort[7].

Whether these data could be extrapolated to ICIs is a matter of debate. Nivolumab was tested in a prospective Child-Pugh B cohort (75% of Child-Pugh B7)[11]. The median overall survival was 7.6 mo, with a disease control rate of 55.1%. Although safety profile may be more favorable, there is a paucity of data on other immune-oncology drugs in the setting of liver dysfunction.

The safety of combined therapies, including ICIs and VEGF targeted pathways (TKIs or anti-VEGF), in patients with unpreserved liver function is a matter to be clarified in prospective real-world data. Tyrosine kinase inhibitors and immunotherapy seems to be feasible in patients with a mild liver alteration. A close follow-up and multidisciplinary management are paramount to secure safety and better outcomes.

Recurrent HCC after liver transplant: An orphan situation in clinical trials

Liver transplantation has been an exclusion criterion in all clinical trials enrolling patients with advanced HCC, TKIs or ICIs. Safety concerns and overall survival in immunosuppressed patients has been one of the main explanations of this exclusion criteria.

However, the use of TKIs has been reported in retrospective cohort studies with acceptable results. Sorafenib was shown to be safe and effective, with a median overall survival of 20.1 mo[34]. The toxicity profile and the risk for liver graft deterioration

have been reported to be similar and lower than patients with no history of transplantation, respectively[35]. Favorable outcomes were observed in a multicenter retrospective study exploring the sorafenib-regorafenib sequencing therapy in the post-transplant setting. The median survival was 12.9 mo (95%CI: 6.7-19.1) since regorafenib initiation and 38.4 mo (95%CI: 18.5-58.4) since sorafenib discontinuation [36]. Other studies have already reported outcomes with lenvatinib in the post-transplant setting.

The risk of allograft rejection with ICI therapy precludes these patients from being treated with ICIs, either monotherapy or in combination with TKIs or anti-VEGF[37]. Therefore, sequencing TKIs is the optimal approach for patients with tumor recurrence after liver transplantation not amenable to local treatment.

Risk of bleeding events associated with systemic treatments

Patients with HCC and coexisting cirrhosis have an increased risk of bleeding events due to portal hypertension. However, the risk of spontaneous bleeding in other organs is rare, and these patients are paradoxically at a higher risk of thrombotic events[38, 39]. The risk of bleeding goes in parallel with the presence and severity of portal hypertension. In patients without prior endoscopy, at least during the last 6 mo, the risk of variceal hemorrhage should be assessed before systemic therapy, particularly with bevacizumab. Primary or secondary prophylaxis of variceal hemorrhage should be implemented according to International Consensus guidelines (*e.g.*, BAVENO VI), either with beta-blockers or endoscopic variceal banding or both for secondary prophylaxis. In some patients without any surrogate marker of clinically significant portal hypertension (*e.g.*, presence of enlarged spleen more than 120 mm, low platelet count < 150000 mm^3 or other abdominal collaterals), upper endoscopy may be replaced by transient elastography as a first or additional approach to rule-out gastroesophageal varices.

Bleeding can occur either due to variceal cause or spontaneous tumor rupture, both dramatic events associated with dismal outcome in patients with advanced HCC. In fact, HCC leads to an increase in portal hypertension, and consequently the risk of bleeding should be reassessed in these patients. Drugs with antiangiogenic activity, TKIs or anti-VEGF are associated with an increased risk of bleeding that usually does not require significant interventions. In pivotal trials, sorafenib was associated with a low risk of severe bleeding events (7% any grade, 1% grade 3) as well as ramucirumab (1% of grade 3-4)[2,8].

On the contrary, the IMBRAVE150 trial did not include patients with untreated or incompletely treated esophageal or gastric varices (according to local clinical practice, either beta-blockers or endoscopic procedures)[13]. This concern was based on the risk of tumor-associated hemorrhage with bevacizumab (3%-5%), with reported fatal bleeding cases in earlier trials[40]. Despite the exclusion of high-risk patients and a well-balanced risk of bleeding (26% of each group had varices), there was a 25.2% rate of any grade bleeding events in the atezolizumab-bevacizumab arm, and fatal bleeding events occurred in 6 patients in the IMBRAVE150 trial (1.8%). Specifically, variceal bleeding occurred more frequently in the atezolizumab plus bevacizumab arm *vs* sorafenib (7% *vs* 4.5%)[13].

The risk of bleeding should be extensively assessed in systemic treatment candidates, and a careful follow-up should be carried out in the real-world setting. Particular attention is required for those patients considered for atezolizumab and bevacizumab, patients using anticoagulants and those with a recent history or higher risk of variceal bleeding (*e.g.*, esophageal or gastric varices with red spots).

Common comorbidities and other conditions excluded in HCC trials

The classes of agents used for treating advanced HCC have particular prescribing concerns due to their mechanism of action. TKIs with antiangiogenic properties may increase the risk of cardiovascular disease and ischemic events. Consequently, patients with risk for cardiovascular events, such as diabetes or prior cardiovascular complications, are underrepresented in clinical trials, although they were not entirely excluded from enrollment. The challenge in such situation relies on the proper management of risk factors. ICIs, on the other hand, carried a low risk of cardiovascular events.

Drug interaction is a crucial topic, particularly with antiretroviral therapy for HIV. Patients with HIV are not included in clinical trials, but a real-world study showed that sorafenib does not impact viral load and CD4-T cell count[41]. Data with immunotherapy for HIV-positive patients lack as they were excluded from pivotal trials with ICIs.

Patients under supportive renal care or hemodialysis have been excluded from clinical trials, and more recent real-world data has been reported with sorafenib treatment[42]. Finally, ICIs may exacerbate autoimmune disorders. Some of these disorders are associated with an increased risk of HCC, such as autoimmune hepatitis.

Exacerbation of immune disorders and immune-related adverse events may occur in up to 75% of the cases. In this regard, ICIs should be used with caution in this population[43]. Many of these events can be managed without discontinuing therapy, but further data are required. Also, there is a deep concern with extrapolating the management of these adverse events in patients with cirrhosis. Most clinical guideline recommendations are based on non-cirrhotic patients[43]. Although immune-related events should be promptly recognized and adequately treated, the use of high steroid doses should be cautiously implemented in cirrhosis[44]. It is already known that the use of steroids may accelerate or result in liver decompensation (*e.g.*, ascites development, among other events).

Sequencing therapies beyond clinical trials

In the second-line setting, all effective options were explored after sorafenib, either intolerance or tumor progression. There is no comparative study that evaluated how second-line drugs perform after lenvatinib or atezolizumab plus bevacizumab. Regorafenib was superior to placebo in sorafenib-tolerant patients[6], ramucirumab was effective in patients with high alpha-fetoprotein (AFP) levels[8], and cabozantinib showed better survival in second or third-lines over placebo[7]. In addition, the combination of nivolumab-ipilimumab (a dual ICI combination) was granted approval after sorafenib based on an encouraging phase II trial[45].

Although more recent retrospective studies have compared nivolumab *vs* regorafenib efficacy, all second-line competitors have not been compared face-to-face in clinical trials[46]. Head-to-head comparisons between all these options are unlikely to be addressed in future trials, so sequencing strategies will be an unmet knowledge requiring real-world data outside clinical trials. Some assumptions are reasonable to be considered when choosing the best strategy (Figure 2).

The selection based on the safety profile is crucial. For example, risk of bleeding, cardiovascular events or immune-related adverse events may impact negatively if not correctly assessed. Survival is the primary objective, but patients with tumor-related symptoms may also benefit from therapies with a higher response rate, such as lenvatinib or atezolizumab plus bevacizumab. Special subgroups not included in trials may be more safely managed based on real-world data showing favorable results. For example, this is the case of sorafenib-regorafenib therapy in transplanted patients.

Alternating treatments with different mechanisms of action instead of using sequences of drugs directed to the same target is a reasonable strategy, although not evidenced-based in clinical trials, particularly for third or even fourth-line therapies. For example, after progression on immunotherapy-based therapy, a TKI is more likely to be effective and vice versa. This issue will be a major discussion when novel therapies are incorporated following the results of ongoing clinical trials.

There is still an unmet need in HCC. The use of biomarkers for treatment selection, except high AFP levels for ramucirumab therapy, is lacking. Even the expression of programmed death ligand 1 in tumor tissue has not been associated with a predictive response. While the neutrophil-lymphocyte ratio has already been associated with better response with sorafenib[20] and lenvatinib[47], other biomarkers in other settings have been extensively explored without clinical implication[48,49].

CONCLUSION

The strict criteria used in clinical trials in advanced HCC have led to a scarcity of available data in a considerable proportion of patients in real-world practice. The daily challenge of treating these underrepresented subgroups can be overcome by future clinical trials addressing special situations, collaborative studies and real-world data [50]. A critical view of study design is essential to avoid excessive extrapolation and not limit efforts to provide better care to some subgroups that are not widely included in clinical research.

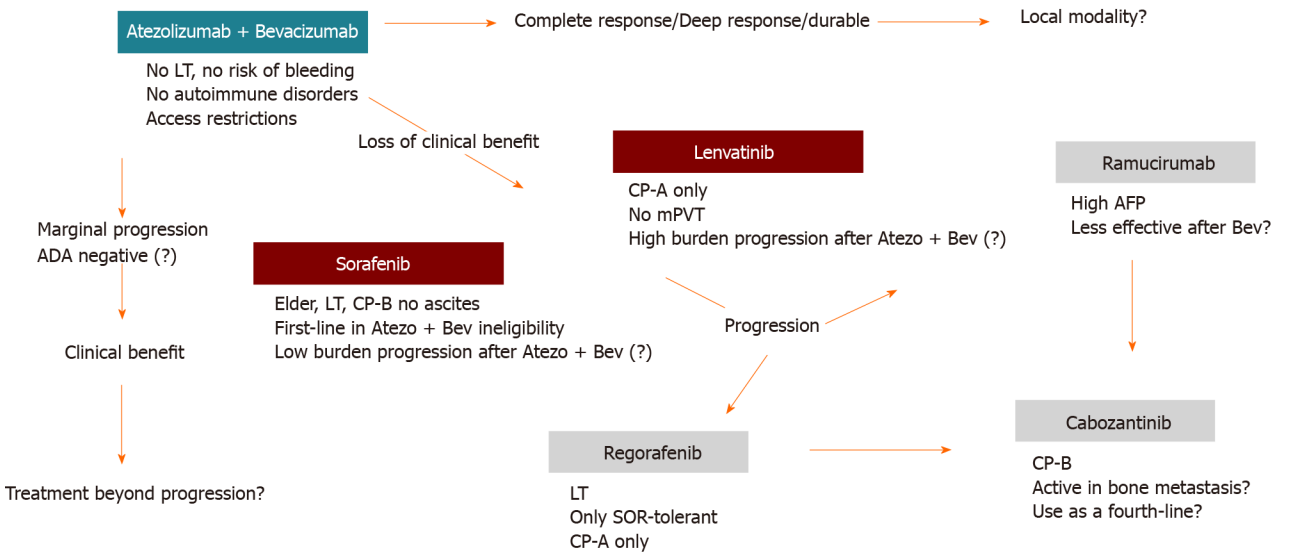


Figure 2 Sequencing systemic therapies in real-world setting. Note: These recommendations should be individualized for each patient. ADA: Anti-drug antibodies; AFP: Alpha-fetoprotein; Atezo + Bev: Atezolizumab + bevacizumab; CP: Child-Pugh; LT: Liver transplantation; mPVT: Main portal vein thrombosis; SOR: Sorafenib.

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REFERENCES

1 **Llovet JM**, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-338 [PMID: 10518312 DOI: 10.1055/s-2007-1007122]

2 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

3 **Cheng AL**, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25-34 [PMID: 19095497 DOI: 10.1016/S1470-2045(08)70285-7]

4 **Bruix J**, da Fonseca LG, Reig M. Insights into the success and failure of systemic therapy for hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 617-630 [PMID: 31371809 DOI: 10.1038/s41575-019-0179-x]

5 **Kudo M**, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, Baron A, Park JW, Han G, Jassem J, Blanc JF, Vogel A, Komov D, Evans TRJ, Lopez C, Dutcus C, Guo M, Saito K, Kraljevic S, Tamai T, Ren M, Cheng AL. Lenvatinib *vs* sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 2018; **391**: 1163-1173 [PMID: 29433850 DOI: 10.1016/S0140-6736(18)30207-1]

6 **Bruix J**, Qin S, Merle P, Granito A, Huang YH, Bodoky G, Pracht M, Yokosuka O, Rosmorduc O, Breder V, Gerolami R, Masi G, Ross PJ, Song T, Bronowicki JP, Ollivier-Hourmand I, Kudo M, Cheng AL, Llovet JM, Finn RS, LeBerre MA, Baumhauer A, Meinhardt G, Han G; RESORCE Investigators. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **389**: 56-66 [PMID: 27932229 DOI: 10.1016/S0140-6736(16)32453-9]

7 **Abou-Alfa GK**, Meyer T, Cheng AL, El-Khoueiry AB, Rimassa L, Ryoo BY, Cicin I, Merle P, Chen Y, Park JW, Blanc JF, Bolondi L, Klümpen HJ, Chan SL, Zagonel V, Pressiani T, Ryu MH, Venook AP, Hessel C, Borgman-Hagey AE, Schwab G, Kelley RK. Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. *N Engl J Med* 2018; **379**: 54-63 [PMID: 29972759 DOI: 10.1056/NEJMoa1717002]

8 **Zhu AX**, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, Assenat E, Brandi G, Pracht M, Lim HY, Rau KM, Motomura K, Ohno I, Merle P, Daniele B, Shin DB, Gerken G, Borg C, Hiriart JB, Okusaka T, Morimoto M, Hsu Y, Abada PB, Kudo M; REACH-2 study investigators. Ramucirumab after

sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2019; **20**: 282-296 [PMID: 30665869 DOI: 10.1016/S1470-2045(18)30937-9]

9 **Finn RS**, Merle P, Granito A, Huang YH, Bodoky G, Pracht M, Yokosuka O, Rosmorduc O, Gerolami R, Caparello C, Cabrera R, Chang C, Sun W, LeBerre MA, Baumhauer A, Meinhardt G, Bruix J. Outcomes of sequential treatment with sorafenib followed by regorafenib for HCC: Additional analyses from the phase III RESORCE trial. *J Hepatol* 2018; **69**: 353-358 [PMID: 29704513 DOI: 10.1016/j.jhep.2018.04.010]

10 **Alsina A**, Kudo M, Vogel A, Cheng AL, Tak WY, Ryoo BY, Evans TRJ, López López C, Daniele B, Misir S, Ren M, Izumi N, Qin S, Finn RS. Effects of Subsequent Systemic Anticancer Medication Following First-Line Lenvatinib: A Post Hoc Responder Analysis from the Phase 3 REFLECT Study in Unresectable Hepatocellular Carcinoma. *Liver Cancer* 2020; **9**: 93-104 [PMID: 32071913 DOI: 10.1159/000504624]

11 **El-Khoueiry AB**, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]

12 **Zhu AX**, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, Verslype C, Zagonel V, Fartoux L, Vogel A, Sarker D, Verset G, Chan SL, Knox J, Daniele B, Webber AL, Ebbinghaus SW, Ma J, Siegel AB, Cheng AL, Kudo M; KEYNOTE-224 investigators. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. *Lancet Oncol* 2018; **19**: 940-952 [PMID: 29875066 DOI: 10.1016/S1470-2045(18)30351-6]

13 **Finn RS**, Qin S, Ikeda M, Galle PR, Ducreux M, Kim TY, Kudo M, Breder V, Merle P, Kaseb AO, Li D, Verret W, Xu DZ, Hernandez S, Liu J, Huang C, Mulla S, Wang Y, Lim HY, Zhu AX, Cheng AL; IMbrave150 Investigators. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *N Engl J Med* 2020; **382**: 1894-1905 [PMID: 32402160 DOI: 10.1056/NEJMoa1915745]

14 **Kim D**, Li AA, Perumpail BJ, Gadiparthi C, Kim W, Cholankeril G, Glenn JS, Harrison SA, Younossi ZM, Ahmed A. Changing Trends in Etiology-Based and Ethnicity-Based Annual Mortality Rates of Cirrhosis and Hepatocellular Carcinoma in the United States. *Hepatology* 2019; **69**: 1064-1074 [PMID: 30014489 DOI: 10.1002/hep.30161]

15 **Fassio E**, Díaz S, Santa C, Reig ME, Martínez Artola Y, Alves de Mattos A, Míguez C, Galizzi J, Zapata R, Ridruejo E, de Souza FC, Hernández N, Pinchuk L; Multicenter Group for Study of Hepatocarcinoma in Latin America; Asociación Latinoamericana para el Estudio del Hígado (ALEH). Etiology of hepatocellular carcinoma in Latin America: a prospective, multicenter, international study. *Ann Hepatol* 2010; **9**: 63-69 [PMID: 20332549]

16 **Carrilho FJ**, Paranaguá-Vezozzo DC, Chagas AL, Alencar RSSM, da Fonseca LG. Epidemiology of Liver Cancer in Latin America: Current and Future Trends. *Semin Liver Dis* 2020; **40**: 101-110 [PMID: 31711252 DOI: 10.1055/s-0039-3399561]

17 **Piñero F**, Costa P, Boteon YL, Duque SH, Marciano S, Anders M, Varón A, Zerega A, Poniachik J, Soza A, Padilla Machaca M, Menéndez J, Zapata R, Vilatoba M, Muñoz L, Maraschio M, Podestá LG, McCormack L, Gadano A, Boin ISFF, García P, Silva M; Latin American Liver Research, Education, Awareness Network (LALREAN). A changing etiologic scenario in liver transplantation for hepatocellular carcinoma in a multicenter cohort study from Latin America. *Clin Res Hepatol Gastroenterol* 2018; **42**: 443-452 [PMID: 29773419 DOI: 10.1016/j.clinre.2018.03.014]

18 **Piñero F**, Pages J, Marciano S, Fernández N, Silva J, Anders M, Zerega A, Ridruejo E, Ameigeiras B, D'Amico C, Gaite L, Bermúdez C, Cobos M, Rosales C, Romero G, McCormack L, Reggiardo V, Colombato L, Gadano A, Silva M. Fatty liver disease, an emerging etiology of hepatocellular carcinoma in Argentina. *World J Hepatol* 2018; **10**: 41-50 [PMID: 29399277 DOI: 10.4254/wjh.v10.i1.41]

19 **Eslam M**, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-Gomez M, Zelber-Sagi S, Wai-Sun Wong V, Dufour JF, Schattenberg JM, Kawaguchi T, Arrese M, Valenti L, Shiha G, Tiribelli C, Yki-Järvinen H, Fan JG, Grønbæk H, Yilmaz Y, Cortez-Pinto H, Oliveira CP, Bedossa P, Adams LA, Zheng MH, Fouad Y, Chan WK, Mendez-Sanchez N, Ahn SH, Castera L, Bugianesi E, Ratziu V, George J. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *J Hepatol* 2020; **73**: 202-209 [PMID: 32278004 DOI: 10.1016/j.jhep.2020.03.039]

20 **Bruix J**, Cheng AL, Meinhardt G, Nakajima K, De Sanctis Y, Llovet J. Prognostic factors and predictors of sorafenib benefit in patients with hepatocellular carcinoma: Analysis of two phase III studies. *J Hepatol* 2017; **67**: 999-1008 [PMID: 28687477 DOI: 10.1016/j.jhep.2017.06.026]

21 **Pfister D**, Núñez NG, Pinyol R, Govaere O, Pinter M, Szydlowska M, Gupta R, Qiu M, Deczkowska A, Weiner A, Müller F, Sinha A, Friebel E, Engleitner T, Lenggenhager D, Moncsek A, Heide D, Stirm K, Kosla J, Kotsiliti E, Leone V, Dudek M, Yousuf S, Inverso D, Singh I, Teijeiro A, Castet F, Montironi C, Haber PK, Tiniakos D, Bedossa P, Cockell S, Younes R, Vacca M, Marra F, Schattenberg JM, Allison M, Bugianesi E, Ratziu V, Pressiani T, D'Alessio A, Personeni N, Rimassa L, Daly AK, Scheiner B, Pomej K, Kirstein MM, Vogel A, Peck-Radosavljevic M, Hucke F, Finkelmeier F, Waidmann O, Trojan J, Schulze K, Wege H, Koch S, Weinmann A, Bueter M, Rössler F, Siebenhüner A, De Dosso S, Mallm JP, Umansky V, Jugold M, Luedde T, Schietinger A,

Schirmacher P, Emu B, Augustin HG, Billeter A, Müller-Stich B, Kikuchi H, Duda DG, Kütting F, Waldschmidt DT, Ebert MP, Rahbari N, Mei HE, Schulz AR, Ringelhan M, Malek N, Spahn S, Bitzer M, Ruiz de Galarreta M, Lujambio A, Dufour JF, Marron TU, Kaseb A, Kudo M, Huang YH, Djouder N, Wolter K, Zender L, Marche PN, Decaens T, Pinato DJ, Rad R, Mertens JC, Weber A, Unger K, Meissner F, Roth S, Jilkova ZM, Claassen M, Anstee QM, Amit I, Knolle P, Becher B, Llovet JM, Heikenwalder M. NASH limits anti-tumour surveillance in immunotherapy-treated HCC. *Nature* 2021; **592**: 450-456 [PMID: 33762733 DOI: 10.1038/s41586-021-03362-0]

22 **Kalbasi A**, Ribas A. Tumour-intrinsic resistance to immune checkpoint blockade. *Nat Rev Immunol* 2020; **20**: 25-39 [PMID: 31570880 DOI: 10.1038/s41577-019-0218-4]

23 **Smith BD**, Smith GL, Hurria A, Hortobagyi GN, Buchholz TA. Future of cancer incidence in the United States: burdens upon an aging, changing nation. *J Clin Oncol* 2009; **27**: 2758-2765 [PMID: 19403886 DOI: 10.1200/JCO.2008.20.8983]

24 **Iavarone M**, Cabibbo G, Piscaglia F, Zavaglia C, Grieco A, Villa E, Cammà C, Colombo M; SOFIA (SOraFenib Italian Assessment) study group. Field-practice study of sorafenib therapy for hepatocellular carcinoma: a prospective multicenter study in Italy. *Hepatology* 2011; **54**: 2055-2063 [PMID: 21898496 DOI: 10.1002/hep.24644]

25 **Tada T**, Kumada T, Hiraoka A, Michitaka K, Atsukawa M, Hirooka M, Tsuji K, Ishikawa T, Takaguchi K, Kariyama K, Itobayashi E, Tajiri K, Shimada N, Shibata H, Ochi H, Toyoda H, Nouso K, Tsutsui A, Nagano T, Itokawa N, Hayama K, Imai M, Joko K, Koizumi Y, Hiasa Y. Safety and efficacy of lenvatinib in elderly patients with unresectable hepatocellular carcinoma: A multicenter analysis with propensity score matching. *Hepatol Res* 2020; **50**: 75-83 [PMID: 31660700 DOI: 10.1111/hepr.13427]

26 **Li D**, Toh H, Merle P, Tsuchiya K, Hernandez S, Shao H, Mulla S, Ding B, Kudo M. Atezolizumab + bevacizumab *vs* sorafenib for unresectable hepatocellular carcinoma: Results from older adults enrolled in IMbrave150. *Ann Oncol* 2020; **31**: 234 [DOI: 10.1016/j.annonc.2020.04.061]

27 **Iavarone M**, Cabibbo G, Biolato M, Della Corte C, Maida M, Barbara M, Basso M, Vavassori S, Craxì A, Grieco A, Cammà C, Colombo M. Predictors of survival in patients with advanced hepatocellular carcinoma who permanently discontinued sorafenib. *Hepatology* 2015; **62**: 784-791 [PMID: 25645399 DOI: 10.1002/hep.27729]

28 **Piñero F**, Marciano S, Fernández N, Silva J, Anders M, Zerega A, Ridruejo E, Romero G, Ameigeiras B, D'Amico C, Gaite L, Bermúdez C, Reggiardo V, Colombato L, Gadano A, Silva M. Intermediate-advanced hepatocellular carcinoma in Argentina: Treatment and survival analysis. *World J Gastroenterol* 2019; **25**: 3607-3618 [PMID: 31367160 DOI: 10.3748/wjg.v25.i27.3607]

29 **Marrero JA**, Kudo M, Venook AP, Ye SL, Bronowicki JP, Chen XP, Dagher L, Furuse J, Geschwind JH, de Guevara LL, Papandreou C, Takayama T, Sanyal AJ, Yoon SK, Nakajima K, Lehr R, Heldner S, Lencioni R. Observational registry of sorafenib use in clinical practice across Child-Pugh subgroups: The GIDEON study. *J Hepatol* 2016; **65**: 1140-1147 [PMID: 27469901 DOI: 10.1016/j.jhep.2016.07.020]

30 **Kim HY**, Park JW, Joo J, Kim H, Woo SM, Lee WJ, Kim CM. Worse outcome of sorafenib therapy associated with ascites and Child-Pugh score in advanced hepatocellular carcinoma. *J Gastroenterol Hepatol* 2013; **28**: 1756-1761 [PMID: 23800278 DOI: 10.1111/jgh.12310]

31 **Hiraoka A**, Kumada T, Kariyama K, Takaguchi K, Atsukawa M, Itobayashi E, Tsuji K, Tajiri K, Hirooka M, Shimada N, Shibata H, Ishikawa T, Ochi H, Tada T, Toyoda H, Nouso K, Tsutsui A, Itokawa N, Imai M, Joko K, Hiasa Y, Michitaka K; Real-life Practice Experts for HCC (RELPEC) Study Group; HCC 48 Group (hepatocellular carcinoma experts from 48 clinics in Japan). Clinical features of lenvatinib for unresectable hepatocellular carcinoma in real-world conditions: Multicenter analysis. *Cancer Med* 2019; **8**: 137-146 [PMID: 30575325 DOI: 10.1002/cam4.1909]

32 **Johnson PJ**, Berhane S, Kagebayashi C, Satomura S, Teng M, Reeves HL, O'Beirne J, Fox R, Skowronska A, Palmer D, Yeo W, Mo F, Lai P, Iñarrairaegui M, Chan SL, Sangro B, Miksad R, Tada T, Kumada T, Toyoda H. Assessment of liver function in patients with hepatocellular carcinoma: a new evidence-based approach-the ALBI grade. *J Clin Oncol* 2015; **33**: 550-558 [PMID: 25512453 DOI: 10.1200/JCO.2014.57.9151]

33 **Hiraoka A**, Kumada T, Atsukawa M, Hirooka M, Tsuji K, Ishikawa T, Takaguchi K, Kariyama K, Itobayashi E, Tajiri K, Shimada N, Shibata H, Ochi H, Tada T, Toyoda H, Nouso K, Tsutsui A, Nagano T, Itokawa N, Hayama K, Imai M, Joko K, Koizumi Y, Hiasa Y, Michitaka K; On behalf of the Real-Life Practice Experts for HCC (RELPEC) Study Group and HCC 48 Group (hepatocellular carcinoma experts from 48 clinics in Japan). Early Relative Change in Hepatic Function with Lenvatinib for Unresectable Hepatocellular Carcinoma. *Oncology* 2019; **97**: 334-340 [PMID: 31466068 DOI: 10.1159/000502095]

34 **Sposito C**, Mariani L, Germini A, Flores Reyes M, Bongini M, Grossi G, Bhoori S, Mazzaferro V. Comparative efficacy of sorafenib *vs* best supportive care in recurrent hepatocellular carcinoma after liver transplantation: a case-control study. *J Hepatol* 2013; **59**: 59-66 [PMID: 23500153 DOI: 10.1016/j.jhep.2013.02.026]

35 **Weinmann A**, Niederle IM, Koch S, Hoppe-Lotichius M, Heise M, Düber C, Schuchmann M, Otto G, Galle PR, Wörns MA. Sorafenib for recurrence of hepatocellular carcinoma after liver transplantation. *Dig Liver Dis* 2012; **44**: 432-437 [PMID: 22265328 DOI: 10.1016/j.dld.2011.12.009]

36 **Iavarone M**, Invernizzi F, Czauderna C, Sanduzzi-Zamparelli M, Bhoori S, Amaddeo G, Manini MA, López MF, Anders M, Pinter M, Rodríguez MJB, Cristóbal MR, Soteras GA, Piñero F, Villadsen GE, Weinmann A, Crespo G, Mazzaferro V, Regnault H, Giorgio M, González-Diéguez

ML, Donato MF, Varela M, Wörns MA, Bruix J, Lampertico P, Reig M. Preliminary experience on safety of regorafenib after sorafenib failure in recurrent hepatocellular carcinoma after liver transplantation. *Am J Transplant* 2019; **19**: 3176-3184 [PMID: 31365177 DOI: 10.1111/ajt.15551]

37 **Aguirre LE**, Guzman ME, Lopes G, Hurley J. Immune Checkpoint Inhibitors and the Risk of Allograft Rejection: A Comprehensive Analysis on an Emerging Issue. *Oncologist* 2019; **24**: 394-401 [PMID: 30413665 DOI: 10.1634/theoncologist.2018-0195]

38 **Tripodi A**, Chantarangkul V, Primignani M, Fabris F, Dell'Era A, Sei C, Mannucci PM. The international normalized ratio calibrated for cirrhosis (INR(liver)) normalizes prothrombin time results for model for end-stage liver disease calculation. *Hepatology* 2007; **46**: 520-527 [PMID: 17659574 DOI: 10.1002/hep.21732]

39 **Tripodi A**, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med* 2011; **365**: 147-156 [PMID: 21751907 DOI: 10.1056/NEJMra1011170]

40 **Siegel AB**, Cohen EI, Ocean A, Lehrer D, Goldenberg A, Knox JJ, Chen H, Clark-Garvey S, Weinberg A, Mandeli J, Christos P, Mazumdar M, Popa E, Brown RS Jr, Rafii S, Schwartz JD. Phase II trial evaluating the clinical and biologic effects of bevacizumab in unresectable hepatocellular carcinoma. *J Clin Oncol* 2008; **26**: 2992-2998 [PMID: 18565886 DOI: 10.1200/JCO.2007.15.9947]

41 **Merchante N**, Ibarra S, Revollo B, Rodríguez-Arrondo F, Merino E, Delgado-Fernández M, Montero-Alonso M, Téllez F, Galindo MJ, Rivero-Juárez A, García MA, Mínguez C, Romero-Palacios A, Garcia-Deltoro M, Pineda JA; GEHEP-002 Study Group. Real-life experience with sorafenib for the treatment of hepatocellular carcinoma in HIV-infected patients. *AIDS* 2017; **31**: 89-95 [PMID: 27755109 DOI: 10.1097/QAD.0000000000001293]

42 **Díaz-González Á**, Sanduzzi-Zamparelli M, da Fonseca LG, Di Costanzo GG, Alves R, Iavarone M, Leal C, Sacco R, Matilla AM, Hernández-Guerra M, Aballay Soteras G, Wörns MA, Pinter M, Varela M, Ladekarl M, Chagas AL, Mínguez B, Arenas JI, Granito A, Sánchez-Torrijos Y, Rojas Á, Rodríguez de Lope C, Alvares-da-Silva MR, Pascual S, Rimassa L, Lledó JL, Huertas C, Sangro B, Giannini EG, Delgado M, Vergara M, Perelló C, Lue A, Sala M, Gallego A, Coll S, Hernáez T, Piñero F, Pereira G, França A, Marín J, Anders M, Mello V, Lozano M, Nault JC, Menéndez J, García Juárez I, Bruix J, Reig M. International and multicenter real-world study of sorafenib-treated patients with hepatocellular carcinoma under dialysis. *Liver Int* 2020; **40**: 1467-1476 [PMID: 32170821 DOI: 10.1111/liv.14436]

43 **Brahmer JR**, Lacchetti C, Schneider BJ, Atkins MB, Brassil KJ, Caterino JM, Chau I, Ernstoff MS, Gardner JM, Ginex P, Hallmeyer S, Holter Chakrabarty J, Leighl NB, Mammen JS, McDermott DF, Naing A, Nastoupil LJ, Phillips T, Porter LD, Puzanov I, Reichner CA, Santomasso BD, Seigel C, Spira A, Suarez-Almazor ME, Wang Y, Weber JS, Wolchok JD, Thompson JA; National Comprehensive Cancer Network. Management of Immune-Related Adverse Events in Patients Treated With Immune Checkpoint Inhibitor Therapy: American Society of Clinical Oncology Clinical Practice Guideline. *J Clin Oncol* 2018; **36**: 1714-1768 [PMID: 29442540 DOI: 10.1200/JCO.2017.77.6385]

44 **Sangro B**, Chan SL, Meyer T, Reig M, El-Khoueiry A, Galle PR. Diagnosis and management of toxicities of immune checkpoint inhibitors in hepatocellular carcinoma. *J Hepatol* 2020; **72**: 320-341 [PMID: 31954495 DOI: 10.1016/j.jhep.2019.10.021]

45 **Yau T**, Kang YK, Kim TY, El-Khoueiry AB, Santoro A, Sangro B, Melero I, Kudo M, Hou MM, Matilla A, Tovoli F, Knox JJ, Ruth He A, El-Rayes BF, Acosta-Rivera M, Lim HY, Neely J, Shen Y, Wisniewski T, Anderson J, Hsu C. Efficacy and Safety of Nivolumab Plus Ipilimumab in Patients With Advanced Hepatocellular Carcinoma Previously Treated With Sorafenib: The CheckMate 040 Randomized Clinical Trial. *JAMA Oncol* 2020; **6**: e204564 [PMID: 33001135 DOI: 10.1001/jamaoncol.2020.4564]

46 **Choi WM**, Choi J, Lee D, Shim JH, Lim YS, Lee HC, Chung YH, Lee YS, Park SR, Ryu MH, Ryoo BY, Lee SJ, Kim KM. Regorafenib Versus Nivolumab After Sorafenib Failure: Real-World Data in Patients With Hepatocellular Carcinoma. *Hepatol Commun* 2020; **4**: 1073-1086 [PMID: 32626838 DOI: 10.1002/hep4.1523]

47 **Tada T**, Kumada T, Hiraoka A, Michitaka K, Atsukawa M, Hirooka M, Tsuji K, Ishikawa T, Takaguchi K, Kariyama K, Itobayashi E, Tajiri K, Shimada N, Shibata H, Ochi H, Yasuda S, Toyoda H, Fukunishi S, Ohama H, Kawata K, Nakamura S, Nouso K, Tsutsui A, Nagano T, Itokawa N, Hayama K, Arai T, Imai M, Joko K, Koizumi Y, Hiasa Y. Neutrophil-to-lymphocyte ratio is associated with survival in patients with unresectable hepatocellular carcinoma treated with lenvatinib. *Liver Int* 2020; **40**: 968-976 [PMID: 32064740 DOI: 10.1111/liv.14405]

48 **Teufel M**, Seidel H, Köchert K, Meinhardt G, Finn RS, Llovet JM, Bruix J. Biomarkers Associated With Response to Regorafenib in Patients With Hepatocellular Carcinoma. *Gastroenterology* 2019; **156**: 1731-1741 [PMID: 30738047 DOI: 10.1053/j.gastro.2019.01.261]

49 **Finn RS**, Kudo M, Cheng A-L, Wyrwicz L, Ngan R, Blanc J-F, Baron AD, Vogel A, Ikeda M, Piscaglia F, Han K-H, Qin S, Minoshima Y, Funahashi Y, Ren M, Dairiki R, Sachdev P, Tamai T, Dutcus C, Evans TRJ. Analysis of serum biomarkers (BM) in patients from a phase 3 study of lenvatinib (LEN) *vs* sorafenib (SOR) as first-line treatment for unresectable hepatocellular carcinoma. *Ann Oncol* 2017; **28**: V617 [DOI: 10.1093/annonc/mdx440]

50 **Mendizabal M**, Silva MO. Developing multicenter consortia in liver disease in Latin America: Challenges and opportunities. *Liver Transpl* 2017; **23**: 1210-1215 [PMID: 28590520 DOI: 10.1002/lt.24793]

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REVIEW

Chronic intestinal failure and short bowel syndrome in Crohn's disease

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Abstract

Chronic intestinal failure (CIF) is a rare but feared complication of Crohn's disease. Depending on the remaining length of the small intestine, the affected intestinal segment, and the residual bowel function, CIF can result in a wide spectrum of symptoms, from single micronutrient malabsorption to complete intestinal failure. Management of CIF has improved significantly in recent years. Advances in home-based parenteral nutrition, in particular, have translated into increased survival and improved quality of life. Nevertheless, 60% of patients are permanently reliant on parenteral nutrition. Encouraging results with new drugs such as teduglutide have added a new dimension to CIF therapy. The outcomes of patients with CIF could be greatly improved by more effective prevention, understanding, and treatment. In complex cases, the care of patients with CIF requires a multidisciplinary approach involving not only physicians but also dietitians and nurses to provide optimal intestinal rehabilitation, nutritional support, and an improved quality of life. Here, we summarize current literature

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on CIF and short bowel syndrome, encompassing epidemiology, pathophysiology, and advances in surgical and medical management, and elucidate advances in the understanding and therapy of CIF-related complications such as catheter-related bloodstream infections and intestinal failure-associated liver disease.

Key Words: Chronic intestinal failure; Short bowel syndrome; Crohn's disease; Inflammatory bowel disease; Parenteral nutrition; Intestinal failure-associated liver disease

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Core Tip: Chronic intestinal failure (CIF) is a rare but feared severe complication of Crohn's disease, with 60% of patients permanently dependent on parenteral nutrition. This review aims to summarize the knowledge available in the current literature describing recent advances in the management and treatment of adult patients with CIF, with emphasis on patients with Crohn's disease. Moreover, it aims to further understanding of modern approaches to CIF complications such as catheter-related bloodstream infections and intestinal failure-associated liver disease.

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INTRODUCTION

Chronic intestinal failure (CIF) is a rare but feared severe complication of Crohn's disease (CD). Sixty percent of patients with CD and CIF are permanently dependent on parenteral nutrition (PN). According to the recommendations of the European Society for Clinical Nutrition and Metabolism (ESPEN)[1], CIF is defined as "a reduction of gut function below the minimum necessary for the absorption of macronutrients and/or water and electrolytes, such that intravenous supplementation is required to maintain health and/or growth". ESPEN suggests classifying CIF on the basis of anatomical, functional, pathophysiological, or clinical characteristics (Figure 1) and has defined five major pathophysiological conditions causing CIF: Short bowel, intestinal fistula, intestinal dysmotility, mechanical obstruction, and extensive mucosal disease[1]. In CD, the most common cause of CIF is short bowel syndrome (SBS), in which the small bowel length, by definition, is less than 200 cm[1]. Concerning nutritional-medicinal management, patients with CIF are most frequently subdivided according to the type and extent of bowel resection and additional surgical procedures (*e.g.*, stomata).

CLASSIFICATION

According to the recommendations of ESPEN, the classification of CIF can be based on pathophysiological, anatomical, functional, or clinical criteria (Figure 1).

From a pathophysiological point of view, a classification into five types (of which SBS is one) has been proposed based on the presence of various gastrointestinal and/or systemic diseases (Table 1)[2].

Anatomically, according to the type and extent of bowel resection and additional surgical procedures (stomata), three different categories of short bowel can be distinguished as prognostic criteria for future disease progression (Figure 2)[1,3]: Type I: Terminal jejunostomy ("very SBS" if remnant bowel length < 50 cm); Type II: Jejunoascendostomy, jejunotransversostomy, or jejunodescendostomy (rarely with colostoma); Type III: Jejunoileotransversostomy (very rarely with colostoma).

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Table 1 Pathophysiological classification of intestinal failure (adapted from Pironi *et al*[2], 2015)

Type	Primary cause	Underlying disease
Short bowel type	Quantitative/qualitative loss of resorptive surface	Post-operative, in patients with mesenteric infarction, Crohn's disease, radiation enteritis, familial polyposis, abdominal traumata, necrotizing enterocolitis[1], bariatric obesity surgery (biliopancreatic diversion with or without duodenal switch), gastroileal anastomosis, extensive tumor resection.
		Congenital, in patients with gastroschisis[1], intestinal atresia[1], intestinal malformation, omphalocele
Fistula type	Bypass of resorptive surface due to jejunocolic fistula	Inflammatory bowel disease (Crohn's disease[2], diverticulitis, radiation enteritis)
		Post-operative[3] in patients with neoplasia (colorectal carcinoma, ovarian carcinoma, Crohn's disease)
		Iatrogenic (post-op, percutaneous drainage)
		Traumata
		Foreign bodies
Dysmotility type	Restricted (insufficient) nutrition intake due to postprandial exacerbation of symptoms, to the point of non-mechanic ileus in severe cases	Ogilvie syndrome (acute non-mechanic obstruction of the colon)
		Chronic intestinal pseudo-obstruction: Primary/idiopathic (neuropathic/myopathic); Secondary (collagenous vascular disease, *e.g.*, PSS, LE, Ehlers-Danlos syndrome; neurological disorders such as Morbus Parkinson, intestinal hypoganglionosis; endocrinopathies)
Obstruction type	Reduced nutrition intake; Increased secretion of liquid and electrolytes in obstructive segments of the intestine; Loss of liquids and nutrients due to recurrent vomiting and/or "overflow sensors"	"Frozen abdomen" in patients with peritoneal carcinomatosis, extensive intestinal adhesion, recurrent peritonitis.
		Neoplastic stenoses and/or strictures
		Incarceration/strangulation of the intestine (hernia)
		Volvulus
Mucosa type	Extensive loss or damage of mucosa results in insufficient resorption of nutrients and pronounced enteral loss	Microvillus inclusion disease[1]
		Tufting enteropathy (intestinal epithelial dysplasia)
		Tricho-hepato-enteric syndrome
		Autoimmune enteropathy
		Intestinal lymphangiectasia
		Protein-losing enteropathy (Morbus Waldman)
		Radiation enteritis
		Chemotherapy-induced/associated enteritis

[1]Causes 70% of pediatric cases[4].
[2]75%-85% of enterocutaneous fistulae (EF).
[3]15%-25% of EF[3].
LE: Lupus erythematosus; PSS: Progressive systemic sclerosis.

The functional classification of intestinal failure, first described by Shaffer[4], is based on its time frame, metabolic course, and long-term progression: Type I: Acute and short-term. Usually a self-limiting condition, often observed in the perioperative setting and/or in association with critical illness, where patients require PN for a few days or weeks; Type II: Prolonged acute condition, often arises in patients with metabolically unstable conditions and requires complex multidisciplinary treatment and intravenous supplementation over a period of weeks to months; Type III: Chronic condition found in metabolically stable patients who need to be intravenously substituted for months or years. May be (partially) reversible or irreversible.

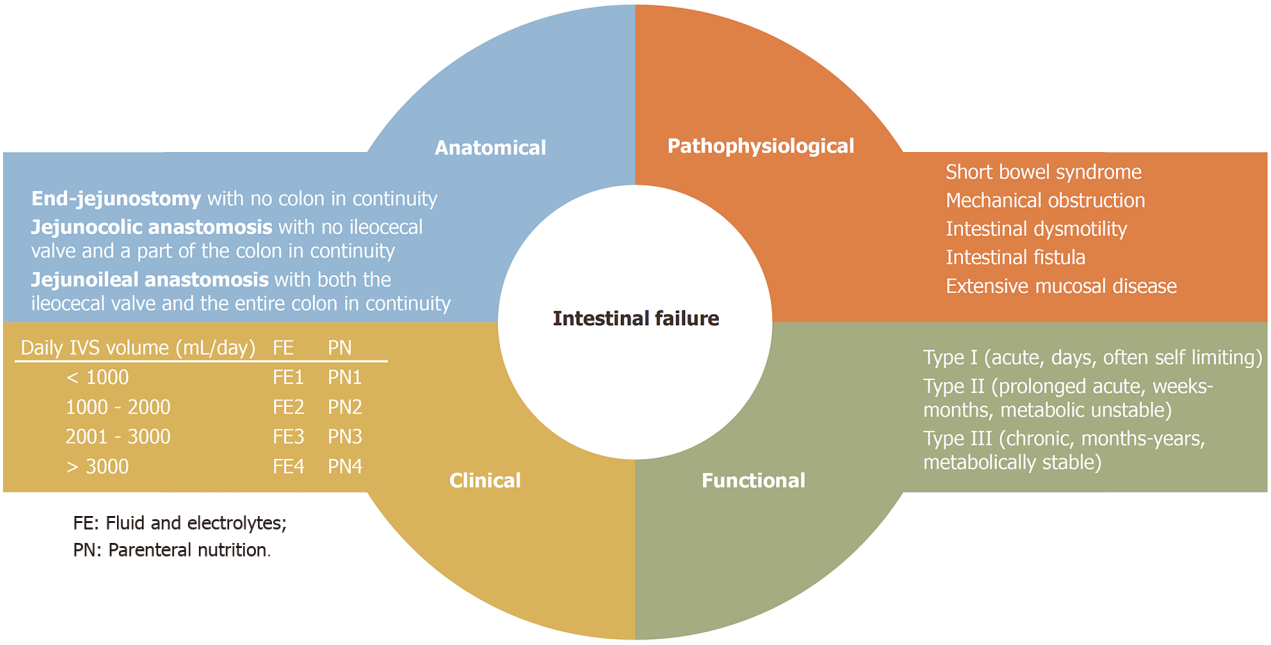


Figure 1 Four domains of chronic intestinal failure. Disease severity is defined by the content of the supplementation — fluid and electrolytes (FE) only, or admixture containing energy (parenteral nutrition (PN)). Each group is subdivided into four categories of mean daily intravenous supplementation (IVS), with volume calculated as average infusion volume per day × number of infusions per week/7.

The clinical classification is based on the patient's energy and fluid requirements for PN in the context of clinical management. It does not aim at risk stratification and is defined according to 16 categories (Figure 1).

EPIDEMIOLOGY

While intestinal failure can occur due to a number of different underlying conditions, CD has been found to be the most common reason for intestinal failure (Figure 3)[5]. However, incidence of CIF as a complication of CD can only be indirectly evaluated based on data from home PN registries. Recent reports from national intestinal failure units in the United Kingdom show that CD is the underlying disease in 30%–32% of patients with long-term intestinal failure[6,7]. Longitudinal data from Japan indicate an incidence of CIF in CD of 8.5%-18.2% during the first 20 years after initial presentation[8,9].

Mechanisms of CIF in CD are poorly understood. Among recognized pre-operative risk factors are systemic steroid treatment[10-13], recent weight loss[11,14], intraabdominal abscess[15-17], and smoking[14,18-20]. Whether recent administration of antitumor necrosis factor medication is associated with post-operative morbidity in CD is currently under investigation.

The peri-/post-operative events that most frequently lead to CIF in CD have been purported to be multiple resections, enterocutaneous fistulation, and malabsorption with multiple resections, leading to cumulative loss of the small bowel. However, Soop *et al*[21], in a longitudinal cohort study of 121 patients referred to a national intestinal failure unit from 2000 to 2018 who were diagnosed with CD and subsequently treated with PN for at least 12 mo, identified septic complications following abdominal surgery to be the most frequent event leading directly to CIF.

ANATOMICAL-PHYSIOLOGICAL ADAPTATION

Three phases of intestinal adaptation can be distinguished. Although it is not possible to define specific biological or functional demarcation points between the hypersecretion, adaptation, and stable (chronically adapted) phases, their differentiation can be a useful aid in therapy planning.

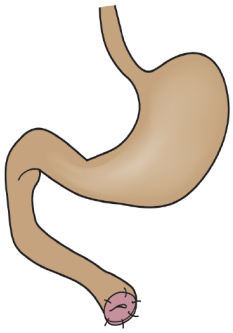
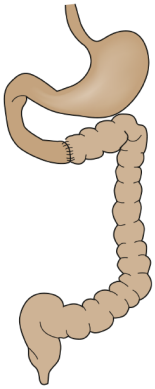
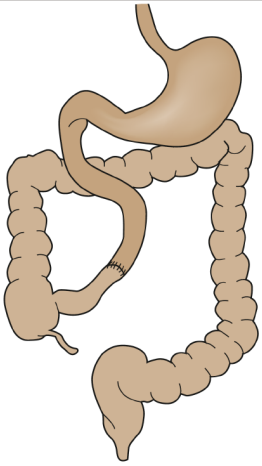
	Type I: Terminal jejunostomy	Type II: Jejunocolic anastomosis	Type III: Jejunoileotransversostomy
Surgical procedure	Complete resection of ileum and colon; Jejunum preserved, represents the end of the intestine	Resection of most of the Ileum, colon preserved	Jejunal resection, at least 10 cm of terminal ileum and colon retained
			
Presence of ileocaecal valve	No	No	Yes
Preservation of colon	No	Partial	Complete
Clinical features	Immediate postsurgical dehydration with risk of electrolyte imbalances; Jejunal output increases after food and fluid intake	Diarrhoea/steatorrhoea; Small intestinal bacterial overgrowth Malabsorption/weight loss Vitamin B_{12} deficiency Oxalate-induced nephrolithiasis	Malnutrition rare
Risk for permanent dependence on parenteral nutrition	Very high	Mostly as supplemental PN (in patients who partially satisfy energy requirements *via* oral/enteral feeding)	Usually no need for parenteral support

Figure 2 Main characteristics of different types of short bowel syndromes according to the anatomical criteria (adapted from Massironi *et al*[3], 2020).

Phase I: Acute (hypersecretory) phase

The hypersecretory phase occurs during the immediate post-operative period and may last for 2 or 3 mo, and in some cases even longer. This phase is characterized by poor absorption of almost all nutrients, including water, electrolytes, proteins, carbohydrates, fats, vitamins, and trace elements. Depending on the extent of resection, daily fluid loss of up to 5 L may occur, while some patients with jejunostomy may lose as much as 6-8 L[22].

Gastric hyperacidity (GH) — increased stomach acidity due to a transient increase in gastric acid secretion (over a period of weeks to months) — affects over half of patients with small bowel resection of 30% or more. GH has been suggested to be caused by the temporary discontinuation of an indirectly or directly acting intestinal inhibitor of gastric secretion (*e.g.*, vasoactive intestinal peptide or gastric inhibitory polypeptide). Alternatively, it may arise due to the diminished breakdown or increased production of a stimulant. Serum levels of gastrin, for example, have been found to be increased after surgery.

Alongside diminished contact time, which leads to inadequate mixing with the chyme, GH-induced inactivation of pancreas ferments is considered vitally important. Following resection of the proximal small bowel, serum levels of cholecystokinin and secretin have been found to be substantially diminished. This generates, in effect, a disturbed positive feedback that leads to a reduction in pancreas stimulation. On the other hand, a reduction in enterokinase activity, observed mainly following duod-

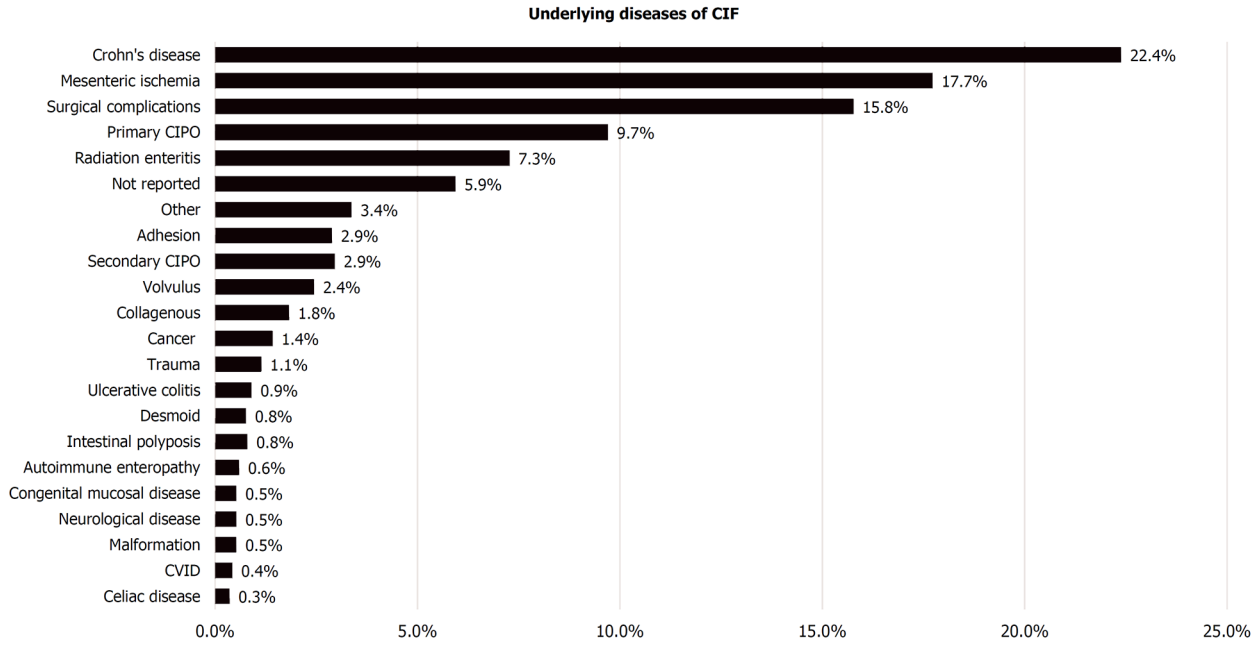


Figure 3 Underlying diseases of chronic intestinal failure. CIF: Chronic intestinal failure; CIPO: Chronic intestinal pseudo-obstruction; CVID: Common variable immunodeficiency.

enum resection, is of no significance.

In patients whose terminal ileum and proximal colon have been surgically removed (type I), a lack of L-cells leads to reduced synthesis of enterohormones [peptide tyrosine-tyrosine, glucagon-like peptide (GLP)-1, GLP-2], effecting an acceleration of gastrointestinal transit duration[23].

Phase II: Adaptation of the residual intestine

Following intestinal resection, structural and functional adaptation processes in the remnant bowel begin even during the early post-operative hypersecretion phase. Distal resection tends to trigger a greater adaptive response than more proximal resection; during this phase, fluid losses should fall to less than 2.5 L. Several years can pass before a stage of maximal adaptation is reached. Generally, 90%-95% of adaptation potential of the remnant bowel is realized within 2 years after resection surgery. In the stabilization phase, improvement of bowel efficiency due to enhanced adaptation leads to a reduction in diarrhea and steatorrhea. Intestinal adaptation is induced by enterotrophic hormones and growth factors, such as epidermal growth factor, growth hormone, insulin-like growth factor-1 (ILG-1) and glucagon-like peptide-2 (GLP-2), an amino acid peptide created by specific post-translational proteolytic cleavage of proglucagon. In addition, intraluminal nutrient supply and the resultant secretion of biliary and pancreatic enzymes play a vital role (for review, see[23]).

Phase III: CIF

Around 50% of patients with prolonged acute intestinal failure go on to develop CIF [24]. Patients with CIF are metabolically stable but need intravenous nutritional support for months or years (reversible CIF) or even lifelong (irreversible CIF). Home-based PN (HPN) is the mainstay of treatment. During this phase, symptoms of SBS are usually addressed with medication. Overall, the probability of weaning off HPN has been reported to be about 50% in adults and up to 73% in children. Complete commutation off HPN in patients with SBS is unlikely[24]. PN can be necessary either to substitute total caloric-nutritional needs when patients are fasting and totally dependent on HPN (total PN) or as supplemental intake in patients who partially satisfy energy requirements *via* oral/enteral feeding (supplemental PN).

DIAGNOSIS AND WORK-UP

Diagnostics include on the one hand the correct diagnosis of SBS/CIF, *i.e.*, determination of the post-operative bowel anatomy (intraoperative bowel length, radiological assessment of bowel length) and the resorptive capacity of the remnant bowel, and on the other hand, the determination of specific nutrient deficiencies and their symptoms and, if applicable, any complications of enteral nutrition (EN)/PN.

As a first step, diarrhea is quantified and further characterized by stool frequency, stool weight, and 24-h steatorrhea/creatorrhea[25,26]. The resorptive surface area can be calculated using the D-xylose absorption test and/or serum citrulline[27,28]. However, data published on the validity of serum citrulline determination as a biomarker for bowel length and a prognostic parameter for successful intestinal adaptation are the subject of controversy. Assessment of early onset nutrient deficiencies requires analysis of iron (ferritin), folic acid, calcium, phosphate, and copper in serum in addition to urine concentrations of zinc and magnesium. In addition, if steatorrhea is present, serum concentrations of the fat-soluble vitamins (A, D, and E) can be utilized for dose-finding and/or correction (cave: Hypervitaminosis). Symptoms of late onset deficiencies, which often remain unnoticed for years before clinical manifestations become apparent, include megaloblastic anemia as a consequence of vitamin B_{12} deficiency (the liver's stores are sufficient for 3-5 years). In this context, and also as an early marker of folic acid or vitamin B_6 deficiency, homocysteine levels serve as a simple and efficient screening parameter (Table 2)[29].

MANAGEMENT OF CIF

Management of CIF focuses on optimizing nutritional status and fluid balance and achieving and/or maintaining adequate weight, while simultaneously minimizing risks associated with long-term complications[30]. The choice of therapy is contingent upon the underlying disease, concomitant disorders, and complications of therapy as well as the localization and extent of resection. Both strategies aim to compensate the diminished resorptive capacity of the remnant bowel, striving in the long-term for oral autonomy. For optimal management, ESPEN recommends that patients with CIF are supported by an expert multidisciplinary team (MDT) addressing all relevant domains of care, such as underlying disease therapy, catheter care, psychological well-being, and PN monitoring[1]. Thus, the ideal MDT should include physicians, surgeons, nurses specializing in stoma and catheter care, dieticians, pharmacists, and psychologists[31].

OPTIMIZING NUTRITIONAL STATUS

There is no specific diet for patients with CIF. Individual remnant intestinal anatomy is a crucial consideration when planning nutritional management and should be clarified prior to commencement of PN. As mentioned above, it is recommended that patients with SBS-IF are divided into three groups based on the absence or presence of the terminal ileum and the colon, since these are the main factors influencing the type and amount of nutrient supplementation required[32].

Specific caloric requirements will vary on an individual basis; however, observational studies have found that patients with SBS consume between 35 and 58 kcal/kg/d in order to meet their nutritional needs[33].

Depending on the adaptation stage, nutritional therapy measures can take the form of overlapping or combined therapy with oral and (long-term) PN/EN, with adjunctive medication as necessary. Patients with jejunostoma (type I) and a residual bowel length of less than 100 cm, as well as those with less than 50 cm continual remnant colon (Table 3), are almost certain to require PN on a permanent basis. If, however, a greater length of remnant bowel is present, it is usually possible to gradually discontinue parenteral feeding or at least progressively convert to partial PN or intravenous fluid supplementation[24,32,34].

There are no controlled data describing the protein requirements of adults with CIF. Prior studies show that protein absorption depends on the length of the small bowel remnant and may increase over time as the bowel adapts, ranging from 61% to 80% [33]. In patients with IBD, supplementation must additionally compensate protein loss *via* the bowel[35]. Current ESPEN guidelines recommend protein substitution of 0.8-2.0 g/kg body weight (BW) per day according to individual needs, representing 20%-

Table 2 Recommended laboratory monitoring for patients receiving parenteral nutrition (adapted from Lappas *et al*[29], 2018)

Parameter	Frequency	
	Initiation of therapy (acute care)	Long-term therapy
Capillary glucose	Every 6 h until advanced to goal and as needed to maintain 140-180 mg/dL	Not routine, as needed basis to coordinate with PN infusion cycle
Basic metabolic panel Phosphorus, magnesium	Daily, until advanced to goal and stable; then 1-2 times/wk	Weekly, then decrease frequency as stable
CBC (with differential)	Baseline; then 1-2 times/wk	Monthly, then decrease frequency as stable
Liver function: ALT, AST, ALP, total bilirubin	Baseline; then weekly	Monthly, then decrease frequency as stable
Serum triglycerides	Baseline if at risk; then as needed	Not routine, as needed
Iron studies, 25-OH vitamin D	Not routine (see Table 4)	Baseline, then every 3-6 mo
Zinc, copper, selenium, manganese	Not routine (see Table 4)	Baseline, then every 6 mo

25-OH vitamin D: 25-hydroxyvitamin D; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; CBC: Complete blood count; PN Parenteral nutrition.

Table 3 Nutrition therapy (macronutrients) according to presence or absence of colon (modified from Pironi *et al*[46], 2016)

Nutrient	Colon present (partial/complete)	Colon removed or disabled
Carbo-hydrates	Slow increase of the proportion of complex carbohydrates up to approx. 40%-50% of total calorie intake, no low molecular weight sugars	Slowly increase the proportion of complex carbohydrates up to approx. 40%-50% of total calorie intake, no low molecular weight sugars, modified FODMAP diet
Protein	Up to 20%-30% of total energy intake	Protein: up to 20%-30% of total energy intake
Fat	Up to 20%-30% of total energy intake	Fat: up to 40% of total energy intake
	Ensure adequate essential fatty acids (high EFA content)	Ensure adequate essential fatty acids (high EFA content)
	Consider MCTs	Limit MCTs
Fiber	Dietary fiber supplements: up to 5-10 g/d	Dietary fiber supplements: up to 5-10 g/d
Oxalate	Diet low in oxalic acid and fats; Replacement of up to 50%-75% of dietary fat with MCTs	No restriction of oxalic acid necessary
Fluids	Even if liquids are well tolerated, iso- and hypotonic drinks are preferable	Oral rehydration solutions frequently required
Lactose	Low lactose diet (10-12 g)	Unrestricted lactose intake

EFA: Essential fatty acids; FODMAP: Fermentable oligo-, di-, monosaccharides and polyols diet; MCT: Medium-chain triglyceride.

30% of daily calorie requirements[1]. The supply of proteins (enteral) and/or amino acids (parenteral) should be chosen to offset undermet needs and ensure the prompt correction of existing deficits (catabolism). The sparse data available on the utility of peptide-based EN are contradictory. However, a considerable increase in protein supply can be achieved through continual nasogastric application of standard polymer feeds, either alone or supplementary to oral (*ad libidum*) nutrition, above and beyond the immediate post-operative phase. Peptide-based enteral feeding is possibly only of additional value in SBS type I[32].

Fat restriction is not necessary in patients who lack a colon. In patients with a preserved colon, it is important to limit fat intake to 20%-25% of total calories in order to reduce the risk of nephrolithiasis from calcium oxalate stones (Table 3). In these patients, a low-fat diet has been shown to increase absorption of calcium, magnesium, and zinc[36] and to improve utilization of medium chain triglycerides for nutrient absorption without increasing stool volume and diminishing calcium and magnesium absorption[37,38]. Fat assimilation can be improved through the addition of pancreas enzyme formulations in powder or granulate form. Controlled studies are not available for CIF[32].

MANAGEMENT OF FLUID AND ELECTROLYTE BALANCE

The primary challenge in patients with jejunostomy is the management of major losses of fluid and electrolytes, particularly zinc, copper, and magnesium (Table 4)[39,40]. It is important for these patients to avoid drinking hypotonic solutions or tap water, as this can lead to worsening electrolyte disturbances by increasing stool sodium content. Conversely, hypertonic solutions such as soda and fruit juice should also be avoided as these solutions are hyperosmolar and can draw water into the gastrointestinal tract and worsen dehydration[33]. Ideally, fluids should be given in small portions between mealtimes, initially in the form of oral rehydration salts (ORS) that offer a sodium-glucose ratio optimized to enhance resorption[41]. In patients with a colon, ORS solutions based on rice starch have been shown to be particularly effective[42]. Sport electrolyte solutions, on the other hand, are not only costly but generally unsuitable, since they contain relatively large amounts of sugar and/or sweeteners in relation to their sodium content. Common sweeteners such as sorbitol or aspartame are known to cause osmotic diarrhea. In addition, fruits, fruit juice, and sweets/candies may increase the frequency of diarrhea. Glutamine-containing ORS solutions are not to be recommended, since glutamine diminishes the intake of sodium and fluids[43].In the adaptation phase (see above), filling foods such as potatoes, rice, oat flakes, or bananas can help solidify the stool. Fluid binding and swelling formulations with pectin may also be beneficial[44].

Ostomy patients should be made aware that every intake of food can be expected to result in stool emptying *via* the ostomy outlet. These patients have a daily fluid requirement of approximately 3 L. Adequate fluid intake can be assessed on the basis of urine volume, with a urine volume of at least 1 L/d considered ideal (urine volume monitoring). Daily intake of 6-9 g salt (*e.g.*, salted meat or vegetable broth) and 25-30 g dietary fiber are also recommended.

Cave: Ileo-/jejunostomy patients (type I) are extremely susceptible to thirst and increased fluid loss due to sweating, diarrhea, *etc.* The resulting rise in oral fluid intake can lead to increased stool volume. Thus, these patients are at increased risk of rapid dehydration, to the point of prerenal kidney failure.

MINERAL SUBSTITUTION

The electrolyte disturbance most frequently seen in SBS is sodium deficiency. As a rough guide, it can be assumed that about 100 mmol/L of sodium are lost with each liter of jejunostomy fluid. Sodium deficiency is better detected by measuring urinary sodium, which decreases before hyponatremia occurs: Urinary sodium concentration below 10 mmol/L is a diagnostic criterion for sodium depletion. Overall, the aims of treatment are to maintain normal hydration and a daily urine volume of at least 800 mL, with a urinary sodium concentration greater than 20 mmol/L[1,40,45,46].

A urine sodium to potassium ratio of ≤ 1 indicates secondary hyperaldosteronism caused by volume depletion as a result of inadequate fluid substitution[47,48]. This marker therefore requires close monitoring and therapy should seek to maintain a sodium to potassium ratio > 1[49].

While hypokalemia may occur in patients with SBS, net intestinal loss occurs only in the case of end-jejunostomy with less than 50 cm remnant jejunum. The effluent from a jejunostoma with a longer remnant jejunum or an ileostoma contains approximately 15 mmol/L potassium. Low serum potassium is most commonly due to urinary losses of potassium associated with secondary hyperaldosteronism elicited by dehydration and sodium depletion. Hypokalemia may also be caused by magnesium depletion, which disrupts many of the potassium transport mechanisms and increases renal potassium excretion; in this case, hypokalemia is resistant to potassium substitution but responds to magnesium replacement[1,50,51].

Due to potassium loss *via* the stool or stoma output, daily potassium requirements of patients with CIF are increased compared to healthy individuals, for whom the recommended intake is 1-1.5 mmol/kg BW per day. Potassium loss in patients with small bowel ostomy amounts to approximately 10-30 mmol/L (Table 4)[49].

Daily calcium requirements are estimated at 0.15-0.25 mmol/kg BW (300-400 mg). Calcium intake should be aimed at maintaining normal levels of parathyroid hormone, with calcium excretion in 24 h urine providing a useful additional marker[49]. Extreme depletion of magnesium stores can disrupt parathyroid hormone excretion, leading to false low measurements[52].

Table 4 Symptoms, prophylactic supplementation, and therapy of frequent (critical) nutrient deficiencies in chronic intestinal failure

Nutrient	Symptoms of deficiency	Normal values (blood/serum)	Additional lab tests	Prophylaxis	Therapy in case of deficiency
Calcium	Neuromuscular hyperarousal, cardiovascular symptoms, osteopathy	2.1-2.7 mmol/L	↑Alkaline phosphatase; ↑intact PTH; ↓Bone mineral density	Calcium citrate, oral 1200–2000 mg/d	Bisphosphonate if T-Score < 2.5
Magnesium	Neuromuscular hyperarousal, osteopathy (PTH effect ↓)	0.75-1.15 mmol/L	↓Magnesium in urine	Magnesium citrate, oral 300 mg/d	10 – 15 mmol magnesium, *e.g.*, in 1000 mL NaCl 0.9 %
Vitamin A	Night blindness, wound healing disorders	1.05-2.80 µmol/L	↓Plasma retinol; ↓ Retinol binding protein	10000-50000 U/d, if liver function normal	No corneal changes: 10000–25000 IU/d oral for 1–2 wk; Corneal changes: 50000–100000 IU i.m. followed by 50000 IU/d i.m. for 2 wk
Vitamin D	Osteopathy, wound healing disorders, immune system disorders	< 20 µg/L: deficiency; 20-30 g/L: insufficiency; > 30 µg/L: sufficient supplies	↑Alkaline phosphatase; ↑intact PTH; ↓Bone mineral density	Oral vitamin D (400–800 U/d) [ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3)] or 100000 U/3–6 mo orally	50000–150000 IU oral 3-5 times a week; If required: calcitriol [1,25(OH)2D] oral
Vitamin K	Hemorrhagic diathesis	INR < 1.2	PIVKA	10 mg/wk	N/A
Vitamin B_1	Polyneuritis ("dry form"), edema, tachycardia, Cardiac insufficiency ("wet form"); Wernicke encephalopathy, ataxia ("central form")	10.64 µg/L	↓Thiamine pyrophosphate; ↓ Erythrocyte transketolase activity	If vomiting, aggressive oral thiamine supplementation with 100 mg/d for 7–14 d	Treatment of Wernicke Encephalopathy: 500 mg i.v. 3 ×/d for 2-3 d; ≥ 250 mg/d i.v. for 5 d; 30 mg oral 2 ×/d
Vitamin B_{12}	Megaloblastic anemia, glossitis, skin and mucous membrane pallor, paresthesia, polyneuropathy, funicular myelosis	156-675 pmol/L	↓Holo-TC; ↓MMA; ↑ Homocysteine	Oral: 1000 µg/wk or 250–350 µg/d, i.m./s.c.: 1000 µg/mo or 3000 µg every 6 mo	1000 or 2000 µg/d oral or 1000 µg/wk i.m.
Zinc	Wound healing disorders, hair loss, taste disturbances, predisposition to infection	11-23 mol/L	↓Zinc in urine	In presence of fistula, diarrhea or stomata: 12 mg; Otherwise: 3-4 mg	30-45 mg (as zinc histidine), 220-440 mg (as zinc sulphate). For each 8–15 mg of elementary zinc, 1 mg copper should be substituted
Iron	Anemia, hair loss, cognitive disorders, predisposition to infection	CRP < 5 µg/L: > 30 µg/L; CRP ≥ 5 µg/L: ≥ 100 g/L	↓Transferrin saturation; ↑Soluble transferrin receptor; ↑ Zinc protoporphyrin	Oral max: 100–150 mg iron	Parenteral, depending on iron status: Aim: normalization of Hb plus transferrin saturation 35%-50% (calculated according to Ganzoni)
Copper	Neutropenia, iron deficiency anemia, central venous development disorders	11-22 µmol/L	↓Copper/zinc superoxide dismutase	Copper gluconate, oxide or sulphate equivalent to 2 mg elementary copper; 1 mg copper for each 8-15 mg zinc	Copper sulphate equivalent to 2.4 mg elementary copper in 100 ml 0.9% NaCl i.v. administered one hour/d for 5 d. Subsequently, oral substitution as required

CRP: C-reactive protein; Holo-TC: Holo-transcobalamin; INR: International normalized ratio; MMA: Methylmalonic acid; PTH: Parathyroid hormone.

Magnesium requirements, estimated at 0.1-0.2 mmol/kg BW (200-300 mg) per day for healthy individuals, can be more than doubled as a consequence of magnesium losses *via* stool or stoma (Table 4). Magnesium supply should be sufficient to maintain normal serum magnesium levels (0.75-1.15 mmol/L). When interpreting serum magnesium values, it is important to consider that magnesium deficiency may be present even at serum levels of up to 0.85 mmol/L[53]. Magnesium secretion of < 9.7 mg/24 h in urine is regarded as a more sensitive parameter for magnesium deficiency than serum magnesium: 24 h urine levels should therefore be determined when serum magnesium is 0.75-0.85 mmol/L[54]. Since magnesium is not well absorbed, oral magnesium substitution is often inadequate. Nocturnal infusions once or twice a week are recommended, each containing 10-15 mmol magnesium, *e.g.*, in 1000 mL NaCl 0.9%[55]. For oral substitution of calcium or magnesium, Ca^{2+} or Mg^{2+} citrate are preferable due to their superior bioavailability[56].

Daily phosphate requirements of healthy persons are estimated at 0.3-0.5 mmol/kg BW. Phosphate supply should be calculated to maintain normal serum phosphate levels while taking account of parathyroid hormone levels (there are no data from controlled trials). When initiating parenteral and/or enteral feeding in undernourished patients, the possibility of nutrition-associated hypophosphatemia must be borne in mind (refeeding syndrome)[57].

MICRONUTRIENTS

The most common micronutrient deficiencies are of vitamin D, zinc, iron, and vitamin B_{12}. These deficiencies can be observed even in patients receiving full ("total") PN, especially during weaning from PN to EN[58].

Vitamin B_{12} malabsorption occurs frequently after terminal ileum resection, even after short resection of only 50 cm. Resection of the ileocecal valve reduces intestinal transit duration and increases the risk of bacterial miscolonization of the small bowel, increasing the likelihood of additional bile acid deconjugation and depletion of the vitamin B_{12} intrinsic factor complex. The resulting pernicious anemia manifests as megaloblastic anemia, thrombocytopenia, and Hunter (atrophic) glossitis, along with neurological disturbances consistent with funicular myelosis (except in folic acid deficiency). To prevent vitamin B_{12} deficiency, 1000 IE should be given prophylactically every 2-3 mo. In case of manifest deficiency, treatment should commence with daily administration of B_{12} at a dose of 1000 IE intramuscular/subcutaneous for 5 d, followed by monthly applications of 1000 IE (Table 4).

Deficiencies of fat-soluble vitamins arise as a direct result of disrupted fat resorption. If untreated, these deficiencies can lead to night blindness (vitamin A), coagulation disturbances (vitamin K), and, in the long term, bone metabolism disorders and even osteoporosis (vitamin D)[59] (Table 4).

Trace element deficiencies may also occur, especially of iron, zinc, and copper[58,60,61]. Patients with intestinal failure are prone to iron deficiency as result of malabsorption, gastrointestinal blood loss, and multiple surgical procedures. Accordingly, iron deficiency is the most common micronutrient deficiency during and after transition from TPN to EN, with reported incidences of 60%-80% for iron deficiency and 30%-37% for iron deficiency anemia[62,63]. A study from the Mayo Clinic (Rochester, MI, United States), including 185 patients, showed that iron deficiency anemia developed much more rapidly in patients with fistula and bowel obstruction than in those with SBS and dysmotility[63].

Despite the high prevalence of iron deficiency, iron is not routinely added to PN formulations because of the risk of anaphylaxis and concerns about incompatibilities. Although data describing the compatibility of iron supplementation with parenteral formulations are conflicting[64], iron dextran has been found to be compatible with lipid-free solutions at an amino acid concentration > 2%. A safer approach would prescribe the intermittent infusion of therapeutic iron doses[65]. Dosage requirements for intravenous iron replacement should be calculated according to Evstatiev *et al*[66].

In patients with diarrhea, large quantities of zinc are lost, with losses of 12 mg zinc per liter stoma output not unusual (Table 4). This considerably exceeds not only normal total zinc requirements but also the content of standard oral mineral supplements[67,68]. In the case of manifest zinc deficiency, 30-45 mg zinc per day can be taken orally (approximately 1 h before breakfast) as zinc histidine or zinc gluconate [69]. However, response to oral supplementation is frequently inadequate, in which case parenteral substitution is indicated[68,70].

PHARMACOLOGICAL TREATMENTS

Analogous to the modified resorption of micro- and macronutrients in CIF, and depending on the underlying disease and the extent and localization of bowel resection, alterations in the bioavailability of any and every kind of drug therapy are to be anticipated. While the scale of these changes is subject to wide interindividual variation, larger drug doses may be required than are typically recommended[30,50]. Data on specific drug classes are scarce, and those that have been published are either case reports or very small cohort studies[71,72]. Therefore, sublingual, transdermal, or transnasal drug application should be chosen whenever suitable options are available [41]. Pharmacological treatments for CIF are primarily based on antisecretory and antimotility treatments intended to minimize gastrointestinal fluid losses. Based on the

identification of GLP-2 as a tissue-specific intestinal growth factor in the late 1990s, hormonal treatment promoting intestinal hyperadaptation has been proposed, aimed at maximizing absorption in the remnant bowel, decreasing intestinal losses, and reducing the need for PN[73,74]. Table 5 shows current drug therapy options for the treatment of CIF.

Antisecretory treatment

For the majority of these medications, only scant scientific evidence is available, or none at all. For example, despite the complete absence of data from controlled studies to endorse the application of proton pump inhibitors in the hypersecretion phase, they are widely recommended as a means of reducing fluid volume and improving the effectiveness of pancreas ferments. Their use in the case of high output stomata is at least supported by two small monocentric studies, whereby intravenous is evidentially superior to oral application[75,76]. Longer-term or permanent intake of proton pump inhibitors is, however, associated with increased risks for osteoporosis and vitamin B_{12} deficiency[41].

Typical second-line agents used to combat gastric hypersecretion include histamine type 2 receptor (H_2) antagonists (*e.g.*, famotidine, ranitidine, cimetidine) and α_2-adrenergic receptor agonists (*e.g.*, clonidine)[75,77]. As with antimotility agents, acid suppressors should be initiated at a low dose and titrated upward to yield maximal efficacy with minimal adverse events. The optimal duration of post-operative antacid therapy is unknown, but it should be discontinued in the event of worsening diarrhea [30].

Data from two smaller trials in patients with CD and ileostoma demonstrated that topically acting steroids may improve the absorptive capacity of the intestinal mucosa for water, independently of their anti-inflammatory effects[78,79].

A number of randomized controlled trials (RCTs) have been published examining the efficacy of crofelemer, a dual inhibitor of cyclic adenosine monophosphate- and calcium-mediated chloride secretion, in chronic acquired immunodeficiency syndrome-associated diarrhea (for reviews, see[80,81]).

Antimotility treatment

The use of antimotility drugs to control diarrhea in CIF has been validated mainly by small studies in patients with ileostomy[59,77]. Recommendations for their use are additionally based on extensive practical experience of their application in diarrhea of infectious and non-infectious etiology[49].

Accelerated intestinal motility is typically treated with opioids or the opioid receptor agonists loperamide and diphenoxylate-atropine as the first-line choices. Unlike diphenoxylate, which crosses the blood-brain barrier, loperamide, a peripherally restricted μ-opioid receptor agonist, does not engender undesirable central nervous system effects such as sedation, euphoria, or addiction[30,82,83]. Because loperamide enters the enterohepatic circulation, higher doses (up to 16 tablets/d) may be needed in patients whose ileum has been shortened or removed. Other antimotility agents include codeine[82,84], morphine, and opium tincture[30]. However, these opioids are not restricted to the peripheral nervous system and can thus generate central nervous system effects[30]. When used together, loperamide and codeine may have a synergistic effect[82].

Besides alleviating gastric hypersecretion, clonidine, a centrally acting α_2 adrenergic and imidazoline receptor agonist (which can also be administered transdermally), has been shown to diminish stomach and colon motility and inhibit intestinal chloride secretion. As with H_2-antagonists, evidence supporting clonidine use in patients with CIF is based only on small case series[85-88]. Dosage of the selected antimotility agent-administered 30 min before meals and at bedtime-should be escalated in a stepwise manner at intervals of 3-5 d until benefit is observed, adverse events occur, or the recommended maximum dosage is reached[30]. Whereas the development of tolerance to analgesic properties of opioids or opioid receptor agonists is well recognized, tolerance to the antidiarrheal effect is rare and the effective dose may remain constant for months to years[30].

Hormonal treatment

In patients with SBS-type CIF, intestinal adaptation can be additionally stimulated by the application of enterotrophic hormones and growth factors, such as growth hormone with or without glutamine or the glucagon-like peptides GLP-1 and GLP-2 [89-93]. Treatment with somatostatin analogues has also been studied.

Table 5 Symptomatic treatment options in short bowel syndrome

Symptom	Drug/therapy	Dose (per day)
Gastral hypersecretion	Proton pump inhibitors	20-40 mg i.v. (p.o.)
	Clonidine	2 × 75-150 μg s.c./p.o.
	Octreotide (sandostatin)[1]	3-4 × 50-100 μg s.c.
Hypermotility	Loperamide	4-6 mg p.o. (max daily dose 16 mg)
	Diphenoxylate	4 × 2.5-7.5 mg (max daily dose 20-25 mg)
	Codeine	30 mg p.o.
	Opium tincture	4 × 0.3-1 mL (10-60 mg) p.o.
Secretory diarrhea	Octreotide (sandostatin)[1]	2-3 × 50-100 μg s.c.
	Budesonide (*e.g.*, entocort)	3 × 3 mg p.o.
	Clonidine	2 × 75-150 μg s.c.
Fat malabsorption	Pancrelipase (*e.g.*, Creon)	40000 IU with main meals (15000 IU with snacks)
Lactose malabsorption	Lactase formulations (L-products)	Depending on severity
Reduced fluid resorption	Locust/carob bean gum flour added to drinks (yoghurt)	Approx. ½-1 tablespoon per glass/pot
	Kaopectate (kaolin/pectin)	4 × 1 tablespoon

[1]Not in the adaptive phase.

Since the pilot trial of Jeppesen *et al*[94] in 2003, a number of trials of teduglutide, a long-acting GLP-2 analogue, have shown an overall 20%-40% reduction in PN dependence, with some patients able to discontinue PN entirely. On the evidence of two 24-wk RCTs, teduglutide is recommended to minimize the number of infusion days in patients with stable infusion-dependent CIF. Improvements in stool consistency and general condition of the patients were demonstrated in both of the aforementioned trials[95,96].

Most recently, first results from a European interdisciplinary center-based retrospective data analysis on teduglutide treatment for SBS in clinical practice have been published. The results show that, even when applied in routine medical care to patients with anatomically and clinically heterogeneous SBS-CIF, teduglutide induced functional and structural changes, allowing a gradual reduction of parenteral support. The data suggest that treatment with teduglutide results in an improved intestinal function and a compensatory effect on nutritional status[97].

It must, however, be kept in mind that all existing GLP-2 trials were conducted in patients at a late phase of intestinal adaptation, months or years after surgery. If therapy were initiated immediately after resection, adaptation would probably be induced more quickly (accelerated) and potentially to a greater degree (supraphysiologic). Conversely, and importantly, because GLP-2 is trophic to the intestinal mucosa, it may carry a risk of promoting or inducing the growth of localized polyps, or more ominously, malignancies. A recent systematic review indicated that treatment with teduglutide for up to 30 mo in individuals without known pre-existing cancer did not confer an increased risk of intestinal neoplasia[98]. However, based on animal data showing that GLP-2 may promote the growth of existing neoplasia, endoscopic examination of the remnant colon is mandatory before beginning teduglutide therapy [74].

In 2013, an open-label, placebo-controlled study showed that GLP-1 decreased diarrhea and fecal excretion in SBS patients. More recently, liraglutide, a GLP-1 analogue, was administered daily subcutaneously in patients with end-jejunostomy over 8 wk in an open-label pilot trial[72]. Liraglutide reduced ostomy wet weight output (by 474 ± 563 g/d), increased intestinal wet weight output (by 464 ± 557 g/d), and improved intestinal energy absorption (by 902 ± 882 kJ/d), with statistical significance in all three instances. Combination therapy with GLP-1 and GLP-2 has been shown to be more effective than GLP-2 alone[99].

Over the past two decades, several clinical studies evaluating the effects of growth hormone, alone or in combination with a high-carbohydrate, low-fat diet and/or glutamine, on intestinal adaptation and absorption in pediatric and adult populations,

have demonstrated conflicting findings[89,100-107]. The positive effects of high-dose growth hormone treatment, mainly on intestinal water absorption, have been described in patients with SBS with a colon in continuity[74]. Although high-dose growth hormone therapy is already in use, no recommendation for routine application of growth hormone is given in the latest guidelines of either the German Nutrition Society[49] or ESPEN[108].

Octreotide, a long-acting analogue of the peptide hormone somatostatin, has been demonstrated to improve diarrhea in patients with CIF by inhibiting gastrin and other gastrointestinal hormones, by inactivating adenylate cyclase, thereby inhibiting intestinal ion secretion and prolonging intestinal transit time[109-111]. Longer-term application of octreotide is associated with an increased risk of gallstones, a recognized complication of SBS, and impairment of intestinal adaptation[30,112].

COMPLICATIONS AND SPECIAL SITUATIONS

Nephrolithiasis and enteric hyperoxaluria

Enteric hyperoxaluria (EH) was first described as a complication in the early 1970s, upon recognition of the association of small bowel resection and subsequent hyperoxaluria and nephrolithiasis: When significant lengths of the ileum are resected (> 60 cm), bypassed, or dysfunctional due to inflammation (*e.g.*, in CD), both fatty acids and bile acids are delivered to the colon at an increased rate, subsequently complexing calcium and thereby reducing free calcium and increasing free oxalate in the colon.

The management of EH focuses on lowering diet oxalate intake and reducing colonic oxalate absorption. However, many patients find restricting oxalate intake to a therapeutic target level of approximately 50 mg/d (< 10 mg oxalate per mealtime) through avoidance of oxalate-rich foods (*e.g.*, rhubarb, spinach, sorrel, cocoa, chocolate, and cola)[58] difficult on an ongoing basis. Since steatorrhea leads to the complexation of calcium by free fatty acids in the colon, and thus to an increase in free oxalate concentration, reducing fat intake is a useful additional dietary intervention. As an alternative, medium chain fatty acids can be used as a substitute for long chain fatty acids[22,113]. As the mainstay of EH therapy, calcium (1000-1200 mg/d) should be orally substituted. If gastric suppression is required, calcium citrate supplements should be favored over the more commonly-used calcium carbonate salts, since the latter are insoluble at a neutral pH[113].

Choleretic diarrhea/bile acid loss syndrome

Colestyramine has been recommended on the basis of clinical consensus and practical experience to treat choleretic diarrhea associated with compensated bile acid malabsorption (*i.e.*, without significant steatorrhea)[49]. While few controlled studies of colestyramine have been conducted, a recent trial comparing the drug to hydroxypropyl cellulose (which is not a placebo) found colestyramine to have a significantly greater effect. A systematic review demonstrated a dose-response relationship between severity of bile acid diarrhea and treatment response, ranging from 96% in patients with severe to 70% in patients with mild bile acid diarrhea[114].

Newer bile acid sequestrants such as colestipol and colesevelam, which are much more selective bile acid binders than colestyramine[115], are reportedly associated with better compliance and fewer side effects[116,117]. The efficacy of colesevelam has now been confirmed in a first RCT[118]. Neither colesevelam nor colestyramine is suitable for the treatment of uncompensated bile acid loss syndrome, since the associated steatorrhea is exacerbated by both compounds (Table 6).

A promising therapeutic approach in the treatment of (decompensated) bile acid loss syndrome is the administration of cholylsarcosine, a conjugated bile acid that has no secretagogue effects. Smaller studies showed a significant increase in calcium and fat absorption in patients treated daily with 4-6 g cholylsarcosine[119-121].

A recent proof-of-concept study assessed the usefulness of obeticholic acid, a potent farnesoid x receptor agonist, in patients with bile acid diarrhea, and found statistically significant increases in fasting serum FGF19, both in patients with idiopathic bile acid diarrhea and in those with secondary disease and ileal resection of less than 45 cm [122].

Gallstones

Gallstones have been reported in 31%-45% of patients with SBS (with or without a colon). Reported risk factors are prolonged periods of starvation, large fluctuations in BW, medications such as opiates, and lipid emulsions (in TPN). In addition, diseases

Table 6 Major complications of short bowel syndrome: risk factors, prevention and treatment (adapted from Pironi *et al*[46], 2016)

	Risk factors	Prevention and/or treatment
Bacterial overgrowth/ miscolonization	Ileocecal valve resection; Reduced intestinal motility (Ogilvie syndrome; chronic intestinal pseudo-obstruction)	Metronidazole (500 mg, 2 times per day), vancomycin (125 mg, 4 times per day), neomycin (500 mg, 3 times per day), clindamycin (300 mg, 3 times per day) tetracycline (500 mg, 3 times per day), rifaximin (550 mg, 2 times per day)
Renal failure	Dehydration; CRBSI; Nephrocalcinosis; Kidney stones	Optimize fluid and sodium balance; Optimize CVC care; Prevent urinary calcium oxalate formation
Calcium oxalate, kidney stones	SBS with colon in continuity and fat malabsorption (enteric hyperoxaluria); Pyridoxine or thiamine deficiency; Excess of ascorbic acid; Dehydration; Low urinary citrate; Low urinary magnesium	Reduce or avoid excess lipid in the diet; Reduce food with high oxalate content; Oral calcium at mealtime (1 g); Oral cholestyramine; Optimize fluid balance; Optimize acid-base balance; Optimize magnesium status; Limit ascorbic acid supplementation
BAMS		
—Compensated	Extent of resection < 100 cm; Fecal bile acid excretion increased; Adequate hepatic compensation of bile acid loss; ≥ reduction of bile acid pool; no or minimal steatorrhea	Colestyramine/Colesevelam
—Decompensated	Extent of resection > 100 cm, fecal bile acid excretion increased; Inadequate hepatic compensation of bile acid loss; ≥ reduction of bile acid pool ≥ steatorrhea	Fat-modified/-reduced diet; Cholylsarcosine/ox gall[1]
Gallstones	Prolonged oral fasting; Interrupted bile acid entero-hepatic circulation; Prolonged treatment with anticholinergic and narcotic drugs	Limit periods of oral fasting; Limit narcotic or anticholinergic treatment; Use oral and/or enteral feeding as much as possible
IFALD-cholestasis	SBS with < 50 cm of residual small bowel; SBS without colon; CRBSI episodes; Chronic intraabdominal inflammation and/or small bowel bacterial overgrowth; Interrupted enterohepatic circulation of bile acid; Oral fasting; PN-overfeeding; i.v. soya-based lipid emulsion ≥ 1 g/kg/d	Avoid oral fasting; Optimize CVC care; Treat intraabdominal inflammation foci; Rehabilitative surgical procedures; Optimize i.v. feeding; i.v. soya-based lipid emulsion < 1 g/kg/d and/or i.v. fish oil lipid emulsion
D-lactic acid acidosis	SBS with a colon in continuity; Carbohydrate and soluble fiber-based diet; Ingestion of rapidly fermentable simple sugars; Feeding D-lactate containing food; High blood and urinary oxalate; Thiamine deficiency; Antibiotic and/or probiotic courses; Dehydration; Decreased renal function; Decreased liver function	Low carbohydrate and simple sugar diet; Antibiotics active against D-lactate-producing bacteria orally, such as metronidazole (500 mg, 2 times per day), vancomycin (125 mg, 4 times per day), neomycin (500 mg, 3 times per day), clindamycin (300 mg, 3 times per day), tetracycline (500 mg, 3 times per day), rifaximin (550 mg, 2 times a day); Thiamine supplementation; Reduction of oxalate absorption; Optimize fluid balance

[1]If colon has been removed or disabled. BAMS: Bile acid malabsorption syndrome; CRBSI: Catheter-related bloodstream infection; CVC: Central venous catheter; i.v.: Intravenous; PN: Parenteral nutrition; PPI: Proton pump inhibitor; PTH: Parathyroid hormone; SBS: Short bowel syndrome.

affecting the ileum (*e.g.*, CD) and absence of ileum and/or ileocecal valve following surgical resection alter enterohepatic circulation and cause a loss of bile salts (Table 7), thus leading to cholesterol supersaturation and sludge formation[123-125].

Small intestinal bacterial overgrowth

Especially after ileocecal valve resection, patients with CIF are at risk of small intestinal bacterial overgrowth (SIBO), *i.e.*, the miscolonization of the upper small bowel by colon-derived bacteria. By promoting bile acid deconjugation, SIBO not only exacerbates steatorrhea, thereby disrupting the absorption of fat and fat-soluble vitamins[126,127], but also hinders intestinal adaptation[127,128]. SIBO is also perceived as a predisposing factor for intestinal failure-associated liver disease (IFALD), since hepatotoxins (*e.g.*, lithocholic acid) produced by anaerobic bacteria in the small bowel may lead to hepatic injury. While diagnosis can be made using H^2 or C^{13} breath tests, their sensitivity is not very high[127]. Some authors therefore suggest a "therapeutic trial" approach on the basis of clinical suspicion, even in non-responders[62,129,130]. Adjunctive intermitting-alternating antibiotic therapy should be administered for a period of 7-10 d (Table 6)[63].

D-lactic acidosis

D-lactic acidosis is a rare, but often overlooked, condition observed only in patients with a preserved colon. It is caused by an increased intake of refined carbohydrates that, having been broken down by bacteria into short-chain fatty acids, lactate when they pass into the colon. The associated decrease in colonic pH promotes the growth of gram-positive, acid-resistant, D-lactate producing anaerobes (*e.g.*, *Bifidobacterium*,

Table 7 Pathophysiological characteristics of short bowel syndrome with and without colon in continuity (adapted from Pironi *et al*[46], 2016)

Characteristic	End-jejunostomy	Jejunocolic or jejunoileal anastomosis
Structural and functional adaptation, to increase nutrient absorption	No evidence thereof at any time after surgery	Possible up to 2 yr after surgery
Gastric hypersecretion (up to 6 mo after resection)	Present	Present
Gastric emptying and small bowel transit	Accelerated gastric emptying for liquids Accelerated small bowel transit	Slowed
GI hormone secretion (PYY, GLP-1, GLP-2)	Decreased/absent	Increased
Energy absorption from microbiota SCFA, production in the colon	Absent	Increased up to 1000 kcal (4.2 MJ) per day
Water and sodium absorption in the remnant small bowel	Possible "net secretion" when jejunum length < 100 cm (more fluid and sodium lost than ingested)	Colon adaptation can increase the absorption of water up to 6 liters and sodium up to 800 mmol per day
Vitamin B12 and bile salt absorption	Absent	Partially conserved or absent
Magnesium absorption	Decreased	Decreased
Remnant small bowel cut-off length for HPN weaning	> 115 cm	Jejunocolic anastomosis > 60 cm Jejunoileal anastomosis with ICV and entire colon > 35 cm

GI: Gastrointestinal; GLP: Glucagon-like peptide; HPN: Home parenteral nutrition; IVC: Ileocecal valve; PYY: Peptide tyrosine-tyrosine; SCFA: Short chain fatty acid.

Lactobacillus, Eubacteriaceae). This results in insufficient metabolism of human D-lactate, and D-lactic acidosis occurs, with associated neurological symptoms such as vision disturbances, confusion, and gait insecurity. Such symptoms are often mistakenly assumed to be signs of alcohol abuse. Diagnosis is made by determining D-lactate levels in the blood[131,132]. The pathogenesis of neurological symptoms associated with D-lactate acidosis remains unclear. One hypothesis is that D-lactate itself is toxic to the brain or alters neuro-transmitter production. Other potentially neurotoxic substances or false neurotransmitters produced in variable quantities during periods of D-lactate elevation may also be involved[46] (Table 6). Due to the similarity of symptoms of D-lactic acidosis to those of Wernicke encephalopathy, prophylactic administration of thiamine has been recommended[131,132].

Diseases of the bone metabolism

Diseases of the bone metabolism, such as osteoporosis and osteomalacia, may occur as long-term complications of TPN and have been reported to occur in 40%-100% of patients with CIF[133-135]. The main causal factors include not only malabsorption of calcium, magnesium[136], and vitamin D but also the presence of underlying inflammatory disease (*e.g.*, CD[137]), combined, as a rule, with many years of steroid intake [135]. Patients on long-term HPN are recommended to undergo bone density testing every 2 years, with frequent assessment of calcium and phosphorus balance, as well as vitamin D levels. Vitamin D substitution should be orientated at 25-hydroxy levels > 30 ng/mL[138].

MANAGEMENT OF THERAPY-RELATED COMPLICATIONS

Therapy-related complications most commonly arise from long-term HPN. While catheter-associated complications are frequent, a variety of organic and metabolic disorders may also occur (for detailed review, see[139]).

Catheter-associated complications

Direct catheter-associated complications in patients receiving long-term HPN can be characterized as either catheter occlusion or thrombotic or infectious complications.

Catheter occlusion can be partial or complete. Factors that influence its occurrence include duration of catheter placement, catheter size and material, type and meticulousness of care of the central venous access route, and the composition of applied infusion solutions. Signs and symptoms of occlusion include resistance when flushing, sluggish flow, inability to infuse fluids, frequent occlusion alarm on the infusion pump, infiltration, extravasation, swelling or leaking at the insertion site upon infusion or during flushing, inability to withdraw blood, and sluggish blood return upon aspiration of blood[140-143].

Possible causes of catheter occlusion include: (1) mechanical problems; (2) non-thrombotic obstruction (precipitate of formulations such as medications or PN constituents within the catheter lumen); and (3) thrombotic obstruction (clot, thrombus, or fibrin deposition).

Any obvious mechanical obstruction should be ruled out before checking for non-thrombotic and thrombotic occlusion[144]. Mechanical occlusions arise from internal or external problems with the catheter, such as kinking in the catheter or tubing, catheter migration or malpositioning, clogging of the cap/needleless connector or filter, or excessive tightness of the retaining suture[145]. Depending on its underlying cause(s), mechanical occlusion can be remedied by removing the venous catheters, avoiding twisting/kinking when dressing, or, if occlusion or malpositioning of the needle is suspected, replacing the non-coring needle.

Catheter-associated infections (CAI) — in particular, catheter-related bloodstream infections (CRBSIs) — remain the "Achilles' heel" of HPN treatment. Infections are classified as follows: Local infections: Catheter exit site infections, port recess infections, and tunnel infections in long-term subcutaneous tunnel catheters (Hickmann catheters); CRBSI: Defined as an infection in which the same organism can be isolated in cultures from the catheter and from peripheral blood, and clinical symptoms of sepsis are present, in the absence of an alternative focus of infection. CRBSI occurs in association with 5%-10% of all central venous catheters, equivalent to 0.3-30 cases/1000 catheter d, and accounts for approximately 70% of all home PN-related hospital admissions[146-148].

More than 50% of all CAIs are caused by gram-positive pathogens, 33% by coagulase-negative staphylococci, and approximately 20%-22% by pathogenic fungi. About a quarter are mixed infections[149]. The chances of saving an infected (tunneled) catheter vary from 50% to 85%, depending on the pathogen responsible[49].

Different catheter-locking solutions have been studied for their effectiveness in preventing CRBSIs, including antiseptic agents, antibiotics, and anticoagulants. Due to the supposed need for an anticoagulant, heparin has most commonly been used as a catheter-locking solution. Its use, however, is no longer recommended, as it has been shown to potentially increase the risk of CRBSI by promoting the formation of an intraluminal biofilm[1,150,151].

Based on its ability to prevent microbial adhesion to the inner catheter surface, and to destroy microbial cell membranes and toxins, use of the broad-spectrum antiseptic agent taurolidine as a catheter-locking solution was described in 1998. Subsequent studies and a meta-analysis confirmed that, compared with heparin, taurolidine decreases CRBSI incidence in patients with catheters. Based on these data and the most recently published RCT by Wouters *et al*[152], and in view of its favorable safety and cost profile, taurolidine solution (*e.g.*, 0.2%) can be applied as a secondary prophylactic locking solution as soon as infection occurs and may also be used for primary CRBSI prophylaxis. However, effective training of the patient (and/or their family) and nursing staff is recognized as the single most effective primary prophylaxis[1,153].

IFALD

Based on the multifactorial nature of IFALD, a recent international position paper suggested defining it as "liver injury as a result of one or more factors relating to intestinal failure including, but not limited to, PN and occurring in the absence of another primary parenchymal liver pathology"[154,155]. Based on retrospective data in adult patients with CIF, Sasdelli *et al*[156] recently reported an IFALD prevalence of 13%-40% for cholestasis, 27%-90% for steatosis, 2%-5% for fibrosis, and 8%-75% for unclassified IFALD, depending on the criteria adopted.

The pathogenesis of IFALD is not yet fully understood and likely multifactorial, with both PN and patient-dependent associated factors playing a role (Table 8)[123,157]. Recommended measures for the prevention and therapy of IFALD are[158,159]: (1) maintenance of suitable proportions of nutrients, avoiding in particular high-calorie and high-glucose feeds, and insulin application, with 20% up to maximum 50% of total energy intake as lipids (approximately 1g fat/kg BW); (2) cyclical infusion feeding periods of 12-16 h, maintaining an overnight fasting period of 8-12 h; (3)

Table 8 Intestinal failure-associated liver disease risk factors in adult patients with home-based parenteral nutrition (adapted from Van Gossum *et al*[157], 2019)

PN-related	Patient-related
Energy overfeeding	Lack of oral feeding
Glucose overload > 7 mg/kg/min	Short bowel syndrome (small bowel remnant < 50 cm)
Lipid emulsion overload	Inflammation/infection
Soya-based lipid emulsion > 1 g/kg/d	Sepsis (*e.g.*, central venous catheter related)
Continuous infusion (24/24 h)	Small intestinal bacterial overgrowth
Contaminants (phytosterols)	Gut inflammation
Antioxidant deficiency	Viral infection (*e.g.*, hepatitis B, C)
Nutrient deficiency (choline, carnitine, methionine, taurine, essential fatty acid deficiency, *etc.*)	Autoimmune
	Hepatotoxic medications

PN: Parenteral nutrition.

enteral feeding, even if the quantities are insufficient to cover requirements, *e.g.*, as a supplement; (4) in patients with cholestasis and/or "sludge" production, administration of ursodesoxycholic acid (10-15 mg/kg BW, *e.g.*, as juice) or cholecystokinin (cave: possibility of anaphylactoid reaction), with additional dose reduction of fat-soluble vitamins, copper, and manganese; and (5) administration of "essential" substrates such as carnitine, taurine, or choline in infants and children. On the basis of affirmative results from a number of randomized studies, fish oil-based lipid solutions are recommended for IFALD therapy in children. Furthermore, an initial study showed a positive effect of fish oil on hepatopathy in adults with CIF[158] (for review, see[160]). Medium-chain triglyceride- and/or olive oil-based lipid solutions are not recommended either for primary or secondary prophylaxis of IFALD[158]. The same applies to probiotics.

SURGICAL PROCEDURES

Reconstructive surgery

A range of operative techniques have been developed over the past years to allow patients with CIF at least the possibility of enteral feeding. These techniques have two common goals: To increase gastrointestinal transit duration and to increase the resorptive surface of the bowel. In principle, three surgical procedures may be applied (for review, see[161-163]): (1) in cases where bowel length is critical, longitudinal lengthening and tailoring, first described by Bianchi in 1980, accomplishes intestinal tapering without loss of surface; (2) in the serial transverse enteroplasty procedure described by Kim *et al*[164] in 2003, the intestinal lumen is narrowed by firing a series of staples perpendicularly to the long axis of the bowel in a zig-zag pattern; (3) if the remaining small bowel is too short (*i.e.*, < 70-80 cm) to achieve weaning from TPN, a reversed antiperistaltic intestinal segment (10-15 cm in adults) can be placed to slow intestinal transit and thereby enhance nutrient and fluid absorption.

Intestinal transplantation

Intestinal transplantation, alone or in combination with other organs (visceral transplantation), is reserved for patients with life-threatening complications of PN defined by significant liver injury with elevated hepatic enzymes, multiple line infections, a single episode of life-threatening catheter-related sepsis, thrombosis of two of the central veins, or frequent episodes of dehydration (for review, see[165]).

CONCLUSION

While considerable advances have been made in recent years with regard to the

understanding and management of CIF and its various related complications, it remains a challenging disorder. Home-based PN has brought distinct benefits, particularly in terms of higher survival rates and improved patient quality of life. Nevertheless, more than half of patients with CIF require PN on a permanent basis. While the traditional mainstays of pharmacological management are antimotility and antisecretory drugs, new hormonally acting drugs that stimulate intestinal adaptation, such as teduglutide, have had encouraging results, adding a new dimension to CIF therapy. More work is needed to improve understanding of CIF mechanisms, and to advance preventive and therapeutic approaches. Optimization of patient outcomes in complex CIF requires a dedicated multidisciplinary team of physicians, dietitians, and nurses, whose foci of care include maximizing intestinal rehabilitation, providing nutritional support, and improving quality of life.

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REFERENCES

1 **Pironi L**, Arends J, Bozzetti F, Cuerda C, Gillanders L, Jeppesen PB, Joly F, Kelly D, Lal S, Staun M, Szczepanek K, Van Gossum A, Wanten G, Schneider SM; Home Artificial Nutrition & Chronic Intestinal Failure Special Interest Group of ESPEN. ESPEN guidelines on chronic intestinal failure in adults. *Clin Nutr* 2016; **35**: 247-307 [PMID: 26944585 DOI: 10.1016/j.clnu.2016.01.020]

2 **Pironi L**, Arends J, Baxter J, Bozzetti F, Peláez RB, Cuerda C, Forbes A, Gabe S, Gillanders L, Holst M, Jeppesen PB, Joly F, Kelly D, Klek S, Irtun Ø, Olde Damink SW, Panisic M, Rasmussen HH, Staun M, Szczepanek K, Van Gossum A, Wanten G, Schneider SM, Shaffer J; Home Artificial Nutrition & Chronic Intestinal Failure; Acute Intestinal Failure Special Interest Groups of ESPEN. ESPEN endorsed recommendations. Definition and classification of intestinal failure in adults. *Clin Nutr* 2015; **34**: 171-180 [PMID: 25311444 DOI: 10.1016/j.clnu.2014.08.017]

3 **Massironi S**, Cavalcoli F, Rausa E, Invernizzi P, Braga M, Vecchi M. Understanding short bowel syndrome: Current status and future perspectives. *Dig Liver Dis* 2020; **52**: 253-261 [PMID: 31892505 DOI: 10.1016/j.dld.2019.11.013]

4 **Shaffer J**. Intestinal failure: definition and service development. *Clin Nutr* 2002; **21**: 144e145 [DOI: 10.1016/S0261-5614(02)80034-6]

5 **Pironi L**, Konrad D, Brandt C, Joly F, Wanten G, Agostini F, Chambrier C, Aimasso U, Zeraschi S, Kelly D, Szczepanek K, Jukes A, Di Caro S, Theilla M, Kunecki M, Daniels J, Serlie M, Poullenot F, Wu J, Cooper SC, Rasmussen HH, Compher C, Seguy D, Crivelli A, Pagano MC, Hughes SJ, Guglielmi FW, Kozjek NR, Schneider SM, Gillanders L, Ellegard L, Thibault R, Matras P, Zmarzly A, Matysiak K, Van Gossum A, Forbes A, Wyer N, Taus M, Virgili NM, O'Callaghan M, Chapman B, Osland E, Cuerda C, Sahin P, Jones L, Lee ADW, Bertasi V, Orlandoni P, Izbéki F, Spaggiari C, Díez MB, Doitchinova-Simeonova M, Garde C, Serralde-Zúñiga AE, Olveira G, Krznaric Z, Czako L, Kekstas G, Sanz-Paris A, Jáuregui EP, Murillo AZ, Schafer E, Arends J, Suárez-Llanos JP, Shaffer J, Lal S. Clinical classification of adult patients with chronic intestinal failure due to benign disease: An international multicenter cross-sectional survey. *Clin Nutr* 2018; **37**: 728-738 [PMID: 28483328 DOI: 10.1016/j.clnu.2017.04.013]

6 **Dibb M**, Soop M, Teubner A, Shaffer J, Abraham A, Carlson G, Lal S. Survival and nutritional dependence on home parenteral nutrition: Three decades of experience from a single referral centre. *Clin Nutr* 2017; **36**: 570-576 [PMID: 26972088 DOI: 10.1016/j.clnu.2016.01.028]

7 **Lloyd DA**, Vega R, Bassett P, Forbes A, Gabe SM. Survival and dependence on home parenteral nutrition: experience over a 25-year period in a UK referral centre. *Aliment Pharmacol Ther* 2006; **24**: 1231-1240 [PMID: 17014582 DOI: 10.1111/j.1365-2036.2006.03106.x]

8 **Watanabe Y**, Miyoshi N, Fujino S, Takahashi H, Haraguchi N, Hata T, Matsuda C, Yamamoto H, Doki Y, Mori M, Mizushima T. Cumulative Inflammation Could Be a Risk Factor for Intestinal Failure in Crohn's Disease. *Dig Dis Sci* 2019; **64**: 2280-2285 [PMID: 30806860 DOI: 10.1007/s10620-019-05553-2]

9 **Watanabe K**, Sasaki I, Fukushima K, Futami K, Ikeuchi H, Sugita A, Nezu R, Mizushima T, Kameoka S, Kusunoki M, Yoshioka K, Funayama Y, Watanabe T, Fujii H, Watanabe M. Long-term incidence and characteristics of intestinal failure in Crohn's disease: a multicenter study. *J Gastroenterol* 2014; **49**: 231-238 [PMID: 23564229 DOI: 10.1007/s00535-013-0797-y]

10 **Fumery M**, Seksik P, Auzolle C, Munoz-Bongrand N, Gornet JM, Boschetti G, Cotte E, Buisson A, Dubois A, Pariente B, Zerbib P, Chafai N, Stefanescu C, Panis Y, Marteau P, Pautrat K, Sabbagh C, Filippi J, Chevrier M, Houze P, Jouven X, Treton X, Allez M; REMIND study group investigators. Postoperative Complications after Ileocecal Resection in Crohn's Disease: A Prospective Study From

the REMIND Group. *Am J Gastroenterol* 2017; **112**: 337-345 [PMID: 27958285 DOI: 10.1038/ajg.2016.541]
11 **Alves A**, Panis Y, Bouhnik Y, Pocard M, Vicaut E, Valleur P. Risk factors for intra-abdominal septic complications after a first ileocecal resection for Crohn's disease: a multivariate analysis in 161 consecutive patients. *Dis Colon Rectum* 2007; **50**: 331-336 [PMID: 17252288 DOI: 10.1007/s10350-006-0782-0]
12 **Yamamoto T**, Allan RN, Keighley MR. Risk factors for intra-abdominal sepsis after surgery in Crohn's disease. *Dis Colon Rectum* 2000; **43**: 1141-1145 [PMID: 10950014 DOI: 10.1007/bf02236563]
13 **Tzivanakis A**, Singh JC, Guy RJ, Travis SP, Mortensen NJ, George BD. Influence of risk factors on the safety of ileocolic anastomosis in Crohn's disease surgery. *Dis Colon Rectum* 2012; **55**: 558-562 [PMID: 22513434 DOI: 10.1097/DCR.0b013e318247c433]
14 **Neary PM**, Aiello AC, Stocchi L, Shawki S, Hull T, Steele SR, Delaney CP, Holubar SD. High-Risk Ileocolic Anastomoses for Crohn's Disease: When Is Diversion Indicated? *J Crohns Colitis* 2019; **13**: 856-863 [PMID: 31329836 DOI: 10.1093/ecco-jcc/jjz004]
15 **Lau C**, Dubinsky M, Melmed G, Vasiliauskas E, Berel D, McGovern D, Ippoliti A, Shih D, Targan S, Fleshner P. The impact of preoperative serum anti-TNFα therapy levels on early postoperative outcomes in inflammatory bowel disease surgery. *Ann Surg* 2015; **261**: 487-496 [PMID: 24950263 DOI: 10.1097/SLA.0000000000000757]
16 **Huang W**, Tang Y, Nong L, Sun Y. Risk factors for postoperative intra-abdominal septic complications after surgery in Crohn's disease: A meta-analysis of observational studies. *J Crohns Colitis* 2015; **9**: 293-301 [PMID: 25572276 DOI: 10.1093/ecco-jcc/jju028]
17 **Brouquet A**, Maggiori L, Zerbib P, Lefevre JH, Denost Q, Germain A, Cotte E, Beyer-Berjot L, Munoz-Bongrand N, Desfourneaux V, Rahili A, Duffas JP, Pautrat K, Denet C, Bridoux V, Meurette G, Faucheron JL, Loriau J, Guillon F, Vicaut E, Benoist S, Panis Y; GETAID chirurgie group. Anti-TNF Therapy Is Associated With an Increased Risk of Postoperative Morbidity After Surgery for Ileocolonic Crohn Disease: Results of a Prospective Nationwide Cohort. *Ann Surg* 2018; **267**: 221-228 [PMID: 29300710 DOI: 10.1097/SLA.0000000000002017]
18 **Sørensen LT**, Jørgensen T, Kirkeby LT, Skovdal J, Vennits B, Wille-Jørgensen P. Smoking and alcohol abuse are major risk factors for anastomotic leakage in colorectal surgery. *Br J Surg* 1999; **86**: 927-931 [PMID: 10417567 DOI: 10.1046/j.1365-2168.1999.01165.x]
19 **Morar PS**, Hodgkinson JD, Thalayasingam S, Koysombat K, Purcell M, Hart AL, Warusavitarne J, Faiz O. Determining Predictors for Intra-abdominal Septic Complications Following Ileocolonic Resection for Crohn's Disease-Considerations in Pre-operative and Peri-operative Optimisation Techniques to Improve Outcome. *J Crohns Colitis* 2015; **9**: 483-491 [PMID: 25796553 DOI: 10.1093/ecco-jcc/jjv051]
20 **Lin YS**, Cheng SW, Wang YH, Chen KH, Fang CJ, Chen C. Systematic review with meta-analysis: risk of post-operative complications associated with pre-operative exposure to anti-tumour necrosis factor agents for Crohn's disease. *Aliment Pharmacol Ther* 2019; **49**: 966-977 [PMID: 30864199 DOI: 10.1111/apt.15184]
21 **Soop M**, Khan H, Nixon E, Teubner A, Abraham A, Carlson G, Lal S. Causes and Prognosis of Intestinal Failure in Crohn's Disease: An 18-year Experience From a National Centre. *J Crohns Colitis* 2020; **14**: 1558-1564 [PMID: 32215559 DOI: 10.1093/ecco-jcc/jjaa060]
22 **Baker ML**, Williams RN, Nightingale JM. Causes and management of a high-output stoma. *Colorectal Dis* 2011; **13**: 191-197 [PMID: 19888956 DOI: 10.1111/j.1463-1318.2009.02107.x]
23 **Tappenden KA**. Intestinal adaptation following resection. *JPEN J Parenter Enteral Nutr* 2014; **38**: 23S-31S [PMID: 24586019 DOI: 10.1177/0148607114525210]
24 **Ukleja A**. Weaning from Parenteral Nutrition. *Gastroenterol Clin North Am* 2019; **48**: 525-550 [PMID: 31668181 DOI: 10.1016/j.gtc.2019.08.007]
25 **Stein J**, Purschian B, Bieniek U, Caspary WF, Lembcke B. Near-infrared reflectance analysis: a new dimension in the investigation of malabsorption syndromes. *Eur J Gastroen Hepat* 1994; **6**: 889-894 [DOI: 10.1097/00042737-199410000-00009]
26 **Picarelli A**, Greco M, Di Giovambattista F, Ramazzotti A, Cedrone C, Corazziari E, Torsoli A. Quantitative determination of faecal fat, nitrogen and water by means of a spectrophotometric technique: near infrared reflectance analysis (NIRA). Assessment of its accuracy and reproducibility compared with chemical methods. *Clin Chim Acta* 1995; **234**: 147-156 [PMID: 7758214 DOI: 10.1016/0009-8981(94)05993-3]
27 **Jeppesen PB**, Gabe SM, Seidner DL, Lee HM, Olivier C. Citrulline correlations in short bowel syndrome-intestinal failure by patient stratification: Analysis of 24 weeks of teduglutide treatment from a randomized controlled study. *Clin Nutr* 2020; **39**: 2479-2486 [PMID: 31784300 DOI: 10.1016/j.clnu.2019.11.001]
28 **Crenn P**, Messing B, Cynober L. Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. *Clin Nutr* 2008; **27**: 328-339 [PMID: 18440672 DOI: 10.1016/j.clnu.2008.02.005]
29 **Lappas BM**, Patel D, Kumpf V, Adams DW, Seidner DL. Parenteral Nutrition: Indications, Access, and Complications. *Gastroenterol Clin North Am* 2018; **47**: 39-59 [PMID: 29413018 DOI: 10.1016/j.gtc.2017.10.001]
30 **Kumpf VJ**. Pharmacologic management of diarrhea in patients with short bowel syndrome. *JPEN J Parenter Enteral Nutr* 2014; **38**: 38S-44S [PMID: 24463352 DOI: 10.1177/0148607113520618]
31 **Dibb M**, Lal S. Monitoring long-term parenteral nutrition. *Curr Opin Gastroenterol* 2019; **35**: 119-

125 [PMID: 30632989 DOI: 10.1097/MOG.0000000000000504]
32 **Jeppesen PB**, Fuglsang KA. Nutritional Therapy in Adult Short Bowel Syndrome Patients with Chronic Intestinal Failure. *Gastroenterol Clin North Am* 2018; **47**: 61-75 [PMID: 29413019 DOI: 10.1016/j.gtc.2017.10.004]
33 **Austin K**, Bonnes S, Daniel H. Controversy in Nutrition Recommendations for Short Bowel Syndrome: How Type of SBS Impacts Response. *Curr Gastroenterol Rep* 2019; **21**: 64 [PMID: 31808005 DOI: 10.1007/s11894-019-0731-4]
34 **Ukleja A**, Scolapio JS, Buchman AL. Nutritional management of short bowel syndrome. *Semin Gastrointest Dis* 2002; **13**: 161-168 [PMID: 12230319]
35 **Levitt DG**, Levitt MD. Protein losing enteropathy: comprehensive review of the mechanistic association with clinical and subclinical disease states. *Clin Exp Gastroenterol* 2017; **10**: 147-168 [PMID: 28761367 DOI: 10.2147/CEG.S136803]
36 **Hessov I**, Andersson H, Isaksson B. Effects of a low-fat diet on mineral absorption in small-bowel disease. *Scand J Gastroenterol* 1983; **18**: 551-554 [PMID: 6669931 DOI: 10.3109/00365528309181636]
37 **Nightingale JM**, Lennard-Jones JE, Gertner DJ, Wood SR, Bartram CI. Colonic preservation reduces need for parenteral therapy, increases incidence of renal stones, but does not change high prevalence of gall stones in patients with a short bowel. *Gut* 1992; **33**: 1493-1497 [PMID: 1452074 DOI: 10.1136/gut.33.11.1493]
38 **Jeppesen PB**, Mortensen PB. Significance of a preserved colon for parenteral energy requirements in patients receiving home parenteral nutrition. *Scand J Gastroenterol* 1998; **33**: 1175-1179 [PMID: 9867096 DOI: 10.1080/00365529850172539]
39 **Miranda SC**, Ribeiro ML, Ferriolli E, Marchini JS. Hypomagnesemia in short bowel syndrome patients. *Sao Paulo Med J* 2000; **118**: 169-172 [PMID: 11120547 DOI: 10.1590/s1516-31802000000600004]
40 **Tappenden KA**. Pathophysiology of short bowel syndrome: considerations of resected and residual anatomy. *JPEN J Parenter Enteral Nutr* 2014; **38**: 14S-22S [PMID: 24500909 DOI: 10.1177/0148607113520005]
41 **Nightingale JM**, Lennard-Jones JE, Walker ER, Farthing MJ. Oral salt supplements to compensate for jejunostomy losses: comparison of sodium chloride capsules, glucose electrolyte solution, and glucose polymer electrolyte solution. *Gut* 1992; **33**: 759-761 [PMID: 1624155 DOI: 10.1136/gut.33.6.759]
42 **Atia AN**, Buchman AL. Oral rehydration solutions in non-cholera diarrhea: a review. *Am J Gastroenterol* 2009; **104**: 2596-604; quiz 2605 [PMID: 19550407 DOI: 10.1038/ajg.2009.329]
43 **Buchman AL**, Scolapio J, Fryer J. AGA technical review on short bowel syndrome and intestinal transplantation. *Gastroenterology* 2003; **124**: 1111-1134 [PMID: 12671904 DOI: 10.1016/s0016-5085(03)70064-x]
44 **Leiss O**. [Clinical management of patients with short bowel syndrome]. *Z Gastroenterol* 2005; **43**: 661-675 [PMID: 16001349 DOI: 10.1055/s-2005-857923]
45 **Jeppesen PB**. Spectrum of short bowel syndrome in adults: intestinal insufficiency to intestinal failure. *JPEN J Parenter Enteral Nutr* 2014; **38**: 8S-13S [PMID: 24486858 DOI: 10.1177/0148607114520994]
46 **Pironi L**. Definitions of intestinal failure and the short bowel syndrome. *Best Pract Res Clin Gastroenterol* 2016; **30**: 173-185 [PMID: 27086884 DOI: 10.1016/j.bpg.2016.02.011]
47 **Ladefoged K**, Olgaard K. Sodium homeostasis after small-bowel resection. *Scand J Gastroenterol* 1985; **20**: 361-369 [PMID: 3890141 DOI: 10.3109/00365528509091665]
48 **Lauverjat M**, Hadj Aissa A, Vanhems P, Boulétreau P, Fouque D, Chambrier C. Chronic dehydration may impair renal function in patients with chronic intestinal failure on long-term parenteral nutrition. *Clin Nutr* 2006; 25: 75-81 [PMID: 16356596 DOI: 10.1016/j.clnu.2005.09.010]
49 **Lamprecht G**, Pape UF, Witte M, Pascher A; Committee udDS. S3-Leitlinie der Deutschen Gesellschaft für Ernährungsmedizin e.V. in Zusammenarbeit mit der AKE, der GESKES und der DGVS Klinische Ernährung in der Gastroenterologie (Teil 3) – Chronisches Darmversagen. *Aktuel Ernahrungsmed* 2014; **39**: e57-e71 [DOI: 10.1055/s-0034-1369922]
50 **Nightingale J**, Woodward JM; Small Bowel and Nutrition Committee of the British Society of Gastroenterology. Guidelines for management of patients with a short bowel. *Gut* 2006; **55** Suppl 4: iv1-i12 [PMID: 16837533 DOI: 10.1136/gut.2006.091108]
51 **Jeppesen PB**, Mortensen PB. Intestinal failure defined by measurements of intestinal energy and wet weight absorption. *Gut* 2000; **46**: 701-706 [PMID: 10764715 DOI: 10.1136/gut.46.5.701]
52 **Martin KJ**, González EA, Slatopolsky E. Clinical consequences and management of hypomagnesemia. *J Am Soc Nephrol* 2009; **20**: 2291-2295 [PMID: 18235082 DOI: 10.1681/ASN.2007111194]
53 **Musso CG**. Magnesium metabolism in health and disease. *Int Urol Nephrol* 2009; 41: 357-362 [PMID: 19274487 DOI: 10.1007/s11255-009-9548-7]
54 **Fleming CR**, George L, Stoner GL, Tarrosa VB, Moyer TP. The importance of urinary magnesium values in patients with gut failure. *Mayo Clin Proc* 1996; **71**: 21-24 [PMID: 8538226 DOI: 10.4065/71.1.21]
55 **Pape UF**, Weylandt KH, Knappe-Drzikova B, Gerlach U, Pascher A. Kurzdarmsyndrom und Darmversagen: Diagnostik und Therapie. *Aktuel Ernahrungsmed* 2013; **38**: 132-146 [DOI: 10.1055/s-0032-1332951]

56 **Walker AF**, Marakis G, Christie S, Byng M. Mg citrate found more bioavailable than other Mg preparations in a randomised, double-blind study. *Magnes Res* 2003; **16**: 183-191 [PMID: 14596323]

57 **Blumenstein I**, Shastri YM, Stein J. Gastroenteric tube feeding: techniques, problems and solutions. *World J Gastroenterol* 2014; **20**: 8505-8524 [PMID: 25024606 DOI: 10.3748/wjg.v20.i26.8505]

58 **Yang CF**, Duro D, Zurakowski D, Lee M, Jaksic T, Duggan C. High prevalence of multiple micronutrient deficiencies in children with intestinal failure: a longitudinal study. *J Pediatr* 2011; **159**: 39-44. e1 [PMID: 21324480 DOI: 10.1016/j.jpeds.2010.12.049]

59 **Ubesie AC**, Heubi JE, Kocoshis SA, Henderson CJ, Mezoff AG, Rao MB, Cole CR. Vitamin D deficiency and low bone mineral density in pediatric and young adult intestinal failure. *J Pediatr Gastroenterol Nutr* 2013; **57**: 372-376 [PMID: 23698025 DOI: 10.1097/MPG.0b013e31829c10eb]

60 **Mziray-Andrew CH**, Sentongo TA. Nutritional deficiencies in intestinal failure. *Pediatr Clin North Am* 2009; **56**: 1185-1200 [PMID: 19931070 DOI: 10.1016/j.pcl.2009.07.005]

61 **Ubesie AC**, Kocoshis SA, Mezoff AG, Henderson CJ, Helmrath MA, Cole CR. Multiple micronutrient deficiencies among patients with intestinal failure during and after transition to enteral nutrition. *J Pediatr* 2013; **163**: 1692-1696 [PMID: 23978355 DOI: 10.1016/j.jpeds.2013.07.015]

62 **Khaodhiar L**, Keane-Ellison M, Tawa NE, Thibault A, Burke PA, Bistrian BR. Iron deficiency anemia in patients receiving home total parenteral nutrition. *JPEN J Parenter Enteral Nutr* 2002; **26**: 114-119 [PMID: 11871735 DOI: 10.1177/0148607102026002114]

63 **Hwa YL**, Rashtak S, Kelly DG, Murray JA. Iron Deficiency in Long-Term Parenteral Nutrition Therapy. *JPEN J Parenter Enteral Nutr* 2016; **40**: 869-876 [PMID: 25972429 DOI: 10.1177/0148607115587329]

64 **Allwood MC**, Martin H, Greenwood M, Maunder M. Precipitation of trace elements in parenteral nutrition mixtures. *Clin Nutr* 1998; **17**: 223-226 [PMID: 10205342 DOI: 10.1016/s0261-5614(98)80063-0]

65 **Robinson CA**, Sawyer JE. Y-site compatibility of medications with parenteral nutrition. *J Pediatr Pharmacol Ther* 2009; **14**: 48-56 [PMID: 23055891 DOI: 10.5863/1551-6776-14.1.48]

66 **Evstatiev R**, Marteau P, Iqbal T, Khalif IL, Stein J, Bokemeyer B, Chopey IV, Gutzwiller FS, Riopel L, Gasche C; FERGI Study Group. FERGIcor, a randomized controlled trial on ferric carboxymaltose for iron deficiency anemia in inflammatory bowel disease. *Gastroenterology* 2011; **141**: 846-853. e1-2 [PMID: 21699794 DOI: 10.1053/j.gastro.2011.06.005]

67 **Wolman SL**, Anderson GH, Marliss EB, Jeejeebhoy KN. Zinc in total parenteral nutrition: requirements and metabolic effects. *Gastroenterology* 1979; **76**: 458-467 [PMID: 107057]

68 **Jeejeebhoy K**. Zinc: an essential trace element for parenteral nutrition. *Gastroenterology* 2009; **137**: S7-12 [PMID: 19874952 DOI: 10.1053/j.gastro.2009.08.014]

69 **Okawada M**, Holst JJ, Teitelbaum DH. Administration of a dipeptidyl peptidase IV inhibitor enhances the intestinal adaptation in a mouse model of short bowel syndrome. *Surgery* 2011; **150**: 217-223 [PMID: 21719060 DOI: 10.1016/j.surg.2011.05.013]

70 **Coughlin S**, Roth L, Lurati G, Faulhaber M. Somatostatin analogues for the treatment of enterocutaneous fistulas: a systematic review and meta-analysis. *World J Surg* 2012; **36**: 1016-1029 [PMID: 22419412 DOI: 10.1007/s00268-012-1494-3]

71 **Stein J**, Stier C, Raab H, Weiner R. Review article: The nutritional and pharmacological consequences of obesity surgery. *Aliment Pharmacol Ther* 2014; **40**: 582-609 [PMID: 25078533 DOI: 10.1111/apt.12872]

72 **Hvistendahl M**, Brandt CF, Tribler S, Naimi RM, Hartmann B, Holst JJ, Rehfeld JF, Hornum M, Andersen JR, Henriksen BM, Brøbech Mortensen P, Jeppesen PB. Effect of Liraglutide Treatment on Jejunostomy Output in Patients With Short Bowel Syndrome: An Open-Label Pilot Study. *JPEN J Parenter Enteral Nutr* 2018; **42**: 112-121 [PMID: 27875281 DOI: 10.1177/0148607116672265]

73 **Pape UF**, Maasberg S, Pascher A. Pharmacological strategies to enhance adaptation in intestinal failure. *Curr Opin Organ Transplant* 2016; **21**: 147-152 [PMID: 26881493 DOI: 10.1097/MOT.0000000000000296]

74 **Billiauws L**, Joly F. Emerging treatments for short bowel syndrome in adult patients. *Expert Rev Gastroenterol Hepatol* 2019; **13**: 241-246 [PMID: 30791759 DOI: 10.1080/17474124.2019.1569514]

75 **Jeppesen PB**, Staun M, Tjellesen L, Mortensen PB. Effect of intravenous ranitidine and omeprazole on intestinal absorption of water, sodium, and macronutrients in patients with intestinal resection. *Gut* 1998; **43**: 763-769 [PMID: 9824602 DOI: 10.1136/gut.43.6.763]

76 **Kunkel D**, Basseri B, Low K, Lezcano S, Soffer EE, Conklin JL, Mathur R, Pimentel M. Efficacy of the glucagon-like peptide-1 agonist exenatide in the treatment of short bowel syndrome. *Neurogastroenterol Motil* 2011; **23**: 739-e328 [PMID: 21557790 DOI: 10.1111/j.1365-2982.2011.01723.x]

77 **de Vries FEE**, Reeskamp LF, van Ruler O, van Arum I, Kuin W, Dijksta G, Haveman JW, Boermeester MA, Serlie MJ. Systematic review: pharmacotherapy for high-output enterostomies or enteral fistulas. *Aliment Pharmacol Ther* 2017; **46**: 266-273 [PMID: 28613003 DOI: 10.1111/apt.14136]

78 **Ecker KW**, Stallmach A, Löffler J, Greinwald R, Achenbach U. Long-term treatment of high intestinal output syndrome with budesonide in patients with Crohn's disease and ileostomy. *Dis Colon Rectum* 2005; **48**: 237-242 [PMID: 15714248 DOI: 10.1007/s10350-004-0768-8]

79 **Ecker KW**, Stallmach A, Seitz G, Gierend M, Greinwald R, Achenbach U. Oral budesonide

significantly improves water absorption in patients with ileostomy for Crohn disease. *Scand J Gastroenterol* 2003; **38**: 288-293 [PMID: 12737444 DOI: 10.1080/00365520310000645a]

80 **Chordia P**, MacArthur RD. Crofelemer, a novel agent for treatment of non-infectious diarrhea in HIV-infected persons. *Expert Rev Gastroenterol Hepatol* 2013; **7**: 591-600 [PMID: 24070150 DOI: 10.1586/17474124.2013.832493]

81 **Crutchley RD**, Miller J, Garey KW. Crofelemer, a novel agent for treatment of secretory diarrhea. *Ann Pharmacother* 2010; 44: 878-884 [PMID: 20388859 DOI: 10.1345/aph.1M658]

82 **King RF**, Norton T, Hill GL. A double-blind crossover study of the effect of loperamide hydrochloride and codeine phosphate on ileostomy output. *Aust N Z J Surg* 1982; **52**: 121-124 [PMID: 7044361 DOI: 10.1111/j.1445-2197.1982.tb06083.x]

83 **Stevens PJ**, Dunbar F, Briscoe P. Potential of Loperamide Oxide in the Reduction of Ileostomy and Colostomy Output. *Clin Drug Investig* 1995; **10**: 158-164 [PMID: 27519199 DOI: 10.2165/00044011-199510030-00004]

84 **Newton CR**. Effect of codeine phosphate, Lomotil, and Isogel on iileostomy function. *Gut* 1978; **19**: 377-383 [PMID: 658767 DOI: 10.1136/gut.19.5.377]

85 **Buchman AL**, Fryer J, Wallin A, Ahn CW, Polensky S, Zaremba K. Clonidine reduces diarrhea and sodium loss in patients with proximal jejunostomy: a controlled study. *JPEN J Parenter Enteral Nutr* 2006; **30**: 487-491 [PMID: 17047172 DOI: 10.1177/0148607106030006487]

86 **McDoniel K**, Taylor B, Huey W, Eiden K, Everett S, Fleshman J, Buchman TG, Alpers D, Klein S. Use of clonidine to decrease intestinal fluid losses in patients with high-output short-bowel syndrome. *JPEN J Parenter Enteral Nutr* 2004; **28**: 265-268 [PMID: 15291409 DOI: 10.1177/0148607104028004265]

87 **Rubinoff MJ**, Piccione PR, Holt PR. Clonidine prolongs human small intestine transit time: use of the lactulose-breath hydrogen test. *Am J Gastroenterol* 1989; **84**: 372-374 [PMID: 2929556]

88 **Baumer P**, Danays T, Lion L, Cosnes J, Gendre JP, Le Quintrec Y. [Effect of clonidine on oro-cecal transit time in normal man]. *Ann Gastroenterol Hepatol (Paris)* 1989; **25**: 5-9 [PMID: 2653174]

89 **Byrne TA**, Wilmore DW, Iyer K, Dibaise J, Clancy K, Robinson MK, Chang P, Gertner JM, Lautz D. Growth hormone, glutamine, and an optimal diet reduces parenteral nutrition in patients with short bowel syndrome: a prospective, randomized, placebo-controlled, double-blind clinical trial. *Ann Surg* 2005; **242**: 655-661 [PMID: 16244538 DOI: 10.1097/01.sla.0000186479.53295.14]

90 **Drucker DJ**. Biologic actions and therapeutic potential of the proglucagon-derived peptides. *Nat Clin Pract Endocrinol Metab* 2005; **1**: 22-31 [PMID: 16929363 DOI: 10.1038/ncpendmet0017]

91 **Drucker DJ**. Gut adaptation and the glucagon-like peptides. *Gut* 2002; **50**: 428-435 [PMID: 11839727 DOI: 10.1136/gut.50.3.428]

92 **Jeppesen PB**. The use of hormonal growth factors in the treatment of patients with short-bowel syndrome. *Drugs* 2006; **66**: 581-589 [PMID: 16620138 DOI: 10.2165/00003495-200666050-00001]

93 **Jeppesen PB**. New approaches to the treatments of short bowel syndrome-associated intestinal failure. *Curr Opin Gastroenterol* 2014; **30**: 182-188 [PMID: 24406477 DOI: 10.1097/MOG.0000000000000046]

94 **Jeppesen PB**, Sanguinetti EL, Buchman A, Howard L, Scolapio JS, Ziegler TR, Gregory J, Tappenden KA, Holst J, Mortensen PB. Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant glucagon-like peptide 2 analogue, improves intestinal function in short bowel syndrome patients. *Gut* 2005; **54**: 1224-1231 [PMID: 16099790 DOI: 10.1136/gut.2004.061440]

95 **Jeppesen PB**, Gilroy R, Pertkiewicz M, Allard JP, Messing B, O'Keefe SJ. Randomised placebo-controlled trial of teduglutide in reducing parenteral nutrition and/or intravenous fluid requirements in patients with short bowel syndrome. *Gut* 2011; **60**: 902-914 [PMID: 21317170 DOI: 10.1136/gut.2010.218271]

96 **Jeppesen PB**, Pertkiewicz M, Messing B, Iyer K, Seidner DL, O'keefe SJ, Forbes A, Heinze H, Joelsson B. Teduglutide reduces need for parenteral support among patients with short bowel syndrome with intestinal failure. *Gastroenterology* 2012; **143**: 1473-1481. e3 [PMID: 22982184 DOI: 10.1053/j.gastro.2012.09.007]

97 **Pevny S**, Maasberg S, Rieger A, Karber M, Blüthner E, Knappe-Drzikova B, Thurmann D, Büttner J, Weylandt KH, Wiedenmann B, Müller VA, Bläker H, Pascher A, Pape UF. Experience with teduglutide treatment for short bowel syndrome in clinical practice. *Clin Nutr* 2019; **38**: 1745-1755 [PMID: 30145039 DOI: 10.1016/j.clnu.2018.07.030]

98 **Ring LL**, Nerup N, Jeppesen PB, Svendsen LB, Achiam MP. Glucagon like peptide-2 and neoplasia; a systematic review. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 257-264 [PMID: 29231791 DOI: 10.1080/17474124.2018.1417032]

99 **Pironi L**, Guidetti C, Incasa E, Poggioli G, Paganelli F, Merli C, Fumi L, Miglioli M. Oral rehydration solution containing rice maltodextrins in patients with total colectomy and high intestinal output. *Int J Clin Pharmacol Res* 2000; **20**: 55-60 [PMID: 11314238]

100 **Szkudlarek J**, Jeppesen PB, Mortensen PB. Effect of high dose growth hormone with glutamine and no change in diet on intestinal absorption in short bowel patients: a randomised, double blind, crossover, placebo controlled study. *Gut* 2000; **47**: 199-205 [PMID: 10896910 DOI: 10.1136/gut.47.2.199]

101 **Jeppesen PB**, Szkudlarek J, Høy CE, Mortensen PB. Effect of high-dose growth hormone and glutamine on body composition, urine creatinine excretion, fatty acid absorption, and essential fatty acids status in short bowel patients: a randomized, double-blind, crossover, placebo-controlled study. *Scand J Gastroenterol* 2001; **36**: 48-54 [PMID: 11218239 DOI: 10.1080/00365520150218057]

102 **Li-Ling**, Irving M. The effectiveness of growth hormone, glutamine and a low-fat diet containing high-carbohydrate on the enhancement of the function of remnant intestine among patients with short bowel syndrome: a review of published trials. *Clin Nutr* 2001; **20**: 199-204 [PMID: 11407865 DOI: 10.1054/clnu.2001.0423]

103 **Seguy D**, Vahedi K, Kapel N, Souberbielle JC, Messing B. Low-dose growth hormone in adult home parenteral nutrition-dependent short bowel syndrome patients: a positive study. *Gastroenterology* 2003; **124**: 293-302 [PMID: 12557135 DOI: 10.1053/gast.2003.50057]

104 **Goulet O**, Dabbas-Tyan M, Talbotec C, Kapel N, Rosilio M, Souberbielle JC, Corriol O, Ricour C, Colomb V. Effect of recombinant human growth hormone on intestinal absorption and body composition in children with short bowel syndrome. *JPEN J Parenter Enteral Nutr* 2010; **34**: 513-520 [PMID: 20852179 DOI: 10.1177/0148607110362585]

105 **Wales PW**, Nasr A, de Silva N, Yamada J. Human growth hormone and glutamine for patients with short bowel syndrome. *Cochrane Database Syst Rev*2010: CD006321 [PMID: 20556765 DOI: 10.1002/14651858.CD006321.pub2]

106 **Peretti N**, Loras-Duclaux I, Kassai B, Restier-Miron L, Guimber D, Gottrand F, Coopman S, Michaud L, Marinier E, Yantren H, Michalski MC, Aubert F, Mercier C, Pelosse M, Lopez M, Chatelain P, Lachaux A. Growth hormone to improve short bowel syndrome intestinal autonomy: a pediatric randomized open-label clinical trial. *JPEN J Parenter Enteral Nutr* 2011; **35**: 723-731 [PMID: 21975668 DOI: 10.1177/0148607111415531]

107 **Seguy D**, Darmaun D, Duhamel A, Thuillier F, Cynober L, Cortot A, Gottrand F, Messing B. Growth hormone enhances fat-free mass and glutamine availability in patients with short-bowel syndrome: an ancillary double-blind, randomized crossover study. *Am J Clin Nutr* 2014; **100**: 850-858 [PMID: 25080462 DOI: 10.3945/ajcn.113.071845]

108 **Van Gossum A**, Cabre E, Hébuterne X, Jeppesen P, Krznaric Z, Messing B, Powell-Tuck J, Staun M, Nightingale J; ESPEN. ESPEN Guidelines on Parenteral Nutrition: gastroenterology. *Clin Nutr* 2009; **28**: 415-427 [PMID: 19515465 DOI: 10.1016/j.clnu.2009.04.022]

109 **Nehra V**, Camilleri M, Burton D, Oenning L, Kelly DG. An open trial of octreotide long-acting release in the management of short bowel syndrome. *Am J Gastroenterol* 2001; **96**: 1494-1498 [PMID: 11374688 DOI: 10.1111/j.1572-0241.2001.03803.x]

110 **Szilagyi A**, Shrier I. Systematic review: the use of somatostatin or octreotide in refractory diarrhoea. *Aliment Pharmacol Ther* 2001; **15**: 1889-1897 [PMID: 11736719 DOI: 10.1046/j.1365-2036.2001.01114.x]

111 **O'Keefe SJ**, Peterson ME, Fleming CR. Octreotide as an adjunct to home parenteral nutrition in the management of permanent end-jejunostomy syndrome. *JPEN J Parenter Enteral Nutr* 1994; **18**: 26-34 [PMID: 8164299 DOI: 10.1177/014860719401800102]

112 **Crenn P**, Morin MC, Joly F, Penven S, Thuillier F, Messing B. Net digestive absorption and adaptive hyperphagia in adult short bowel patients. *Gut* 2004; **53**: 1279-1286 [PMID: 15306586 DOI: 10.1136/gut.2003.030601]

113 **Asplin JR**. The management of patients with enteric hyperoxaluria. *Urolithiasis* 2016; **44**: 33-43 [PMID: 26645872 DOI: 10.1007/s00240-015-0846-5]

114 **Wedlake L**, A'Hern R, Russell D, Thomas K, Walters JR, Andreyev HJ. Systematic review: the prevalence of idiopathic bile acid malabsorption as diagnosed by SeHCAT scanning in patients with diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2009; **30**: 707-717 [PMID: 19570102 DOI: 10.1111/j.1365-2036.2009.04081.x]

115 **Mottacki N**, Simrén M, Bajor A. Review article: bile acid diarrhoea - pathogenesis, diagnosis and management. *Aliment Pharmacol Ther* 2016; **43**: 884-898 [PMID: 26913381 DOI: 10.1111/apt.13570]

116 **Orekoya O**, McLaughlin J, Leitao E, Johns W, Lal S, Paine P. Quantifying bile acid malabsorption helps predict response and tailor sequestrant therapy. *Clin Med (Lond)* 2015; **15**: 371 [PMID: 26407388 DOI: 10.7861/clinmedicine.15-4-371]

117 **Wedlake L**, Thomas K, Lalji A, Anagnostopoulos C, Andreyev HJ. Effectiveness and tolerability of colesevelam hydrochloride for bile-acid malabsorption in patients with cancer: a retrospective chart review and patient questionnaire. *Clin Ther* 2009; **31**: 2549-2558 [PMID: 20109999 DOI: 10.1016/j.clinthera.2009.11.027]

118 **Sueyoshi R**, Woods Ignatoski KM, Okawada M, Hartmann B, Holst J, Teitelbaum DH. Stimulation of intestinal growth and function with DPP4 inhibition in a mouse short bowel syndrome model. *Am J Physiol Gastrointest Liver Physiol* 2014; **307**: G410-G419 [PMID: 24970775 DOI: 10.1152/ajpgi.00363.2013]

119 **Heydorn S**, Jeppesen PB, Mortensen PB. Bile acid replacement therapy with cholylsarcosine for short-bowel syndrome. *Scand J Gastroenterol* 1999; **34**: 818-823 [PMID: 10499484 DOI: 10.1080/003655299750025769]

120 **Gruy-Kapral C**, Little KH, Fordtran JS, Meziere TL, Hagey LR, Hofmann AF. Conjugated bile acid replacement therapy for short-bowel syndrome. *Gastroenterology* 1999; **116**: 15-21 [PMID: 9869597 DOI: 10.1016/s0016-5085(99)70223-4]

121 **Fürst T**, Bott C, Stein J, Dressman JB. Enteric-coated cholylsarcosine microgranules for the treatment of short bowel syndrome. *J Pharm Pharmacol* 2005; **57**: 53-60 [PMID: 15638993 DOI: 10.1211/0022357055155]

122 **Walters JR**, Johnston IM, Nolan JD, Vassie C, Pruzanski ME, Shapiro DA. The response of patients with bile acid diarrhoea to the farnesoid X receptor agonist obeticholic acid. *Aliment Pharmacol*

Ther 2015; **41**: 54-64 [PMID: 25329562 DOI: 10.1111/apt.12999]
123 **Bond A**, Huijbers A, Pironi L, Schneider SM, Wanten G, Lal S. Review article: diagnosis and management of intestinal failure-associated liver disease in adults. *Aliment Pharmacol Ther* 2019; **50**: 640-653 [PMID: 31342540 DOI: 10.1111/apt.15432]
124 **Bond A**, Taylor M, Abraham A, Teubner A, Soop M, Carlson G, Lal S. Examining the pathophysiology of short bowel syndrome and glucagon-like peptide 2 analogue suitability in chronic intestinal failure: experience from a national intestinal failure unit. *Eur J Clin Nutr* 2019; **73**: 751-756 [PMID: 30111847 DOI: 10.1038/s41430-018-0278-8]
125 **Appleton ND**, Lal S, Carlson GL, Shaw S, Stevens P, Peristerakis I, Soop M. Cholelithiasis and Related Morbidity in Chronic Intestinal Failure: a Longitudinal Cohort Study from a National Specialized Centre. *J Gastrointest Surg* 2019; **23**: 2002-2006 [PMID: 30324401 DOI: 10.1007/s11605-018-3979-3]
126 **Grace E**, Shaw C, Whelan K, Andreyev HJ. Review article: small intestinal bacterial overgrowth--prevalence, clinical features, current and developing diagnostic tests, and treatment. *Aliment Pharmacol Ther* 2013; **38**: 674-688 [PMID: 23957651 DOI: 10.1111/apt.12456]
127 **Stein JM**, Schneider AR. [Bacterial overgrowth syndrome]. *Z Gastroenterol* 2007; **45**: 620-628 [PMID: 17620228 DOI: 10.1055/s-2007-963045]
128 **O'Keefe SJ**. Bacterial overgrowth and liver complications in short bowel intestinal failure patients. *Gastroenterology* 2006; **130**: S67-S69 [PMID: 16473075 DOI: 10.1053/j.gastro.2005.09.067]
129 **Bohm M**, Siwiec RM, Wo JM. Diagnosis and management of small intestinal bacterial overgrowth. *Nutr Clin Pract* 2013; **28**: 289-299 [PMID: 23614961 DOI: 10.1177/0884533613485882]
130 **Capron JP**, Gineston JL, Herve MA, Braillon A. Metronidazole in prevention of cholestasis associated with total parenteral nutrition. *Lancet* 1983; **1**: 446-447 [PMID: 6131169 DOI: 10.1016/s0140-6736(83)91442-3]
131 **Petersen C**. D-lactic acidosis. *Nutr Clin Pract* 2005; **20**: 634-645 [PMID: 16306301 DOI: 10.1177/0115426505020006634]
132 **Fabian E**, Kramer L, Siebert F, Högenauer C, Raggam RB, Wenzl H, Krejs GJ. D-lactic acidosis - case report and review of the literature. *Z Gastroenterol* 2017; **55**: 75-82 [PMID: 27723911 DOI: 10.1055/s-0042-117647]
133 **Pironi L**, Tjellesen L, De Francesco A, Pertkiewicz M, Morselli Labate AM, Staun M, Przedlacki J, Lezo A, Orlandoni P, Pasanisi F; ESPEN-home artificial nutrition working group. Bone mineral density in patients on home parenteral nutrition: a follow-up study. *Clin Nutr* 2004; **23**: 1288-1302 [PMID: 15556251 DOI: 10.1016/j.clnu.2004.04.003]
134 **Pironi L**, Labate AM, Pertkiewicz M, Przedlacki J, Tjellesen L, Staun M, De Francesco A, Gallenca P, Guglielmi FW, Van Gossum A, Orlandoni P, Contaldo F, Villares JM; Espen-Home Artificial Nutrition Working Group. Prevalence of bone disease in patients on home parenteral nutrition. *Clin Nutr* 2002; **21**: 289-296 [PMID: 12135588 DOI: 10.1054/clnu.2002.0548]
135 **Nightingale JM**. Hepatobiliary, renal and bone complications of intestinal failure. *Best Pract Res Clin Gastroenterol* 2003; **17**: 907-929 [PMID: 14642857 DOI: 10.1016/s1521-6918(03)00108-2]
136 **Castiglioni S**, Cazzaniga A, Albisetti W, Maier JA. Magnesium and osteoporosis: current state of knowledge and future research directions. *Nutrients* 2013; **5**: 3022-3033 [PMID: 23912329 DOI: 10.3390/nu5083022]
137 **Gupta S**, Wu X, Moore T, Shen B. Frequency, risk factors, and adverse sequelae of bone loss in patients with ostomy for inflammatory bowel diseases. *Inflamm Bowel Dis* 2014; **20**: 259-264 [PMID: 24378598 DOI: 10.1097/01.MIB.0000439065.92211.d3]
138 **Bischoff-Ferrari HA**, Willett WC, Orav EJ, Lips P, Meunier PJ, Lyons RA, Flicker L, Wark J, Jackson RD, Cauley JA, Meyer HE, Pfeifer M, Sanders KM, Stähelin HB, Theiler R, Dawson-Hughes B. A pooled analysis of vitamin D dose requirements for fracture prevention. *N Engl J Med* 2012; **367**: 40-49 [PMID: 22762317 DOI: 10.1056/NEJMoa1109617]
139 **Stein J**, Böhles H. Lipid and Lipoprotein Metabolism. In: Stein J, Jauch KW, editors. Clinical Nutrition and Infusion Therapy [Klinische Ernährung und Infusionstherapie]. 1st ed. Berlin, Heidelberg, New York: Springer-Verlag, 2003: 171-190
140 **Cook N**. Central venous catheters: preventing infection and occlusion. *Br J Nurs* 1999; **8**: 980-982, 984, 986 [PMID: 10711027 DOI: 10.12968/bjon.1999.8.15.6524]
141 **Haire WD**, Herbst SLJNiCP. Invited Review: Use of Alteplase (t-PA) for the Management of Thrombotic Catheter Dysfunction: Guidelines From a Consensus Conference of the National Association of Vascular Access Networks (NAVAN). 2000; 15: 265-275 [DOI: 10.1177/088453360001500602]
142 **Bhutta ST**, Culp WC. Evaluation and management of central venous access complications. *Tech Vasc Interv Radiol* 2011; **14**: 217-224 [PMID: 22099014 DOI: 10.1053/j.tvir.2011.05.003]
143 **Reed T**, Phillips S. Management of central venous catheter occlusions and repairs. *J Intraven Nurs* 1996; **19**: 289-294 [PMID: 9060354]
144 **Baskin JL**, Pui CH, Reiss U, Wilimas JA, Metzger ML, Ribeiro RC, Howard SC. Management of occlusion and thrombosis associated with long-term indwelling central venous catheters. *Lancet* 2009; **374**: 159-169 [PMID: 19595350 DOI: 10.1016/S0140-6736(09)60220-8]
145 **Kerner JA Jr**, Garcia-Careaga MG, Fisher AA, Poole RL. Treatment of catheter occlusion in pediatric patients. *JPEN J Parenter Enteral Nutr* 2006; **30**: S73-S81 [PMID: 16387916 DOI: 10.1177/01486071060300s1s73]
146 **Tribler S**, Brandt CF, Fuglsang KA, Staun M, Broebech P, Moser CE, Scheike T, Jeppesen PB.

Catheter-related bloodstream infections in patients with intestinal failure receiving home parenteral support: risks related to a catheter-salvage strategy. *Am J Clin Nutr* 2018; **107**: 743-753 [PMID: 29722835 DOI: 10.1093/ajcn/nqy010]

147 **Wanten G**, Calder PC, Forbes A. Managing adult patients who need home parenteral nutrition. *BMJ* 2011; **342**: d1447 [PMID: 21421667 DOI: 10.1136/bmj.d1447]

148 **Dibb MJ**, Abraham A, Chadwick PR, Shaffer JL, Teubner A, Carlson GL, Lal S. Central Venous Catheter Salvage in Home Parenteral Nutrition Catheter-Related Bloodstream Infections: Long-Term Safety and Efficacy Data. *JPEN J Parenter Enteral Nutr* 2016; **40**: 699-704 [PMID: 25224728 DOI: 10.1177/0148607114549999]

149 **Thompson JS**, Rochling FA, Weseman RA, Mercer DF. Current management of short bowel syndrome. *Curr Probl Surg* 2012; **49**: 52-115 [PMID: 22244264 DOI: 10.1067/j.cpsurg.2011.10.002]

150 **Shanks RM**, Donegan NP, Graber ML, Buckingham SE, Zegans ME, Cheung AL, O'Toole GA. Heparin stimulates Staphylococcus aureus biofilm formation. *Infect Immun* 2005; **73**: 4596-4606 [PMID: 16040971 DOI: 10.1128/IAI.73.8.4596-4606.2005]

151 **Allon M**. Prophylaxis against dialysis catheter-related bacteremia: a glimmer of hope. *Am J Kidney Dis* 2008; **51**: 165-168 [PMID: 18215693 DOI: 10.1053/j.ajkd.2007.12.003]

152 **Wouters Y**, Theilla M, Singer P, Tribler S, Jeppesen PB, Pironi L, Vinter-Jensen L, Rasmussen HH, Rahman F, Wanten GJA. Randomised clinical trial: 2% taurolidine versus 0.9% saline locking in patients on home parenteral nutrition. *Aliment Pharmacol Ther* 2018; **48**: 410-422 [PMID: 29978597 DOI: 10.1111/apt.14904]

153 **Barco S**, Serlie MJ, Coppens M. Primary thromboprophylaxis for adult patients on home parenteral nutrition: A comment on the 2016 ESPEN guideline. *Clin Nutr* 2016; **35**: 1579-1580 [PMID: 27546797 DOI: 10.1016/j.clnu.2016.08.003]

154 **Lal S**, Pironi L, Wanten G, Arends J, Bozzetti F, Cuerda C, Joly F, Kelly D, Staun M, Szczepanek K, Van Gossum A, Schneider SM; Home Artificial Nutrition & Chronic Intestinal Failure Special Interest Group of the European Society for Clinical Nutrition and Metabolism (ESPEN). Clinical approach to the management of Intestinal Failure Associated Liver Disease (IFALD) in adults: A position paper from the Home Artificial Nutrition and Chronic Intestinal Failure Special Interest Group of ESPEN. *Clin Nutr* 2018; **37**: 1794-1797 [PMID: 30017241 DOI: 10.1016/j.clnu.2018.07.006]

155 **Morgan J**, Dibb M, Lal S. Intestinal failure-associated liver disease in adult patients. *Curr Opin Clin Nutr Metab Care* 2019; **22**: 383-388 [PMID: 31313688 DOI: 10.1097/MCO.0000000000000594]

156 **Sasdelli AS**, Agostini F, Pazzeschi C, Guidetti M, Lal S, Pironi L. Assessment of Intestinal Failure Associated Liver Disease according to different diagnostic criteria. *Clin Nutr* 2019; **38**: 1198-1205 [PMID: 29778510 DOI: 10.1016/j.clnu.2018.04.019]

157 **Van Gossum A**, Demetter P. Hepatobiliary Complications of Chronic Intestinal Failure. *Gastroenterol Clin North Am* 2019; **48**: 551-564 [PMID: 31668182 DOI: 10.1016/j.gtc.2019.08.008]

158 **Barco S**, Heuschen CB, Salman B, Brekelmans MP, Serlie MJ, Middeldorp S, Coppens M. Home parenteral nutrition-associated thromboembolic and bleeding events: results of a cohort study of 236 individuals. *J Thromb Haemost* 2016; **14**: 1364-1373 [PMID: 27122107 DOI: 10.1111/jth.13351]

159 **Lacaille F**, Gupte G, Colomb V, D'Antiga L, Hartman C, Hojsak I, Kolacek S, Puntis J, Shamir R; ESPGHAN Working Group of Intestinal Failure and Intestinal Transplantation. Intestinal failure-associated liver disease: a position paper of the ESPGHAN Working Group of Intestinal Failure and Intestinal Transplantation. *J Pediatr Gastroenterol Nutr* 2015; **60**: 272-283 [PMID: 25272324 DOI: 10.1097/MPG.0000000000000586]

160 **Cuerda C**, Pironi L; Home Artificial Nutrition and Chronic Intestinal Failure Special Interest Group of ESPEN. Reply, Letter to the Editor - Primary thromboprophylaxis for adult patients on home parenteral nutrition: A comment on the 2016 ESPEN guideline. *Clin Nutr* 2016; **35**: 1581-1582 [PMID: 27544409 DOI: 10.1016/j.clnu.2016.08.001]

161 **Rege AS**, Sudan DL. Autologous gastrointestinal reconstruction: review of the optimal nontransplant surgical options for adults and children with short bowel syndrome. *Nutr Clin Pract* 2013; **28**: 65-74 [PMID: 23087264 DOI: 10.1177/0884533612460405]

162 **Rege A**. The Surgical Approach to Short Bowel Syndrome - Autologous Reconstruction versus Transplantation. *Viszeralmedizin* 2014; **30**: 179-189 [PMID: 26288592 DOI: 10.1159/000363589]

163 **Abu-Elmagd K**. The concept of gut rehabilitation and the future of visceral transplantation. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 108-120 [PMID: 25601664 DOI: 10.1038/nrgastro.2014.216]

164 **Kim HB**, Fauza D, Garza J, Oh JT, Nurko S, Jaksic T. Serial transverse enteroplasty (STEP): a novel bowel lengthening procedure. *J Pediatr Surg* 2003; **38**: 425-429 [PMID: 12632361 DOI: 10.1053/jpsu.2003.50073]

165 **Kahn AB**, Tulla KA, Tzvetanov IG. Indications of Intestinal Transplantation. *Gastroenterol Clin North Am* 2019; **48**: 575-583 [PMID: 31668184 DOI: 10.1016/j.gtc.2019.08.010]

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REVIEW

Non-cirrhotic hepatocellular carcinoma in chronic viral hepatitis: Current insights and advancements

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Abstract

Primary liver cancers carry significant morbidity and mortality. Hepatocellular carcinoma (HCC) develops within the hepatic parenchyma and is the most common malignancy originating from the liver. Although 80% of HCCs develop within background cirrhosis, 20% may arise in a non-cirrhotic milieu and are referred to non-cirrhotic-HCC (NCHCC). NCHCC is often diagnosed late due to lack of surveillance. In addition, the rising prevalence of non-alcoholic fatty liver disease and diabetes mellitus have increased the risk of developing HCC on non-cirrhotic patients. Viral infections such as chronic Hepatitis B and less often chronic hepatitis C with advance fibrosis are associated with NCHCC. NCHCC individuals may have Hepatitis B core antibodies and occult HBV infection, signifying the role of Hepatitis B infection in NCHCC. Given the effectiveness of current antiviral therapies, surgical techniques and locoregional treatment options, nowadays such patients have more options and potential for cure. However, these lesions need early identification with diagnostic models and multiple surveillance strategies to improve overall outcomes. Better understanding of the NCHCC risk factors, tumorigenesis, diagnostic tools and treatment options are critical to improving prognosis and overall outcomes on these patients. In this review, we aim to discuss NCHCC epidemiology, risk

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factors, and pathogenesis, and elaborate on NCHCC diagnosis and treatment strategies.

Key Words: Cirrhosis; Hepatic fibrosis; Non-alcoholic liver disease; Primary liver cancer; Hepatocellular carcinoma; Hepatoma; Liver cancer; Hepatitis B virus; Hepatitis C virus; Liver resection; Liver transplantation

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Core Tip: Non-cirrhotic hepatocellular carcinoma (HCC) accounts for 20% of reported HCCs. Such tumors are typically diagnosed late, compromising the outcome. The discovery of direct antivirals, loco-regional treatments and systemic novel immune-chemotherapies, along with advancements of complex hepatobiliary surgery, and the genesis of transplant oncology have revolutionized the management of these aggressive primary liver tumors. Coordinated care at tertiary high-volume HCC, preferably liver transplant centers, remains critical. It is time the stakeholders pursued a consensus approach in developing universal HCC surveillance and treatment strategies on non-cirrhotic patients at risk, such as patients with non-alcoholic steatohepatitis and/or patients with advanced fibrosis.

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INTRODUCTION

Primary liver cancer originates from the liver parenchyma, the bile ducts or both. Worldwide, as per 2018 statistics, primary liver cancer is the second most lethal cancer (only next to pancreatic cancer), fourth leading cause of cancer-related mortality and the sixth most frequently diagnosed with an incidence of 841000 cases per year[1]. Hepatocellular carcinoma (HCC) is the most common primary malignant tumor (90%) originating from the liver[2]. HCC commonly develops within a background of chronic liver disease, characterized by progressive hepatic fibrosis, loss of architecture and formation of regenerative nodules (cirrhosis). This is referred to as cirrhotic-HCC and is present in the majority of the cases (80%). However, 20% of HCC cases develop on a non-cirrhotic background, and therefore referred to as non-cirrhotic-HCC (NCHCC)[3]. Fibrolamellar HCC, angiosarcoma, lymphoma, embryonic sarcoma are other non-cirrhotic liver malignancies, but are rare in occurrence. Due to the lack of surveillance strategies, NCHCC is often diagnosed late, leading to poor prognosis[4,5].

NCHCC risk factors include alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), hepatitis B [hepatitis B virus, (HBV)], hepatitis C [hepatitis C virus (HCV)], hepatitis D virus, tobacco abuse, diabetes mellitus, genetic factors and environmental toxins. Among these, NAFLD and viral hepatitis (HBV and HCV) have been the most common. Given the obesity pandemic, NAFLD burden on the health systems has been expanding. Chronic HBV can be associated with high viral loads, hepatitis D coinfection, prolonged replication phase, concomitant tobacco use and alcohol intake, which can predispose to NCHCC pathogenesis. Similarly, chronic HCV can be associated with carcinogenesis due to multiple gene products development during viremia, altered cell cycle resulting in NCHCC. Further, this risk can continue even after eradication of HCV. It is critical to understand the underlying mechanisms associated with chronic viral hepatitis leading to NCHCC. A comprehensive strategy is needed for surveillance, diagnosis, and management of these tumors[6].

In this review, we discuss NCHCC epidemiology, risk factors, and pathogenesis, and elaborate on NCHCC diagnosis and treatment strategies.

METHODS

An online search was performed using databases PubMed/Medline, EMBASE, Cochrane, Web of Science and CINAHL from January 1, 2000 to January 1, 2021 to identify published reports on HCC in chronic viral hepatitis without cirrhosis. We used following search terms- "carcinoma, hepatocellular" or "cancer, hepatocellular" and "viral hepatitis" and "HBV" and "HCV" excluding "liver cirrhosis." This resulted in 705 published reports. With use of filters (human species and English language), 648 published reports were obtained. After removing articles not relevant/duplicates/non-English language including a manual search, 677 published articles were reviewed.

EPIDEMIOLOGY

HCC is the most common primary liver cancer and a leading cause of cancer-related deaths[5]. HCC-related deaths have been increasing globally. In the United States, in 2001-2006, average HCC incidence increased from 2.7 (2001) per 100,000 to 3.2 per 100,000 (2006)[7]. During this time, HCC was considered ninth leading cause of cancer death. From 2011-2014, mortality from HCC increased by 2.7%[8]. Geographically, HCC is 72% in Asia[9]. World's highest incidence is noted in Mongolia with an incidence of 93.7 per 100,000[1]. Racial and ethnic variations are present in HCC with Asian/Pacific Islands, Blacks, Native Americans having a higher prevalence compared to whites[7,10].

Up to 20% of HCC can grow in a non-cirrhotic liver[11]. While HCC shows a unimodal distribution with peak at the seventh decade of life, NCHCC shows bimodal age distribution, with peaks at second and seventh decade of life[11,12]. This could be related to HBV infection at birth and during adult life[13]. Fibrolamellar HCC is commonly seen in younger adults (second and third decades of life). Male to female ratio is 3:2 in HCC *vs* 1.3-2:1 for NCHCC[14]. Fibrolamellar HCC does not show any sex predilection. Contrary to HCC which is seen frequently in Asians, prevalence of fibrolamellar HCC is higher in Europe and North America[15]. Shim *et al*[16] reported that in Korea, prevalence of HBV infection among NCHCC can range up to 77%. NCHCC individuals, even without active HBV infection, can have antibodies against HBV core (indicative of prior HBV infection) and occult HBV infection [HBV DNA in the liver/blood without hepatitis B surface antigen (HBsAg)], signifying the role of HBV infection in NCHCC[16]. Despite the disappearance of HBV antigen (with treatment or spontaneous regression), some patients continue to be at risk of developing HCC[17].

NON-VIRAL RISK FACTORS FOR NCHCC

Obesity, overweight, and diabetes mellitus are considered NCHCC risk factors. Fatty liver disease can lead to inflammation and hepatocellular carcinogenesis (Table 1)[18]. Tumor suppressor gene dysregulation plays an important role in steatosis development, hepatocyte injury, and NCHCC tumorigenesis[4,19]. Increased tumor necrosis factor-alfa, interleukin-6, leptin, resistin and decreased adiponectin contribute to carcinogenesis in non-cirrhotic livers[20]. Recently, Sydor *et al*[21] reported an association with gut microbiota and primary conjugated bile acid composition in CHCC and NCHCC carcinogenesis among NASH patients[21]. Microbiota-associated changes in bile acid homeostasis and farnesoid X receptor signaling *via* fibroblast growth factor-19 might contribute to the tumorigenesis in these patients[21]. Given the promising results obtained from direct-acting antiviral (DAA) agents in the treatment of HCV, metabolic syndrome and NAFLD will likely predominate and may become the leading risk factor for the pathogenesis of HCC and NCHCC.

Other risk factors include toxin exposure (alcohol, aflatoxin B1, industrial agents, genotoxins, anabolic steroids, iron excess)[22-25], genetic conditions, such as Wilson's disease, glycogen storage disease, Alpha-1 antitrypsin deficiency, hereditary hemochromatosis, acute hepatic porphyria's, hypercitrullinemia, Budd-Chiari syndrome, nodular regenerative hyperplasia[26-30], and germline mutations (telomerase reverse transcriptase gene mutation is the most commonly described mutation in NCHCC)[31]. Excess alcohol intake may play a role in NCHCC carcinogenesis[32]. Studies reported that prevalence of alcohol abuse among patients with NCHCC range up to 12%-21%[4,11,33,34]. If alcohol leads to significant inflammation

Table 1 Key differences between non cirrhotic hepatocellular carcinoma and hepatocellular carcinoma

	HCC	NCHCC
Epidemiology	Eighty percent of HCC develops with a cirrhotic background. A unimodal age distribution (peak in 7th decade) noted. Male:female ratio - 3:2	Twenty percent of tumors develop in non-cirrhotic liver. A bimodal age distribution (peak in 2nd and 7th decade) noted. Male:female ratio-2:1
Risk factors	Development of cirrhosis from any etiology can progress to HCC. Hepatotropic viruses, environmental and life-style factors (alcohol, tobacco), metabolic conditions (nonalcoholic fatty liver disease, diabetes mellitus, obesity) play a predominant role	NCHCC develops without a background of underlying cirrhosis. Viral (HBV, HCV infection) and non-viral risk factors (obesity, diabetes mellitus, toxin exposure, germline mutations and genetic disorders) noted
Clinical features	Symptoms could be related to underlying cirrhosis (from portal hypertension) or HCC (early satiety, upper abdominal pain) itself. Paraneoplastic signs such as hypercalcemia, hypoglycemia have been reported	Generalized fatigue, abdominal pain and weight loss are common symptoms. Can present at late stage with large tumor burden, extrahepatic metastasis
Diagnosis	High quality cross-sectional imaging (CT/MRI) are used with typical arterial phase hyper-enhancement and portal venous washout. LI-RADS classification is used in classification of radiological findings in HCC	Although CT and MRI are increasingly utilized for diagnosis, liver biopsy are utilized in patients when cross-sectional imaging is equivocal. LI-RADS classification cannot be utilized for NCHCC and instead tumor characteristics (size, imaging features) are utilized for staging
Treatment	Given the underlying cirrhosis, liver transplant candidacy need to be evaluated for HCC patients. Resectability of the lesion, amount of liver reserve, vascular invasion, performance status determines the treatment outcomes	Antiviral treatment recommended when etiology of NCHCC is HBV/HCV. Surgery remains the main treatment modality. Systemic and local therapy options are increasingly being utilized for NCHCC

Key differences in epidemiology, risk factors, clinical presentations, diagnosis and treatment for non-cirrhotic hepatocellular carcinoma and hepatocellular carcinoma. A multidisciplinary team evaluation is frequently utilized for diagnosis and treatment. CT: Computed tomography; MRI: Magnetic resonance; HCC: Hepatocellular carcinoma; NCHCC: Non cirrhotic hepatocellular carcinoma; LI-RADS: Liver Reporting and Data System; HBV: Hepatitis B; HCV: Hepatitis C.

with or without fibrosis in these NCHCC is unclear. Further, a synergism with other risk factors, such as viral hepatitis and metabolic syndrome could likely play a role in hepatic carcinogenesis.

VIRAL RISK FACTORS FOR NCHCC

Hepatotropic viral infections such as HBV and less often HCV are major contributors to NCHCC. Hepatitis D can cause coinfection with HBV and lead to high risk of HCC compared to HBV alone [odds ratio (OD) 1.28, 95% confidence interval (CI) 1.05-1.57] [35]. Few studies report hepatitis E may affect chronic HBV infection as they already have compromised liver function[36,37]. If similar risk exists for NCHCC is unclear. Additionally, NCHCC can exhibit more genomic variants, suggestive of a separate tumor biology in these patients[38,39]. Further, HBV-human immunodeficiency virus (HIV) coinfection has been reported to cause higher risk of HCC (OD 7.1, 95%CI: 2.8-17.9)[40].

HBV AND NON-CIRRHOTIC HCC CARCINOGENESIS

HBV virus is a DNA virus (Figure 1), a member of the *Hepadnaviridae* family. It is composed of viral core [nucleocapsid, HBV core antigen (HBcAg) and DNA polymerase] and surface [formed by surface antigen (HBsAg)]. The genes coding for HBcAg code for HBV e antigen (HBeAg). Worldwide, there are 292 million people living with chronic HBV, with 2.2 million reported in the United States[41,42]. HBV (+/- HDV) associated HCC is more common in low and middle socio-economic population. Chronic HBV coinfection can been seen with HDV and/ or HCV.

Ten genotype variants (A to J) are known for HBV of which genotype B and C are highly relevant to HCC development[43]. In general, patients with genotype B have lower risk of progression towards cirrhosis (due to less active disease and earlier HBeAg seroconversion)[44], compared to genotype C. Few studies from Japan demonstrated that genotype B is more common in younger NCHCC[45]. Some studies suggest that individuals infected with either B or C HBV genotype with high T1762/A1764 basal core promotor mutation have a higher risk of HCC development, especially among non-cirrhotic younger adults (< 50 years)[46,47]. Notably, most of

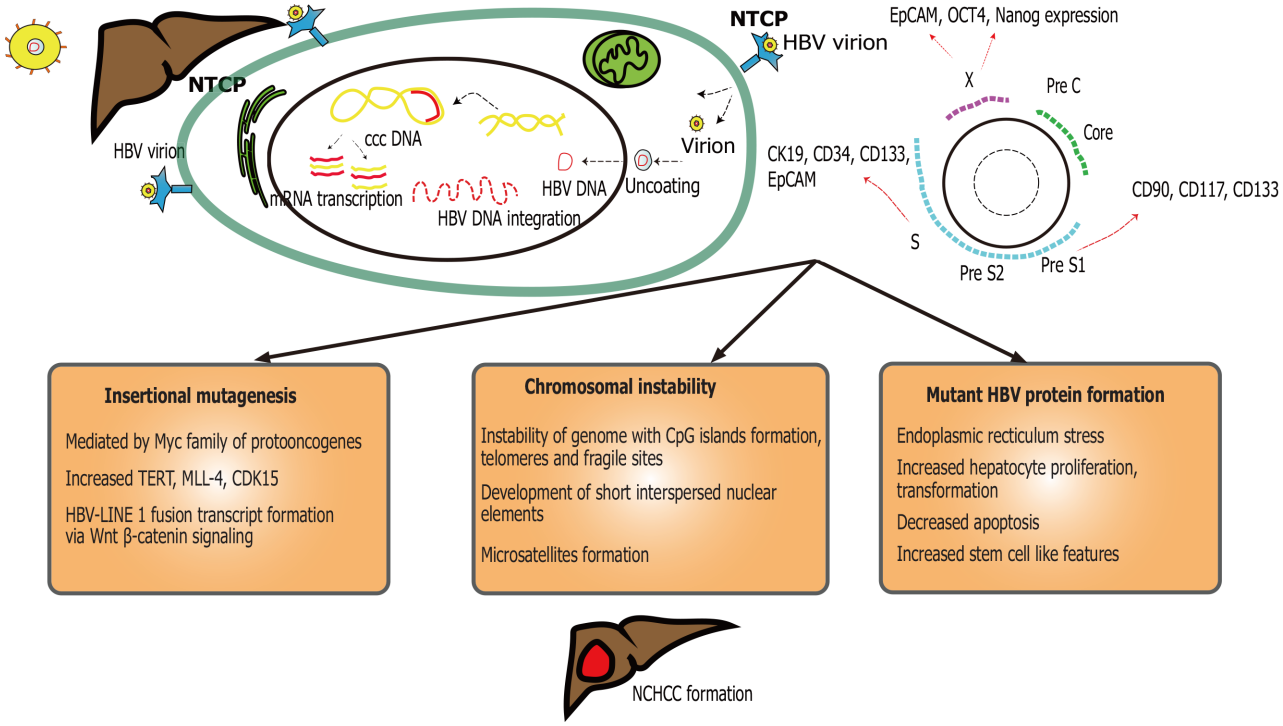


Figure 1 Mechanisms of oncogenesis in hepatitis B virus induced non-cirrhotic hepatocellular carcinoma. Entry of hepatitis B virus (HBV) virion into hepatocytes results incorporation of DNA into the host genome resulting in covalently closed circular DNA. Integration of HBV DNA leads to expression of multiple stem cell markers. These markers differ based on the region of the HBV genome (preS1, S, core, X). Three mechanisms of oncogenesis with insertional mutagenesis, chromosomal instability and mutant protein formation shown. HBV: Hepatitis B virus; NTCP: Na+-taurocholate co-transporting polypeptide; cccDNA: Covalently closed circular DNA; EpCAM: Epithelial cell adhesion molecule; OCT4: Octamer-binding transcription factor 4; Nanog: Homeobox protein; CK19: Cytokeratin 19; CD: Cluster of differentiation; TERT: Telomerase reverse transcriptase; MLL4: Myeloid lymphoid leukemia 4; CKD15: Cyclin dependent kinase 15; NCHCC: Noncirrhotic hepatocellular carcinoma.

these studies were of Asian origin, known to have higher HBV prevalence compared to the West. Mutations are frequently noticed during HBV replication, due to its lack of proofreading activity during reverse transcription. Girones *et al*[48] reported that hepadnavirus mutation rate is 100 times higher than other DNA viruses[48]. Thirty percent of these HCC patients have no underlying cirrhosis (NCHCC)[49]. Pooled HCC surveillance adherence is much lower in non-cirrhotics (23%) compared to their cirrhotic counterparts, despite recommendations[50]. This can lead to tumors, which are advanced at the time of diagnosis, limiting treatment options and outcomes.

Transforming hepatocytes without significant fibrosis or inflammation remains a hallmark of HBV-induced NCHCC[51]. The underlying mechanism remains unclear, even though related to cellular transformation with epigenetic alteration, telomere shortening and instability of the genome system, insertional proto-oncogenes/tumor suppressor mutagenesis, and expression of mutant HBV proteins following integration [52,53]. A variety of factors can predispose HBV positive individuals to develop HCC through either cirrhosis or non-cirrhotic pathways. HBV genome, due to its ability to integrate into the host, can cause genomic instability, disrupting normal regulatory mechanisms. Further, and most interestingly, these mechanisms continue to exist even after seroconversion, due to HBV genome integration. This has been related to HBV highly stable minichromosome and/ or covalently closed circular DNA (HBV cccDNA) which resides inside the hepatocyte nucleus[54]. Tu *et al*[53] compared HBV integrated sites between tumor and matched non-tumor tissues and found that HCC tumors have higher integration event frequency in coding/promoter regions. In the non-cirrhotic setting, recurrent enhancer II/core HBV promoter integration near telomerase reverse transcriptase, myeloid/lymphoid or mixed leukemia 4 genes cause upregulation of oncogenes during early and late tumorigenesis[53,55]. This is critical for HBV non-cirrhotic carcinogenesis, even after HBV treatment or spontaneous resolution. High viral loads of HBV are independently associated with NCHCC, indicative of genetic transformation of the hepatocytes induced by the HBV[47].

Many cellular processes modulate the HBV cccDNA such as histone acetylation, epigenetic modification and activation of signal transduction[56]. These processes could be a target for pharmaceutical agents, however remaining challenges are

cccDNA longevity and stability in the host and viremia recurrence once the antiviral therapy is stopped[57]. The host immune response is unable to eliminate infected cells, resulting instead in immune-mediated damage. This can lead to repeat bouts of hepatitis and inflammation-necrosis-proliferation cycles, resulting in production of reaction oxygen species, genetic mutations and carcinogenesis[58].

NCHCC individuals are more likely African American (OR 6.8, 95%CI: 2.1-22.4), Asian (OR 11.6, 95%CI 2.63-50.8) or have a family history of HCC (OR 32.9, 95%CI: 3.76-288)[51]. In addition, some of the NCHCC from chronic HBV can have multiple risk factors, such as HCV coinfection, alcohol abuse and cryptogenic etiologies[16]. It has been widely contested that viral infection alone might not be sufficient for carcinogenesis; an interplay of host factors, environment and time are needed for full blown cancer development. This probably explains the variation in HCC prevalence among different population groups, and the protracted timeline for HBV-related tumorigenesis, which may span at least one to two decades of life.

HCV AND NON-CIRROTIC HCC CARCINOGENESIS

HCV is a part of *Flaviviridae* family. It is an single-stranded enveloped RNA virus which primarily infects hepatocytes (Figure 2) and targets liver-specific cellular host factors[59]. Precise HCV NCHCC prevalence is unclear. Few reports suggested a possible incidence of up to 10.6%[60]. This risk decreases to 4.2% after achieved sustained viral response (SVR)[61]. Few risk factors, such as male sex, advance age (> 60 years), F3 fibrosis, steatosis, elevated ALT at the end of treatment and history of alcohol abuse contribute to this risk. Furthermore, HCV genotype 3 with steatosis may correlate with HCC[62].

Similar to HBV, successful HCV treatment would not completely eliminate HCC risk, even though risk would be much lower compared to HBV[63]. Attributed risk factors are HCV-genotype 1b[64], viral co-infection infections (HBV, HIV), alcohol or tobacco abuse, and metabolic syndrome. HCV core protein can alter the telomerase activity and immortalize the hepatocytes (along with loss of tumor suppressor function)[65]. Upon hepatocyte entry, the viral RNA undergoes replication and translation in the rough endoplasmic reticulum[66]. This translated product is cleaved by host and viral proteases to form different proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B). Among these, NS3, NS4B, NS5A can induce carcinogenesis coupled with host cellular proteins[65]. Release of reactive oxygen species remains a critical part for HCV-induced HCC. Chronic HCV infection leads to milder liver inflammation, facilitating release of pro-inflammatory cytokines and growth factors, and the generation of inflammation-necrosis-proliferation cycles[67,68]. This is precipitated by increased reactive oxygen species production, mediated by calcium release and high expression of HCV core protein, and cytochrome p450 2E1 oxidase [69]. Decades of this cycles lead to fibrosis, cirrhosis and, eventually HCC.

Due to the ongoing risk of carcinogenesis despite achieving SVR, active evaluation for non-viral risk factors (co-infection, alcohol, tobacco, fatty liver, and metabolic syndrome) should be performed. Recommendations on the surveillance of non-cirrhotic patients with stage 2 or 3 fibrosis and history of treatment-naïve or SVR HCV infection have been unclear. The American Association for the Study of Liver Diseases do not recommend active surveillance. The European Association of the Study of Liver recommended that surveillance would be useful[70].

VIRAL CO-INFECTION AND HCC

Co-infection of hepatotropic viruses can occur among each other and rarely with other virus infection such as the HIV. Due to identical modes of transmission (contaminated needles, blood and sexual routes), co-infection with viral hepatitis and HIV have been observed in certain populations (sub-Saharan Africa)[71]. Although the rates of infection differ based on region and risk-based groups, 5%-20% of HIV patients could be co-infected with HBV. Further, the rates of morbidity and mortality are significantly higher among HIV and HBV co-infection even after adequate viral suppression[72]. HCV and HIV co-infection, although lower (1%-7%) compared to HBV, can lead to evolution of fibrosis (especially with low CD4 counts) and early onset cirrhosis[73]. Chronic viral hepatitis D, being a defective virus, depends on HBV for propagation and occurs in patients concomitantly infected with HDV and HBV. HDV could infect up to 5%-15% of all HBV carriers[74]. This co-infection can cause severe form of

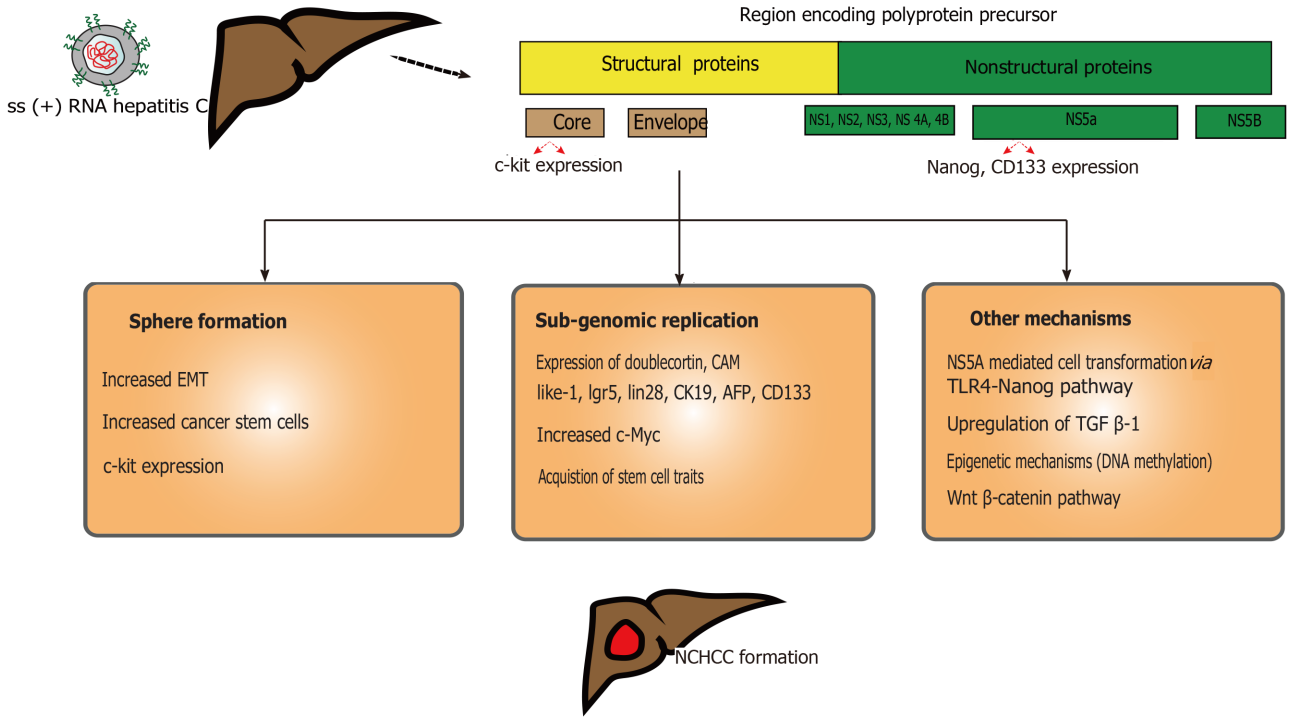


Figure 2 Mechanisms of oncogenesis in hepatitis C virus induced non-cirrhotic hepatocellular carcinoma. Entry of single stranded hepatitis C virus (HBV) RNA into hepatocytes leads to expression of multiple oncogenic proteins. The core section and NS5A sections of the HCV genome produces c-Kit and Nanog-CD133 proteins respectively. HCV infected hepatocytes can lead to sphere formation, sub-genomic replication formation and multiple other mechanism are noted in the schema. Ss: Single stranded; NS: Non-structural; c-Kit: Proto-oncogene, Cam Kinase-like-1; Lgr5: Leucine rich G-protein receptor; CD: Cluster of differentiation; Lin28: RNA binding protein; CK19: Cytokeratin 19; AFP: Alfa fetoprotein; TLR4: Toll-like receptor 4; TGFβ-1: Transforming growth factor-1; NCHCC: Noncirrhotic hepatocellular carcinoma.

chronic viral hepatitis, with 2-fold risk of mortality and 3-fold risk of cirrhosis[75]. The risk of HCC increased 1.2 fold with this co-infection and even higher (7.1%) with HIV-infection. HDV-associated HCC can be due to oncogenesis induced by impact of viral replication/epigenetic events and abnormal cell methylation processes[76]. Despite these findings, future studies are needed to evaluate the carcinogenesis induced by co-infection and long-term effects in the patients.

SYMPTOMS AND SIGNS

NCHCC usually presents at late stage, with a large tumor size, extrahepatic metastases, and heavy tumor burden. Symptoms may include malaise, fatigue, weight loss, abdominal pain, gastrointestinal bleeding and distension, even though often patients are asymptomatic[77-79].

DIAGNOSIS

Imaging

Diagnosis of HCC is mostly performed with non-invasive techniques and rarely histological diagnosis is needed (Table 2). Imaging has replaced histology and conventional angiography[80]. Most of the radiological features of CHCC and NCHCC are similar, except, of course, for the background of cirrhosis among the CHCC patients. Multiple imaging modalities are available, including ultrasound (US), contrast enhanced US, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography scan[81], CT-based radiomics nomogram [82,83], and angiography-assisted CT hepatic angiography[84,85]. Classical CT/MRI findings are arterial phase hyper-enhancement (due to hepatic arterial supply of the tumor), followed by portal venous washout.

Table 2 Imaging characteristics of cirrhotic and non-cirrhotic hepatocellular carcinoma

	CHCC	NCHCC
Imaging modality	Background of advance fibrosis (cirrhosis)	No background of advance fibrosis (cirrhosis)
CT	Homogenous with irregular but well defined margin	Initially hypoattenuating mass which can be come heterogenous (areas of necrosis/hemorrhage within the tumor) when tumor attains bigger size
	Multiple masses	Large solitary mass (/dominant mass) with satellite nodules
	Extrahepatic extension less common	Extrahepatic extension (with direct adjacent organ) is more often seen
	Vascular invasion (encasement) more common (85%)	Metastasis frequently seen, vascular invasion less common (15%)
		Lymphadenopathy seen in 20% of cases.
MR	T1: Variable but mostly hypointense. T2: Hyperintense/isointense compared to surrounding liver	Unenhanced T1 image - Hypointense lesion (presence of hemorrhage/fat can increase the signal). T2 - Hyperintense (low grade/well differentiated can be iso/hypointense)
	DWI-high ADC when lesion is well differentiated	DWI - Used for small lesions. Shows low ADC

CHCC: Cirrhotic hepatocellular carcinoma; NCHCC: Non-cirrhotic hepatocellular carcinoma; CT: Computed tomography; MRI: Magnetic resonance; DWI: Diffuse weighted imaging; ADC: Apparent diffusion coefficient.

Few studies evaluated clinico-radiological characteristics in NCHCC and found that they differ compared to CHCC[86]. Time of contrast washout correlates with histopathological grade in HCC[86]. US is rarely used for diagnosis of the NCHCC due to its non-specific findings, especially for tumors which are less than 2 cm in size, obesity and background of cirrhosis[87]. In rare conditions, such as undifferentiated embryonal sarcoma, ultrasound shows solid component of the tumor (and can exclude purely cystic lesions which are benign)[88]. The sensitivity of US can be significantly limited (up to 21%) due to abdominal fat and obesity; CT/MR is frequently used in these situations[89].

Computed tomography

CT is one of the most commonly used modality of imaging for diagnosis of HCC and can be critical in identifying salient features of HCC. Patients with NCHCC can present with large tumor size lesions, solitary mass (with or without satellite lesions)[90]. They can have well-defined margins with mosaic pattern of enhancement, complete capsule and delayed washout on the CT[86]. This in comparison to CHCC which showed smaller lesions, ill-defined margins, heterogeneous enhancement, no capsule and portal venous phase washout (Table 2). Extrahepatic lesions are more commonly noted in NCHCC (20.5%) compared to CHCC (6.5%). Calcifications are rare in HCC, but in fibrolamellar subtype, they can be found up to 70% and can be associated with central scar.

Magnetic resonance

MRI imaging is superior and often diagnostic. Although several imaging protocols and contrast media are available, hepatocyte specific contrast media are beneficial for accurate diagnosis in NCHCC. MRI Imaging characteristics of NCHCC can differ from classical CHCC in few important aspects (Table 2). Some of independent predictors that were utilized in prior studies include T1-hypointensity, T2-hypo/hyperintensity, lack of central tumor-enhancement, and satellite lesions. Use of these features improved the sensitivity and specificity of identifying lesions as high as 91% and 98% respectively[91]. Although superior in nature, MRI features can differ. T1 images are mostly hypointense. Degree of tumor differentiation, iron or glycogen, lipid, copper could make the lesion appear hyperintense. Further lipid could be distributed diffusely, which can manifest as signal drop outs on the MRI[92]. The mosaic pattern often seen in the NCHCC may be better seen on MRI compared to CT, due to better soft tissue enhancement. HCCs are mildly hyperintense or isointense compared to the surrounding liver on T2- weighted images.

Liver biopsy

Histological diagnosis for CHCC is rarely needed due to availability of high quality imaging such as CT and MRI. However, NCHCC often requires biopsy confirmation as the standard LIRADS classification can only be applied in the presence of cirrhosis.

If lesions are smaller (< 1 cm) especially in NCHCC with atypical features, it is reasonable to repeat imaging in 6 months or obtain histological diagnosis with a liver biopsy based upon the pretest probably and HCC risk factors. Multiple routes of liver biopsy can tried; percutaneous or endoscopic ultrasound guided[93]. Risk of bleeding, pain, tumor seeding of the needle track should be weighed against obtaining adequate tissue for histological diagnosis[94]. Multidisciplinary team HCC diagnosis and management has now become the standard of care. Multidisciplinary approach can improve the overall care in these patients[95].

TREATMENT

Treatment options for NCHCC depend on the etiology, tumor size, extent, vascular invasion, performance status of the individual, and their transplant candidacy (Table 3). Surgery remains the mainstay, if the lesion is resectable. With the introduction of antiviral agents for HCV and HBV, there has been significant improvement on HCC occurrence. Advanced surgical techniques have improved survivability in these patients. In recent years, the implementation of locoregional and novel systemic therapies have been added to the armamentarium of the treatment options in these patients.

Antiviral therapy

All patients with NCHCC should be evaluated for underlying cause. If HBV or HCV infection is detected, the patient should be offered the option of antiviral treatment.

HBV

Entecavir and tenofovir are used as first-line therapies for CHB infection. Multiple risk prediction models have been developed for the study of HCC prevalence in HBV patients[96]. Some of these models include individual prediction model, Chinese University-HCC, Guide with Age, Gender, HBV DNA, Core Promoter Mutation and Cirrhosis-HCC, Risk Estimation for Hepatocellular Carcinoma in Chronic HBV and Nomogram-HCC[97]. Arends *et al*[98] noted that HCC incidence is lower in patients who have received antiviral therapy, even though it did not eliminate the risk: The reported 5-year cumulative incidence rate of NCHCC was 2.1%. Although both therapeutic agents reduce the risk of HCC, few studies compared their effectiveness. A systematic review reported that tenofovir was associated with lower risk of HCC development [adjusted hazard ratio (HR) 0.81; 95%CI: 0.62-0.85], however the beneficial effect did not reach statistical significance for non-cirrhotic patients (adjusted HR 0.83; 95%CI: 0.51-1.35)[99].

HCV

DAA agents have revolutionized HCV care. This in turn, has decreased the risk of HCC on both cirrhotics and non-cirrhotics. Tanaka *et al*[100] analyzed 5814 patients (5646 SVR and, 168 non-SVR) from Asia and noted that HCC incidence was higher in the non-SVR group (5.26 *vs* 1.94, $P < 0.001$). Among the SVR group, in non-cirrhotic SVR patients, baseline alfa fetoprotein of ≥ 10 ng/mL was significant (adjusted HR: 4.26, $P = 0.005$)[100]. SVR in HCV patients depended on the type of treatment regimen. Few studies showed that these regimens might achieve SVR (especially in genotype-2) at higher rate in non-cirrhotics compared to cirrhotics (98.2% *vs* 89.4%)[101]

Surgery

Surgical resection remains the mainstay of NCHCC treatment. Survival of these patients is excellent if the lesions are at an early stage[5]. Unfortunately, NCHCC lesions are usually large due to their late diagnosis[102]. Long-term overall survival (OS) after hepatic resection for NCHCC showed an OS rate of 62%-100% (1 year), 46%-78% (3 year) and 30%-64% (5 year)[103,104]. Multiple synchronous lesions, large tumor size, non-clear resection margin, poor tumor staging and lymphatic invasion were indicators of poor prognosis[104]. Subject to the presence of adequate future liver remnant, these patients may undergo repeat resections. Advanced surgical resection modalities, such as non-anatomical (parenchymal sparing liver resections), ex-situ resections along with the application of future liver remnant growth techniques, such as portal venous embolization, two-stage hepatectomies, associating liver partition with portal vein ligation, hepatic venous deprivation with/without locoregional treatments, have allowed for extended repeat resections on these patients, extending survival.

Table 3 Treatment options for non-cirrhotic hepatocellular carcinoma

Treatment	Comments
Antiviral therapy	If HBV or HCV are identified as potential causes of NCHCC, aggressive treatment should be pursued. Entecavir, tenofovir have been used for HBV and DAA agents are used for HCV infection
Surgery	Mainstay for the treatment of NCHCC. BCLC staging cannot be used for NCHCC patients. Tumor size, elevated bilirubin level, low platelet count, vascular invasion can predict prognosis in NCHCC individuals
Locoregional therapy	Limited data available in NCHCC patients. Isolated cases and case series showed improved prognosis with these treatment options
Systemic therapy	Multikinase inhibitors (sorafenib, regorafenib), immunotherapy (nivolumab), chemotherapeutic agents (epirubicin, cisplatin, 5-fluororuacil, capecitabine, docetaxel, GEMOX) have been used in NCHCC with various success

Potential treatment options for non-cirrhotic hepatocellular carcinoma. Antiviral therapy is indicated if hepatitis C virus or hepatitis C virus is identified as a potential cause. While surgery remains the mainstay of the treatment, locoregional and systemic therapy options have been tried. HCC: Hepatocellular carcinoma; DAA: Direct acting antiviral; NCHCC: Non cirrhotic hepatocellular carcinoma; BCLC: Barcelona-Clinic Liver Cancer; HBV: Hepatitis B virus; HCV: Hepatitis C virus; GEMOX: Gemcitabine and oxaliplatin regimen.

Tumor size (> 5 cm), elevated bilirubin levels (> 5.6 mg/L), low platelet count, portal vein thrombus development can predict vascular invasion and prognosis in the patients[105,106]. Few studies in the past debated if tumor size along predicts survival in these patients. Pommergaard *et al*[39] studied 22787 HCC patients from European Liver Transplant Registry transplanted between 1990 and 2016 and noted that HCC in non-cirrhotic livers had similar overall mortality (adjusted HR 1.11, 95%CI: 0.99-1.25), but higher HCC-specific mortality (adjusted HR 1.62, 95%CI: 1.31-2); perhaps due to a more aggressive biology of the tumors in the non-cirrhotic livers. NCHCC patients were younger, had lower MELD scores and higher risk of microvascular invasion and received more locoregional treatment. Irrespective of the background liver quality, vascular invasion remains critical, underlining the need for early diagnosis and management strategies[107].

In the absence of extrahepatic disease or vascular invasion, liver transplantation (LT) has been the gold standard of HCC treatment among cirrhotics based on the Milan criteria, with the number and size of lesions the being the main determinants [108]. If outside Milan criteria, the patients' lesions may be down-staged under specific guidelines (University of California San Francisco criteria), and then be reassessed for transplantability. On patients beyond the established criteria, novel surgical approaches can be pursued, such as living donor transplantation, split transplantation or the use of marginal grafts, such as donation after circulatory death allografts[109]. On non-cirrhotics, macrovascular invasion and extrahepatic spread are the only recommended exclusion criteria for LT[39]. As per Mazzaferro *et al*[108] if transplanted within Milan criteria, the four-year actuarial survival is 76%, and the recurrence free survival 83%. The recurrence risk increases if the pre-transplant lesions is higher: 78% recurrence is noted with lesions higher than 8 cm compared to 40% with 4-8 cm lesions[110,111]. Higher NCHCC recurrence may reflect more advanced tumors and multicentric carcinogenesis[39,112].

Locoregional therapies

Locoregional therapies such as radiofrequency ablation or transarterial embolization modalities, have been highly successful in the management of HCC on cirrhotics. However, little has been reported in the non-cirrhotic setting. Wagle *et al*[79] retrospectively noted ten NCHCC patients with outcomes with LRT and surgery. One patient underwent trans-arterial embolization and three underwent sequential TACE-portal vein embolization. No mortality was noted in these patients and risk-free survival in all these ten patients was 100% at year 1, 62% at year 3 and OS was 100% at year 1, 72% at year 3 and 72% at year 5. These findings indicate the developing role for LRTs for NCHCC and overall improved prognosis.

Systemic treatment

Systemic therapy with multikinase inhibitors (Sorafenib, regorafenib) and programmed cell death protein 1 inhibitor (nivolumab) have been tried for HCC and extended their use in NCHCC[113]. Sorafenib is effective in advanced HCC and can prolong survival in these patients. However, owing to its adverse events (nausea,

excessive fatigue, diarrhea and skin reactions), its widespread use is limited[114]. Use of chemotherapeutic agents such as epirubicin (E), cisplatin (C), 5-fluororuacil (F), capecitabine, docetaxel, GEMOX haven tried in NCHCC and fibrolamellar HCC. Variable responses have noted with these regiments. For instance, epirubicin, cisplatin, capecitabine showed 52% disease control and epirubicin, cisplatin and 5-flurouracil. GEMOX showed complete response in fibrolamellar HCC. Combination therapy such as use of Sorafenib and mammalian target of rapamycin inhibitors[115] has been attempted with one year survival of 82% and five year survival of 33%[116]. New immunotherapy protocols such as atezolizumab-bevacizumab combination has not been studied in NCHCC, however future trials can be expected with these agents in NCHCC[117]. Although these modalities seem promising, further studies are needed to evaluate their role in survivability in NCHCC patients.

FUTURE RESEARCH DIRECTION

Improved cross-sectional imaging characteristics are expected to identify NCHCC at an earlier stage and provide increasing treatment options in the future. Survival and recurrence rates in NCHCC have been improving and expected to reach HCC patients with efficacy of antiviral treatment options, living donor liver transplantation, parenchymal sparing liver resection and two stage liver resections. Use of artificial intelligence, deep learning models (convolutional neural network) are being utilized for identification of NCHCC[118]. Messenger RNA (mRNA) is a family of RNA molecules which are involved in coding proteins and convey genetic information. On the contrary, microRNAs (miRNAs) are non-coding molecules (22 nucleotide length) that regulate gene expression especially in post-transcriptional state[119]. Dysregulated miRNA can lead to DNA damage with alter gene expression playing a role in NCHCC tumor pathogenesis. Use of micro RNA and messenger RNA (miRNA-mRNA) networks with bioinformatic analysis and experimental validation are being development for therapeutics for NHCCC[119]. Use of miRNA as a potential serum biomarker for diagnosis, prognostication, survival after liver resection and systemic therapy have been studied[120,121]. Despite these advances, further research on molecular mechanism of mRNA and miRNA regulation in NCHCC, and validation of genes involved in NCHCC are urgently needed.

CONCLUSION

Following the introduction of direct antiviral treatments and the increasing prevalence of NAFLD, NCHCC has been on the rise. Even though less common than the hepatocellular carcinoma encountered on cirrhotics, NCHCC still accounts for 20% of reported hepatomas. Such tumors are typically diagnosed late, thus compromising the outcome. The discovery and generalization of use of direct antivirals, loco-regional treatments and systemic novel immune-chemotherapies, the advancements of complex hepatobiliary surgery and the genesis of transplant oncology have added to the treatment armamentarium of these aggressive primary liver tumors. Multidisciplinary approach and coordinated care at tertiary high-volume HCC, preferably liver transplant centers, remain critical. It is time the stakeholders pursued a consensus approach in developing universal HCC surveillance and treatment strategies on non-cirrhotic patients at risk, such as NAFLD and/or patients with advanced fibrosis.

REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Davis GL**, Dempster J, Meler JD, Orr DW, Walberg MW, Brown B, Berger BD, O'Connor JK, Goldstein RM. Hepatocellular carcinoma: management of an increasingly common problem. *Proc (Bayl Univ Med Cent)* 2008; **21**: 266-280 [PMID: 18628926 DOI: 10.1080/08998280.2008.11928410]

3 **Lee DH**, Lee JM. Primary malignant tumours in the non-cirrhotic liver. *Eur J Radiol* 2017; **95**: 349-361 [PMID: 28987692 DOI: 10.1016/j.ejrad.2017.08.030]

4 **Schütte K**, Schulz C, Poranzke J, Antweiler K, Bornschein J, Bretschneider T, Arend J, Ricke J, Malfertheiner P. Characterization and prognosis of patients with hepatocellular carcinoma (HCC) in

the non-cirrhotic liver. *BMC Gastroenterol* 2014; **14**: 117 [PMID: 24990270 DOI: 10.1186/1471-230X-14-117]

5 **Sigurdsson B**, Sigurdardottir R, Arnardottir MB, Lund SH, Jonasson JG, Björnsson ES. A nationwide study on hepatocellular carcinoma. *Cancer Epidemiol* 2020; **69**: 101835 [PMID: 33068877 DOI: 10.1016/j.canep.2020.101835]

6 **Papatheodoridis GV**, Sypsa V, Dalekos GN, Yurdaydin C, Van Boemmel F, Buti M, Calleja JL, Chi H, Goulis J, Manolakopoulos S, Loglio A, Voulgaris T, Gatselis N, Keskin O, Veelken R, Lopez-Gomez M, Hansen BE, Savvidou S, Kourikou A, Vlachogiannakos J, Galanis K, Idilman R, Esteban R, Janssen HLA, Berg T, Lampertico P. Hepatocellular carcinoma prediction beyond year 5 of oral therapy in a large cohort of Caucasian patients with chronic hepatitis B. *J Hepatol* 2020; **72**: 1088-1096 [PMID: 31981727 DOI: 10.1016/j.jhep.2020.01.007]

7 **Centers for Disease Control and Prevention (CDC)**. Hepatocellular carcinoma - United States, 2001-2006. *Morb Mortal Wkly Rep* 2010; **59**: 517-520 [PMID: 20448528]

8 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

9 **Singal AG**, Lampertico P, Nahon P. Epidemiology and surveillance for hepatocellular carcinoma: New trends. *J Hepatol* 2020; **72**: 250-261 [PMID: 31954490 DOI: 10.1016/j.jhep.2019.08.025]

10 **Kao JH**. Hepatitis B vaccination and prevention of hepatocellular carcinoma. *Best Pract Res Clin Gastroenterol* 2015; **29**: 907-917 [PMID: 26651252 DOI: 10.1016/j.bpg.2015.09.011]

11 **Trevisani F**, D'Intino PE, Caraceni P, Pizzo M, Stefanini GF, Mazziotti A, Grazi GL, Gozzetti G, Gasbarrini G, Bernardi M. Etiologic factors and clinical presentation of hepatocellular carcinoma. Differences between cirrhotic and noncirrhotic Italian patients. *Cancer* 1995; **75**: 2220-2232 [PMID: 7536121 DOI: 10.1002/1097-0142(19950501)75:9<2220::aid-cncr2820750906>3.0.co;2-4]

12 **Smalley SR**, Moertel CG, Hilton JF, Weiland LH, Weiand HS, Adson MA, Melton LJ 3rd, Batts K. Hepatoma in the noncirrhotic liver. *Cancer* 1988; **62**: 1414-1424 [PMID: 2843280 DOI: 10.1002/1097-0142(19881001)62:7<1414::aid-cncr2820620729>3.0.co;2-7]

13 **Schmit N**, Nayagam S, Thursz MR, Hallett TB. The global burden of chronic hepatitis B virus infection: comparison of country-level prevalence estimates from four research groups. *Int J Epidemiol* 2021; **50**: 560-569 [PMID: 33367672 DOI: 10.1093/ije/dyaa253]

14 **Gaddikeri S**, McNeeley MF, Wang CL, Bhargava P, Dighe MK, Yeh MM, Dubinsky TJ, Kolokythas O, Lalwani N. Hepatocellular carcinoma in the noncirrhotic liver. *AJR Am J Roentgenol* 2014; **203**: W34-W47 [PMID: 24951228 DOI: 10.2214/AJR.13.11511]

15 **Houben KW**, McCall JL. Liver transplantation for hepatocellular carcinoma in patients without underlying liver disease: a systematic review. *Liver Transpl Surg* 1999; **5**: 91-95 [PMID: 10071346 DOI: 10.1002/lt.500050201]

16 **Shim CW**, Park JW, Kim SH, Kim JS, Kim BH, Hong EK. Noncirrhotic hepatocellular carcinoma: etiology and occult hepatitis B virus infection in a hepatitis B virus-endemic area. *Therap Adv Gastroenterol* 2017; **10**: 529-536 [PMID: 28804513 DOI: 10.1177/1756283X17710247]

17 **Tamori A**, Nishiguchi S, Kubo S, Narimatsu T, Habu D, Takeda T, Hirohashi K, Shiomi S. HBV DNA integration and HBV-transcript expression in non-B, non-C hepatocellular carcinoma in Japan. *J Med Virol* 2003; **71**: 492-498 [PMID: 14556260 DOI: 10.1002/jmv.10514]

18 **Billeter AT**, Müller PC, Albrecht T, Roessler S, Löffler M, Lemekhova A, Mehrabi A, Müller-Stich BP, Hoffmann K. Impact of Type 2 Diabetes on Oncologic Outcomes of Hepatocellular Carcinomas in Non-Cirrhotic, Non-alcoholic Steatohepatitis: a Matched-Pair Analysis. *J Gastrointest Surg* 2021; **25**: 1193-1202 [PMID: 32378092 DOI: 10.1007/s11605-020-04628-0]

19 **Ertle J**, Dechêne A, Sowa JP, Penndorf V, Herzer K, Kaiser G, Schlaak JF, Gerken G, Syn WK, Canbay A. Non-alcoholic fatty liver disease progresses to hepatocellular carcinoma in the absence of apparent cirrhosis. *Int J Cancer* 2011; **128**: 2436-2443 [PMID: 21128245 DOI: 10.1002/ijc.25797]

20 **Perumpail RB**, Liu A, Wong RJ, Ahmed A, Harrison SA. Pathogenesis of hepatocarcinogenesis in non-cirrhotic nonalcoholic fatty liver disease: Potential mechanistic pathways. *World J Hepatol* 2015; **7**: 2384-2388 [PMID: 26464753 DOI: 10.4254/wjh.v7.i22.2384]

21 **Sydor S**, Best J, Messerschmidt I, Manka P, Vilchez-Vargas R, Brodesser S, Lucas C, Wegehaupt A, Wenning C, Aßmuth S, Hohenester S, Link A, Faber KN, Moshage H, Cubero FJ, Friedman SL, Gerken G, Trauner M, Canbay A, Bechmann LP. Altered Microbiota Diversity and Bile Acid Signaling in Cirrhotic and Noncirrhotic NASH-HCC. *Clin Transl Gastroenterol* 2020; **11**: e00131 [PMID: 32352707 DOI: 10.14309/ctg.0000000000000131]

22 **Desai A**, Sandhu S, Lai JP, Sandhu DS. Hepatocellular carcinoma in non-cirrhotic liver: A comprehensive review. *World J Hepatol* 2019; **11**: 1-18 [PMID: 30705715 DOI: 10.4254/wjh.v11.i1.1]

23 **Hardt A**, Stippel D, Odenthal M, Hölscher AH, Dienes HP, Drebber U. Development of hepatocellular carcinoma associated with anabolic androgenic steroid abuse in a young bodybuilder: a case report. *Case Rep Pathol* 2012; **2012**: 195607 [PMID: 22934212 DOI: 10.1155/2012/195607]

24 **Perisetti A**, Raghavapuram S, Sheikh AB, Yendala R, Rahman R, Shanshal M, Thein KZ, Farooq A. Mushroom Poisoning Mimicking Painless Progressive Jaundice: A Case Report with Review of the Literature. *Cureus* 2018; **10**: e2436 [PMID: 29896444 DOI: 10.7759/cureus.2436]

25 **Chu YJ**, Yang HI, Wu HC, Liu J, Wang LY, Lu SN, Lee MH, Jen CL, You SL, Santella RM, Chen CJ. Aflatoxin B_1 exposure increases the risk of cirrhosis and hepatocellular carcinoma in chronic hepatitis B virus carriers. *Int J Cancer* 2017; **141**: 711-720 [PMID: 28509392 DOI: 10.1002/ijc.30782]

26 **Britto MR**, Thomas LA, Balaratnam N, Griffiths AP, Duane PD. Hepatocellular carcinoma arising in non-cirrhotic liver in genetic haemochromatosis. *Scand J Gastroenterol* 2000; **35**: 889-893 [PMID: 10994630 DOI: 10.1080/003655200750023282]

27 **Evert M**, Dombrowski F. [Hepatocellular carcinoma in the non-cirrhotic liver]. *Pathologe* 2008; **29**: 47-52 [PMID: 18057936 DOI: 10.1007/s00292-007-0953-3]

28 **Topic A**, Ljujic M, Radojkovic D. Alpha-1-antitrypsin in pathogenesis of hepatocellular carcinoma. *Hepat Mon* 2012; **12**: e7042 [PMID: 23162602 DOI: 10.5812/hepatmon.7042]

29 **Deybach JC**, Puy H. Hepatocellular carcinoma without cirrhosis: think acute hepatic porphyrias and vice versa. *J Intern Med* 2011; **269**: 521-524 [PMID: 21323767 DOI: 10.1111/j.1365-2796.2011.02358.x]

30 **Nakayama M**, Okamoto Y, Morita T, Matsumoto M, Fukui H, Nakano H, Tsujii T. Promoting effect of citrulline in hepatocarcinogenesis: possible mechanism in hypercitrullinemia. *Hepatology* 1990; **11**: 819-823 [PMID: 2347556 DOI: 10.1002/hep.1840110517]

31 **Müller M**, Bird TG, Nault JC. The landscape of gene mutations in cirrhosis and hepatocellular carcinoma. *J Hepatol* 2020; **72**: 990-1002 [PMID: 32044402 DOI: 10.1016/j.jhep.2020.01.019]

32 **Tobari M**, Hashimoto E, Taniai M, Kodama K, Kogiso T, Tokushige K, Yamamoto M, Takayoshi N, Satoshi K, Tatsuo A. The characteristics and risk factors of hepatocellular carcinoma in nonalcoholic fatty liver disease without cirrhosis. *J Gastroenterol Hepatol* 2020; **35**: 862-869 [PMID: 31597206 DOI: 10.1111/jgh.14867]

33 **Wörns MA**, Bosslet T, Victor A, Koch S, Hoppe-Lotichius M, Heise M, Hansen T, Pitton MB, Niederle IM, Schuchmann M, Weinmann A, Düber C, Galle PR, Otto G. Prognostic factors and outcomes of patients with hepatocellular carcinoma in non-cirrhotic liver. *Scand J Gastroenterol* 2012; **47**: 718-728 [PMID: 22472070 DOI: 10.3109/00365521.2012.677952]

34 **Giannini EG**, Marenco S, Bruzzone L, Savarino V, Farinati F, Del Poggio P, Rapaccini GL, Di Nolfo MA, Benvegnù L, Zoli M, Borzio F, Caturelli E, Chiaramonte M, Trevisani F. Hepatocellular carcinoma in patients without cirrhosis in Italy. *Dig Liver Dis* 2013; **45**: 164-169 [PMID: 23047000 DOI: 10.1016/j.dld.2012.08.018]

35 **Alfaiate D**, Clément S, Gomes D, Goossens N, Negro F. Chronic hepatitis D and hepatocellular carcinoma: A systematic review and meta-analysis of observational studies. *J Hepatol* 2020; **73**: 533-539 [PMID: 32151618 DOI: 10.1016/j.jhep.2020.02.030]

36 **Tseng TC**, Liu CJ, Chang CT, Su TH, Yang WT, Tsai CH, Chen CL, Yang HC, Liu CH, Chen PJ, Chen DS, Kao JH. HEV superinfection accelerates disease progression in patients with chronic HBV infection and increases mortality in those with cirrhosis. *J Hepatol* 2020; **72**: 1105-1111 [PMID: 32006586 DOI: 10.1016/j.jhep.2020.01.012]

37 **Perisetti A**, Laoveeravat P, Inamdar S, Tharian B, Thandassery R, Goyal H. Hepatitis E virus infection in liver transplant recipients: a descriptive literature review. *Eur J Gastroenterol Hepatol* 2020; **32**: 916-922 [PMID: 32091436 DOI: 10.1097/MEG.0000000000001682]

38 **Wong N**, Lai P, Lee SW, Fan S, Pang E, Liew CT, Sheng Z, Lau JW, Johnson PJ. Assessment of genetic changes in hepatocellular carcinoma by comparative genomic hybridization analysis: relationship to disease stage, tumor size, and cirrhosis. *Am J Pathol* 1999; **154**: 37-43 [PMID: 9916916 DOI: 10.1016/S0002-9440(10)65248-0]

39 **Pommergaard HC**, Rostved AA, Adam R, Rasmussen A, Salizzoni M, Bravo MAG, Cherqui D, De Simone P, Houssel-Debry P, Mazzaferro V, Soubrane O, García-Valdecasas JC, Prous JF, Pinna AD, O'Grady J, Karam V, Duvoux C, Thygesen LC. Mortality after Transplantation for Hepatocellular Carcinoma: A Study from the European Liver Transplant Registry. *Liver Cancer* 2020; **9**: 455-467 [PMID: 32999871 DOI: 10.1159/000507397]

40 **Béguelin C**, Moradpour D, Sahli R, Suter-Riniker F, Lüthi A, Cavassini M, Günthard HF, Battegay M, Bernasconi E, Schmid P, Calmy A, Braun DL, Furrer H, Rauch A, Wandeler G; Swiss HIV Cohort Study. Hepatitis delta-associated mortality in HIV/HBV-coinfected patients. *J Hepatol* 2017; **66**: 297-303 [PMID: 27746337 DOI: 10.1016/j.jhep.2016.10.007]

41 **Polaris Observatory Collaborators**. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol* 2018; **3**: 383-403 [PMID: 29599078 DOI: 10.1016/S2468-1253(18)30056-6]

42 **Lim JK**, Nguyen MH, Kim WR, Gish R, Perumalswami P, Jacobson IM. Prevalence of Chronic Hepatitis B Virus Infection in the United States. *Am J Gastroenterol* 2020; **115**: 1429-1438 [PMID: 32483003 DOI: 10.14309/ajg.0000000000000651]

43 **Stuyver L**, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; **81**: 67-74 [PMID: 10640543 DOI: 10.1099/0022-1317-81-1-67]

44 **Chan HL**, Wong GL, Tse CH, Chim AM, Yiu KK, Chan HY, Sung JJ, Wong VW. Hepatitis B virus genotype C is associated with more severe liver fibrosis than genotype B. *Clin Gastroenterol Hepatol* 2009; **7**: 1361-1366 [PMID: 19683072 DOI: 10.1016/j.cgh.2009.08.004]

45 **Chen CH**, Eng HL, Lee CM, Kuo FY, Lu SN, Huang CM, Tung HD, Chen CL, Changchien CS. Correlations between hepatitis B virus genotype and cirrhotic or non-cirrhotic hepatoma. *Hepatogastroenterology* 2004; **51**: 552-555 [PMID: 15086200]

46 **Kao JH**, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. *Gastroenterology* 2003; **124**: 327-334 [PMID: 12557138 DOI: 10.1053/gast.2003.50053]

47 **Do AL**, Wong CR, Nguyen LH, Nguyen VG, Trinh H, Nguyen MH. Hepatocellular carcinoma incidence in noncirrhotic patients with chronic hepatitis B and patients with cirrhosis of all etiologies. *J Clin Gastroenterol* 2014; **48**: 644-649 [PMID: 24201999 DOI: 10.1097/MCG.0000000000000015]

48 **Girones R**, Miller RH. Mutation rate of the hepadnavirus genome. *Virology* 1989; **170**: 595-597 [PMID: 2728351 DOI: 10.1016/0042-6822(89)90455-8]

49 **Sanyal AJ**, Yoon SK, Lencioni R. The etiology of hepatocellular carcinoma and consequences for treatment. *Oncologist* 2010; **15** Suppl 4: 14-22 [PMID: 21115577 DOI: 10.1634/theoncologist.2010-S4-14]

50 **Wang C**, Chen V, Vu V, Le A, Nguyen L, Zhao C, Wong CR, Nguyen N, Li J, Zhang J, Trinh H, Nguyen MH. Poor adherence and low persistency rates for hepatocellular carcinoma surveillance in patients with chronic hepatitis B. *Medicine (Baltimore)* 2016; **95**: e4744 [PMID: 27583921 DOI: 10.1097/MD.0000000000004744]

51 **Chayanupatkul M**, Omino R, Mittal S, Kramer JR, Richardson P, Thrift AP, El-Serag HB, Kanwal F. Hepatocellular carcinoma in the absence of cirrhosis in patients with chronic hepatitis B virus infection. *J Hepatol* 2017; **66**: 355-362 [PMID: 27693539 DOI: 10.1016/j.jhep.2016.09.013]

52 **Mani SKK**, Andrisani O. Hepatitis B Virus-Associated Hepatocellular Carcinoma and Hepatic Cancer Stem Cells. *Genes (Basel)* 2018; **9**: 137 [PMID: 29498629 DOI: 10.3390/genes9030137]

53 **Tu T**, Budzinska MA, Shackel NA, Urban S. HBV DNA Integration: Molecular Mechanisms and Clinical Implications. *Viruses* 2017; **9**: 75 [PMID: 28394272 DOI: 10.3390/v9040075]

54 **Turton KL**, Meier-Stephenson V, Badmalia MD, Coffin CS, Patel TR. Host Transcription Factors in Hepatitis B Virus RNA Synthesis. *Viruses* 2020; **12**: 160 [PMID: 32019103 DOI: 10.3390/v12020160]

55 **Dong H**, Zhang L, Qian Z, Zhu X, Zhu G, Chen Y, Xie X, Ye Q, Zang J, Ren Z, Ji Q. Identification of HBV-MLL4 Integration and Its Molecular Basis in Chinese Hepatocellular Carcinoma. *PLoS One* 2015; **10**: e0123175 [PMID: 25901726 DOI: 10.1371/journal.pone.0123175]

56 **Belloni L**, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, Petersen J, Raimondo G, Dandri M, Levrero M. IFN-α inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. *J Clin Invest* 2012; **122**: 529-537 [PMID: 22251702 DOI: 10.1172/JCI58847]

57 **Iwamoto M**, Saso W, Sugiyama R, Ishii K, Ohki M, Nagamori S, Suzuki R, Aizaki H, Ryo A, Yun JH, Park SY, Ohtani N, Muramatsu M, Iwami S, Tanaka Y, Sureau C, Wakita T, Watashi K. Epidermal growth factor receptor is a host-entry cofactor triggering hepatitis B virus internalization. *Proc Natl Acad Sci USA* 2019; **116**: 8487-8492 [PMID: 30952782 DOI: 10.1073/pnas.1811064116]

58 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

59 **Lindenbach BD**, Rice CM. Unravelling hepatitis C virus replication from genome to function. *Nature* 2005; **436**: 933-938 [PMID: 16107832 DOI: 10.1038/nature04077]

60 **Alotaibi AS**, Alghamdi W, Marotta P, Qumosani K. A266 Hepatocellular carcinoma prevalence in non-cirrhotic hepatitis c patients. *J Can Assoc Gastroenterol* 2018; **1** suppl 2: 383-384 [DOI: 10.1093/jcag/gwy009.266]

61 **Ikeda M**, Fujiyama S, Tanaka M, Sata M, Ide T, Yatsuhashi H, Watanabe H. Risk factors for development of hepatocellular carcinoma in patients with chronic hepatitis C after sustained response to interferon. *J Gastroenterol* 2005; **40**: 148-156 [PMID: 15770398 DOI: 10.1007/s00535-004-1519-2]

62 **Ioannou GN**. HCC surveillance after SVR in patients with F3/F4 fibrosis. *J Hepatol* 2021; **74**: 458-465 [PMID: 33303216 DOI: 10.1016/j.jhep.2020.10.016]

63 **Mattos AA**, Marcon Pdos S, Araújo FS, Coral GP, Tovo CV. Hepatocellular carcinoma in a non-cirrhotic patient with sustained virological response after hepatitis c treatment. *Rev Inst Med Trop Sao Paulo* 2015; **57**: 519-522 [PMID: 27049708 DOI: 10.1590/S0036-46652015000600011]

64 **Lee MH**, Yang HI, Lu SN, Jen CL, You SL, Wang LY, L'Italien G, Chen CJ, Yuan Y; REVEAL-HCV Study Group. Hepatitis C virus genotype 1b increases cumulative lifetime risk of hepatocellular carcinoma. *Int J Cancer* 2014; **135**: 1119-1126 [PMID: 24482200 DOI: 10.1002/ijc.28753]

65 **Ray RB**, Meyer K, Ray R. Hepatitis C virus core protein promotes immortalization of primary human hepatocytes. *Virology* 2000; **271**: 197-204 [PMID: 10814584 DOI: 10.1006/viro.2000.0295]

66 **D'souza S**, Lau KC, Coffin CS, Patel TR. Molecular mechanisms of viral hepatitis induced hepatocellular carcinoma. *World J Gastroenterol* 2020; **26**: 5759-5783 [PMID: 33132633 DOI: 10.3748/wjg.v26.i38.5759]

67 **Valgimigli M**, Valgimigli L, Trerè D, Gaiani S, Pedulli GF, Gramantieri L, Bolondi L. Oxidative stress EPR measurement in human liver by radical-probe technique. Correlation with etiology, histology and cell proliferation. *Free Radic Res* 2002; **36**: 939-948 [PMID: 12448819 DOI: 10.1080/1071560210000006653]

68 **Kim CW**, Chang KM. Hepatitis C virus: virology and life cycle. *Clin Mol Hepatol* 2013; **19**: 17-25 [PMID: 23593605 DOI: 10.3350/cmh.2013.19.1.17]

69 **Smirnova OA**, Ivanova ON, Bartosch B, Valuev-Elliston VT, Mukhtarov F, Kochetkov SN, Ivanov AV. Hepatitis C Virus NS5A Protein Triggers Oxidative Stress by Inducing NADPH Oxidases 1 and 4 and Cytochrome P450 2E1. *Oxid Med Cell Longev* 2016; **2016**: 8341937 [PMID: 27200149 DOI: 10.1155/2016/8341937]

70 **European Association for the Study of the Liver**. European Organisation for Research and Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908-943 [PMID: 22424438 DOI: 10.1016/j.jhep.2011.12.001]

71 **Matthews PC**, Geretti AM, Goulder PJ, Klenerman P. Epidemiology and impact of HIV coinfection with hepatitis B and hepatitis C viruses in Sub-Saharan Africa. *J Clin Virol* 2014; **61**: 20-33 [PMID: 24973812 DOI: 10.1016/j.jcv.2014.05.018]

72 **Rajbhandari R**, Jun T, Khalili H, Chung RT, Ananthakrishnan AN. HBV/HIV coinfection is associated with poorer outcomes in hospitalized patients with HBV or HIV. *J Viral Hepat* 2016; **23**: 820-829 [PMID: 27291562 DOI: 10.1111/jvh.12555]

73 **Akinyemi JO**, Ogunbosi BO, Fayemiwo AS, Adesina OA, Obaro M, Kuti MA, Awolude OA, Olaleye DO, Adewole IF. Demographic and epidemiological characteristics of HIV opportunistic infections among older adults in Nigeria. *Afr Health Sci* 2017; **17**: 315-321 [PMID: 29062325 DOI: 10.4314/ahs.v17i2.4]

74 **Miao Z**, Zhang S, Ou X, Li S, Ma Z, Wang W, Peppelenbosch MP, Liu J, Pan Q. Estimating the Global Prevalence, Disease Progression, and Clinical Outcome of Hepatitis Delta Virus Infection. *J Infect Dis* 2020; **221**: 1677-1687 [PMID: 31778167 DOI: 10.1093/infdis/jiz633]

75 **Sureau C**, Negro F. The hepatitis delta virus: Replication and pathogenesis. *J Hepatol* 2016; **64**: S102-S116 [PMID: 27084031 DOI: 10.1016/j.jhep.2016.02.013]

76 **Diaz G**, Engle RE, Tice A, Melis M, Montenegro S, Rodriguez-Canales J, Hanson J, Emmert-Buck MR, Bock KW, Moore IN, Zamboni F, Govindarajan S, Kleiner DE, Farci P. Molecular Signature and Mechanisms of Hepatitis D Virus-Associated Hepatocellular Carcinoma. *Mol Cancer Res* 2018; **16**: 1406-1419 [PMID: 29858376 DOI: 10.1158/1541-7786.MCR-18-0012]

77 **Augustine T**, Perisetti A, Tharian B. Unusual Cause of Upper Gastrointestinal Bleeding in Cirrhosis. *Clin Gastroenterol Hepatol* 2020; **18**: e60-e61 [PMID: 30902600 DOI: 10.1016/j.cgh.2019.03.026]

78 **Polavarapu AD**, Ahmed M, Samaha G, Weerasinghe CK, Deeb L, Sokoloff A. Spontaneous Rupture of Fibrolamellar Variant Hepatocellular Carcinoma. *Gastroenterology Res* 2019; **12**: 166-170 [PMID: 31236158 DOI: 10.14740/gr1148]

79 **Wagle P**, Narkhede R, Desai G, Pande P, Kulkarni DR, Varty P. Surgical management of large hepatocellular carcinoma: the first single-center study from western India. *Arq Bras Cir Dig* 2020; **33**: e1505 [PMID: 33237158 DOI: 10.1590/0102-672020190001e1505]

80 **Huo TI**, Ho SY, Liu PH. Changing faces of hepatocellular carcinoma: East vs West. *Liver Int* 2021; **41**: 1430-1431 [PMID: 33283446 DOI: 10.1111/liv.14753]

81 **Chalaye J**, Costentin CE, Luciani A, Amaddeo G, Ganne-Carrié N, Baranes L, Allaire M, Calderaro J, Azoulay D, Nahon P, Seror O, Mallat A, Soussan M, Duvoux C, Itti E, Nault JC. Positron emission tomography/computed tomography with 18F-fluorocholine improve tumor staging and treatment allocation in patients with hepatocellular carcinoma. *J Hepatol* 2018; **69**: 336-344 [PMID: 29518452 DOI: 10.1016/j.jhep.2018.02.018]

82 **Nie P**, Wang N, Pang J, Yang G, Duan S, Chen J, Xu W. CT-Based Radiomics Nomogram: A Potential Tool for Differentiating Hepatocellular Adenoma From Hepatocellular Carcinoma in the Noncirrhotic Liver. *Acad Radiol* 2021; **28**: 799-807 [PMID: 32386828 DOI: 10.1016/j.acra.2020.04.027]

83 **Nie P**, Yang G, Guo J, Chen J, Li X, Ji Q, Wu J, Cui J, Xu W. A CT-based radiomics nomogram for differentiation of focal nodular hyperplasia from hepatocellular carcinoma in the non-cirrhotic liver. *Cancer Imaging* 2020; **20**: 20 [PMID: 32093786 DOI: 10.1186/s40644-020-00297-z]

84 **Guniganti P**, Kierans AS. PET/MRI of the hepatobiliary system: Review of techniques and applications. *Clin Imaging* 2021; **71**: 160-169 [PMID: 33285404 DOI: 10.1016/j.clinimag.2020.10.056]

85 **Liang Y**, Xu F, Guo Y, Lai L, Jiang X, Wei X, Wu H, Wang J. Diagnostic performance of LI-RADS for MRI and CT detection of HCC: A systematic review and diagnostic meta-analysis. *Eur J Radiol* 2021; **134**: 109404 [PMID: 33276248 DOI: 10.1016/j.ejrad.2020.109404]

86 **Jamwal R**, Krishnan V, Kushwaha DS, Khurana R. Hepatocellular carcinoma in non-cirrhotic versus cirrhotic liver: a clinico-radiological comparative analysis. *Abdom Radiol (NY)* 2020; **45**: 2378-2387 [PMID: 32372205 DOI: 10.1007/s00261-020-02561-z]

87 **Marrero JA**, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; **68**: 723-750 [PMID: 29624699 DOI: 10.1002/hep.29913]

88 **Psatha EA**, Semelka RC, Fordham L, Firat Z, Woosley JT. Undifferentiated (embryonal) sarcoma of the liver (USL): MRI findings including dynamic gadolinium enhancement. *Magn Reson Imaging* 2004; **22**: 897-900 [PMID: 15234460 DOI: 10.1016/j.mri.2004.02.003]

89 **Esfeh JM**, Hajifathalian K, Ansari-Gilani K. Sensitivity of ultrasound in detecting hepatocellular carcinoma in obese patients compared to explant pathology as the gold standard. *Clin Mol Hepatol* 2020; **26**: 54-59 [PMID: 31726817 DOI: 10.3350/cmh.2019.0039]

90 **Di Martino M**, Saba L, Bosco S, Rossi M, Miles KA, Di Miscio R, Lombardo CV, Tamponi E, Piga M, Catalano C. Hepatocellular carcinoma (HCC) in non-cirrhotic liver: clinical, radiological and pathological findings. *Eur Radiol* 2014; **24**: 1446-1454 [PMID: 24770466 DOI: 10.1007/s00330-014-3173-2]

91 **Fischer MA**, Raptis DA, Donati OF, Hunziker R, Schade E, Sotiropoulos GC, McCall J, Bartlett A, Bachellier P, Frilling A, Breitenstein S, Clavien PA, Alkadhi H, Patak MA. MR imaging features for

improved diagnosis of hepatocellular carcinoma in the non-cirrhotic liver: Multi-center evaluation. *Eur J Radiol* 2015; **84**: 1879-1887 [PMID: 26194029 DOI: 10.1016/j.ejrad.2015.06.029]

92 **Mitchell DG**, Palazzo J, Hann HW, Rifkin MD, Burk DL Jr, Rubin R. Hepatocellular tumors with high signal on T1-weighted MR images: chemical shift MR imaging and histologic correlation. *J Comput Assist Tomogr* 1991; **15**: 762-769 [PMID: 1653279 DOI: 10.1097/00004728-199109000-00007]

93 **Johnson KD**, Laoveeravat P, Yee EU, Perisetti A, Thandassery RB, Tharian B. Endoscopic ultrasound guided liver biopsy: Recent evidence. *World J Gastrointest Endosc* 2020; **12**: 83-97 [PMID: 32218888 DOI: 10.4253/wjge.v12.i3.83]

94 **Palmer QD**, Perisetti A, Broadfoot B, Johnson KD, Garcia-Saenz-de-Sicilia M. Recurrent Hepatocellular Carcinoma From Needle Tract Seeding 13 Years Post-Transplant: 2218. *Am J Gastroenterol* 2019; **114**: S1242-S1243

95 **Lhewa D**, Green EW, Naugler WE. Multidisciplinary Team Management of Hepatocellular Carcinoma Is Standard of Care. *Clin Liver Dis* 2020; **24**: 771-787 [PMID: 33012458 DOI: 10.1016/j.cld.2020.07.009]

96 **Dasari BV**, Kamarajah SK, Hodson J, Pawlik TM, Vauthey JN, Ma YT, Punia P, Coldham C, Abradelo M, Roberts KJ, Marudanayagam R, Sutcliffe RP, Muiesan P, Mirza DF, Isaac J. Development and validation of a risk score to predict the overall survival following surgical resection of hepatocellular carcinoma in non-cirrhotic liver. *HPB (Oxford)* 2020; **22**: 383-390 [PMID: 31416786 DOI: 10.1016/j.hpb.2019.07.007]

97 **Chon HY**, Lee JS, Lee HW, Chun HS, Kim BK, Park JY, Kim DY, Ahn SH, Kim SU. Impact of antiviral therapy on risk prediction model for hepatocellular carcinoma development in patients with chronic hepatitis B. *Hepatol Res* 2021; **51**: 406-416 [PMID: 33242365 DOI: 10.1111/hepr.13600]

98 **Arends P**, Sonneveld MJ, Zoutendijk R, Carey I, Brown A, Fasano M, Mutimer D, Deterding K, Reijnders JG, Oo Y, Petersen J, van Bömmel F, de Knegt RJ, Santantonio T, Berg T, Welzel TM, Wedemeyer H, Buti M, Pradat P, Zoulim F, Hansen B, Janssen HL; VIRGIL Surveillance Study Group. Entecavir treatment does not eliminate the risk of hepatocellular carcinoma in chronic hepatitis B: limited role for risk scores in Caucasians. *Gut* 2015; **64**: 1289-1295 [PMID: 25011935 DOI: 10.1136/gutjnl-2014-307023]

99 **Cheung KS**, Mak LY, Liu SH, Cheng HM, Seto WK, Yuen MF, Lai CL. Entecavir vs Tenofovir in Hepatocellular Carcinoma Prevention in Chronic Hepatitis B Infection: A Systematic Review and Meta-Analysis. *Clin Transl Gastroenterol* 2020; **11**: e00236 [PMID: 33031195 DOI: 10.14309/ctg.0000000000000236]

100 **Tanaka Y**, Ogawa E, Huang CF, Toyoda H, Jun DW, Tseng CH, Hsu YC, Enomoto M, Takahashi H, Furusyo N, Yeh ML, Iio E, Yasuda S, Lam CP, Lee DH, Haga H, Yoon EL, Ahn SB, Wong G, Nakamuta M, Nomura H, Tsai PC, Jung JH, Song DS, Dang H, Maeda M, Henry L, Cheung R, Yuen MF, Ueno Y, Eguchi Y, Tamori A, Yu ML, Hayashi J, Nguyen MH; REAL-C Investigators. HCC risk post-SVR with DAAs in East Asians: findings from the REAL-C cohort. *Hepatol Int* 2020; **14**: 1023-1033 [PMID: 33277685 DOI: 10.1007/s12072-020-10105-2]

101 **Cheng TS**, Liang PC, Huang CF, Yeh ML, Huang CI, Lin ZY, Chen SC, Huang JF, Dai CY, Hsieh PH, Chuang WL, Yu ML. Real-world effectiveness of direct-acting antiviral agents for chronic hepatitis C patients with genotype-2 infection after completed treatment. *Kaohsiung J Med Sci* 2021; **37**: 334-345 [PMID: 33151016 DOI: 10.1002/kjm2.12315]

102 **Cipriani F**, Fantini C, Ratti F, Lauro R, Tranchart H, Halls M, Scuderi V, Barkhatov L, Edwin B, Troisi RI, Dagher I, Reggiani P, Belli G, Aldrighetti L, Abu Hilal M. Laparoscopic liver resections for hepatocellular carcinoma. Can we extend the surgical indication in cirrhotic patients? *Surg Endosc* 2018; **32**: 617-626 [PMID: 28717870 DOI: 10.1007/s00464-017-5711-x]

103 **Xie QS**, Chen ZX, Zhao YJ, Gu H, Geng XP, Liu FB. Systematic review of outcomes and meta-analysis of risk factors for prognosis after liver resection for hepatocellular carcinoma without cirrhosis. *Asian J Surg* 2021; **44**: 36-45 [PMID: 32988708 DOI: 10.1016/j.asjsur.2020.08.019]

104 **Martínez-Mier G**, Esquivel-Torres S, Casanova-Sánchez IE, Escobar-Ríos AY, Troche-Gutiérrez JM, Yoldi-Aguirre CA. Hepatocellular carcinoma in the noncirrhotic liver: Clinical features and outcomes in Veracruz, Mexico. *Rev Gastroenterol Mex (Engl Ed)* 2021; **86**: 4-12 [PMID: 32345506 DOI: 10.1016/j.rgmx.2019.11.007]

105 **Lim C**, Compagnon P, Sebagh M, Salloum C, Calderaro J, Luciani A, Pascal G, Laurent A, Levesque E, Maggi U, Feray C, Cherqui D, Castaing D, Azoulay D. Hepatectomy for hepatocellular carcinoma larger than 10 cm: preoperative risk stratification to prevent futile surgery. *HPB (Oxford)* 2015; **17**: 611-623 [PMID: 25980326 DOI: 10.1111/hpb.12416]

106 **Abdalla EK**, Denys A, Hasegawa K, Leung TW, Makuuchi M, Murthy R, Ribero D, Zorzi D, Vauthey JN, Torzilli G. Treatment of large and advanced hepatocellular carcinoma. *Ann Surg Oncol* 2008; **15**: 979-985 [PMID: 18236115 DOI: 10.1245/s10434-007-9727-7]

107 **Panwar R**, Pal S, Dash NR, Shalimar, Sahni P, Acharya SK, Pande GK, Chattopadhyay TK, Nundy S. Hepatic resection for predominantly large size hepatocellular carcinoma: Early and long-term results from a tertiary care center in India. *Indian J Gastroenterol* 2016; **35**: 280-286 [PMID: 27515744 DOI: 10.1007/s12664-016-0678-4]

108 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699 [PMID: 8594428 DOI: 10.1056/NEJM199603143341104]

109 **Sapisochin G**, Bruix J. Liver transplantation for hepatocellular carcinoma: outcomes and novel surgical approaches. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 203-217 [PMID: 28053342 DOI: 10.1038/nrgastro.2016.193]

110 **McPeake JR**, O'Grady JG, Zaman S, Portmann B, Wight DG, Tan KC, Calne RY, Williams R. Liver transplantation for primary hepatocellular carcinoma: tumor size and number determine outcome. *J Hepatol* 1993; **18**: 226-234 [PMID: 7691926 DOI: 10.1016/s0168-8278(05)80250-8]

111 **Halazun KJ**, Sapisochin G, von Ahrens D, Agopian VG, Tabrizian P. Predictors of outcome after liver transplantation for hepatocellular carcinoma (HCC) beyond Milan criteria. *Int J Surg* 2020; **82S**: 61-69 [PMID: 32707331 DOI: 10.1016/j.ijsu.2020.07.029]

112 **Taura K**, Ikai I, Hatano E, Yasuchika K, Nakajima A, Tada M, Seo S, Machimoto T, Uemoto S. Influence of coexisting cirrhosis on outcomes after partial hepatic resection for hepatocellular carcinoma fulfilling the Milan criteria: an analysis of 293 patients. *Surgery* 2007; **142**: 685-694 [PMID: 17981188 DOI: 10.1016/j.surg.2007.05.009]

113 Nivolumab Approved for Liver Cancer. *Cancer Discov* 2017; **7**: OF3 [PMID: 28978557 DOI: 10.1158/2159-8290.CD-NB2017-138]

114 **Hussaarts KGAM**, van Doorn L, Bins S, Sprengers D, de Bruijn P, van Leeuwen RWF, Koolen SLW, van Gelder T, Mathijssen RHJ. Combining Sorafenib and Immunosuppression in Liver Transplant Recipients with Hepatocellular Carcinoma. *Pharmaceuticals (Basel)* 2021; **14**: 46 [PMID: 33435321 DOI: 10.3390/ph14010046]

115 **Li T**, Zhang G, Wang L, Li S, Xu X, Gao Y. Defects in mTORC1 Network and mTORC1-STAT3 Pathway Crosstalk Contributes to Non-inflammatory Hepatocellular Carcinoma. *Front Cell Dev Biol* 2020; **8**: 225 [PMID: 32363190 DOI: 10.3389/fcell.2020.00225]

116 **Invernizzi F**, Iavarone M, Zavaglia C, Mazza S, Maggi U, Cesarini L, Antonelli B, Airoldi A, Manini MA, Sangiovanni A, Rossi G, Donato MF, Saverio Belli L, Lampertico P. Experience With Early Sorafenib Treatment With mTOR Inhibitors in Hepatocellular Carcinoma Recurring After Liver Transplantation. *Transplantation* 2020; **104**: 568-574 [PMID: 31517781 DOI: 10.1097/TP.0000000000002955]

117 **Finn RS**, Qin S, Ikeda M, Galle PR, Ducreux M, Kim TY, Kudo M, Breder V, Merle P, Kaseb AO, Li D, Verret W, Xu DZ, Hernandez S, Liu J, Huang C, Mulla S, Wang Y, Lim HY, Zhu AX, Cheng AL; IMbrave150 Investigators. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *N Engl J Med* 2020; **382**: 1894-1905 [PMID: 32402160 DOI: 10.1056/NEJMoa1915745]

118 **Oestmann PM**, Wang CJ, Savic LJ, Hamm CA, Stark S, Schobert I, Gebauer B, Schlachter T, Lin M, Weinreb JC, Batra R, Mulligan D, Zhang X, Duncan JS, Chapiro J. Deep learning-assisted differentiation of pathologically proven atypical and typical hepatocellular carcinoma (HCC) versus non-HCC on contrast-enhanced MRI of the liver. *Eur Radiol* 2021; epub ahead of print [PMID: 33409782 DOI: 10.1007/s00330-020-07559-1]

119 **Ding B**, Lou W, Liu J, Li R, Chen J, Fan W. In silico analysis excavates potential biomarkers by constructing miRNA-mRNA networks between non-cirrhotic HCC and cirrhotic HCC. *Cancer Cell Int* 2019; **19**: 186 [PMID: 31346321 DOI: 10.1186/s12935-019-0901-3]

120 **Zhang Y**, Li T, Qiu Y, Zhang T, Guo P, Ma X, Wei Q, Han L. Serum microRNA panel for early diagnosis of the onset of hepatocellular carcinoma. *Medicine (Baltimore)* 2017; **96**: e5642 [PMID: 28079796 DOI: 10.1097/MD.0000000000005642]

121 **Nishida N**, Arizumi T, Hagiwara S, Ida H, Sakurai T, Kudo M. MicroRNAs for the Prediction of Early Response to Sorafenib Treatment in Human Hepatocellular Carcinoma. *Liver Cancer* 2017; **6**: 113-125 [PMID: 28275578 DOI: 10.1159/000449475]

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REVIEW

Natural killer cells in pancreatic cancer stroma

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Abstract

Pancreatic cancer remains one of medicine's largest areas of unmet need. With five-year survival rates of < 8%, little improvement has been made in the last 50 years. Typically presenting with advance stage disease, treatment options are limited. To date, surgery remains the only potentially curative option, however, with such late disease presentation, the majority of patients are unresectable. Thus, new therapeutic options and a greater understanding of the complex stromal interactions within the tumour microenvironment are sorely needed to revise the dismal outlook for pancreatic cancer patients. Natural killer (NK) cells are crucial effector units in cancer immunosurveillance. Often used as a prognostic biomarker in a range of malignancies, NK cells have received much attention as an attractive target for immunotherapies, both as cell therapy and as a pharmaceutical target. Despite this interest, the role of NK cells in pancreatic cancer remains poorly defined. Nevertheless, increasing evidence of the importance of NK cells in this dismal prognosis disease is beginning to come to light. Here, we review the role of NK cells in pancreatic cancer, examine the complex interactions of these crucial effector units within pancreatic cancer stroma and shed light on the increasingly attractive use of NK cells as therapy.

Key Words: Pancreatic cancer; Natural killer cells; Tumour microenvironment; Pancreatic cancer stroma; Stromal cells

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Core Tip: Increasing evidence has found natural killer (NK) cells to be crucial players in the prognosis and progression of cancer. Whilst pancreatic cancer remains one of medicine's largest areas of unmet need, NK cells may prove to be an exciting new therapeutic option for pancreatic cancer patients. Here we provide an overview of the complex interactions between NK cells and pancreatic cancer stroma, suggest a role for NK cells as prognostic biomarkers and highlight exciting new NK cell-based treatment options which may transform the therapeutic landscape of pancreatic cancer.

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INTRODUCTION

Pancreatic cancer

Pancreatic cancer is a malignancy with a dismal prognosis. Set to become the second leading cause of cancer-related death worldwide by 2030, little progress has been made in the treatment of pancreatic cancer over the past five decades[1,2]. Surgery remains the only potentially curative option, however, with the majority of patients typically presenting with advance stage disease, most cases are unresectable. Further to this, with approximately 80% of surgery patients relapsing, frequently within two years, pancreatic cancer has solidified itself as one of medicine's most urgent areas of unmet need[3].

Pancreatic ductal adenocarcinoma tumour microenvironment

Characterised by its strong desmoplastic reaction, the pancreatic ductal adenocarcinoma (PDAC) tumour microenvironment (TME) plays a crucial role in disease progression[4,5]. Primary tumour sites display extensive fibrosis characterised by overexpression of extracellular matrix proteins (such as laminin, collagen and fibronectin) and activation of fibroblastic cells. Multiple cell types, both cancer and stromal, are present in the pancreatic TME, including; pancreatic stellate cells (PSCs), myeloid-derived suppressor cells (MDSCs), tumour associated macrophages (TAMs) and regulatory T-cells (Tregs), amongst many other cell types[6,7]. The dense fibrosis associated with PDAC (largely orchestrated by PSCs) results in tumour hypoxia, a feature characteristic of PDAC, which is exacerbated by the secretion of anti-angiogenic factors (such as endostatin and angiostatin) by both pancreatic cancer and stellate cells[6,8]. Development of a hypoxic TME has been linked to disease aggressiveness and progression, as well as chemotherapy resistance. Importantly, in addition to developing resistance to chemotherapeutics, the pancreatic TME is highly immunosuppressive, limiting the efficacy of the immune-mediated cancer surveillance [6]. MDSCs release reactive oxygen species (ROS) and reactive nitrogen species which have been shown to inhibit T cell proliferation and migration into the TME. In addition, release of immunosuppressive cytokines including interleukin (IL)-10 and transforming growth factor beta (TGF-β) sustains the development of Tregs which further modulate the TME[9]. Tregs secrete immunosuppressive cytokines such as IL-10 and TGF-β which recruit additional immunosuppressive cells to the TME and stimulate the transition of $CD4^+$ T cells to $FoxP3^+$ regulatory cells, facilitating immune evasion[6,10]. Through the release of IL-10 and TGF-β, tumour associated macrophages are also able to induce T-cell anergy leading to the development of an immune-privileged microenvironment[11]. Finally, pancreatic cancer cells can downregulate Fas, resulting in resistance to $CD8^+$ T cell-induced Fas/FasL apoptosis[6,12]. Key components of the PDAC TME are shown in Figure 1.

NATURAL KILLER CELLS

NK cells are large granular lymphocytes which are key components of the innate immune system, and are poorly understood compared to other lymphocytes (T and B

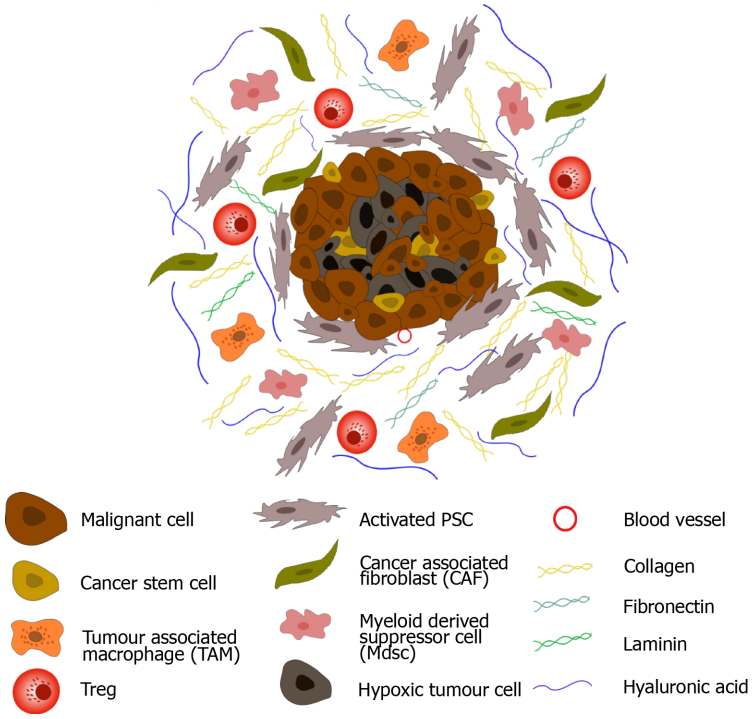


Figure 1 Pancreatic ductal adenocarcinoma tumour microenvironment. Upon activation, pancreatic stellate cells secrete an abundance of extracellular matrix proteins including collagen, fibronectin, laminin, and hyaluronic acid, leading to dense desmoplasia. In addition, fibroblastic cells (CAFs) become active, and immune suppressive cells (myeloid-derived suppressor cells, regulatory T-cells and tumour associated macrophages) are sequestered to the tumour microenvironment (TME). Secretion of anti-angiogenic factors, in addition to dense desmoplasia, results in the development of a hypoxic TME. Cancer stem cells are also observed in pancreatic ductal adenocarcinoma.

cells) belonging to the adaptive immune system. Acting as the first line of defence against viral infected and malignant cells[13,14], NK cells are classified as $CD56^{+}CD3^{-}$ cells. This classification can be further sub-divided into two main effector populations: immunomodulatory $CD56^{bright}CD16^{-}$ cells which regulate their function through cytokine release [specifically interferon (IFN)-γ], and cytotoxic $CD56^{dim}CD16^{+}$ effector cells[14]. Activation of NK cells relies on the balance of signals received from inhibitory and activating cell surface receptors (Figure 2)[13]. Inhibitory receptors, comprised of killer cell immunoglobulin-like receptors (KIRs) and C-type lectin-like receptors, including natural killer group 2 member A (NKG2A), specifically recognisemajor histocompatibility complex (MHC) class 1 molecules. These ligands are highly expressed on non-transformed 'self' cells, and consequently prevent harmful activation of NK cells against host cells. Conversely, malignant cells often downregulate the expression of surface MHC-1 molecules to evade detection by T cells. This 'missing self' signal prevents the inhibition of the NK cells, resulting in cytotoxic efficacy[13,14]. NK cells can also be negatively regulated by checkpoint proteins such as programme death 1 (PD-1), which binds to its' ligands programme death ligand 1 and 2 (PDL-1, PDL-2)[13]. Activating receptors include the type 1 transmembrane natural cytotoxicity receptors (NCRs) NKp46 (which is exclusive to NK cells), NKp30 and NKp44 (which are also expressed on T cells), the C-type lectin-like receptors NKG2C and NKG2D, the activating KIRs and the DNAX accessory molecule 1 (DNAM1)[13-15]. Ligands for the activating KIR receptors include the HLA-C2 and HLA-A ligands, however, the interaction between ligand and receptor is less well understood for activating KIRs than for their inhibitory counterparts[16]. NKG2D receptors recognise stress-induced proteins on transformed and virally infected cells. These include the MHC class 1 related genes MICA and MICB and UL-16 binding proteins[16]. Whilst recognising nectin adhesion molecule and the poliovirus receptor, DNAM1 also interacts with the β2 integrin leukocyte function-associated antigen 1 (LFA-1) which is involved in the formation of the immunological synapse[15]. It is important to note that in addition to independent activation, specific combinations of activating receptors are synergistic, increasing the overall signal received by the effector cell and consequently, increasing its cytotoxic response[17].

$CD16^{+}$ NK cells, in addition to direct receptor-ligand binding, express a propensity to carry out antibody-dependent cellular cytotoxicity (ADCC)[18]. CD16 (FcγRIII) is a transmembrane receptor which can bind the Fc region of IgG1 and IgG3 antibodies,

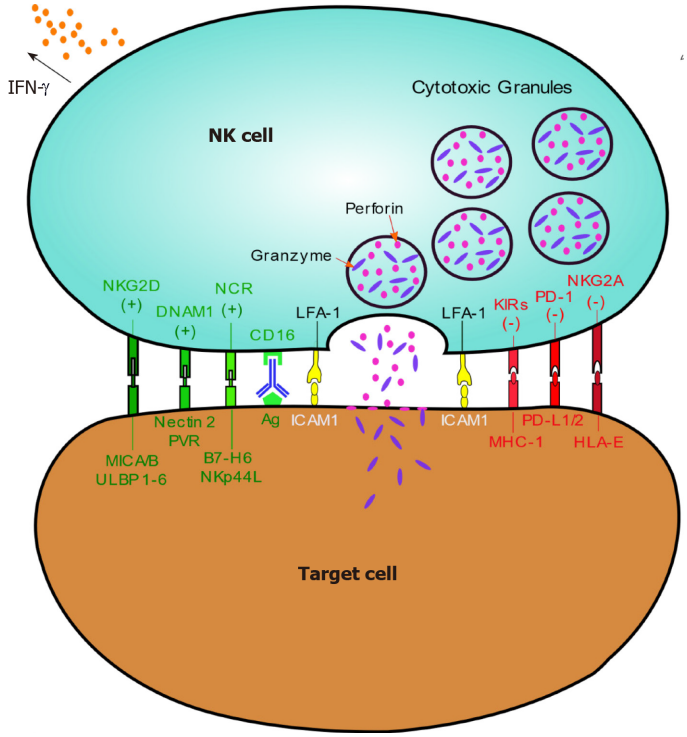


Figure 2 Activation and cytotoxicity of natural killer cells. Natural killer (NK) cells recognise a multitude of ligands on both healthy and transformed cells. Inhibitory receptors (red) recognise 'self-antigens' on healthy tissue preventing activation. However, these molecules are lost on aberrant cells as a result of viral transformation or malignancy ('missing-self') leading to NK cell activation. Alternatively, NK cells may become active through engagement of activating receptors (green) *via* stress ligands expressed on transformed cells. Binding of leukocyte function-associated antigen 1 to ICAM1 stabilises the immunological synapse between NK and target cells and ensures effective cytotoxicity. Upon activation NK cells release cytotoxic granules which contain perforin and granzymes to initiate target cell death *via* necrotic or apoptotic pathways. NK cells can also execute antibody dependent cellular cytotoxicity through Fc engagement of the CD16 receptor. Finally, NK cells secrete cytokines, such as interferon-γ, facilitating crosstalk between the adaptive and innate immune system, resulting in dendritic and T cell recruitment.

enabling NK cells to kill immunoglobulin labelled cells[16], a crucial concept for monoclonal antibody-based therapies[13].

NK Cytotoxicity

Following initial interactions between activating/inhibitory receptors and target cells, the balance of signals received by the NK cell determines its activation status. If activated, NK cells must form an immunological synapse with the target cell. Formation of this synapse enables stable adhesion, polarisation of cytotoxic granules and subsequent lysis of the target cell. This is achieved through binding of the β2 integrin, LFA-1[17]. Consisting of two chains, α_L and β_2, LFA-1 is a heterodimer whose reactivity to its ligands (ICAM family members) can be modified *via* conformational changes. Specifically, a bent conformation exhibits a low affinity for its ligands, intermediate affinity can be achieved through a closed/extended conformation, whilst an open/extended conformation results in high-affinity binding (Figure 3)[17,19,20].

In contrast to T cells, NK cells do not require inside-out signals (such as chemokines and T cell receptor activation) to stimulate LFA-1 binding and can signal autonomously[15,21]. Following integrin activation, a signalling network is employed to facilitate the convergence of cytotoxic granules to the microtubule organising centre (MTOC) which is subsequently polarised towards the target cell, allowing degranulation (Figure 4)[21-26]. Actin remodelling is also carried out at the immunological synapse in response to NK cell activation, facilitating docking of the cytolytic granules [27]. Integrin-linked kinase, paxillin, Pyk2 and RhoGEF7 signalling cascades are employed for the polarisation of MTOC, with Cdc42, CLIP-170, Par6 and APC components of this signalling cascade being key for granule polarisation[21,28]. During this process, the mitochondria and Golgi complex have also been shown to polarise towards the immunological synapse[29,30]. Following polarisation of the cytotoxic granules, the pore-forming protein, perforin, and the serine proteases, granzymes, are exocytosed leading to target cell death *via* perforin induced necrosis, or granzyme stimulated caspase-dependent apoptosis[28,31,32]. Non-granule dependent cytotoxic mechanisms include the binding of death ligands such as TNF-related

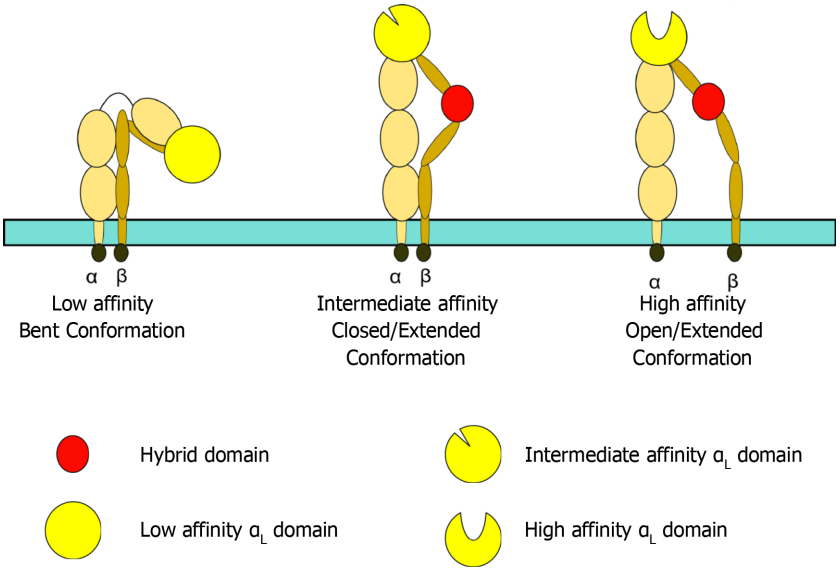


Figure 3 Leukocyte function-associated antigen 1 conformation. Leukocyte function-associated antigen 1 (LFA-1) affinity can be altered by its conformation. When bent, LFA-1 exhibits low affinity for its ligand, ICAM1. Intermediate affinity is achieved through a closed/extended conformation, whilst an open/extended conformation results in high affinity binding to ICAM1 and generation of an effective, stable immunological synapse.

apoptosis-inducing ligand and FasL to their receptor on target cells result in extrinsic apoptosis *via* caspase-8 activation[33] and release of IFN-γ[13,34,35].

NK cells in cancer

Initially identified as a result of their 'natural' cytotoxicity towards both syngeneic and allogeneic tumour cells, NK cells have widely demonstrated potent anti-tumoral cytotoxicity[36-38]. However, they are highly heterogeneous both between cancer types and intra-tumourally[36]. Moreover, the variable functional status of NK cells is seen to greatly impact their anti-tumoral efficacy[36]. Upregulation of the inhibitory receptor NKG2A has been associated with NK cell exhaustion and poorer prognosis in patients with liver cancer[39], whilst PD-1 engagement of NK cells has been shown to block the polarisation of lytic granules and impair outside-in integrin signalling[40]. Increased expression of PD-1 on NK cells has also been associated with poorer overall survival in patients with hepatocellular carcinoma[41]. Conversely, high proportions of functionally active NK cells result in favourable outcomes in many cancer types [42]. Retrospective flow cytometric assessment of peripheral blood samples from metastatic prostate cancer patients (Gleason scores between 6-9) followed by univariate Cox regression analysis demonstrated a significant correlation between expression of the activating receptors NKp46 and NKp30, and longer overall survival [42]. Similarly, immunostaining of tissue samples from 98 patients with gastric cancer (stage 1-4) combined with multivariate analysis revealed a positive correlation between NKG2D expression in tumour infiltrating lymphocytes and prolonged OS (hazard ratio 0.34)[43]. With the propensity to exhibit potent anti-tumoral activity, NK cell-based immunotherapy research has flourished in the past few years[36].

NK cells as prognostic markers in PDAC

NK cell number has been found to convey prognostic significance. Through flow cytometric analysis of PBMCs from resectable PDAC patients (stage Ib-III) both pre- and post-surgery, Hoshikawa *et al*[44] demonstrated a positive correlation between the percentage of NK cells in peripheral blood and recurrence-free survival, with patients who exhibited high NK levels expressing later disease recurrence. Moreover, univariate and multivariate analysis using the Cox proportional hazard regression model demonstrated NK cell frequency to be the only favourable prognostic factor for recurrence-free survival. Additional factors tested included tumour stage, N status, radicality, age and gender. Importantly, no additional circulating mononuclear cells were included in this analysis. NK cell infiltrate within the TME (assessed by gene expression profile) was also found to be associated with later disease recurrence, however, this was not statistically significant[44]. Assessment of a larger cohort of patients would improve the power of this assessment and may provide further insight into the role of tumour infiltrating NK cells in PDAC.

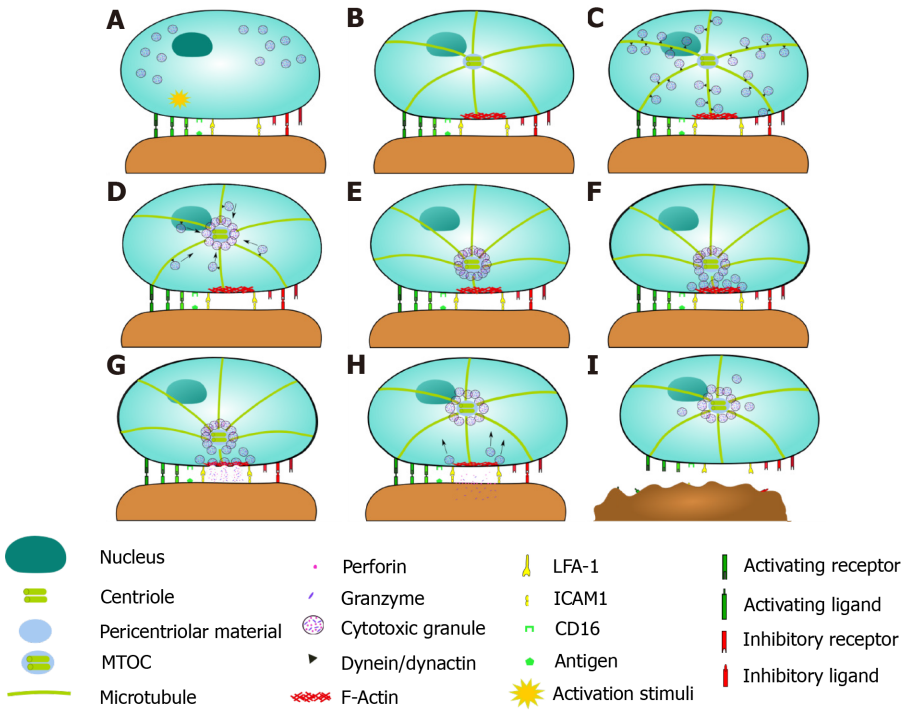


Figure 4 Cytotoxic granule convergence and microtubule organising centre polarisation. A: Following receptor stimulation leading to natural killer (NK) cell activation, leukocyte function-associated antigen 1 engages with its ligand ICAM1 on the malignant cell, forming a stable immunological synapse; B: F-actin accumulates and polymerises at the immune synapse, forming a filamentous mesh which modulates the release of cytolytic granules. Tubulin microtubules then form from the microtubule organising centre (MTOC); C and D: Cytotoxic granules converge on the microtubules (C) and are polarised towards the MTOC where they converge (D); E: This granule movement is dependent on dynein/dynactin motor function. Dynamic rearrangement of the microtubules facilitates polarisation of MTOC towards the immunological synapse; F and G: This polarisation is stimulated *via* Integrin-linked kinase, paxillin, Pyk2 and RhoGEF7 signalling. Following polarisation to the immunological synapse, a subsection of cytotoxic granules fuse with the plasma membrane (F) (a process largely regulated by Munc 13-4 and Rab27a) and undergo degranulation *via* either complete or incomplete fusion (G); H: Cytotoxic granules which do not degranulate are recycled and are hypothesised to remain converged at the MTOC to facilitate serial NK cell killing. Granules which undergo incomplete fusion are rapidly recycled through clathrin mediated endocytosis of granule membrane proteins, further facilitating serial killing; I: Finally, the malignant cell undergoes perforin induced necrosis or granule dependent apoptosis. NK cells detach from the malignant cell and move on to the next target.

Finally, gene set enrichment analysis revealed that patients with enriched type I and II IFN signatures within tumour tissues had later disease recurrence. Type I and II IFNs are closely associated with NK cell function. Type I IFNs induce NK cell activation both directly, through binding to type I IFN receptor, or indirectly, through stimulating dendritic cell release of IL-15, whilst type II IFNs (namely IFN-γ) are produced by activated NK cells[45,46]. Moreover, IFNs can induce CXCL10 release, leading to the further recruitment of NK cells to the tumour tissue[44]. Thus, enriched IFN signatures were also concluded to be a positive prognostic biomarker in PDAC [44].

Yang *et al*[47] also suggest prognostic implication of NK cells in PDAC. High densities of NK cells in peripheral blood samples (analysed by flow cytometry) were found to correlate with poor overall survival in patients with advanced PDAC (stage III/IV) when accompanied by a high neutrophil: lymphocyte ratio (obtained from routine hospital data). NK cell number (HR 1.45), as well as patient age (HR 1.34), neutrophil to lymphocyte ratio (HR 1.48) and absence of metastasis (HR 0.72) were found to be independent prognostic markers following univariate and multivariate Cox regression analysis. It is perhaps prudent to note that subsequent ELISAs to measure release of IFN-γ and TNF-α from NK cells as well as serum IL-2 Levels, a known activator of NK cells, demonstrated lower levels of all three markers in patients with high NK cell densities, although only IFN-γ reached significance. These results suggest a distinct subtype of NK cells with impaired function in PDAC patients and thus prognostic significance may rely not just on NK cell numbers, but on functional subtypes[47]. The results obtained within this study focus solely on peripheral blood circulating NK cells. Further work to classify functional NK cell subtypes within PDAC tumour tissue would provide more conclusive insights into the prognostic significance of these effector cells in PDAC.

NK cells in murine models of PDAC

NK cells have demonstrated potent anti-tumoral efficacy in murine models of PDAC. Using a transgenic mouse model in which oncogenic transposons for KrasG12V and myristoylated Akt2 were introduced into p53fl/fl mice *via* intra-pancreatic injection and electroporation, along with plasmids for Cre recombinase and sleeping beauty transposase, Brooks *et al*[48] demonstrated that neo-adjuvant PD-1 blockade plus adjuvant CD96 inhibition in combination with gemcitabine prevented relapse and facilitated long term remission following resection surgery. To further investigate the role of immune cells in neo-adjuvant and adjuvant treatment of PDAC, mice were injected (intraperitoneal) with anti-CD8 and anti-NK.1.1 depletion antibodies. Pre-operatively, depletion of both $CD8^+$ T cells and NK cells significantly reduced survival when compared to the control. Furthermore, adjuvant NK but not $CD8^+$ depletion was found to impair survival and resulted in an increase in local disease recurrence. *In vitro* luciferase cytotoxicity assays confirmed NK cell cytolytic efficacy against tumour derived cancer cells, a finding consistent with the results obtained for depletion experiments. Thus, targeting both T and NK cells through immune checkpoint inhibition may confer long term survival benefits in metastatic cases of PDAC[48]. Similarly, in a $Kras^{LSL\text{-}G12D}$ $p53^{LSL\text{-}R172H}$ *Pdx1-Cre* (KPC) model of PDAC, NK cell-based adoptive transfer immunotherapy was found to significantly delay tumour growth [49]. In addition, immunostaining of KPC tumours at end time-points revealed significantly elevated areas of necrosis in mice treated with adoptive transfer of NK cells compared to control mice, demonstrating the cytotoxic efficacy of NK cell treatment, a finding replicated through *in vitro* flow cytometry cytotoxicity assays[49].

Xenograft models of PDAC have also been used to demonstrate the efficacy of *ex vivo* expansion of NK cells. NOD *scid* gamma (NSG) mice injected subcutaneously with MiaPaca2 cells demonstrated a significant reduction in tumour growth when treated with adoptive transfer of *ex vivo* expanded NK cells (versus control group)[50]. This finding demonstrated both successful trafficking of NK cells to the tumour site and tumour control following intravenous injection, suggesting that NK cells may prove an effective systemic treatment in xenograft models of PDAC[50]. It is prudent to note that the nature of this model requires the mice included within the study to be immuno-compromised, and this must be taken into consideration when reviewing the data presented. Employing additional models would add further validity to the therapeutic impact of *ex vivo* expanded NK cells in murine models of PDAC[50].

Notorious for their resistance to typical anti-proliferative/cytotoxic therapies, cancer stem cells are an important subpopulation involved in cancer progression, metastasis and recurrence. Despite their notoriety, CSCs are preferentially targeted by NK cells in both the autologous and allogeneic setting; an effect which was found to be NKG2D dependent[51]. Metastatic, intra-pancreatic and subcutaneous models of PDAC were established in NSG mice using the PANC-1 cancer cell line. Mice treated with adoptively transferred NK cells were found to have substantial reductions in tumour volume when compared to untreated mice. Flow cytometric analysis also demonstrated a significant reduction in CSC populations in mice treated with adoptive NK cell transfer (denoted by aldehyde and CD24 expression). Moreover, immunostaining revealed co-localisation of NK cells and CSCs within tumour tissues. Ames *et al*[51] conclude that NK cells possess the ability to identify and preferentially target CSCs in solid tumours. As such, further work investigating the impact of systemic adoptive transfer of NK cells on CSC populations may yield exciting new therapeutic insights into the treatment of this dismal prognosis disease.

Tumour induced NK cell dysfunction and immune evasion in PDAC

Pancreatic tumour cells have developed several methods of NK-cell immune evasion. Murine models of pancreatic cancer specifically designed to express MYC at the Rosa26 Locus, either with or without the *Lsl-Kras*^{G12D} allele, were used to determine the role of MYC in pancreatic tumour progression. MYC expression was found to drive pancreatic cancer development and accelerate disease progression in precursor lesions initiated by KRAS. Moreover, bulk RNA sequencing of end stage tumours revealed reduced expression of T-cell, B-cell and NK cell markers in tumour tissue, suggesting that immune cell infiltration may be regulated by MYC. GeneGo analysis revealed that allelic activation of both KRAS and MYC resulted in significant downregulation of type I IFN, an effect that was found to be dependent on repressional binding of MYC-interacting zinc finger protein, MIZ1. Deletion of MIZ1 resulted in restoration of NK and B cell infiltration into tumour tissues, an effect ablated upon antibody dependent blockade of type I IFN[52]. Similarly, the oncoprotein Sloan-Kettering Institute has been shown to inhibit SMAD association with the acetyltransferases CBP and p300,

which are key regulators of inducible expression of NKG2D ligands on cancer cells, and facilitate repression of gene transcription *via* histone deacetylases. This results in downregulation of NKG2D ligands on tumoral cells, reducing NKG2D dependent cytotoxicity and facilitating immune evasion[53]. In addition, tumoral cells are seen to exhibit intricate crosstalk with NK effector cells and have been shown to induce functional deregulation[54]. Co-culture with the pancreatic cancer cell line MiaPaca2 was found to induce NK cell anergy *via* fibrinogen-like protein 2 and was characterised through decreased expression of DNAM-1, IFN-γ and CD107a and increased expression of PD-1[55]. Importantly, when co-cultured, anergic NK cells were seen to induce anergy in naïve NK cells, reducing IFN-γ and CD107a expression[55]. Further work investigating the reversal of NK cell anergy, both tumour-dependent and bystander anergy may lead to novel therapeutic insights, and provide a baseline from which to further challenge PDAC immune evasion.

MHC class-I chain-related molecules A/B (MICA/B) are crucial ligands for the activating receptor NKG2D. Several studies have demonstrated immune evasion as a result of MICA/B shedding by tumour cells. Duan *et al*[56] demonstrated that high glucose levels, which are widely correlated with pancreatic cancer, could facilitate immune evasion. Specifically, *in vitro* assays demonstrated a decrease in NK cell induced lysis of pancreatic cancer cell lines (demonstrated by lactate dehydrogenase release assays) when cultured in high glucose conditions. Moreover, western blot and quantitative real-time polymerase chain reaction (PCR) analysis revealed that this decrease in function was a result of reduced expression of MICA/B in cancer cell lines at both the protein and mRNA level. Mechanistically, high glucose was found to inhibit AMP-activated protein kinase signalling, leading to upregulation of the polycomb group protein (PcG) Bmi1, and subsequent promotion of GATA2 expression. This augmentation inhibited expression of MICA/B on pancreatic cancer cells and led to their immune evasion[56]. Similar shedding of the NKG2D ligand has been observed in response to hypoxia. High levels of HIF1α have been correlated with decreased expression of MICA/B on pancreatic tumour cells and also with increased internalisation of the activating receptor NKG2D, suggesting a dual role for the hypoxic TME in NK cell dysfunction[57]. Specifically, immune-histochemical analysis of PDAC patient tumour tissues revealed a significant correlation between MICA surface expression and high HIF1α. Moreover, immune-fluorescent staining of NK cells isolated from patient PBMCs demonstrated clear internalisation of the activating NKG2D receptors as well as MICA/B[57].

Receptor expression is found to be largely augmented in pancreatic cancer patients. Flow cytometric analysis of participant blood samples revealed that expression of the activating receptors DNAM-1 (CD226) and CD96 were significantly reduced in pancreatic cancer patients (stage I-IV) when compared to healthy controls. This downregulation was suggested to lead to NK cell dysfunction and tumour evasion.[58] Downregulation of NKG2D in pancreatic cancer patients has also been correlated to reduced cytotoxicity of the effector cells. *In vitro* blockade of NKG2D using neutralising antibodies was found to significantly decrease killing of MiaPaca2, BxPC3 and Capan2 cell lines by IL-15 stimulated NK cells[59]. This finding highlights the functional importance of NKG2D expression on NK cell function and may prove to be a crucial marker of NK cell efficacy against malignancy.

Additionally, flow cytometric analysis of cell surface markers following *in vitro* co-cultures of NK cells derived from healthy PBMCs and pancreatic cancer cell lines revealed downregulation of the NK activating receptors NKG2D, NKp30, NKp46 and DNAM-1. ELISA analysis suggested that this dysfunction was induced as a result of matrix metalloproteinase 9 (MMP9) and Indoleamine 2,3 dioxygenase (IDO) signalling cascades.[60] In addition to downregulation of activating receptors, exposure to MMP9 and IDO also led to decreased TNF-α and IFN-γ production by NK cells, an effect that was reversed upon blockade of MMP9 and IDO using tissue inhibitor of metalloproteinases 1 and 1-Methyl-DL-tryptopan, respectively[60]. This finding was replicated in a comprehensive study of surface receptor expression and cytotoxic granule positive cells in pancreatic (stage II and IV), gastric (stage 0-IV) and colorectal cancer (stage I-IV) patients. Using flow cytometry, Peng *et al*[61] identified significant downregulation of the activating receptors NKG2D, NKp30, NKp46 and DNAM-1 on NK cells identified in peripheral blood samples from pancreatic cancer patients when compared to healthy controls, whilst expression of the inhibitory receptor KIR3DL1 was significantly upregulated. Moreover, the percentage of perforin positive circulating NK cells was found to be significantly reduced in pancreatic cancer patients. Taken together, these alterations evidence the dysfunction of NK cells observed in malignancy.

Impairment in degranulation was also identified by Jun *et al*[62]. In an *in vitro* flow cytometry-based degranulation assay, PBMCs derived from malignant patients, non-malignant patients or healthy controls were mixed with target cells before staining with CD107a. NK cells derived from pancreatic cancer patients showed significantly impaired degranulation when compared to non-malignant and/or healthy control samples. Despite demonstrating impaired cytotoxic capabilities, no significant difference in NK cell IFN-γ production was observed between cancer patients and healthy controls. Importantly, multivariate analysis revealed that tumour-induced NK cell dysfunction correlated with disease stage, suggesting progressive impairment of NK cells with advanced-stage disease. Thus, as previously suggested, NK functional status may prove an attractive prognostic marker in PDAC.

NK cell interaction with stromal cells

NK cell-stromal cell interactions have been shown to result in significant cellular dysfunction and exclusion of NK cells from tumour tissues, suggesting that NK cells can be educated by the TME[47,63]. As crucial players in the development of the pre-metastatic niche, extracellular vesicles are key to the progression of PDAC and contain large numbers of immune regulatory factors including TGF-β, nectin-2 and PVR. Flow cytometric analysis following *in vitro* co-culture of NK cells and extracellular vesicles demonstrated downregulation of multiple NK cell receptors and cytokines, specifically, IFN-γ, TNFα, CD107a and NKG2D, resulting in gross cytotoxic impairment (demonstrated through tumour sphere cytotoxicity assays) and NK cell dysfunction. This impairment was further associated with the activation of the TGF-β-Smad3/4 signalling pathway[64]. It should also be noted that NK cell dysfunction is heavily regulated by the soluble factors and cytokines secreted by both tumoural and stromal cells, with TGF-β, IDO, MMPs and interleukins proving largely responsible for this impairment (Figure 5)[50,60,65-67].

NK cell interaction with suppressive immunoregulatory cells has a significant impact on cytotoxic effector function. Both MDSCs and TAMs produce IL-23, IL-6, IL10 and TGF-β which results in the downregulation of IFN-γ, perforin and IL-12 production by NK cells, decreasing cytotoxicity and NK cell proliferation within the TME[65]. TAMs also inhibit NK cell function through cell-cell interactions. M2 macrophages express PDL-1 which binds to PD-1 expressed on NK cells. This checkpoint protein prevents NK cell engagement and induces downregulation of the activating receptors NKG2D, natural cytotoxicity receptors and DNAM1, leading to reduced cytotoxicity[67]. Immunosuppressive Tregs release IL-10 and TGF-β into the TME. This cytokine release decreases NK cell cytotoxicity through the downregulation of activating receptors and decreased production of anti-tumour cytokines such as IFN-γ[65].

Moreover, MDSCs have also been shown to augment FcR mediate NK cell functions. Adoptive transfer of MDSC in a Panc02-EGFR$^+$ murine model of pancreatic cancer significantly inhibited the efficacy of monoclonal antibody therapy, with mice receiving Cetuximab + MDSCs expressing significantly larger tumour volumes than did mice treated with Cetuximab + splenocytes. Furthermore, NK cells co-cultured with tumour derived (melanoma) MDSCs were found to express significantly reduced phospho-ERK than did though cultured alone (as measured by flow cytometry). Thus, it was concluded that MDSCs inhibit FcR mediated signal transduction, resulting in impaired cytokine production, ADCC dysfunction and reduced anti-tumour activity. An effect that was found to be, at least in part, in response to MDSC nitric oxide production[68].

Likewise, tumour-associated neutrophils have been shown to impair NK cell function through the release of arginase and ROS[67]. *In vitro* co-culture assays using PBMCs derived from healthy donors demonstrated that activated granulocytes (stimulated with Phorbol 12-myristate 13-acetate) produced ROS including hydrogen peroxide, which was found to be cytotoxic to CD56^{dim} CD16$^+$ NK cells, but not CD56^{bright} CD16$^-$ subsets, suggesting that interactions with stromal cells may result in differential effects on cytotoxic effector subset[69].

Tumour-associated macrophages and cancer-associated fibroblasts have also been shown to produce growth arrest-specific gene 6 (Gas6) in the pancreatic TME. As a negative regulator of the immune system, the Gas6-AXL pathway is seen to prevent NK cell activation. Orthotopic syngeneic models of pancreatic cancer in which tumour cells derived from KPC mice were transduced with zsGreen/Luciferase and orthotopically injected into the pancreas of immune-compromised mice were used to investigate the role of Gas6 in NK cell activation and pancreatic cancer development. Pharmacological blockade of Gas6 signalling using neutralising antibodies was found to inhibit pancreatic cancer metastasis. Moreover, immunohistochemical analysis of

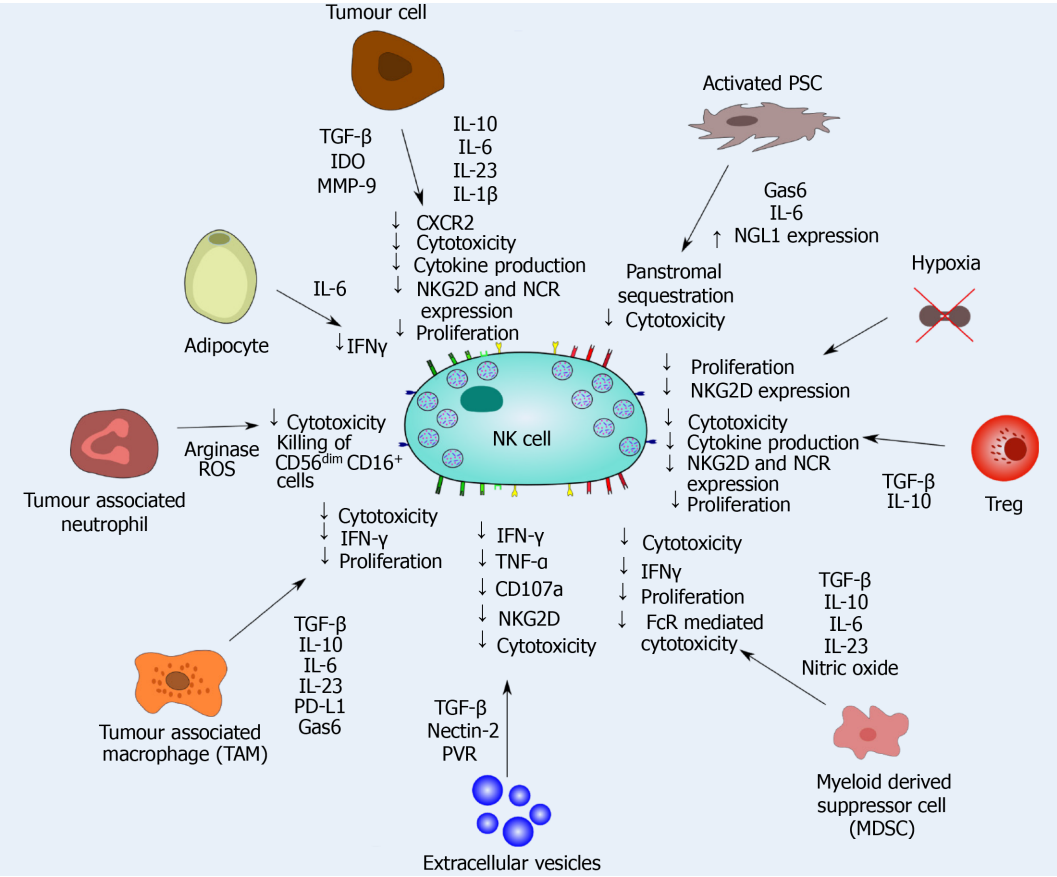


Figure 5 Natural killer cell dysfunction caused by tumoral and stromal cells in pancreatic ductal adenocarcinoma. Natural killer (NK) cell interactions with multiple stromal and tumour cells significantly impacts the cytotoxic efficacy of NK cells in pancreatic ductal adenocarcinoma. Transforming growth factor -β, interleukin (IL)-10, IL-6, IL-23 and IL-1β release significantly dampens NK cell cytotoxicity and function, and inhibits intratumoural proliferation of NK cells. NK cell mediated cytokine release is also inhibited within the immunosuppressive tumour microenvironment. Finally, chemokine release may also sequester NK cells in the panstromal compartment, preventing engagement with tumour cells.

NK cell infiltrates revealed a significant increase in the number of NKp46^{+} NK cells in lung metastasis in mice treated with anti-Gas6 therapy when compared to the control. This finding was also observed in the tumour draining lymph nodes; however no significant difference was observed in NK cell infiltration in primary tumour sites between treatment groups[70]. As such, Gas6 blockade may prove a promising new therapeutic target for the treatment of metastatic lesions in pancreatic cancer.

NK cells have been shown to be excluded from tumour tissue as a result of NK-stromal cell interactions. Through *ex vivo* immunostaining and Ariol image analysis of tissue micro-arrays, Ene-Obong *et al*[4] demonstrated that infiltrates of CD56^{+} natural killer cells were significantly lower in the juxtatumoural compartment of PDAC tissues (stage I-III) when compared to panstroma. This finding suggests PSCs sequester NK cells in the panstromal compartment of the TME, preventing NK induced cancer cell death. Similarly, Lim *et al*[50] demonstrated that NK cell frequencies in malignant tissue from PDAC patients (stage I-IV) were as low as < 0.5% (as assessed by flow cytometry). This low infiltration was attributed to reduced expression of the CXCR2 receptor on NK cells which resulted in poor chemotaxis into tumour tissues.

Known orchestrators of the TME, pancreatic stellate cells are crucial regulators of immune cell infiltrates in pancreatic cancer and have been shown to promote tumour progression through the development of an immunosuppressive environment[5,71-73]. In an orthotopic mouse model of pancreatic cancer in which either Panc02 cells alone or in combination with activated PSCs were injected into the pancreas of C57BL/6 mice, Li *et al*[74] report a significant reduction in the number of NK cells in tumour tissue in mice co-transplanted with PSCs when compared to mice injected with Panc02 cells alone. This finding is consistent with *ex vivo* analysis of human PDAC samples[4]. Further work is needed to demonstrate the mechanistic link between PSCs and NK cell tumoural exclusion.

In a recent study, Francescone *et al*[75] demonstrated dynamic crosstalk between PDAC cancer cells, CAFs and immune cells. Specifically, in *in vitro* co-culture assays

CAFs were found to ectopically express the neural presynaptic protein NetrinG1 (NG1) whilst its binding partner, NGL1 was identified on PDAC cells. This interaction was found to convey protection on cancer cells from NK cell-driven elimination,a fact quantified through CRISPR/Cas9 NG1 knockout which resulted in decreased expression of the immunosuppressive cytokines TGF-β, IL-8 and IL-6, and restored NK cell anti-tumour activation. This finding was reproduced in murine orthotopic models of PDAC in which NGL1 was knocked out of syngeneic mouse cells. This work provides a potential novel target in PDAC in which the immunosuppressive nature of PDAC induce by CAF cells can be reverted.

Finally, *in vitro* and xenograft models of PDAC in NOD SCID mice demonstrated that stromal TGF-β signalling stimulates CAF cells to secrete IL-6, which in turn suppresses NK cell function as assessed through NK cell killing assays. Concomitantly, stromal TGF-β limits NK cell function itself[76]. *In vivo* TGFβ blockade using the anti-mouse TGFβR2 mAb, 2G8, was found to reduce tumour progression, an effect that was reversed upon NK cell depletion[76]. Furthermore, a conditional KRAS (G12D) model of pancreatic cancer combined with a high-fat calorie diet demonstrated that stromal adipocytes produce IL-6, reducing NK cell function through IL-6 mediated downregulation of IFN-γ production. Thus, it has been suggested that adipocytes and PSCs may provide tumour sanctuaries within the TME through the immunosuppression of NK cells[77,78].

Taken together these findings demonstrate the complex interactions between NK cells and the PDAC microenvironment. Despite their cytotoxic efficacy against malignancy, the dysfunction induced by stromal cells greatly augments the natural anti-tumoral activity of NK cells. As such, a deeper understanding of these interactions is crucial to fully unlock the potential of NK cells in the treatment of PDAC.

NK CELLS AS THERAPY IN PDAC

Despite the known dysfunction of NK cells associated with PDAC, these cytotoxic effector units display evident cytotoxic capabilities and as such have received much attention as a potential immunotherapeutic tool in the treatment of PDAC. One of the immunotherapeutic routes explored is the use of NK cells as cellular immunotherapy [79]. When compared to T cells, the use of NK cells as cellular immune-therapeutics express several advantages. Firstly, NK cells present a much lower risk of on-target/off-tumour toxicity, rendering NK cells a relatively safe treatment option. Moreover, neurotoxicity, cytokine release syndrome and graft-versus-host disease are much less likely to occur in chimeric antigen receptor (CAR)-NK treatment than observed with the use of CAR-T therapy. Secondly, due to this reduced risk of graft-versus-host disease from allogenic NK sources, NK cells have the potential to be derived from multiple sources, including cell lines such as NK92. This facilitates the development of a truly 'off-the-shelf' cellular immunotherapeutic, eliminating the need for personalised therapeutics and the subsequent challenges these treatments invoke[79]. Thirdly, CAR-NK cells retain their natural cytotoxic capabilities and consequently can eliminate malignant cells in both a CAR dependent and independent manner[79,80].

Several studies have focused on harnessing the anti-tumoral efficacy of NK cells against PDAC. Lee *et al*[81], rationally designed a CAR-NK to specifically target folate receptor alpha (FRα) and death receptor 4 (DR4) which were found to be highly expressed on tumour cells. In addition, the CAR-NK was loaded with an apoptosis-inducing death ligand to further induce anti-tumoral cytotoxicity. Treatment with the FRα/DR4 targeting NK cells significantly increased tumour-selective apoptosis and NK cell infiltration in tumour tissue in sub-cutaneous models of pancreatic cancer. Similarly, Xia *et al*[82] investigated the efficacy of a Robo1 bi-specific CAR-NK cell treatment in combination with ^{125}I seed radiotherapy in orthotopic murine models of pancreatic cancer. Expressed on pancreatic cancer cells, Robo-1 is a member of the axon guidance receptor family which has been found modulate T-cell chemotaxis into the TME. Tumour size was significantly reduced in mice treated with combination therapy when compared to ^{125}I seed treatment alone. Moreover, tumours of mice receiving combination therapy displayed a significantly higher greyscale value than did ^{125}I treatment alone. Thus, it was concluded that bi-specific CAR-NK cells may prove a promising immunotherapeutic when combined with ^{125}I seed therapy in treatment of pancreatic cancer. Identification of novel antigens for therapy are useful strategies for cell-based therapy[83,84].

Table 1 Current clinical trials employing natural killer cells therapy in pancreatic cancer

Primary therapy	Additional intervention	Tumour type	Phase	NCT number	Status	Ref.
NK cell infusions	Irreversible electroporation	Advanced pancreatic cancer	I/II	NCT02718859	Completed	[90, 91]
Dendritic cell activated, cytokine induced killer treatment	S-1 (drug)	Advanced pancreatic cancer	I/II	NCT01781520	Completed	[87, 92]
BiCAR NK cells (ROBO1 CAR-NK cells)		Pancreatic cancer	I/II	NCT03941457	Recruiting	[93]
Ex-vivo expanded autologous NK cells (SNK01)	Trastuzumab; Cetuximab	Advanced solid tumour; metastatic cancer; HER-2+ breast cancer; HER-2 positive gastric cancer; HER-2 protein overexpression; oesophageal cancer; ovarian cancer; endometrium cancer; bladder cancer; pancreatic cancer; colorectal cancer; NSCLC; EGFR+ NSCLC; head and neck squamous cell carcinoma; triple-negative breast cancer; cervical cancer; sarcoma	I/IIa	NCT04464967	Not yet recruiting	[94]
High activity NK cells		Pancreatic cancer	I/II	NCT03008304	Completed	[95]
Activated NK cells		Lung cancer; breast cancer; colon cancer; pancreatic cancer; ovarian cancer	I/II	NCT03634501	Recruiting	[96]
FT500-an allogenic, iPSC derived NK cell immunotherapy	Nivolumab; pembrolizumab; atezolizumab; cyclophosphamide; fludarabine; IL-2	Advanced solid tumours; lymphoma; gastric cancer; colorectal cancer; head and neck cancer; squamous cell carcinoma; EGFR positive solid tumour; HER2 positive breast cancer; hepatocellular carcinoma; small-cell lung cancer; renal cell carcinoma; pancreas cancer; melanoma; NSCLC; urothelial carcinoma, cervical cancer; microsatellite instability; merkel cell carcinoma	I	NCT03841110	Recruiting	[97]
FT500-101 allogenic NK cell immunotherapy		Advanced solid tumours; lymphoma; gastric cancer; colorectal cancer; head and neck cancer; squamous cell carcinoma; EGFR positive solid tumour; HER2 positive breast cancer; hepatocellular carcinoma; small-cell lung cancer; renal cell carcinoma; pancreas cancer; melanoma; NSCLC; urothelial carcinoma, cervical cancer; microsatellite instability; merkel cell carcinoma	Observational study	NCT04106167	Recruiting	[98]
FATE-NK100 (donor-derived *ex-vivo* activated immunotherapy)	Trastuzumab; Cetuximab	HER2+ gastric cancer; colorectal cancer; head and neck squamous cell carcinoma; $EGFR^+$ solid tumours; advanced solid tumours; HER2 postie breast cancer; hepatocellular carcinoma; NSCLC; renal cell carcinoma; pancreatic cancer; melanoma	I	NCT03319459	Active, not recruiting	[99]
Autologous dendritic cell vaccine loaded with personalised peptides to stimulate innate and adaptive immune response *via* activating T and NK cells	Standard of care; Nivolumab	Pancreatic adenocarcinoma	Ib	NCT04627246	Recruiting	[100]
ACE1702 cellular therapy (anti-HER2 NK cells)	Cyclophosphamide; Fludarabine	Locally advanced solid tumours; metastatic cancer; solid tumour; HER-2+ gastric cancer; HER-2 + metastatic breast cancer	I	NCT04319757	Recruiting	[101]
NK cells	Bortezomib	CML; pancreatic cancer; colon/rectal cancer; multiple myeloma; carcinoma-NSCLC	I	NCT00720785	Recruiting	[102]
Cytokine-induced killer cells	Tegafur; Gimeracil; Oteracil potassium	Advanced cancer	II	NCT03002831	Terminated	[103, 104]
Anti-MUC1 CAR-pNK cells		Hepatocellular carcinoma; NSCLC; pancreatic cancer; triple negative invasive breast carcinoma; malignant glioma of the brain; colorectal carcinoma; gastric carcinoma	I/II	NCT02839954	Unknown	[105]

Autologous NK/NK T cell immunotherapy	Breast cancer; glioma; hepatocellular carcinoma; squamous cell lung cancer; pancreatic cancer; colon cancer; prostate cancer	I	NCT00909558	Suspended	[106]

NK cell: Natural killer cell; CAR: Chimeric antigen receptor; NSCLC: Non-small cell lung cancer; CML: Chronic myeloid leukemia; iPSC: Induced pluripotent stem cells.

In addition to CAR therapies, cytokine supplement either alone or in combination with additional therapeutics has demonstrated significant benefit in both preclinical studies and clinical trials. In combination with the immune priming CD40 agonist, supplementation of IL-15 in orthotopic and KPC mouse models of pancreatic cancer resulted in a significant increase in immune cell infiltration into tumour tissue (assessed through multi-colour flow cytometry), particularly of NK and $CD8^+$ T cells. This infiltration resulted in enhanced anti-tumour effect and significantly improved long term survival (log-rank tests; $P \leq 0.0001$)[85]. Furthermore, Lin *et al*[86] demonstrated that allogeneic NK cell transfer in combination with irreversible electroporation significantly increased median progression-free and overall survival in stage III PDAC patients and increased median overall survival in stage IV patients. Moreover, multiple allogenic transfers correlated with better prognosis in stage III patients. Similarly, treatment with a dendritic cell/cytokine-induced killer cell vaccine was found increase both overall and progression-free survival in advanced pancreatic cancer patients[87]. Taken together these results highlight the therapeutic potential of NK cell-based therapies. Current clinical trials employing NK cell therapy in pancreatic cancer (identified through entering the search terms 'natural killer cells' and 'pan-creatic cancer' at clinicaltrials.gov) are highlighted in Table 1.

CONCLUSION

NK cells are powerful effector units, which, when harnessed, can confer striking therapeutic benefit. With the innate effector units demonstrating strong efficacy against malignancy, both in preclinical studies, and clinical trials, the potential for NK cell therapy in PDAC is only just beginning to come to light. Additional research is needed to fully elucidate the role of NK cells in pancreatic cancer and deconvolute the intricate relationships between NK and stromal cells within the TME, facilitating co-targeting of tumour stroma[73,88]. Through defining these relationships, novel functional and mechanistic insights into this devastating disease can be achieved[89] and the full therapeutic potential of NK cells can be harnessed.

REFERENCES

1 **Neuzillet C**, Rousseau B, Kocher H, Bourget P, Tournigand C. Unravelling the pharmacologic opportunities and future directions for targeted therapies in gastro-intestinal cancers Part 1: GI carcinomas. *Pharmacol Ther* 2017; **174**: 145-172 [PMID: 28223233 DOI: 10.1016/j.pharmthera.2017.02.028]

2 **Rahib L**, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; **74**: 2913-2921 [PMID: 24840647 DOI: 10.1158/0008-5472.CAN-14-0155]

3 **Young K**, Hughes DJ, Cunningham D, Starling N. Immunotherapy and pancreatic cancer: unique challenges and potential opportunities. *Ther Adv Med Oncol* 2018; **10**: 1758835918816281 [PMID: 30574212 DOI: 10.1177/1758835918816281]

4 **Ene-Obong A**, Clear AJ, Watt J, Wang J, Fatah R, Riches JC, Marshall JF, Chin-Aleong J, Chelala C, Gribben JG, Ramsay AG, Kocher HM. Activated pancreatic stellate cells sequester CD8+ T cells to reduce their infiltration of the juxtatumoral compartment of pancreatic ductal adenocarcinoma. *Gastroenterology* 2013; **145**: 1121-1132 [PMID: 23891972 DOI: 10.1053/j.gastro.2013.07.025]

5 **Froeling FE**, Kocher HM. Homeostatic restoration of desmoplastic stroma rather than its ablation slows pancreatic cancer progression. *Gastroenterology* 2015; **148**: 849-850 [PMID: 25724458 DOI: 10.1053/j.gastro.2015.02.043]

6 **Ren B**, Cui M, Yang G, Wang H, Feng M, You L, Zhao Y. Tumor microenvironment participates in metastasis of pancreatic cancer. *Mol Cancer* 2018; **17**: 108 [PMID: 30060755 DOI: 10.1186/s12943-018-0858-1]

7 **Uzunparmak B**, Sahin IH. Pancreatic cancer microenvironment: a current dilemma. *Clin Transl*

Med 2019; **8**: 2 [PMID: 30645701 DOI: 10.1186/s40169-019-0221-1]
8 **Di Maggio F**, Arumugam P, Delvecchio FR, Batista S, Lechertier T, Hodivala-Dilke K, Kocher HM. Pancreatic stellate cells regulate blood vessel density in the stroma of pancreatic ductal adenocarcinoma. *Pancreatology* 2016; **16**: 995-1004 [PMID: 27288147 DOI: 10.1016/j.pan.2016.05.393]
9 **Trovato R**, Fiore A, Sartori S, Canè S, Giugno R, Cascione L, Paiella S, Salvia R, De Sanctis F, Poffe O, Anselmi C, Hofer F, Sartoris S, Piro G, Carbone C, Corbo V, Lawlor R, Solito S, Pinton L, Mandruzzato S, Bassi C, Scarpa A, Bronte V, Ugel S. Immunosuppression by monocytic myeloid-derived suppressor cells in patients with pancreatic ductal carcinoma is orchestrated by STAT3. *J Immunother Cancer* 2019; **7**: 255 [PMID: 31533831 DOI: 10.1186/s40425-019-0734-6]
10 **Principe DR**, DeCant B, Mascariñas E, Wayne EA, Diaz AM, Akagi N, Hwang R, Pasche B, Dawson DW, Fang D, Bentrem DJ, Munshi HG, Jung B, Grippo PJ. TGFβ Signaling in the Pancreatic Tumor Microenvironment Promotes Fibrosis and Immune Evasion to Facilitate Tumorigenesis. *Cancer Res* 2016; **76**: 2525-2539 [PMID: 26980767 DOI: 10.1158/0008-5472.CAN-15-1293]
11 **Xu JY**, Wang WS, Zhou J, Liu CY, Shi JL, Lu PH, Ding JL. The Importance of a Conjoint Analysis of Tumor-Associated Macrophages and Immune Checkpoints in Pancreatic Cancer. *Pancreas* 2019; **48**: 904-912 [PMID: 31268976 DOI: 10.1097/MPA.0000000000001364]
12 **Tesfaye AA**, Kamgar M, Azmi A, Philip PA. The evolution into personalized therapies in pancreatic ductal adenocarcinoma: challenges and opportunities. *Expert Rev Anticancer Ther* 2018; **18**: 131-148 [PMID: 29254387 DOI: 10.1080/14737140.2018.1417844]
13 **Gonzalez-Rodriguez AP**, Villa-Álvarez M, Sordo-Bahamonde C, Lorenzo-Herrero S, Gonzalez S. NK Cells in the Treatment of Hematological Malignancies. *J Clin Med* 2019; **8** [PMID: 31569769 DOI: 10.3390/jcm8101557]
14 **Granzin M**, Wagner J, Köhl U, Cerwenka A, Huppert V, Ullrich E. Shaping of Natural Killer Cell Antitumor Activity by *Ex Vivo* Cultivation. *Front Immunol* 2017; **8**: 458 [PMID: 28491060 DOI: 10.3389/fimmu.2017.00458]
15 **Kumar S**. Natural killer cell cytotoxicity and its regulation by inhibitory receptors. *Immunology* 2018; **154**: 383-393 [PMID: 29512837 DOI: 10.1111/imm.12921]
16 **Mehta RS**, Randolph B, Daher M, Rezvani K. NK cell therapy for hematologic malignancies. *Int J Hematol* 2018; **107**: 262-270 [PMID: 29383623 DOI: 10.1007/s12185-018-2407-5]
17 **Urlaub D**, Höfer K, Müller ML, Watzl C. LFA-1 Activation in NK Cells and Their Subsets: Influence of Receptors, Maturation, and Cytokine Stimulation. *J Immunol* 2017; **198**: 1944-1951 [PMID: 28100681 DOI: 10.4049/jimmunol.1601004]
18 **Seidel UJ**, Schlegel P, Lang P. Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies. *Front Immunol* 2013; **4**: 76 [PMID: 23543707 DOI: 10.3389/fimmu.2013.00076]
19 **Lefort CT**, Ley K. Neutrophil arrest by LFA-1 activation. *Front Immunol* 2012; **3**: 157 [PMID: 22701459 DOI: 10.3389/fimmu.2012.00157]
20 **Yuki K**, Bu W, Xi J, Sen M, Shimaoka M, Eckenhoff RG. Isoflurane binds and stabilizes a closed conformation of the leukocyte function-associated antigen-1. *FASEB J***26**: 4408-4417 [PMID: 22815384 DOI: 10.1096/fj.12-212746]
21 **Zhang M**, March ME, Lane WS, Long EO. A signaling network stimulated by β2 integrin promotes the polarization of lytic granules in cytotoxic cells. *Sci Signal* 2014; **7**: ra96 [PMID: 25292215 DOI: 10.1126/scisignal.2005629]
22 **Chaldakov GN**. Colchicine, Inflammation and Fibrosis in Cardiovascular Disease: Merging Three Classical Tales. *Bulgarian Society for Cell Biology* 2017; **28**: 105-110 [DOI: 10.14748/bmr.v28.4456]
23 **Paul S**, Lal G. The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. *Front Immunol* 2017; **8**: 1124 [PMID: 28955340 DOI: 10.3389/fimmu.2017.01124]
24 **Mentlik AN**, Sanborn KB, Holzbaur EL, Orange JS. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Mol Biol Cell* 2010; **21**: 2241-2256 [PMID: 20444980 DOI: 10.1091/mbc.e09-11-0930]
25 **Mukherjee M**, Mace EM, Carisey AF, Ahmed N, Orange JS. Quantitative Imaging Approaches to Study the CAR Immunological Synapse. *Mol Ther* 2017; **25**: 1757-1768 [PMID: 28663103 DOI: 10.1016/j.ymthe.2017.06.003]
26 **Viswanath DI**, Mace EM, Hsu HT, Orange JS. Quantification of natural killer cell polarization and visualization of synaptic granule externalization by imaging flow cytometry. *Clin Immunol* 2017; **177**: 70-75 [PMID: 26948929 DOI: 10.1016/j.clim.2016.03.004]
27 **Brown AC**, Dobbie IM, Alakoskela JM, Davis I, Davis DM. Super-resolution imaging of remodeled synaptic actin reveals different synergies between NK cell receptors and integrins. *Blood* 2012; **120**: 3729-3240 [PMID: 22966166 DOI: 10.1182/blood-2012-05-429977]
28 **Mace EM**, Dongre P, Hsu HT, Sinha P, James AM, Mann SS, Forbes LR, Watkin LB, Orange JS. Cell biological steps and checkpoints in accessing NK cell cytotoxicity. *Immunol Cell Biol* 2014; **92**: 245-255 [PMID: 24445602 DOI: 10.1038/icb.2013.96]
29 **Stinchcombe JC**, Salio M, Cerundolo V, Pende D, Arico M, Griffiths GM. Centriole polarisation to the immunological synapse directs secretion from cytolytic cells of both the innate and adaptive immune systems. *BMC Biol* 2011; **9**: 45 [PMID: 21711522 DOI: 10.1186/1741-7007-9-45]

30 **Abarca-Rojano E**, Muñiz-Hernández S, Moreno-Altamirano MM, Mondragón-Flores R, Enriquez-Rincón F, Sánchez-García FJ. Re-organization of mitochondria at the NK cell immune synapse. *Immunol Lett* 2009; **122**: 18-25 [PMID: 19038287 DOI: 10.1016/j.imlet.2008.10.008]

31 **Capuano C**, Paolini R, Molfetta R, Frati L, Santoni A, Galandrini R. PIP2-dependent regulation of Munc13-4 endocytic recycling: impact on the cytolytic secretory pathway. *Blood* 2012; **119**: 2252-2262 [PMID: 22271450 DOI: 10.1182/blood-2010-12-324160]

32 **Backes CS**, Friedmann KS, Mang S, Knörck A, Hoth M, Kummerow C. Natural killer cells induce distinct modes of cancer cell death: Discrimination, quantification, and modulation of apoptosis, necrosis, and mixed forms. *J Biol Chem* 2018; **293**: 16348-16363 [PMID: 30190323 DOI: 10.1074/jbc.RA118.004549]

33 **Zhu Y**, Huang B, Shi J. Fas ligand and lytic granule differentially control cytotoxic dynamics of natural killer cell against cancer target. *Oncotarget* 2016; **7**: 47163-47172 [PMID: 27323411 DOI: 10.18632/oncotarget.9980]

34 **Allen F**, Bobanga ID, Rauhe P, Barkauskas D, Teich N, Tong C, Myers J, Huang AY. CCL3 augments tumor rejection and enhances CD8+ T cell infiltration through NK and CD103+ dendritic cell recruitment *via* IFNγ. *Oncoimmunology* 2017; **7**: e1393598 [PMID: 29399390 DOI: 10.1080/2162402X.2017.1393598]

35 **Mishra HK**, Pore N, Michelotti EF, Walcheck B. Anti-ADAM17 monoclonal antibody MEDI3622 increases IFNγ production by human NK cells in the presence of antibody-bound tumor cells. *Cancer Immunol Immunother* 2018; **67**: 1407-1416 [PMID: 29978334 DOI: 10.1007/s00262-018-2193-1]

36 **Wu SY**, Fu T, Jiang YZ, Shao ZM. Natural killer cells in cancer biology and therapy. *Mol Cancer* 2020; **19**: 120 [PMID: 32762681 DOI: 10.1186/s12943-020-01238-x]

37 **Chiossone L**, Dumas PY, Vienne M, Vivier E. Natural killer cells and other innate lymphoid cells in cancer. *Nat Rev Immunol* 2018; **18**: 671-688 [PMID: 30209347 DOI: 10.1038/s41577-018-0061-z]

38 **Trinchieri G**. Biology of natural killer cells. *Adv Immunol* 1989; **47**: 187-376 [PMID: 2683611 DOI: 10.1016/s0065-2776(08)60664-1]

39 **Sun C**, Xu J, Huang Q, Huang M, Wen H, Zhang C, Wang J, Song J, Zheng M, Sun H, Wei H, Xiao W, Sun R, Tian Z. High NKG2A expression contributes to NK cell exhaustion and predicts a poor prognosis of patients with liver cancer. *Oncoimmunology* 2016; **6**: e1264562 [PMID: 28197391 DOI: 10.1080/2162402X.2016.1264562]

40 **Huang Y**, Chen Z, Jang JH, Baig MS, Bertolet G, Schroeder C, Huang S, Hu Q, Zhao Y, Lewis DE, Qin L, Zhu MX, Liu D. PD-1 blocks lytic granule polarization with concomitant impairment of integrin outside-in signaling in the natural killer cell immunological synapse. *J Allergy Clin Immunol* 2018; **142**: 1311-1321. e8 [PMID: 29679656 DOI: 10.1016/j.jaci.2018.02.050]

41 **Liu Y**, Cheng Y, Xu Y, Wang Z, Du X, Li C, Peng J, Gao L, Liang X, Ma C. Increased expression of programmed cell death protein 1 on NK cells inhibits NK-cell-mediated anti-tumor function and indicates poor prognosis in digestive cancers. *Oncogene* 2017; **36**: 6143-6153 [PMID: 28692048 DOI: 10.1038/onc.2017.209]

42 **Pasero C**, Gravis G, Granjeaud S, Guerin M, Thomassin-Piana J, Rocchi P, Salem N, Walz J, Moretta A, Olive D. Highly effective NK cells are associated with good prognosis in patients with metastatic prostate cancer. *Oncotarget* 2015; **6**: 14360-14373 [PMID: 25961317 DOI: 10.18632/oncotarget.3965]

43 **Kamei R**, Yoshimura K, Yoshino S, Inoue M, Asao T, Fuse M, Wada S, Kuramasu A, Furuya-Kondo T, Oga A, Iizuka N, Suzuki N, Maeda N, Watanabe Y, Matsukuma S, Iida M, Takeda S, Ueno T, Yamamoto N, Fukagawa T, Katai H, Sasaki H, Hazama S, Oka M, Nagano H. Expression levels of UL16 binding protein 1 and natural killer group 2 member D affect overall survival in patients with gastric cancer following gastrectomy. *Oncol Lett* 2018; **15**: 747-754 [PMID: 29391893 DOI: 10.3892/ol.2017.7354]

44 **Hoshikawa M**, Aoki T, Matsushita H, Karasaki T, Hosoi A, Odaira K, Fujieda N, Kobayashi Y, Kambara K, Ohara O, Arita J, Hasegawa K, Kakimi K, Kokudo N. NK cell and IFN signatures are positive prognostic biomarkers for resectable pancreatic cancer. *Biochem Biophys Res Commun* 2018; **495**: 2058-2065 [PMID: 29253566 DOI: 10.1016/j.bbrc.2017.12.083]

45 **Paolini R**, Bernardini G, Molfetta R, Santoni A. NK cells and interferons. *Cytokine Growth Factor Rev* 2015; **26**: 113-120 [PMID: 25443799 DOI: 10.1016/j.cytogfr.2014.11.003]

46 **Lee AJ**, Ashkar AA. The Dual Nature of Type I and Type II Interferons. *Front Immunol* 2018; **9**: 2061 [PMID: 30254639 DOI: 10.3389/fimmu.2018.02061]

47 **Yang C**, Cheng H, Zhang Y, Fan K, Luo G, Fan Z, Huang Q, Lu Y, Jin K, Wang Z, Yu X, Liu C. Anergic natural killer cells educated by tumor cells are associated with a poor prognosis in patients with advanced pancreatic ductal adenocarcinoma. *Cancer Immunol Immunother* 2018; **67**: 1815-1823 [PMID: 30167864 DOI: 10.1007/s00262-018-2235-8]

48 **Brooks J**, Fleischmann-Mundt B, Woller N, Niemann J, Ribback S, Peters K, Demir IE, Armbrecht N, Ceyhan GO, Manns MP, Wirth TC, Kubicka S, Bernhardt G, Smyth MJ, Calvisi DF, Gürlevik E, Kühnel F. Perioperative, Spatiotemporally Coordinated Activation of T and NK Cells Prevents Recurrence of Pancreatic Cancer. *Cancer Res* 2018; **78**: 475-488 [PMID: 29180478 DOI: 10.1158/0008-5472.CAN-17-2415]

49 **Hu S**, Yang J, Shangguan J, Eresen A, Li Y, Ma Q, Yaghmai V, Velichko Y, Hu C, Zhang Z. Natural killer cell-based adoptive transfer immunotherapy for pancreatic ductal adenocarcinoma in a *Kras*$^{LSL-G12D}$ *p53*$^{LSL-R172H}$ *Pdx1-Cre* mouse model. *Am J Cancer Res* 2019; **9**: 1757-1765 [PMID:

31497356]
50 **Lim SA**, Kim J, Jeon S, Shin MH, Kwon J, Kim TJ, Im K, Han Y, Kwon W, Kim SW, Yee C, Kim SJ, Jang JY, Lee KM. Defective Localization With Impaired Tumor Cytotoxicity Contributes to the Immune Escape of NK Cells in Pancreatic Cancer Patients. *Front Immunol* 2019; **10**: 496 [PMID: 31024520 DOI: 10.3389/fimmu.2019.00496]
51 **Ames E**, Canter RJ, Grossenbacher SK, Mac S, Chen M, Smith RC, Hagino T, Perez-Cunningham J, Sckisel GD, Urayama S, Monjazeb AM, Fragoso RC, Sayers TJ, Murphy WJ. NK Cells Preferentially Target Tumor Cells with a Cancer Stem Cell Phenotype. *J Immunol* 2015; **195**: 4010-4019 [PMID: 26363055 DOI: 10.4049/jimmunol.1500447]
52 **Muthalagu N**, Monteverde T, Raffo-Iraolagoitia X, Wiesheu R, Whyte D, Hedley A, Laing S, Kruspig B, Upstill-Goddard R, Shaw R, Neidler S, Rink C, Karim SA, Gyuraszova K, Nixon C, Clark W, Biankin AV, Carlin LM, Coffelt SB, Sansom OJ, Morton JP, Murphy DJ. Repression of the Type I Interferon Pathway Underlies MYC- and KRAS-Dependent Evasion of NK and B Cells in Pancreatic Ductal Adenocarcinoma. *Cancer Discov* 2020; **10**: 872-887 [PMID: 32200350 DOI: 10.1158/2159-8290.CD-19-0620]
53 **Ponath V**, Frech M, Bittermann M, Al Khayer R, Neubauer A, Brendel C, Pogge von Strandmann E. The Oncoprotein SKI Acts as A Suppressor of NK Cell-Mediated Immunosurveillance in PDAC. *Cancers (Basel)* 2020; **12** [PMID: 33023028 DOI: 10.3390/cancers12102857]
54 **Van Audenaerde JRM**, Roeyen G, Darcy PK, Kershaw MH, Peeters M, Smits ELJ. Natural killer cells and their therapeutic role in pancreatic cancer: A systematic review. *Pharmacol Ther* 2018; **189**: 31-44 [PMID: 29660367 DOI: 10.1016/j.pharmthera.2018.04.003]
55 **Ostapchuk YO**, Perfilyeva YV, Kali A, Tleulieva R, Yurikova OY, Stanbekova GE, Karalnik BV, Belyaev NN. Fc Receptor is Involved in Nk Cell Functional Anergy Induced by Miapaca2 Tumor Cell Line. *Immunol Invest* 2020; 1-16 [PMID: 32865068 DOI: 10.1080/08820139.2020.1813757]
56 **Duan Q**, Li H, Gao C, Zhao H, Wu S, Wu H, Wang C, Shen Q, Yin T. High glucose promotes pancreatic cancer cells to escape from immune surveillance *via* AMPK-Bmi1-GATA2-MICA/B pathway. *J Exp Clin Cancer Res* 2019; **38**: 192 [PMID: 31088566 DOI: 10.1186/s13046-019-1209-9]
57 **Ou ZL**, Luo Z, Wei W, Liang S, Gao TL, Lu YB. Hypoxia-induced shedding of MICA and HIF1A-mediated immune escape of pancreatic cancer cells from NK cells: role of circ_0000977/miR-153 axis. *RNA Biol* 2019; **16**: 1592-1603 [PMID: 31402756 DOI: 10.1080/15476286.2019.1649585]
58 **Peng YP**, Xi CH, Zhu Y, Yin LD, Wei JS, Zhang JJ, Liu XC, Guo S, Fu Y, Miao Y. Altered expression of CD226 and CD96 on natural killer cells in patients with pancreatic cancer. *Oncotarget* 2016; **7**: 66586-66594 [PMID: 27626490 DOI: 10.18632/oncotarget.11953]
59 **Van Audenaerde JRM**, De Waele J, Marcq E, Van Loenhout J, Lion E, Van den Bergh JMJ, Jesenofsky R, Masamune A, Roeyen G, Pauwels P, Lardon F, Peeters M, Smits ELJ. Interleukin-15 stimulates natural killer cell-mediated killing of both human pancreatic cancer and stellate cells. *Oncotarget* 2017; **8**: 56968-56979 [PMID: 28915646 DOI: 10.18632/oncotarget.18185]
60 **Peng YP**, Zhang JJ, Liang WB, Tu M, Lu ZP, Wei JS, Jiang KR, Gao WT, Wu JL, Xu ZK, Miao Y, Zhu Y. Elevation of MMP-9 and IDO induced by pancreatic cancer cells mediates natural killer cell dysfunction. *BMC Cancer* 2014; **14**: 738 [PMID: 25274283 DOI: 10.1186/1471-2407-14-738]
61 **Peng YP**, Zhu Y, Zhang JJ, Xu ZK, Qian ZY, Dai CC, Jiang KR, Wu JL, Gao WT, Li Q, Du Q, Miao Y. Comprehensive analysis of the percentage of surface receptors and cytotoxic granules positive natural killer cells in patients with pancreatic cancer, gastric cancer, and colorectal cancer. *J Transl Med* 2013; **11**: 262 [PMID: 24138752 DOI: 10.1186/1479-5876-11-262]
62 **Jun E**, Song AY, Choi JW, Lee HH, Kim MY, Ko DH, Kang HJ, Kim SW, Bryceson Y, Kim SC, Kim HS. Progressive Impairment of NK Cell Cytotoxic Degranulation Is Associated With TGF-β1 Deregulation and Disease Progression in Pancreatic Cancer. *Front Immunol* 2019; **10**: 1354 [PMID: 31281312 DOI: 10.3389/fimmu.2019.01354]
63 **Barrow AD**, Colonna M. Tailoring Natural Killer cell immunotherapy to the tumour microenvironment. *Semin Immunol* 2017; **31**: 30-36 [PMID: 28935344 DOI: 10.1016/j.smim.2017.09.001]
64 **Zhao J**, Schlößer HA, Wang Z, Qin J, Li J, Popp F, Popp MC, Alakus H, Chon SH, Hansen HP, Neiss WF, Jauch KW, Bruns CJ, Zhao Y. Tumor-Derived Extracellular Vesicles Inhibit Natural Killer Cell Function in Pancreatic Cancer. *Cancers (Basel)* 2019; **11** [PMID: 31234517 DOI: 10.3390/cancers11060874]
65 **Konjević GM**, Vuletić AM, Mirjačić Martinović KM, Larsen AK, Jurišić VB. The role of cytokines in the regulation of NK cells in the tumor environment. *Cytokine* 2019; **117**: 30-40 [PMID: 30784898 DOI: 10.1016/j.cyto.2019.02.001]
66 **Ostrand-Rosenberg S**, Sinha P, Beury DW, Clements VK. Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression. *Semin Cancer Biol* 2012; **22**: 275-281 [PMID: 22313874 DOI: 10.1016/j.semcancer.2012.01.011]
67 **Bassani B**, Baci D, Gallazzi M, Poggi A, Bruno A, Mortara L. Natural Killer Cells as Key Players of Tumor Progression and Angiogenesis: Old and Novel Tools to Divert Their Pro-Tumor Activities into Potent Anti-Tumor Effects. *Cancers (Basel)* 2019; **11**: 461 [PMID: 30939820 DOI: 10.3390/cancers11040461]
68 **Stiff A**, Trikha P, Mundy-Bosse B, McMichael E, Mace TA, Benner B, Kendra K, Campbell A, Gautam S, Abood D, Landi I, Hsu V, Duggan M, Wesolowski R, Old M, Howard JH, Yu L, Stasik

N, Olencki T, Muthusamy N, Tridandapani S, Byrd JC, Caligiuri M, Carson WE. Nitric Oxide Production by Myeloid-Derived Suppressor Cells Plays a Role in Impairing Fc Receptor-Mediated Natural Killer Cell Function. *Clin Cancer Res* 2018; **24**: 1891-1904 [PMID: 29363526 DOI: 10.1158/1078-0432.CCR-17-0691]

69 **Harlin H**, Hanson M, Johansson CC, Sakurai D, Poschke I, Norell H, Malmberg KJ, Kiessling R. The CD16- CD56(bright) NK cell subset is resistant to reactive oxygen species produced by activated granulocytes and has higher antioxidative capacity than the CD16+ CD56(dim) subset. *J Immunol* 2007; **179**: 4513-4519 [PMID: 17878347 DOI: 10.4049/jimmunol.179.7.4513]

70 **Ireland L**, Luckett T, Schmid MC, Mielgo A. Blockade of Stromal Gas6 Alters Cancer Cell Plasticity, Activates NK Cells, and Inhibits Pancreatic Cancer Metastasis. *Front Immunol* 2020; **11**: 297 [PMID: 32174917 DOI: 10.3389/fimmu.2020.00297]

71 **Kadaba R**, Birke H, Wang J, Hooper S, Andl CD, Di Maggio F, Soylu E, Ghallab M, Bor D, Froeling FE, Bhattacharya S, Rustgi AK, Sahai E, Chelala C, Sasieni P, Kocher HM. Imbalance of desmoplastic stromal cell numbers drives aggressive cancer processes. *J Pathol* 2013; **230**: 107-117 [PMID: 23359139 DOI: 10.1002/path.4172]

72 **Froeling FE**, Feig C, Chelala C, Dobson R, Mein CE, Tuveson DA, Clevers H, Hart IR, Kocher HM. Retinoic acid-induced pancreatic stellate cell quiescence reduces paracrine Wnt-β-catenin signaling to slow tumor progression. *Gastroenterology* 2011; **141**: 1486-1497, 1497.e1-1497. 14 [PMID: 21704588 DOI: 10.1053/j.gastro.2011.06.047]

73 **Neuzillet C**, Tijeras-Raballand A, Ragulan C, Cros J, Patil Y, Martinet M, Erkan M, Kleeff J, Wilson J, Apte M, Tosolini M, Wilson AS, Delvecchio FR, Bousquet C, Paradis V, Hammel P, Sadanandam A, Kocher HM. Inter- and intra-tumoural heterogeneity in cancer-associated fibroblasts of human pancreatic ductal adenocarcinoma. *J Pathol* 2019; **248**: 51-65 [PMID: 30575030 DOI: 10.1002/path.5224]

74 **Li C**, Cui L, Yang L, Wang B, Zhuo Y, Zhang L, Wang X, Zhang Q, Zhang S. Pancreatic Stellate Cells Promote Tumor Progression by Promoting an Immunosuppressive Microenvironment in Murine Models of Pancreatic Cancer. *Pancreas* 2020; **49**: 120-127 [PMID: 31856087 DOI: 10.1097/MPA.0000000000001464]

75 **Francescone R**, Vendramini-Costa DB, Franco-Barraza J, Wagner J, Muir A, Gabitova L, Pazina T, Luong T, Shah N, Rollins D, Malik R, Gupta S, Thapa R, Restifo D, Lau A, Zhou Y, Cai KQ, Hensley HH, Nicolas E, Kruger WD, Devarajan K, Balachandran S, El-Deiry WS, Heiden MV, Campbell K, Astsaturov I, Cukierman E. Abstract 2038: NG1/NGL1 engagement supports PDAC development *via* CAF to PDAC nutrition and CAF-regulated immunosuppression. *Cancer Research* 2019; **79** (13 Supplement): 2038 [DOI: 10.1158/1538-7445.AM2019-2038]

76 **Huang H**, Zhang Y, Gallegos V, Sorrelle N, Zaid MM, Toombs J, Du W, Wright S, Hagopian M, Wang Z, Hosein AN, Sathe AA, Xing C, Koay EJ, Driscoll KE, Brekken RA. Targeting TGFβR2-mutant tumors exposes vulnerabilities to stromal TGFβ blockade in pancreatic cancer. *EMBO Mol Med* 2019; **11**: e10515 [PMID: 31609088 DOI: 10.15252/emmm.201910515]

77 **Jewett A**, Kos J, Fong Y, Ko MW, Safaei T, Perišić Nanut M, Kaur K. NK cells shape pancreatic and oral tumor microenvironments; role in inhibition of tumor growth and metastasis. *Semin Cancer Biol* 2018; **53**: 178-188 [PMID: 30081230 DOI: 10.1016/j.semcancer.2018.08.001]

78 **Kaur K**, Chang HH, Topchyan P, Cook JM, Barkhordarian A, Eibl G, Jewett A. Deficiencies in Natural Killer Cell Numbers, Expansion, and Function at the Pre-Neoplastic Stage of Pancreatic Cancer by KRAS Mutation in the Pancreas of Obese Mice. *Front Immunol* 2018; **9**: 1229 [PMID: 29977235 DOI: 10.3389/fimmu.2018.01229]

79 **Xie G**, Dong H, Liang Y, Ham JD, Rizwan R, Chen J. CAR-NK cells: A promising cellular immunotherapy for cancer. *EBioMedicine* 2020; **59**: 102975 [PMID: 32853984 DOI: 10.1016/j.ebiom.2020.102975]

80 **Oei VYS**, Siernicka M, Graczyk-Jarzynka A, Hoel HJ, Yang W, Palacios D, Almåsbak H, Bajor M, Clement D, Brandt L, Önfelt B, Goodridge J, Winiarska M, Zagozdzon R, Olweus J, Kyte JA, Malmberg KJ. Intrinsic Functional Potential of NK-Cell Subsets Constrains Retargeting Driven by Chimeric Antigen Receptors. *Cancer Immunol Res* 2018; **6**: 467-480 [PMID: 29459477 DOI: 10.1158/2326-6066.CIR-17-0207]

81 **Lee YE**, Ju A, Choi HW, Kim JC, Kim EE, Kim TS, Kang HJ, Kim SY, Jang JY, Ku JL, Kim SC, Jun E, Jang M. Rationally designed redirection of natural killer cells anchoring a cytotoxic ligand for pancreatic cancer treatment. *J Control Release* 2020; **326**: 310-323 [PMID: 32682905 DOI: 10.1016/j.jconrel.2020.07.016]

82 **Xia N**, Haopeng P, Gong JU, Lu J, Chen Z, Zheng Y, Wang Z, Sun YU, Yang Z, Hoffman RM, Liu F. Robo1-specific CAR-NK Immunotherapy Enhances Efficacy of ^{125}I Seed Brachytherapy in an Orthotopic Mouse Model of Human Pancreatic Carcinoma. *Anticancer Res* 2019; **39**: 5919-5925 [PMID: 31704816 DOI: 10.21873/anticanres.13796]

83 **Raj D**, Nikolaidi M, Garces I, Lorizio D, Castro NM, Caiafa SG, Moore K, Brown NF, Kocher HM, Duan X, Nelson BH, Lemoine NR, Marshall JF. CEACAM7 Is an Effective Target for CAR T-cell Therapy of Pancreatic Ductal Adenocarcinoma. *Clin Cancer Res* 2021; **27**: 1538-1552 [PMID: 33479048 DOI: 10.1158/1078-0432.CCR-19-2163]

84 **Raj D**, Yang MH, Rodgers D, Hampton EN, Begum J, Mustafa A, Lorizio D, Garces I, Propper D, Kench JG, Kocher HM, Young TS, Aicher A, Heeschen C. Switchable CAR-T cells mediate remission in metastatic pancreatic ductal adenocarcinoma. *Gut* 2019; **68**: 1052-1064 [PMID: 30121627 DOI: 10.1136/gutjnl-2018-316595]

85 **Van Audenaerde JR**, Marcq E, von Scheidt B, Davey AS, Oliver AJ, De Waele J, Quatannens D, Van Loenhout J, Pauwels P, Roeyen G, Lardon F, Slaney CY, Peeters M, Kershaw MH, Darcy PK, Smits EL. Novel combination immunotherapy for pancreatic cancer: potent anti-tumor effects with CD40 agonist and interleukin-15 treatment. *Clin Transl Immunology* 2020; **9**: e1165 [PMID: 32821382 DOI: 10.1002/cti2.1165]

86 **Lin M**, Alnaggar M, Liang S, Wang X, Liang Y, Zhang M, Chen J, Niu L, Xu K. An important discovery on combination of irreversible electroporation and allogeneic natural killer cell immunotherapy for unresectable pancreatic cancer. *Oncotarget* 2017; **8**: 101795-101807 [PMID: 29254205 DOI: 10.18632/oncotarget.21974]

87 **Jiang N**, Qiao G, Wang X, Morse MA, Gwin WR, Zhou L, Song Y, Zhao Y, Chen F, Zhou X, Huang L, Hobeika A, Yi X, Xia X, Guan Y, Song J, Ren J, Lyerly HK. Dendritic Cell/Cytokine-Induced Killer Cell Immunotherapy Combined with S-1 in Patients with Advanced Pancreatic Cancer: A Prospective Study. *Clin Cancer Res* 2017; **23**: 5066-5073 [PMID: 28611200 DOI: 10.1158/1078-0432.CCR-17-0492]

88 **Kocher HM**, Basu B, Froeling FEM, Sarker D, Slater S, Carlin D, deSouza NM, De Paepe KN, Goulart MR, Hughes C, Imrali A, Roberts R, Pawula M, Houghton R, Lawrence C, Yogeswaran Y, Mousa K, Coetzee C, Sasieni P, Prendergast A, Propper DJ. Phase I clinical trial repurposing all-trans retinoic acid as a stromal targeting agent for pancreatic cancer. *Nat Commun* 2020; **11**: 4841 [PMID: 32973176 DOI: 10.1038/s41467-020-18636-w]

89 **Sivapalan L**, Kocher HM, Ross-Adams H, Chelala C. Molecular profiling of ctDNA in pancreatic cancer: Opportunities and challenges for clinical application. *Pancreatology* 2021; **21**: 363-378 [PMID: 33451936 DOI: 10.1016/j.pan.2020.12.017]

90 **Niu L**. Combination of Irreversible Electroporation and NK Immunotherapy for Advanced Pancreatic Cancer [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02718859 ClinicalTrials.gov Identifier: NCT0271885987

91 **Lin M**, Liang S, Wang X, Liang Y, Zhang M, Chen J, Niu L, Xu K. Percutaneous irreversible electroporation combined with allogeneic natural killer cell immunotherapy for patients with unresectable (stage III/IV) pancreatic cancer: a promising treatment. *J Cancer Res Clin Oncol* 2017; **143**: 2607-2618 [PMID: 28871458 DOI: 10.1007/s00432-017-2513-4]

92 **Jiang N**. Study of S-1 Plus DC-CIK for Patients With Unresectable Locally Advanced Pancreatic Cancer [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT01781520 ClinicalTrials.gov Identifier: NCT01781520

93 **Wang Z**. Clinical Research of ROBO1 Specific BiCAR-NK Cells on Patients With Pancreatic Cancer Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT03941457 ClinicalTrials.gov Identifier: NCT03941457

94 **Song P**. Safety and Preliminary Efficacy of SNK01 in Combination With Trastuzumab or Cetuximab in Subjects With Advanced HER2 or EGFR Cancers Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT04464967 ClinicalTrials.gov Identifier: NCT04464967

95 **Fuda-Cancer-Hospital**. High-activity Natural Killer Immunotherapy for Small Metastases of Pancreatic Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT03008304 ClinicalTrials.gov Identifier: NCT03008304

96 **Chen Z**. Clinical Study on Anti-tumor Effect Induced by Activated Primary Natural Killer (NK) Cells. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT03634501 ClinicalTrials.gov Identifier: NCT03634501

97 **Chou J**. FT500 as Monotherapy and in Combination With Immune Checkpoint Inhibitors in Subjects With Advanced Solid Tumors Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT03841110 ClinicalTrials.gov Identifier: NCT03841110

98 **Chu W**. Long-term, Non-interventional, Observational Study Following Treatment With Fate Therapeutics FT500 Cellular Immunotherapy. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT04106167 ClinicalTrials.gov Identifier: NCT04106167

99 **Cooley S**. FATE-NK100 as Monotherapy and in Combination With Monoclonal Antibody in Subjects With Advanced Solid Tumors. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT03319459 ClinicalTrials.gov Identifier: NCT03319459

100 **Digklia A**. Personalized Vaccine With SOC Chemo Followed by Nivo in Pancreatic Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT04627246 ClinicalTrials.gov Identifier: NCT04627246

101 **Kurman M**. ACE1702 in Subjects With Advanced or Metastatic HER2-expressing Solid Tumors. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT04319757 ClinicalTrials.gov Identifier:

NCT04319757

102 **Childs R**. Natural Killer Cells and Bortezomib to Treat Cancer Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT00720785 ClinicalTrials.gov Identifier: NCT00720785

103 **Wang Z**, Liu Y, Li R, Shang Y, Zhang Y, Zhao L, Li W, Yang Y, Zhang X, Yang T, Nie C, Han F, Luo S, Gao Q, Song Y. Autologous cytokine-induced killer cell transfusion increases overall survival in advanced pancreatic cancer. *J Hematol Oncol* 2016; **9**: 6 [PMID: 26842696 DOI: 10.1186/s13045-016-0237-6]

104 **Gao Q**. Autologous Cytokine-induced Killer Cells Combined Chemotherapy in Advanced Pancreatic Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT03002831 ClinicalTrials.gov Identifier: NCT03002831

105 **Yang L**. CAR-pNK Cell Immunotherapy in MUC1 Positive Relapsed or Refractory Solid Tumor Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT02839954 ClinicalTrials.gov Identifier: NCT02839954

106 **Korn D**. Safety and Effectiveness Study of Autologous Natural Killer and Natural Killer T Cells on Cancer. [accessed 27 Jan 2021]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: http://clinicaltrials.gov/show/NCT00909558 ClinicalTrials.gov Identifier: NCT00909558

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REVIEW

COVID-19 and its effects on the digestive system

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Abstract

Coronavirus disease 2019 (COVID-19) is caused by infection of the coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) with typical respiratory symptoms. SARS-CoV-2 invades not only the respiratory system, but also other organs expressing the cell surface receptor angiotensin converting enzyme 2. In particular, the digestive system is a susceptible target of SARS-CoV-2. Gastrointestinal symptoms of COVID-19 include anorexia, nausea, vomiting, diarrhea, abdominal pain, and liver damage. Patients with digestive damage have a greater chance of progressing to severe or critical illness, a poorer prognosis, and a higher risk of death. This paper aims to summarize the digestive system symptoms of COVID-19 and discuss fecal-oral contagion of SARS-CoV-2. It also describes the characteristics of inflammatory bowel disease patients with SARS-CoV-2 infection and discusses precautions for preventing SARS-CoV-2 infection during gastrointestinal endoscopy procedures. Improved attention to digestive system abnormalities and gastrointestinal symptoms of COVID-19 patients may aid health care providers in the process of clinical diagnosis, treatment, and epidemic prevention and control.

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Core Tip: Coronavirus infections can cause a series of digestive diseases and may also be accompanied by digestive manifestations. Furthermore, the potential mechanisms of coronavirus disease 2019 (COVID-19) on the digestive system, the fecal-oral contagion of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the characteristics of inflammatory bowel disease patients with SARS-CoV-2 infection, and the management during gastrointestinal endoscopy procedures are also discussed. This review provides a new perspective to clinicians for the prevention and treatment of COVID-19.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a neo-type respiratory infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; previously known as 2019-nCoV). SARS-CoV-2 emerged in Wuhan, Hubei Province in late December 2019[1] and quickly spread throughout China and subsequently throughout over 213 countries, evolving into a pandemic and threatening global public health by human-to-human transmission. By December 29, 2020, over 79 million reported cases and over 1.7 million deaths have been confirmed globally since the start of the pandemic[2].

SARS-CoV-2 is a single-stranded positive-sense RNA virus belonging to the β-coronavirus family. SARS-CoV-2 shows over 88% homology with two bat-derived severe acute respiratory syndrome (SARS)-related coronaviruses[3] and is identified as the eighth coronavirus with human infection capacity[4]. Other similar coronaviruses with this capacity include SARS-CoV, which causes severe acute respiratory syndrome, and Middle East respiratory syndrome (MERS-CoV), which causes Middle East respiratory syndrome. In contrast to SARS-CoV and MERS-CoV, the new virus is highly transmissible between individuals even during the pre-clinical phase. It has higher transmission and infection potentiality but a reported lower mortality rate as compared to SARS-CoV and MERS-CoV[5]. Although respiratory compromise with dominant symptoms of fever and cough is the cardinal feature of the disease, involvement of the gastrointestinal (GI) tract and the hepatic system has been increasingly reported. In this review article, we discuss in detail GI symptoms and the role of liver involvement in COVID-19. We also discuss the possible effects of COVID-19 in inflammatory bowel disease (IBD) patients and precautions to be taken during GI endoscopy procedures.

DIGESTIVE TRANSMISSION OF COVID-19 OUTBREAKS

SARS-CoV-2 is spread and transmitted mainly through direct or indirect droplet exposure. The finding of SARS-CoV-2 nucleic acid in patients' feces indicates that SARS-CoV-2 has the potential to be transmitted through the fecal-oral route. Several studies have reported the presence of viral RNA in feces or anal/rectal swabs of patients with COVID-19[6-9]. In a study about SARS-CoV-2 detection in the specimens of 205 COVID-19 patients, the live virus was detected in 29% of fecal specimens, implying that SARS-CoV-2 may be transmitted by the fecal route[10]. Guan *et al*[11] found that SARS-CoV-2 RNA was detected in four (6.5%) of 62 stool specimens, and four rectal swabs were positive for SARS-CoV-2 RNA. The percentage of positive stool samples has been reported up to 53.42% among hospitalized patients confirmed with

COVID-19[12]. Chen *et al*[13] reported a special case of an infected COVID-19 patient with a positive virus nucleic acid result in a fecal specimen and negative findings on several pharyngeal and sputum samples. This case report contributes to the understanding of the infection route of SARS-CoV-2 by demonstrating that the virus can grow in the digestive tract and may be capable of spreading through fecal-oral transmission[13]. The duration time of positive stool results ranged from 1 to 12 d and 17 patients continued to have positive results in stool after showing negative results in respiratory samples[12]. Another systematic review and meta-analysis found that viral RNA was detected in stool samples from 48.1% of patients in the study sample, even in stool collected after respiratory samples had produced negative test results[14]. These findings indicate that viral gastrointestinal tract infections and potential fecal transmission may have persisted even after the virus was cleared in the respiratory tract. This positive detection of SARS-CoV-2 in stool specimens was a breakthrough because it demonstrated that the virus could replicate and exist in the digestive tract. The duration of viral nucleic acid in feces is longer than that in respiratory specimens, and the peak of viral load is later. Therefore, clinicians should consider the possibility of viral transmission through the fecal-oral route in the management of COVID-19. The importance of the high detection rate of viral RNA in fecal samples needs to be more carefully considered so that fecal-oral transmission of SARS-CoV-2 can be better controlled and prevented.

GASTROINTESTINAL SYMPTOMS OF COVID-19 PATIENTS

The most prominent clinical presentations of COVID-19 in the existing literature are respiratory symptoms such as fever, cough and sputum, and dyspnea. Digestive system symptoms in COVID-19 patients have been increasingly reported with the accumulation of case data as the pandemic continues to evolve[1,15-21] (Table 1). The most commonly reported gastrointestinal symptoms of the disease are diarrhea, anorexia, nausea, vomiting, abdominal discomfort, and gastrointestinal bleeding. Loss of appetite, diarrhea, and vomiting were the three most frequent digestive symptoms in patients with COVID-19. There have been some reports of a small number of patients presenting only with diarrhea and vomiting without fever or cough[22,23]. In a meta-analysis of 60 studies including 4243 patients, the pooled prevalence of all gastrointestinal symptoms was 17.6%[14]. Eleven studies in the meta-analysis compared the prevalence of gastrointestinal symptoms to COVID-19 disease severity; 11.8% of patients with non-severe COVID-19 and 17.1% of patients with severe COVID-19 had gastrointestinal symptoms, indicating that the prevalence of severe disease was more common in patients with gastrointestinal symptoms[14]. Redd *et al*[24] similarly found that gastrointestinal symptoms occurred in 61.3% of patients included in the study, and they were the predominant presenting complaint amongst 20.3% of patients. Importantly, gastrointestinal manifestations may be the only initial symptoms in some patients with COVID-19. In the study by An *et al*[25], 54 patients reported only gastrointestinal symptoms without fever or respiratory symptoms. Six (66.7%) patients had anorexia. None of the cases presented with fever at onset, and all cases presented with digestive symptoms occurring 1-3 d prior to admission[25]. Another study involving 1141 COVID-19 patients found that 183/1141 (16%) presented with GI symptoms only[18]. In addition, digestive symptoms appeared to be associated with worse prognoses. Multiple studies have reported a higher incidence of diarrhea, nausea, or vomiting in patients with severe disease as compared to those with non-severe disease. In addition, there is a connection between the presence of diarrhea and respiratory symptom severity; Li *et al*[26] reported that more patients with diarrhea required ventilator support and were admitted to the intensive care unit (ICU) than those without diarrhea. Pan *et al*[27] found that patients with digestive symptoms had a longer time from onset to hospital admission as compared to patients without digestive symptoms. Sixty percent of patients without digestive symptoms recovered and were discharged, while only 34.3% of patients with digestive symptoms recovered[27]. Digestive symptoms appeared to be tied to worse COVID-19 outcomes.

This bank of published literature provides robust evidence for GI symptoms as common clinical manifestations of COVID-19. GI symptoms should attract the attention of both patients and doctors. It is crucial for clinicians to be on the alert for atypical symptoms to avoid missed COVID-19 diagnosis.

Table 1 Summary of clinical features of coronavirus disease 2019 patients with digestive symptoms, *n* (%)

Ref.	Total patients	Diarrhea	Anorexia	Nausea	Vomiting	Abdominal pain
Huang *et al*[1], 2020	38	1 (2.6)				
Chen *et al*[15], 2020	99	2 (2.0)		1 (1.0)	1 (1.0)	
Zhou *et al*[105], 2020	141	9 (4.7)		7 (3.7)	7 (3.7)	
Mao *et al*[19], 2020	6686	601 (9)	1404 (21)	401 (6)	401 (6)	200 (3)
Guan *et al*[11], 2020	1999	42 (3.8)		55 (5.0)	55 (5.0)	
Pan *et al*[27], 2020	204	35 (17.2)	4 (2.0)		81 (39.7)	2 (0.98)
Holshue *et al*[20], 2020	1 (first case)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Luo *et al*[18], 2020	1141	68 (6.0)	180 (15.8)	134 (11.7)	119 (10.4)	45 (3.9)
Han *et al*[9], 2020	206	67 (32.5)	112 (49.5)		24 (11.7)	9 (4.4)
Lin *et al*[106], 2020	95	23 (24.2)	17 (17.9)	17 (17.9)	4 (4.2)	2 (2.1)
Wang *et al*[32], 2020	138	55 (39.9)	14 (10.1)	14 (10.1)	5 (3.6)	3 (2.2)
Zhang *et al*[107], 2020	140	17 (12.2)	18 (12.9)	24 (17.3)	7 (5)	8 (5.8)
Liu *et al*[23], 2020	137	11 (8.0)				

MECHANISMS OF GASTROINTESTINAL TRACT INVOLVEMENT

Intestinal damage caused by SARS-CoV-2 infection has been verified by autopsy and biopsy. A recent report described the intestinal autopsy from a COVID-19 patient who developed alternating segmental dilatation and stenosis of the small intestine[28]. However, the mechanism by which SARS-CoV-2 causes gastrointestinal symptoms remains unclear. The pathogenicity of COVID-19 is thought to be related to the angiotensin converting enzyme 2 (ACE2) receptor[29-32]. The SARS-CoV-2 virus consists of four main structural proteins: The spike (S) protein, membrane (M) proteins, nucleocapsid (N) proteins, envelope (E) proteins[33,34]. The S protein is the key component that mediates the entry of the virus into the host cell[35,36]. In the process of infecting cells, the S proteins of most coronaviruses are cleaved into S1 receptor-binding subunit and S2 fusion subunit by host cell Furin-like protease. S1 contains a receptor-binding domain (RBD) that plays an important role in recognizing and binding to the ACE2. Epigallocatechin gallate (EGCG), an active constituent of green tea, has been identified as a potential inhibitor of the RBD domain and other proteins of SARS-CoV-2[37]. The effect of green tea consumption may be explored for inhibition of S protein domains to prevent its binding with ACE2[38]. This may be particularly effective in the gastrointestinal tract, as the oral route implies maximum availability in the digestive tract. The S2 subunit is associated with the fusion of the viral membrane and the host cell membrane. The entry of coronavirus into susceptible cells is a complex process that requires ACE2 receptor binding and the enhancement of proteolytic distribution of protein S[36]. ACE2 is also closely related to the physiological processes of virus infection, virus killing, and natural immunity in the immune system (Figure 1). Transmembrane serine proteinase 2 (TMPRSS2) plays an indispensable role in the invasion and transmission of the virus for S protein priming. ACE2 is distributed in many tissues and organs of the human body. It is not only expressed in the heart, lung, kidney, and blood vessels but also in the digestive system, such as in the duodenum, jejunum, and liver[26]. Hoffmann *et al*[35] confirmed that the host cell entry of SARS-CoV-2 depends on the SARS-CoV receptor ACE2 and that the entry can be blocked by a clinically proven inhibitor of the cellular serine protease TMPRSS2. Xiao *et al*[12] observed the staining for viral nucleocapsid protein in the cytoplasm of gastric, duodenal, and rectum glandular epithelial cells and the positive staining for ACE2 and SARS-CoV-2 in the gastrointestinal epithelium from patients who tested positive for SARS-CoV-2 RNA in feces. Another study by Qian *et al*[39] also observed typical SARS-CoV-2 virus particles in the intestinal epithelial cells of a patient under electron microscopy and obtained direct evidence of active SARS-CoV-2 viral replication in the intestine. Virus particles were found in the cytoplasm of intestinal epithelial cells in a rectal adenocarcinoma patient with co-existing COVID-19 and the virions showed a typical morphology of coronavirus under electron microscopy[39]. These findings indicate that SARS-CoV-2 could directly target

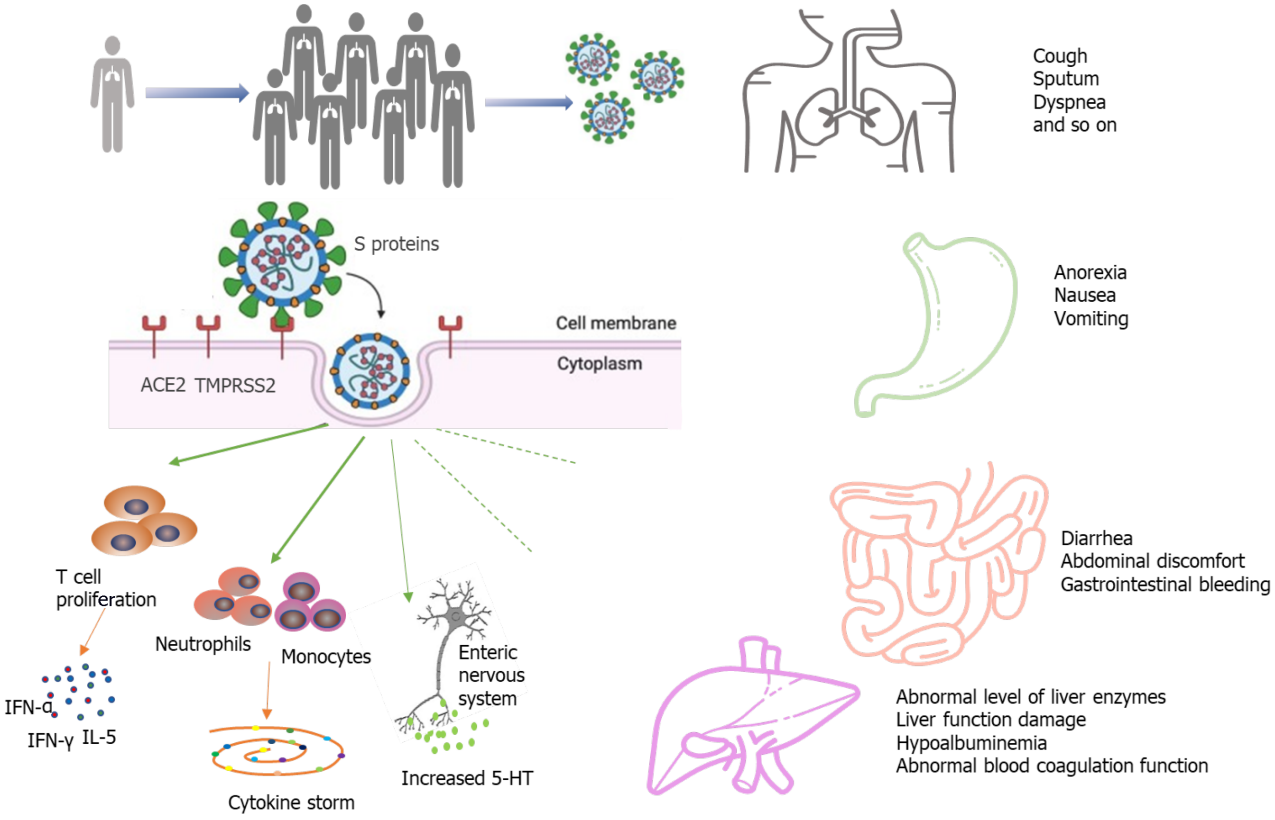


Figure 1 A simplified diagram of the potential pathological mechanisms for gastrointestinal symptoms in severe acute respiratory syndrome coronavirus 2 infection. ACE2: Angiotensin converting enzyme-2; TMPRSS2: Transmembrane serine protease-2.

gastrointestinal cells, especially gastric and intestinal epithelial cells, leading to inflammatory reactions.

The "cytokine storm" has been considered as an essential mechanism of multiple organ dysfunction in COVID-19 patients. High expression of pro-inflammatory cytokines was detected in the serum of most COVID-19 patients, including interferon-γ, interferon-α, and interleukin (IL)-5[1]. The cytokine storm in COVID-19 infection is mainly caused by inducing varieties of pro-inflammatory signals through antigen-presenting cells and T cells against viral infection, and activated macrophages and dendritic cells[40-42]. These cells release chemokines and cytokines to attract more inflammatory cells, such as neutrophils, monocytes, and dendritic cells, which aggregate to the site of inflammation and activate the cascade of inflammatory response. The activated inflammatory cells release more cytokines, further worsening injury to the organs. The fierce cytokine-induced immunopathological attack contributes to the aggravation of patient's condition with a poorer prognosis.

LIVER INJURY IN COVID-19 PATIENTS

An increasing number of studies have reported liver damage in patients with COVID-19 and several have reported COVID-19 patients to have an increased risk of liver dysfunction[1,15,22,43,44]. COVID-19 patients may incur different degrees of liver function damage with elevated aspartate amino transaminase (AST), glutamate moderately amino transaminase (ALT), and total bilirubin (TBil)[45,46]. The risk of liver damage in severe and critically ill patients was higher than in mild patients in most studies. However, there was a subtle difference in the prevalence of lung injury and COVID-19 disease severity across studies, and the exact extent of liver involvement in the COVID-19 disease course remains uncertain[19,46]. In a meta-analysis of 12 studies comprised of 1267 patients, the pooled prevalence of liver injury was 19%, the prevalence of ALT elevation was 18%, the prevalence of AST elevation was 21%, and the prevalence of total bilirubin elevation was 6%[47]. Another cohort study of 1992 patients observed that 215 (11%) patients had an abnormal level of ALT or TBil[41]. Among patients with an abnormal ALT or TBil test result, 77% had a mild

increase, 17% had a moderate increase, and 6% had a severe increase[48]. Additionally, the degree of liver function damage appears to correlate with the occurrence of gastrointestinal symptoms. Jin *et al*[47] reported that the incidence rate of elevated AST was significantly higher in patients with GI symptoms than in those without. Xu *et al*[49] observed moderate microvesicular steatosis and mild lobular and portal activity in the liver biopsy specimens of the patient with COVID-19, which provided evidence of liver injury. It is worth noting that the elevated prothrombin time among COVID-19 patients with digestive symptoms is common, and several studies have reported thromboembolism as a presenting clinical feature of COVID-19[50-53]. Therefore, liver function and the level of liver enzymes should be monitored early in COVID-19 patients with digestive symptoms (Figure 1).

COVID-19 may promote deterioration of liver function in patients who had been diagnosed with chronic liver disease previously and predict an increased risk for severe illness. Several studies have demonstrated that baseline liver disease severity is strongly associated with COVID-19-related morbidity and mortality; additionally, decompensated cirrhosis, hepatocellular carcinoma, and alcohol-related liver disease are risk factors for adverse outcomes from COVID-19[54-58]. A multi-center study involving 867 patients with chronic liver disease and COVID-19 reported that 14.0% of patients died, 60.4% were hospitalized, 23% were admitted to the ICU, and 7.7% developed hepatic decompensation[55]. Another study indicated that mortality was 32% in COVID-19 patients with a previous history of cirrhosis compared to 8% in those without ($P < 0.001$)[59]. Moon *et al*[60] found that 23.3% of patients with cirrhosis and COVID-19 were admitted to the ICU, 17.5% were treated with invasive ventilation, 18.6% were given non-invasive ventilatory support, 4.9% were given renal replacement therapy, and 39.8% died. Nowadays, accumulated data suggest that SARS-CoV-2 infection in patients with cirrhosis appears to be a particularly lethal combination. Compared to the patients without baseline liver disease, the patients with baseline liver disease are prone to unfavorable prognoses.

The mechanisms of liver injury in COVID-19 patients are complex. The higher overall mortality among patients with CLD and COVID-19 may be due to cirrhosis-associated immune dysfunction and metabolic syndrome[61,62], while it needs more research to confirm and explore.

IBD PATIENTS WITH SARS-COV-2 INFECTION

IBD, which mainly includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, nonspecific inflammatory disease with unclear etiology and pathogenesis. At present, intestinal microbial growth disorder, intestinal mucosal barrier injury, abnormal immune response in intestinal mucosal tissue, genetic susceptibility, and environmental factors are considered to be involved in the occurrence of intestinal inflammation. Patients with IBD are prone to frequent and severe infections and are often treated with corticosteroids, which are immunosuppressive agents. Whether the clinical presentation in patients with IBD differs from that of non-IBD people and the risk of SARS-CoV-2 infection or development of COVID-19 in patients with active IBD are uncertain[63]. GI symptoms including diarrhea and abdominal pain appear to be more frequent in COVID-19 IBD patients than in the COVID-19 non-IBD group. Aziz *et al*[64] reported an incidence of 0.3% for COVID-19 in their cohort of 9177 patients with IBD, an ICU admission rate of 8.6% in IBD patients with COVID-19, and a mortality rate of 6.3% in IBD patients with COVID-19. In a multicenter study from eight major gastrointestinal centers in Lombardy, Italy, IBD patients were not reported to have an increased risk of COVID-19 specific symptoms or more severe disease as compared with a control group of gastroenterology patients[65]. Another observational study confirmed that patients with IBD had a higher risk of COVID-19 incidence as compared to the general population and that tumor necrosis factor (TNF) antagonists may reduce the severity of COVID-19[66]. Singh *et al*[67] indicated no significant differences in the composite outcome of hospitalization or mortality between COVID-19 patients with IBD and those without. In addition, IBD patients with COVID-19 on long-term biologics or non-steroid immunomodulatory therapies did not have a higher risk of worse COVID-19 outcomes[67]. As the pandemic continues, it is essential to continue the collection of descriptive data to determine if patients with IBD are more vulnerable to SARS-CoV-2 infection.

Due to the frequent use of biologics and immune suppressors in patients with IBD, questions arose on whether IBD treatment can be continued during SARS-CoV-2 infection and whether a potential threat from the application of biologics and immune

suppressors would be present for patients. Several studies implied that thiopurines, steroids, and oral salicylates could be associated with an increased risk of developing severe COVID-19[68,69]. Feldmann *et al*[70] indicated that anti-TNF antibodies might reduce some of the processes that occur during COVID-19 lung inflammation. As well, Brenner *et al*[68] found that tumor necrosis factor antagonists did not appear to be associated with severe COVID-19. Future studies are required to investigate the impact of anti-TNF antibodies. The American Gastroenterological Association (AGA) recommended that patients with IBD who have been infected with SARS-CoV-2 but have not developed COVID-19 should hold thiopurines, methotrexate, and tofacitinib and should delay the biological therapies; on the other hand, patients with IBD who develop COVID-19 should hold thiopurines, methotrexate, tofacitinib, and biological therapies during the viral illness as well and restart previous treatment after complete symptom resolution or negative PCR tests for SARS-CoV-2[63]. Olendzki *et al*[71] propounded that the Anti-Inflammatory Diet (IBD-AID) was effective in some IBD patients as an adjunct dietary therapy. In the absence of biological therapies, IBD-AID and some identified anti-inflammatory aliments such as ginger, turmeric, and cinnamon may be provided to IBD patients due to their potential inhibitory effects on key pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6[72].

MALNUTRITIONAL RISK AND NUTRITIONAL INTERVENTION IN COVID-19 PATIENTS

Malnutrition has been associated with outcomes of COVID-19 patients and is correlated with a higher risk of mortality[73]. In a study of 348 severe patients with COVID-19, 161 patients had mild malnutrition and 139 suffered moderate-severe malnutrition. The patients with moderate-severe malnutrition had a lower survival rate and higher mortality compared to those with normal or mild malnutrition[74]. One of the reasons is that COVID-19 induces an acute inflammatory response, which accelerates the consumption of nutrients, such as protein, micronutrients, and glucose [75,76]. Gastrointestinal symptoms caused by SARS-CoV-2 further impacted the nutrition absorption and exacerbated malnutrition. In addition, patients' anxiety and poor appetite were also potential contributors to malnutrition[11,77,78]. Some studies show that elderly patients with COVID-19 have a higher risk of developing moderate-severe malnutrition and multi-system organ dysfunction due to their weaker immune function and chronic comorbidities[79,80].

The albumin levels are also decreased in COVID-19 patients and are positively correlated with the infection severity[81]. Zhang *et al*[82] found that 54.78% of COVID-19 patients (63/115) present hypoalbuminemia and among the severe COVID-19 patients, the percentage is 90.32%. Decreased protein synthesis, inadequate nutrition intake, increased tissue catabolism, and nutrient overconsumption may be potential contributors to hypoalbuminemia. Albumin is synthesized in the liver and has several key physiologic functions, such as maintaining colloid oncotic pressure, binding and transporting substances, and sustaining acid-base equilibrium[83]. During the acute stress response, inflammatory mediators such as C-reactive protein, fibrinogen, and alpha 1-antitrypsin are increased, whereas albumin synthesis is decreased[84,85]. Due to the inflammation-induced higher endothelial permeability, albumin can escape to the extravascular space through capillaries, leading to lower serum albumin levels. Hypoalbuminemia may be related to a worse clinical outcomes for COVID-19 patients [73,86-89]. In a retrospective analysis of 181 patients with COVID-19, patients with higher albumin levels on admission were associated with a lower risk of developing ARDS, admission to the ICU, and for every 1 g/dL increase of albumin, there is a 72% decreased risk of developing venous thromboembolism[90]. In another retrospective analysis, the researchers showed significant differences in the rates of hypoalbuminemia (odds ratio = 5.68) between deceased and recovered patients[91].

Previous studies also show that micronutrients play important roles in boosting the immune system[92-94]. Several studies have demonstrated that low levels or decreased intake of micronutrients such as vitamins A, E, B6, B12, Zn, and Se were associated with adverse clinical outcomes of COVID-19[95-99]. Im *et al*[100] reported that 24.0% of the patients with COVID-19 had severe vitamin D deficiency, and among 12 patients with respiratory distress, 91.7% were deficient in at least one nutrient. They speculated that vitamin D deficiency might be an important risk factor for the poor prognosis of COVID-19. In order to maximize anti-infection nutritional defense, we suggest the daily provision of vitamins and trace elements to malnourished patients with COVID-19. The European Society for Clinical Nutrition and Metabolism (ESPEN)

recommended reasonable provision of vitamins and trace elements to benefit anti-infection nutritional defense[101].

Plenty of scientists have emphasized the significance of various nutritional interventions to regulate immune function[102]. Nutrition support is of great importance for patients with severe COVID-19, which can elevate immune function, decrease the incidence of multiple organ failure, and improve the prognosis of the disease. Therefore, the nutritional status of COVID-19 patients should be taken into consideration after admission.

PRECAUTIONS FOR PREVENTING SARS-COV-2 INFECTION DURING GI ENDOSCOPY PROCEDURES

With the growing spread of COVID-19, concerns should be raised with respect to guaranteeing safety for endoscopy operators. Exposure of the respiratory tract and/or digestive tract during endoscopic examination inevitably provides the risk for patients' secretions and excretions to become potential sources of infection, which will significantly increase the probability of nosocomial cross-infection during the pandemic. Therefore, it is important that endoscopy centers pay great attention to indications that most vitally warrant digestive endoscopy procedures. Iacucci *et al*[103] recommended four different urgent scenarios that could necessitate endoscopy: Confirmation of a new diagnosis, especially in a moderate-to-severe scenario; a severe acute flare-up in patients with ulcerative colitis; partial bowel obstruction in patients with IBD, which could be secondary to neoplasia or ileocolonic anastomotic stricture; and cholangitis and jaundice in patients with known primary sclerosing cholangitis with dominant bile duct stricture. Further, endoscopists and assistants must strictly execute hospital infection control requirements and take corresponding classified protective measures according to the actual situation of patients. The AGA recommended the use of N95 (or N99 or PAPR) masks instead of surgical masks as part of appropriate personal protective equipment[104]. It is essential to evaluate the risk of patients with suspected or confirmed COVID-19 before endoscopy, in order to defer unnecessary endoscopies to minimize concomitant exposure.

CONCLUSION

Patients with COVID-19 may also develop various gastrointestinal symptoms, which may be pre-existing or not accompanied by respiratory symptoms. The importance of recognizing and diagnosing the occurrence of gastrointestinal symptoms is immeasurable. Clinicians should be cautious in the management of this highly infectious disease and recognize that gastrointestinal symptoms may be characteristic of COVID-19. Careful consideration of gastrointestinal symptoms may enable early COVID-19 detection, diagnosis, isolation, and intervention. Evidence on fecal-oral contagion of SARS-CoV-2 continues to increase. It is therefore important to step up infection control measures to avoid fecal-oral transmission and standardize health care operational processes.

REFERENCES

1 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

2 **National Comprehensive Cancer Network**. NCCN Clinical Practice Guidelines in Oncology: Hepatobiliary Cancers. [cited 7 July 2020]. Available from: http://www.nccn.org/professionals/physician_gls

3 **Lu R**, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 2020; **395**: 565-574 [PMID: 32007145 DOI: 10.1016/S0140-6736(20)30251-8]

4 **Zheng J**. SARS-CoV-2: an Emerging Coronavirus that Causes a Global Threat. *Int J Biol Sci* 2020;

16: 1678-1685 [PMID: 32226285 DOI: 10.7150/ijbs.45053]
5 **Liu Y**, Gayle AA, Wilder-Smith A, Rocklöv J. The reproductive number of COVID-19 is higher compared to SARS coronavirus. *J Travel Med* 2020; **27** [PMID: 32052846 DOI: 10.1093/jtm/taaa021]
6 **Wu Y**, Guo C, Tang L, Hong Z, Zhou J, Dong X, Yin H, Xiao Q, Tang Y, Qu X, Kuang L, Fang X, Mishra N, Lu J, Shan H, Jiang G, Huang X. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *Lancet Gastroenterol Hepatol* 2020; **5**: 434-435 [PMID: 32199469 DOI: 10.1016/S2468-1253(20)30083-2]
7 **Xu Y**, Li X, Zhu B, Liang H, Fang C, Gong Y, Guo Q, Sun X, Zhao D, Shen J, Zhang H, Liu H, Xia H, Tang J, Zhang K, Gong S. Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. *Nat Med* 2020; **26**: 502-505 [PMID: 32284613 DOI: 10.1038/s41591-020-0817-4]
8 **Young BE**, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, Tan TY, Ng TY, Cui L, Said Z, Kurupatham L, Chen MI, Chan M, Vasoo S, Wang LF, Tan BH, Lin RTP, Lee VJM, Leo YS, Lye DC; Singapore 2019 Novel Coronavirus Outbreak Research Team. Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore. *JAMA* 2020; **323**: 1488-1494 [PMID: 32125362 DOI: 10.1001/jama.2020.3204]
9 **Han C**, Duan C, Zhang S, Spiegel B, Shi H, Wang W, Zhang L, Lin R, Liu J, Ding Z, Hou X. Digestive Symptoms in COVID-19 Patients With Mild Disease Severity: Clinical Presentation, Stool Viral RNA Testing, and Outcomes. *Am J Gastroenterol* 2020; **115**: 916-923 [PMID: 32301761 DOI: 10.14309/ajg.0000000000000664]
10 **Wang W**, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. *JAMA* 2020; **323**: 1843-1844 [PMID: 32159775 DOI: 10.1001/jama.2020.3786]
11 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]
12 **Xiao F**, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for Gastrointestinal Infection of SARS-CoV-2. *Gastroenterology* 2020; **158**: 1831-1833. e3 [PMID: 32142773 DOI: 10.1053/j.gastro.2020.02.055]
13 **Chen L**, Lou J, Bai Y, Wang M. COVID-19 Disease With Positive Fecal and Negative Pharyngeal and Sputum Viral Tests. *Am J Gastroenterol* 2020; **115**: 790 [PMID: 32205644 DOI: 10.14309/ajg.0000000000000610]
14 **Cheung KS**, Hung IFN, Chan PPY, Lung KC, Tso E, Liu R, Ng YY, Chu MY, Chung TWH, Tam AR, Yip CCY, Leung KH, Fung AY, Zhang RR, Lin Y, Cheng HM, Zhang AJX, To KKW, Chan KH, Yuen KY, Leung WK. Gastrointestinal Manifestations of SARS-CoV-2 Infection and Virus Load in Fecal Samples From a Hong Kong Cohort: Systematic Review and Meta-analysis. *Gastroenterology* 2020; **159**: 81-95 [PMID: 32251668 DOI: 10.1053/j.gastro.2020.03.065]
15 **Chen N**, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020; **395**: 507-513 [PMID: 32007143 DOI: 10.1016/S0140-6736(20)30211-7]
16 **Zhou Z**, Zhao N, Shu Y, Han S, Chen B, Shu X. Effect of Gastrointestinal Symptoms in Patients With COVID-19. *Gastroenterology* 2020; **158**: 2294-2297 [PMID: 32199880 DOI: 10.1053/j.gastro.2020.03.020]
17 **Wu Z**, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA* 2020; **323**: 1239-1242 [PMID: 32091533 DOI: 10.1001/jama.2020.2648]
18 **Luo S**, Zhang X, Xu H. Don't Overlook Digestive Symptoms in Patients With 2019 Novel Coronavirus Disease (COVID-19). *Clin Gastroenterol Hepatol* 2020; **18**: 1636-1637 [PMID: 32205220 DOI: 10.1016/j.cgh.2020.03.043]
19 **Mao R**, Qiu Y, He JS, Tan JY, Li XH, Liang J, Shen J, Zhu LR, Chen Y, Iacucci M, Ng SC, Ghosh S, Chen MH. Manifestations and prognosis of gastrointestinal and liver involvement in patients with COVID-19: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* 2020; **5**: 667-678 [PMID: 32405603 DOI: 10.1016/S2468-1253(20)30126-6]
20 **Holshue ML**, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, Spitters C, Ericson K, Wilkerson S, Tural A, Diaz G, Cohn A, Fox L, Patel A, Gerber SI, Kim L, Tong S, Lu X, Lindstrom S, Pallansch MA, Weldon WC, Biggs HM, Uyeki TM, Pillai SK; Washington State 2019-nCoV Case Investigation Team. First Case of 2019 Novel Coronavirus in the United States. *N Engl J Med* 2020; **382**: 929-936 [PMID: 32004427 DOI: 10.1056/NEJMoa2001191]
21 **Zhang G**, Hu C, Luo L, Fang F, Chen Y, Li J, Peng Z, Pan H. Clinical features and short-term outcomes of 221 patients with COVID-19 in Wuhan, China. *J Clin Virol* 2020; **127**: 104364 [PMID: 32311650 DOI: 10.1016/j.jcv.2020.104364]
22 **Wang D**, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y,

Wang X, Peng Z. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 2020; **323**: 1061-1069 [PMID: 32031570 DOI: 10.1001/jama.2020.1585]

23 **Liu K**, Fang YY, Deng Y, Liu W, Wang MF, Ma JP, Xiao W, Wang YN, Zhong MH, Li CH, Li GC, Liu HG. Clinical characteristics of novel coronavirus cases in tertiary hospitals in Hubei Province. *Chin Med J (Engl)* 2020; **133**: 1025-1031 [PMID: 32044814 DOI: 10.1097/CM9.0000000000000744]

24 **Redd WD**, Zhou JC, Hathorn KE, McCarty TR, Bazarbashi AN, Thompson CC, Shen L, Chan WW. Prevalence and Characteristics of Gastrointestinal Symptoms in Patients With Severe Acute Respiratory Syndrome Coronavirus 2 Infection in the United States: A Multicenter Cohort Study. *Gastroenterology* 2020; **159**: 765-767. e2 [PMID: 32333911 DOI: 10.1053/j.gastro.2020.04.045]

25 **An P**, Chen H, Jiang X, Su J, Xiao Y, Ding Y, Ren H, Ji M, Feiyi Chen, Lv X, Chen M, Li J, Yin A, Kang J, Chen W, Liu S, Tan W, Wu L, Dong W, Cao J, Zhou Z, Shen L, Tan S, Chen G, Zhou J, Yang Y, Yu H. Clinical Features of 2019 Novel Coronavirus Pneumonia Presented Gastrointestinal Symptoms But Without Fever Onset (2/2/2020). *Lancet* 2020 [DOI: 10.2139/ssrn.3532530]

26 **Li MY**, Li L, Zhang Y, Wang XS. Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. *Infect Dis Poverty* 2020; **9**: 45 [PMID: 32345362 DOI: 10.1186/s40249-020-00662-x]

27 **Pan L**, Mu M, Yang P, Sun Y, Wang R, Yan J, Li P, Hu B, Wang J, Hu C, Jin Y, Niu X, Ping R, Du Y, Li T, Xu G, Hu Q, Tu L. Clinical Characteristics of COVID-19 Patients With Digestive Symptoms in Hubei, China: A Descriptive, Cross-Sectional, Multicenter Study. *Am J Gastroenterol* 2020; **115**: 766-773 [PMID: 32287140 DOI: 10.14309/ajg.0000000000000620]

28 **Liu Q**, Wang RS, Qu GQ, Wang YY, Liu P, Zhu YZ, Fei G, Ren L, Zhou YW, Liu L. Gross examination report of a COVID-19 death autopsy. *Fa Yi Xue Za Zhi* 2020; **36**: 21-23 [PMID: 32198987 DOI: 10.12116/j.issn.1004-5619.2020.01.005]

29 **Hikmet F**, Méar L, Edvinsson Å, Micke P, Uhlén M, Lindskog C. The protein expression profile of ACE2 in human tissues. *Mol Syst Biol* 2020; **16**: e9610 [PMID: 32715618 DOI: 10.15252/msb.20209610]

30 **Xu J**, Chu M, Zhong F, Tan X, Tang G, Mai J, Lai N, Guan C, Liang Y, Liao G. Digestive symptoms of COVID-19 and expression of ACE2 in digestive tract organs. *Cell Death Discov* 2020; **6**: 76 [PMID: 32818075 DOI: 10.1038/s41420-020-00307-w]

31 **Chen L**, Marishta A, Ellison CE, Verzi MP. Identification of Transcription Factors Regulating SARS-CoV-2 Entry Genes in the Intestine. *Cell Mol Gastroenterol Hepatol* 2021; **11**: 181-184 [PMID: 32810597 DOI: 10.1016/j.jcmgh.2020.08.005]

32 **Wang C**, Wang S, Li D, Wei DQ, Zhao J, Wang J. Human Intestinal Defensin 5 Inhibits SARS-CoV-2 Invasion by Cloaking ACE2. *Gastroenterology* 2020; **159**: 1145-1147. e4 [PMID: 32437749 DOI: 10.1053/j.gastro.2020.05.015]

33 **Chan JF**, Kok KH, Zhu Z, Chu H, To KK, Yuan S, Yuen KY. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. *Emerg Microbes Infect* 2020; **9**: 221-236 [PMID: 31987001 DOI: 10.1080/22221751.2020.1719902]

34 **Tortorici MA**, Veesler D. Structural insights into coronavirus entry. *Adv Virus Res* 2019; **105**: 93-116 [PMID: 31522710 DOI: 10.1016/bs.aivir.2019.08.002]

35 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280. e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]

36 **Li W**, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003; **426**: 450-454 [PMID: 14647384 DOI: 10.1038/nature02145]

37 **Chourasia M**, Koppula PR, Battu A, Ouseph MM, Singh AK. EGCG, a Green Tea Catechin, as a Potential Therapeutic Agent for Symptomatic and Asymptomatic SARS-CoV-2 Infection. *Molecules* 2021; **26** [PMID: 33668085 DOI: 10.3390/molecules26051200]

38 **Henss L**, Auste A, Schürmann C, Schmidt C, von Rhein C, Mühlebach MD, Schnierle BS. The green tea catechin epigallocatechin gallate inhibits SARS-CoV-2 infection. *J Gen Virol* 2021; **102** [PMID: 33830908 DOI: 10.1099/jgv.0.001574]

39 **Qian Q**, Fan L, Liu W, Li J, Yue J, Wang M, Ke X, Yin Y, Chen Q, Jiang C. Direct evidence of active SARS-CoV-2 replication in the intestine. *Clin Infect Dis* 2020 [PMID: 32638022 DOI: 10.1093/cid/ciaa925]

40 **Ward SG**, Westwick J. Chemokines: understanding their role in T-lymphocyte biology. *Biochem J* 1998; **333** (Pt 3): 457-470 [PMID: 9677302 DOI: 10.1042/bj3330457]

41 **Moore JB**, June CH. Cytokine release syndrome in severe COVID-19. *Science* 2020; **368**: 473-474 [PMID: 32303591 DOI: 10.1126/science.abb8925]

42 **Tisoncik JR**, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the eye of the cytokine storm. *Microbiol Mol Biol Rev* 2012; **76**: 16-32 [PMID: 22390970 DOI: 10.1128/MMBR.05015-11]

43 **Gao Y**, Li T, Han M, Li X, Wu D, Xu Y, Zhu Y, Liu Y, Wang X, Wang L. Diagnostic utility of clinical laboratory data determinations for patients with the severe COVID-19. *J Med Virol* 2020; **92**: 791-796 [PMID: 32181911 DOI: 10.1002/jmv.25770]

44 **Yang X**, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, Zou X, Yuan S, Shang Y. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med* 2020; **8**: 475-481 [PMID: 32105632 DOI: 10.1016/S2213-2600(20)30079-5]

45 **Yao N**, Wang SN, Lian JQ, Sun YT, Zhang GF, Kang WZ, Kang W. [Clinical characteristics and influencing factors of patients with novel coronavirus pneumonia combined with liver injury in Shaanxi region]. *Zhonghua Gan Zang Bing Za Zhi* 2020; **28**: 234-239 [PMID: 32153170 DOI: 10.3760/cma.j.cn501113-20200226-00070]

46 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]

47 **Jin X**, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, Hao SR, Jia HY, Cai H, Zhang XL, Yu GD, Xu KJ, Wang XY, Gu JQ, Zhang SY, Ye CY, Jin CL, Lu YF, Yu X, Yu XP, Huang JR, Xu KL, Ni Q, Yu CB, Zhu B, Li YT, Liu J, Zhao H, Zhang X, Yu L, Guo YZ, Su JW, Tao JJ, Lang GJ, Wu XX, Wu WR, Qv TT, Xiang DR, Yi P, Shi D, Chen Y, Ren Y, Qiu YQ, Li LJ, Sheng J, Yang Y. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. *Gut* 2020; **69**: 1002-1009 [PMID: 32213556 DOI: 10.1136/gutjnl-2020-320926]

48 **Chan KH**, Poon LL, Cheng VC, Guan Y, Hung IF, Kong J, Yam LY, Seto WH, Yuen KY, Peiris JS. Detection of SARS coronavirus in patients with suspected SARS. *Emerg Infect Dis* 2004; **10**: 294-299 [PMID: 15030700 DOI: 10.3201/eid1002.030610]

49 **Xu Z**, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med* 2020; **8**: 420-422 [PMID: 32085846 DOI: 10.1016/S2213-2600(20)30076-X]

50 **Oxley TJ**, Mocco J, Majidi S, Kellner CP, Shoirah H, Singh IP, De Leacy RA, Shigematsu T, Ladner TR, Yaeger KA, Skliut M, Weinberger J, Dangayach NS, Bederson JB, Tuhrim S, Fifi JT. Large-Vessel Stroke as a Presenting Feature of Covid-19 in the Young. *N Engl J Med* 2020; **382**: e60 [PMID: 32343504 DOI: 10.1056/NEJMc2009787]

51 **Zhang L**, Feng X, Zhang D, Jiang C, Mei H, Wang J, Zhang C, Li H, Xia X, Kong S, Liao J, Jia H, Pang X, Song Y, Tian Y, Wang B, Wu C, Yuan H, Zhang Y, Li Y, Sun W, Zhu S, Wang S, Xie Y, Ge S, Zhang L, Hu Y, Xie M. Deep Vein Thrombosis in Hospitalized Patients With COVID-19 in Wuhan, China: Prevalence, Risk Factors, and Outcome. *Circulation* 2020; **142**: 114-128 [PMID: 32421381 DOI: 10.1161/CIRCULATIONAHA.120.046702]

52 **Cao T**, Zhang G, Xie H, Pellegrini E, Li J, Chen X, Pan H. Case Report: The Coronavirus Disease 2019 (COVID-19) Pneumonia With Multiple Thromboembolism. *Front Neurol* 2020; **11**: 625272 [PMID: 33551979 DOI: 10.3389/fneur.2020.625272]

53 **Chen S**, Liu H, Li T, Huang R, Gui R, Zhang J. Correlation analysis of coagulation dysfunction and liver damage in patients with novel coronavirus pneumonia: a single-center, retrospective, observational study. *Ups J Med Sci* 2020; **125**: 293-296 [PMID: 32990149 DOI: 10.1080/03009734.2020.1822960]

54 **Qi X**, Liu Y, Wang J, Fallowfield JA, Li X, Shi J, Pan H, Zou S, Zhang H, Chen Z, Li F, Luo Y, Mei M, Liu H, Wang Z, Li J, Yang H, Xiang H, Liu T, Zheng MH, Liu C, Huang Y, Xu D, Kang N, He Q, Gu Y, Zhang G, Shao C, Liu D, Zhang L, Kawada N, Jiang Z, Wang F, Xiong B, Takehara T, Rockey DC; COVID-Cirrhosis-CHESS Group. Clinical course and risk factors for mortality of COVID-19 patients with pre-existing cirrhosis: a multicentre cohort study. *Gut* 2021; **70**: 433-436 [PMID: 32434831 DOI: 10.1136/gutjnl-2020-321666]

55 **Kim D**, Adeniji N, Latt N, Kumar S, Bloom PP, Aby ES, Perumalswami P, Roytman M, Li M, Vogel AS, Catana AM, Wegermann K, Carr RM, Aloman C, Chen V, Rabiee A, Sadowski B, Nguyen V, Dunn W, Chavin K, Zhou K, Lizaola-Mayo B, Moghe A, Debes J, Lee TH, Branch A, Viveiros K, Chan W, Chascsa D, Kwo P, Dhanasekaran R. Predictors of Outcomes of COVID-19 in Patients with Chronic Liver Disease: US Multi-center Study. *Clin Gastroenterol Hepatol* 2020 [PMID: 32950749 DOI: 10.1016/j.cgh.2020.09.027]

56 **Kuderer NM**, Choueiri TK, Shah DP, Shyr Y, Rubinstein SM, Rivera DR, Shete S, Hsu CY, Desai A, de Lima Lopes G Jr, Grivas P, Painter CA, Peters S, Thompson MA, Bakouny Z, Batist G, Bekaii-Saab T, Bilen MA, Bouganim N, Larroya MB, Castellano D, Del Prete SA, Doroshow DB, Egan PC, Elkrief A, Farmakiotis D, Flora D, Galsky MD, Glover MJ, Griffiths EA, Gulati AP, Gupta S, Hafez N, Halfdanarson TR, Hawley JE, Hsu E, Kasi A, Khaki AR, Lemmon CA, Lewis C, Logan B, Masters T, McKay RR, Mesa RA, Morgans AK, Mulcahy MF, Panagiotou OA, Peddi P, Pennell NA, Reynolds K, Rosen LR, Rosovsky R, Salazar M, Schmidt A, Shah SA, Shaya JA, Steinharter J, Stockerl-Goldstein KE, Subbiah S, Vinh DC, Wehbe FH, Weissmann LB, Wu JT, Wulff-Burchfield E, Xie Z, Yeh A, Yu PP, Zhou AY, Zubiri L, Mishra S, Lyman GH, Rini BI, Warner JL; COVID-19 and Cancer Consortium. Clinical impact of COVID-19 on patients with cancer (CCC19): a cohort study. *Lancet* 2020; **395**: 1907-1918 [PMID: 32473681 DOI: 10.1016/S0140-6736(20)31187-9]

57 **Singh S**, Khan A. Clinical Characteristics and Outcomes of Coronavirus Disease 2019 Among Patients With Preexisting Liver Disease in the United States: A Multicenter Research Network Study. *Gastroenterology* 2020; **159**: 768-771. e3 [PMID: 32376408 DOI: 10.1053/j.gastro.2020.04.064]

58 **Bajaj JS**, Garcia-Tsao G, Biggins SW, Kamath PS, Wong F, McGeorge S, Shaw J, Pearson M,

Chew M, Fagan A, de la Rosa Rodriguez R, Worthington J, Olofson A, Weir V, Trisolini C, Dwyer S, Reddy KR. Comparison of mortality risk in patients with cirrhosis and COVID-19 compared with patients with cirrhosis alone and COVID-19 alone: multicentre matched cohort. *Gut* 2021; **70**: 531-536 [PMID: 32660964 DOI: 10.1136/gutjnl-2020-322118]

59 **Marjot T**, Moon AM, Cook JA, Abd-Elsalam S, Aloman C, Armstrong MJ, Pose E, Brenner EJ, Cargill T, Catana MA, Dhanasekaran R, Eshraghian A, García-Juárez I, Gill US, Jones PD, Kennedy J, Marshall A, Matthews C, Mells G, Mercer C, Perumalswami PV, Avitabile E, Qi X, Su F, Ufere NN, Wong YJ, Zheng MH, Barnes E, Barritt AS 4th, Webb GJ. Outcomes following SARS-CoV-2 infection in patients with chronic liver disease: An international registry study. *J Hepatol* 2021; **74**: 567-577 [PMID: 33035628 DOI: 10.1016/j.jhep.2020.09.024]

60 **Moon AM**, Webb GJ, Aloman C, Armstrong MJ, Cargill T, Dhanasekaran R, Genescà J, Gill US, James TW, Jones PD, Marshall A, Mells G, Perumalswami PV, Qi X, Su F, Ufere NN, Barnes E, Barritt AS, Marjot T. High mortality rates for SARS-CoV-2 infection in patients with pre-existing chronic liver disease and cirrhosis: Preliminary results from an international registry. *J Hepatol* 2020; **73**: 705-708 [PMID: 32446714 DOI: 10.1016/j.jhep.2020.05.013]

61 **Albillos A**, Lario M, Álvarez-Mon M. Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance. *J Hepatol* 2014; **61**: 1385-1396 [PMID: 25135860 DOI: 10.1016/j.jhep.2014.08.010]

62 **Mehta P**, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ; HLH Across Speciality Collaboration, UK. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet* 2020; **395**: 1033-1034 [PMID: 32192578 DOI: 10.1016/S0140-6736(20)30628-0]

63 **Rubin DT**, Feuerstein JD, Wang AY, Cohen RD. AGA Clinical Practice Update on Management of Inflammatory Bowel Disease During the COVID-19 Pandemic: Expert Commentary. *Gastroenterology* 2020; **159**: 350-357 [PMID: 32283100 DOI: 10.1053/j.gastro.2020.04.012]

64 **Aziz M**, Fatima R, Haghbin H, Lee-Smith W, Nawras A. The Incidence and Outcomes of COVID-19 in IBD Patients: A Rapid Review and Meta-analysis. *Inflamm Bowel Dis* 2020; **26**: e132-e133 [PMID: 32619003 DOI: 10.1093/ibd/izaa170]

65 **Maconi G**, Bosetti C, De Monti A, Boyapati RK, Shelton E, Piazza N, Carvalhas Gabrielli AM, Lenti MV, Bezzio C, Ricci C, Greco S, Romeo S, Giangregorio F, Gridavilla D, Tagliani F, Massari A, Pastorelli L, Di Sabatino A, Saibeni S, Alicante S, Ferretti F, Rizzardini G, Galli M, Ardizzone S. Risk of COVID 19 in patients with inflammatory bowel diseases compared to a control population. *Dig Liver Dis* 2021; **53**: 263-270 [PMID: 33483259 DOI: 10.1016/j.dld.2020.12.013]

66 **Rizzello F**, Calabrese C, Salice M, Calandrini L, Privitera H, Melotti L, Peruzzi G, Dussias N, Belluzzi A, Scaioli E, Decorato A, Siniscalchi A, Filippone E, Laureti S, Rottoli M, Poggioli G, Gionchetti P. COVID-19 in IBD: The experience of a single tertiary IBD center. *Dig Liver Dis* 2021; **53**: 271-276 [PMID: 33451910 DOI: 10.1016/j.dld.2020.12.012]

67 **Singh S**, Khan A, Chowdhry M, Bilal M, Kochhar GS, Clarke K. Risk of Severe Coronavirus Disease 2019 in Patients With Inflammatory Bowel Disease in the United States: A Multicenter Research Network Study. *Gastroenterology* 2020; **159**: 1575-1578. e4 [PMID: 32522507 DOI: 10.1053/j.gastro.2020.06.003]

68 **Brenner EJ**, Ungaro RC, Gearry RB, Kaplan GG, Kissous-Hunt M, Lewis JD, Ng SC, Rahier JF, Reinisch W, Ruemmele FM, Steinwurz F, Underwood FE, Zhang X, Colombel JF, Kappelman MD. Corticosteroids, But Not TNF Antagonists, Are Associated With Adverse COVID-19 Outcomes in Patients With Inflammatory Bowel Diseases: Results From an International Registry. *Gastroenterology* 2020; **159**: 481-491. e3 [PMID: 32425234 DOI: 10.1053/j.gastro.2020.05.032]

69 **Ungaro RC**, Brenner EJ, Gearry RB, Kaplan GG, Kissous-Hunt M, Lewis JD, Ng SC, Rahier JF, Reinisch W, Steinwurz F, Underwood FE, Zhang X, Colombel JF, Kappelman MD. Effect of IBD medications on COVID-19 outcomes: results from an international registry. *Gut* 2021; **70**: 725-732 [PMID: 33082265 DOI: 10.1136/gutjnl-2020-322539]

70 **Feldmann M**, Maini RN, Woody JN, Holgate ST, Winter G, Rowland M, Richards D, Hussell T. Trials of anti-tumour necrosis factor therapy for COVID-19 are urgently needed. *Lancet* 2020; **395**: 1407-1409 [PMID: 32278362 DOI: 10.1016/S0140-6736(20)30858-8]

71 **Olendzki BC**, Silverstein TD, Persuitte GM, Ma Y, Baldwin KR, Cave D. An anti-inflammatory diet as treatment for inflammatory bowel disease: a case series report. *Nutr J* 2014; **13**: 5 [PMID: 24428901 DOI: 10.1186/1475-2891-13-5]

72 **Coelho MR**, Romi MD, Ferreira DMTP, Zaltman C, Soares-Mota M. The Use of Curcumin as a Complementary Therapy in Ulcerative Colitis: A Systematic Review of Randomized Controlled Clinical Trials. *Nutrients* 2020; **12** [PMID: 32751776 DOI: 10.3390/nu12082296]

73 **Huang J**, Cheng A, Kumar R, Fang Y, Chen G, Zhu Y, Lin S. Hypoalbuminemia predicts the outcome of COVID-19 independent of age and co-morbidity. *J Med Virol* 2020; **92**: 2152-2158 [PMID: 32406952 DOI: 10.1002/jmv.26003]

74 **Wei C**, Liu Y, Li Y, Zhang Y, Zhong M, Meng X. Evaluation of the nutritional status in patients with COVID-19. *J Clin Biochem Nutr* 2020; **67**: 116-121 [PMID: 33041506 DOI: 10.3164/jcbn.20-91]

75 **Stachowska E**, Folwarski M, Jamioł-Milc D, Maciejewska D, Skonieczna-Żydecka K. Nutritional Support in Coronavirus 2019 Disease. *Medicina (Kaunas)* 2020; **56** [PMID: 32545556 DOI: 10.3390/medicina56060289]

76 **Singer P**, Blaser AR, Berger MM, Alhazzani W, Calder PC, Casaer MP, Hiesmayr M, Mayer K, Montejo JC, Pichard C, Preiser JC, van Zanten ARH, Oczkowski S, Szczeklik W, Bischoff SC.

ESPEN guideline on clinical nutrition in the intensive care unit. *Clin Nutr* 2019; **38**: 48-79 [PMID: 30348463 DOI: 10.1016/j.clnu.2018.08.037]

77 **Khan MA**, Khan ZA, Charles M, Pratap P, Naeem A, Siddiqui Z, Naqvi N, Srivastava S. Cytokine Storm and Mucus Hypersecretion in COVID-19: Review of Mechanisms. *J Inflamm Res* 2021; **14**: 175-189 [PMID: 33519225 DOI: 10.2147/JIR.S271292]

78 **Akhtar S**, Das JK, Ismail T, Wahid M, Saeed W, Bhutta ZA. Nutritional perspectives for the prevention and mitigation of COVID-19. *Nutr Rev* 2021; **79**: 289-300 [PMID: 33570583 DOI: 10.1093/nutrit/nuaa063]

79 **Liu K**, Chen Y, Lin R, Han K. Clinical features of COVID-19 in elderly patients: A comparison with young and middle-aged patients. *J Infect* 2020; **80**: e14-e18 [PMID: 32171866 DOI: 10.1016/j.jinf.2020.03.005]

80 **Recinella G**, Marasco G, Serafini G, Maestri L, Bianchi G, Forti P, Zoli M. Prognostic role of nutritional status in elderly patients hospitalized for COVID-19: a monocentric study. *Aging Clin Exp Res* 2020; **32**: 2695-2701 [PMID: 33034016 DOI: 10.1007/s40520-020-01727-5]

81 **Viana-Llamas MC**, Arroyo-Espliguero R, Silva-Obregón JA, Uribe-Heredia G, Núñez-Gil I, García-Magallón B, Torán-Martínez CG, Castillo-Sandoval A, Díaz-Caraballo E, Rodríguez-Guinea I, Domínguez-López J. Hypoalbuminemia on admission in COVID-19 infection: An early predictor of mortality and adverse events. A retrospective observational study. *Med Clin (Barc)* 2021; **156**: 428-436 [PMID: 33627230 DOI: 10.1016/j.medcli.2020.12.018]

82 **Zhang Y**, Zheng L, Liu L, Zhao M, Xiao J, Zhao Q. Liver impairment in COVID-19 patients: A retrospective analysis of 115 cases from a single centre in Wuhan city, China. *Liver Int* 2020; **40**: 2095-2103 [PMID: 32239796 DOI: 10.1111/liv.14455]

83 **Soni N**. Wonderful albumin? *BMJ* 1995; **310**: 887-888 [PMID: 7677837 DOI: 10.1136/bmj.310.6984.887]

84 **Kim S**, McClave SA, Martindale RG, Miller KR, Hurt RT. Hypoalbuminemia and Clinical Outcomes: What is the Mechanism behind the Relationship? *Am Surg* 2017; 83: 1220-1227 [PMID: 29183523 DOI: 10.1177/000313481708301123]

85 **Margarson MP**, Soni N. Serum albumin: touchstone or totem? *Anaesthesia* 1998; 53: 789-803 [PMID: 9797524 DOI: 10.1046/j.1365-2044.1998.00438.x]

86 **Chen S**, Zhang D, Zheng T, Yu Y, Jiang J. DVT incidence and risk factors in critically ill patients with COVID-19. *J Thromb Thrombolysis* 2021; **51**: 33-39 [PMID: 32607652 DOI: 10.1007/s11239-020-02181-w]

87 **Aziz M**, Fatima R, Lee-Smith W, Assaly R. The association of low serum albumin level with severe COVID-19: a systematic review and meta-analysis. *Crit Care* 2020; **24**: 255 [PMID: 32456658 DOI: 10.1186/s13054-020-02995-3]

88 **Huang W**, Li C, Wang Z, Wang H, Zhou N, Jiang J, Ni L, Zhang XA, Wang DW. Decreased serum albumin level indicates poor prognosis of COVID-19 patients: hepatic injury analysis from 2,623 hospitalized cases. *Sci China Life Sci* 2020; **63**: 1678-1687 [PMID: 32567003 DOI: 10.1007/s11427-020-1733-4]

89 **Chen B**, Gu HQ, Liu Y, Zhang G, Yang H, Hu H, Lu C, Li Y, Wang L, Zhao Y, Pan H. A model to predict the risk of mortality in severely ill COVID-19 patients. *Comput Struct Biotechnol J* 2021; **19**: 1694-1700 [PMID: 33777331 DOI: 10.1016/j.csbj.2021.03.012]

90 **Kheir M**, Saleem F, Wang C, Mann A, Chua J. Higher albumin levels on admission predict better prognosis in patients with confirmed COVID-19. *PLoS One* 2021; **16**: e0248358 [PMID: 33725003 DOI: 10.1371/journal.pone.0248358]

91 **Zheng T**, Liu X, Wei Y, Li X, Zheng B, Gong Q, Dong L, Zhong J. Laboratory Predictors of COVID-19 Mortality: A Retrospective Analysis from Tongji Hospital in Wuhan. *Mediators Inflamm* 2021; **2021**: 6687412 [PMID: 33679237 DOI: 10.1155/2021/6687412]

92 **Gombart AF**, Pierre A, Maggini S. A Review of Micronutrients and the Immune System-Working in Harmony to Reduce the Risk of Infection. *Nutrients* 2020; **12** [PMID: 31963293 DOI: 10.3390/nu12010236]

93 **Elmadfa I**, Meyer AL. The Role of the Status of Selected Micronutrients in Shaping the Immune Function. *Endocr Metab Immune Disord Drug Targets* 2019; **19**: 1100-1115 [PMID: 31142256 DOI: 10.2174/1871530319666190529101816]

94 **Wintergerst ES**, Maggini S, Hornig DH. Immune-enhancing role of vitamin C and zinc and effect on clinical conditions. *Ann Nutr Metab* 2006; **50**: 85-94 [PMID: 16373990 DOI: 10.1159/000090495]

95 **Pizzini A**, Aichner M, Sahanic S, Böhm A, Egger A, Hoermann G, Kurz K, Widmann G, Bellmann-Weiler R, Weiss G, Tancevski I, Sonnweber T, Löffler-Ragg J. Impact of Vitamin D Deficiency on COVID-19-A Prospective Analysis from the CovILD Registry. *Nutrients* 2020; **12** [PMID: 32932831 DOI: 10.3390/nu12092775]

96 **Semba RD**, Tang AM. Micronutrients and the pathogenesis of human immunodeficiency virus infection. *Br J Nutr* 1999; **81**: 181-189 [PMID: 10434844 DOI: 10.1017/s0007114599000379]

97 **Papadimitriou-Olivgeris M**, Gkikopoulos N, Wüst M, Ballif A, Simonin V, Maulini M, Nusbaumer C, Bertaiola Monnerat L, Tschopp J, Kampouri EE, Wilson P, Duplain H. Predictors of mortality of influenza virus infections in a Swiss Hospital during four influenza seasons: Role of quick sequential organ failure assessment. *Eur J Intern Med* 2020; **74**: 86-91 [PMID: 31899057 DOI: 10.1016/j.ejim.2019.12.022]

98 **Goncalves-Mendes N**, Talvas J, Dualé C, Guttmann A, Corbin V, Marceau G, Sapin V, Brachet P,

Evrard B, Laurichesse H, Vasson MP. Impact of Vitamin D Supplementation on Influenza Vaccine Response and Immune Functions in Deficient Elderly Persons: A Randomized Placebo-Controlled Trial. *Front Immunol* 2019; **10**: 65 [PMID: 30800121 DOI: 10.3389/fimmu.2019.00065]

99 **Zhang L**, Liu Y. Potential interventions for novel coronavirus in China: A systematic review. *J Med Virol* 2020; **92**: 479-490 [PMID: 32052466 DOI: 10.1002/jmv.25707]

100 **Im JH**, Je YS, Baek J, Chung MH, Kwon HY, Lee JS. Nutritional status of patients with COVID-19. *Int J Infect Dis* 2020; **100**: 390-393 [PMID: 32795605 DOI: 10.1016/j.ijid.2020.08.018]

101 **Barazzoni R**, Bischoff SC, Breda J, Wickramasinghe K, Krznaric Z, Nitzan D, Pirlich M, Singer P; endorsed by the ESPEN Council. ESPEN expert statements and practical guidance for nutritional management of individuals with SARS-CoV-2 infection. *Clin Nutr* 2020; **39**: 1631-1638 [PMID: 32305181 DOI: 10.1016/j.clnu.2020.03.022]

102 **Butler MJ**, Barrientos RM. The impact of nutrition on COVID-19 susceptibility and long-term consequences. *Brain Behav Immun* 2020; **87**: 53-54 [PMID: 32311498 DOI: 10.1016/j.bbi.2020.04.040]

103 **Iacucci M**, Cannatelli R, Labarile N, Mao R, Panaccione R, Danese S, Kochhar GS, Ghosh S, Shen B. Endoscopy in inflammatory bowel diseases during the COVID-19 pandemic and post-pandemic period. *Lancet Gastroenterol Hepatol* 2020; **5**: 598-606 [PMID: 32305075 DOI: 10.1016/S2468-1253(20)30119-9]

104 **Sultan S**, Lim JK, Altayar O, Davitkov P, Feuerstein JD, Siddique SM, Falck-Ytter Y, El-Serag HB; AGA Institute. AGA Rapid Recommendations for Gastrointestinal Procedures During the COVID-19 Pandemic. *Gastroenterology* 2020; **159**: 739-758. e4 [PMID: 32247018 DOI: 10.1053/j.gastro.2020.03.072]

105 **Zhou F**, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* 2020; **395**: 1054-1062 [PMID: 32171076 DOI: 10.1016/S0140-6736(20)30566-3]

106 **Lin L**, Jiang X, Zhang Z, Huang S, Fang Z, Gu Z, Gao L, Shi H, Mai L, Liu Y, Lin X, Lai R, Yan Z, Li X, Shan H. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. *Gut* 2020; **69**: 997-1001 [PMID: 32241899 DOI: 10.1136/gutjnl-2020-321013]

107 **Zhang JJ**, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, Akdis CA, Gao YD. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy* 2020; **75**: 1730-1741 [PMID: 32077115 DOI: 10.1111/all.14238]

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REVIEW

Neuropilin-1: A feasible link between liver pathologies and COVID-19

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Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has a tremendous impact on the health of millions of people worldwide. Unfortunately, those suffering from previous pathological conditions are more vulnerable and tend to develop more severe disease upon infection with the new SARS-CoV-2. This coronavirus interacts with the angiotensin-converting enzyme 2 receptor to invade the cells. Recently, another receptor, neuropilin-1 (NRP-1), has been reported to amplify the viral infection. Interestingly, NRP-1 is expressed in nonparenchymal liver cells and is related to and upregulated in a wide variety of liver-related pathologies. It has been observed that SARS-CoV-2 infection promotes liver injury through several pathways that may be influenced by the previous pathological status of the patient and liver expression of NRP-1. Moreover, coronavirus disease 2019 causes an inflammatory cascade called cytokine storm in patients with severe disease. This cytokine storm may influence liver sinusoidal-cell phenotype, facilitating viral invasion. In this review, the shreds of evidence linking NRP-1 with liver pathologies such as hepatocellular carcinoma, liver fibrosis, nonalcoholic fatty liver disease and inflammatory disorders are discussed in the context of SARS-CoV-2 infection. In addition, the involvement of the infection-related cytokine storm in NRP-1 overexpression and the subsequent increased risk of SARS-CoV-2 infection are also analyzed. This review aims to shed some light on the involvement of liver NRP-1 during SARS-CoV-2 infection and emphasizes the possible involvement this receptor with the observed liver damage.

Key Words: Liver; Liver sinusoidal endothelial cells; Hepatic stellate cells; SARS-CoV-2; COVID-19; Pathology

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Core Tip: Severe acute respiratory syndrome coronavirus 2 uses angiotensin-converting enzyme 2 and neuropilin-1 (NRP-1) receptors to infect cells. NRP-1 expression is upregulated in several liver pathologies, which may facilitate viral infection. Moreover, the cytokine storm might increase liver permeability and NRP-1 expression, giving rise to an increased severity of infection and a worse prognosis.

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INTRODUCTION

The identification of a new coronavirus from patients suffering from an outbreak of pneumonia of unknown origin in the city of Wuhan, China, in December 2019[1], alarmed the scientific and medical community. This coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), spread all over the globe, becoming a public health emergency and a pandemic that has paralyzed the world for a year. Moreover, the high incidence of newly infected individuals has pushed health systems worldwide to the limit, with a continuous influx of patients requiring hospitalization because of complications related to this new coronavirus. After the chaotic initial months of the pandemic, an increasing number of studies have shown that the severity of SARS-CoV-2 is directly related to the health status of the infected individuals[2] and also with gender[3]. Severe complications and an increased risk of mortality have been linked with various pathologies[2,4] including obesity, diabetes, lung disease, and hypertension[2,4-7]. SARS-CoV-2 invades the mucosal cells of the host, mainly through the nose and mouth. Once in the body, SARS-CoV-2 infects the epithelial cells of the nasal cavity using a specific receptor present in those cells[8]. In detail, angiotensin-converting enzyme 2 (ACE2) protein expressed in the membrane of the epithelial cell surface serves as the entry for the spike protein of the coronavirus, facilitating the infection[2,8]. Once in the cell, SARS-CoV-2 kidnaps the genetic machinery of the host cell to increase its copy number, leading to virus amplification.

Interestingly, there is recent evidence of another SARS-CoV-2 receptor expressed on the surface of host cells during infection, neuropilin-1 (NRP-1)[9,10]. NRP-1 is a non-tyrosine kinase receptor isoform of the NRP protein family, which also includes NRP2. These transmembrane glycoproteins consist of a common short cytoplasmic domain and a large extracellular domain of 840 amino acid residues[11]. NRPs are present in all vertebrates and initially found in neurons, where they function as adhesion molecules[12]. NRPs lack catalytic activity, but they closely interact with the cytosolic adaptor protein synectin or GIPC1[13] to participate in signaling events. NRPs play a critical role during vasculogenesis[14,15] because of their ability to interact with vascular endothelial growth factor (VEGF)[16]. NRP-1 also interacts with transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF)[17] to regulate a broad spectrum of processes under both normal physiological conditions and pathological responses. The findings led to an increasing number of studies of NRPs in health and disease that have shown their involvement in diverse diseases, including pathologic angiogenesis, fibrosis, cirrhosis, and cancer[18-22]. The expression of NRPs is upregulated in those diseases, which suggests that they are potential therapeutic targets.

NRP-1 was initially found in the nervous system, but is expressed in many cell types in tissues of the heart, lung, pancreas, skeletal muscle, and liver. This widespread expression has put NRP-1 under the spotlight because of evidence that it facilitates the entry of SARS-CoV-2 into host cells[9,10] and increases the extent of infection.

The pathologies associated with NRP-1 are common to several organs, and many involve the liver, including fibrosis, cirrhosis, malignancies (hepatocellular carcinoma, liver metastasis, cholangiocarcinoma, and others), and angiogenesis. NRP-1 is expressed in liver-resident cells, especially nonparenchymal cells, such as liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSC). Some NRP-1 ligands are involved in liver pathologies. For example, VEGF mediates the angiogenic

response of LSECs[23] and is associated with metastatic growth[24]. Another NRP-1 ligand, TGF-β, mediates the activation of HSCs during fibrogenesis[25,26], leading to liver fibrosis and extracellular matrix (ECM) remodeling during liver metastasis and the creation of a premetastatic niche[27]. Platelet-derived growth factor (PDGF) is also involved in HSC activation, a required step in the pathogenesis of liver fibrosis, cholangiocarcinoma, liver metastasis, and HCC[28-31]. It is tempting to hypothesize that the increased expression of NRP-1 in the liver under both physiological conditions and in patients with liver diseases modulates coronavirus disease 2019 (COVID-19) infection. This review summarizes the potential implications of liver expression of NRP-1 during SARS-CoV-2 infection and its possible role in COVID-19 disease progres -sion and severity.

NRP-1 AND THE LIVER: FOCUS ON NONPARENCHYMAL CELLS

The liver is a functionally complex organ that not only maintains metabolic homeostasis but also has immune functions, such as the elimination of pathogens. Recently, NRP-1 has been identified as a facilitating receptor for SARS-CoV-2 infection. As it is expressed in some types of liver cells, it may affect the status of liver disease in COVID-19 patients[10]. Liver functions are carried out by a number of different cell populations, including hepatocytes, which make up about 92.5% of the liver volume[32], and nonparenchymal cells that include LSECs, Kupffer cells (KCs) [33], and HSCs[34]. Small, but important percentages of leukocytes, such as natural killer (NK) cells, natural killer T (NKT) cells, myeloid-derived suppressor cells, and T cells[35,36].

NRP-1 expression has been detected in LSECs[37] and HSCs[38] in the adult liver. Although the expression of NRP-1 is weak in HSCs, it increases following activation associated with diseases with various etiologies. NRP-1 expression in HSCs will be discussed in later sections. Bergé *et al*[39] reported that NRP-1 was not expressed in the hepatocytes of healthy adult livers[39]. Aung *et al*[40] reported weak expression in the cytoplasm of adult hepatocytes but no expression in KCs[40]. There is no doubt of the NRP-1 expression in fetal hepatic monocytes observed by Rantakari *et al*[41] and the absence of NRP-1 in adult hepatic macrophages.

Hepatic sinusoidal endothelium is characterized by the presence of fenestrae and the absence of a true basement membrane. This characteristic of LSECs allows direct contact between blood components and other hepatic cell types[42]. In the fetal liver, NRP-1 is expressed in LSECs in close association with plasmalemma vesicle associated protein (also known as PV-1 and MECA32) during biogenesis of the fenestra, an association that is lost in the adult liver[43]. In fetal LSECs, plasmalemma vesicle associated protein forms additional associations with components of the VEGF signaling pathway. Despite the unknown functional implication of this association, mice deficient in this protein present with significant leukocyte infiltration and an evident steatosis[44].

NRP-1 is expressed in HSCs, but its expression is largely confined to LSECs and co-distributed with that of VEGFR in normal liver tissue. NRP-1 regulates the expression of VEGFR2 at both the transcriptional and post-translational levels by the activation of focal adhesion kinase. The activation of NRP-1 in LSECs thus initiates multiple intracellular signal transduction pathways that regulate cell proliferation, survival, and migration, which are essential for angiogenesis[20].

During physiological aging, the expression of NRP-1 increases in LSECs and is associated with factors present in the lumens of the sinusoids. NRP-1 interacts with hypoxic inducing factor (HIF)-2α to suppress anti-thrombotic and anti-inflammatory pathways that are correlated with profibrotic aggregation of macrophages and platelets. The inhibition of NRP-1 or its association with HIF-2α normalizes the profibrotic niche, with restores the regenerative ability of the liver[45]. It is interesting to note that during aging, LSECs undergo a pseudo capillarization associated with a decrease in endocytic capacity and an increase in leukocyte adhesion, with reduced liver perfusion[46]. However, whether there is a direct relationship between increased expression of NRP-1 in LSECs or pseudo capillarization with a decrease in endocytic capacity is unknown at this time.

The presence of multiple ligands for NRP-1 underscores the importance of this receptor in the liver environment[47]. During adulthood, liver vascularization is stimulated by low blood flow associated with an increase in VEGF and the consequent proliferation of LSECs[48]. Under physiological conditions, hepatic angiogenesis that takes place during regeneration contributes to the formation of new functional

sinusoids. As a VEGF coreceptor NRP-1 is the main mediator of angiogenesis[48], although the role of this coreceptor in intrahepatic angiogenesis is currently unclear. NRP-1 may regulate the action of VEGF on vascular permeability, the proliferation of endothelial cells, and leukocyte adhesion. Some studies suggest that NRP-1 acts independently of VEGFR2 or modulates its activity by stimulating cell migration and adhesion, which are essential for the development of an angiogenic response. In addition to its direct action on VEGFR phosphorylation, NRP-1 indirectly stimulates the VEGFR-dependent angiogenic pathway by preventing the binding of VEGF and its decoy receptor, placental growth factor[16,49].

The interaction of NRP-1 with VEGFR and VEGF is dependent on heparin, which can alter VEGF signaling in endothelial cells to either stimulate or inhibit angiogenesis [50]. Heparin is an anticoagulant that is synthesized in the body, eliminated by receptor-mediated endocytosis in LSECs, and accumulates in the liver. The formation of complexes consisting of heparin and other proteins has important clinical implications[51]. Heparin increases the binding of VEGF with the VEGFR/NRP-1[52] complex and the binding of NRP-1 to placental growth factor-2[53,54] by increasing the number of binding sites without affecting the affinity itself.

As mentioned previously, hepatic macrophages in the adult liver do not express NRP-1. However, they do produce a wide range of angiogenic factors that interact with NRP-1, including HIF-2α and VEGF in addition to TGFβ[55], which requires extracellular activation by NRP-1 to increase its affinity for its receptors. The effects of TGFβ on angiogenesis depend on the receptor with which it interacts. Both receptors are expressed in LSECs, suggesting a balancing function in the angiogenic process. The signaling pathways initiated after the interaction of TGFβ with its receptor are highly dependent on the specific microenvironment in the liver at the time. Indeed, signaling *via* TFGβ receptor binding of anaplastic lymphoma kinase promotes angiogenesis; while the interaction with activin receptor-like kinase 5 inhibits angiogenesis. Although this factor has long been considered profibrogenic in cooperation with other NRP-1 ligands such as PDGF-B, its inhibition causes undesired effects. Expression of both NRP-1 and TGF-β is low in quiescent HSCs in the normal liver and both increase immediately upon liver damage[56]. That suggests a complex scenario for NRP-1 as a coreceptor with angiogenic and profibrogenic receptors.

We must not forget the functions of the liver as an immune organ, in which NRP-1 participates. In addition to the liver lymphocyte population, which is selectively enriched with NK and NKT cells, circulating lymphocytes interact closely with LSECs, KCs, and dendritic cells present in liver sinusoids[57]. NRP-1 has been associated with inhibition of immune responses[58], which may also occur in the liver, an organ that is part of the innate immune system.

FEASIBLE INVOLVEMENT OF NRP-1 DURING LIVER PATHOLOGIES AND COVID-19

COVID-19 affects mainly the upper airways. In some cases, involvement of the lower airways, gives rise to pneumonia[59]. Along with the ability to infect other tissues in addition to the airways, the impact of SARS-CoV-2 infection is detectable in various organs even without local viral invasion[59]. Liver injury occurs in a large proportion of COVID-19 patients and is characterized by elevated levels of gamma-glutamyl transferase, alanine aminotransferase, and/or aspartate aminotransferase enzymes; and occasionally, moderate hyperbilirubinemia[60-63]. The persistence and degree of liver damage caused by COVID-19 seem to vary. In most cases, liver function recovers soon after viral infection, but patients with severe disease may develop irreversible hepatic injury[64-66]. In line with that observation, increased severity of SARS-CoV-2 infection is related to reduced hepatic function[62,67]. A recent postmortem analysis found microvesicular steatosis along with lobular and portal activity in a CODIV-19 patient[68].

To date, apart from immune-related inflammation and hypoxia generated by airway malfunction, the mechanisms proposed to explain the reported liver damage were drug toxicity, inflammation, and hypoxia resulting from lung malfunction[60,69]. The expression of ACE2 in cholangiocytes has also been proposed as a possible mechanism of liver injury. NRP-1 expression in LSECs and HSCs may thus act to amplify the liver damage as a consequence of SARS-CoV-2 infection (Figure 1).

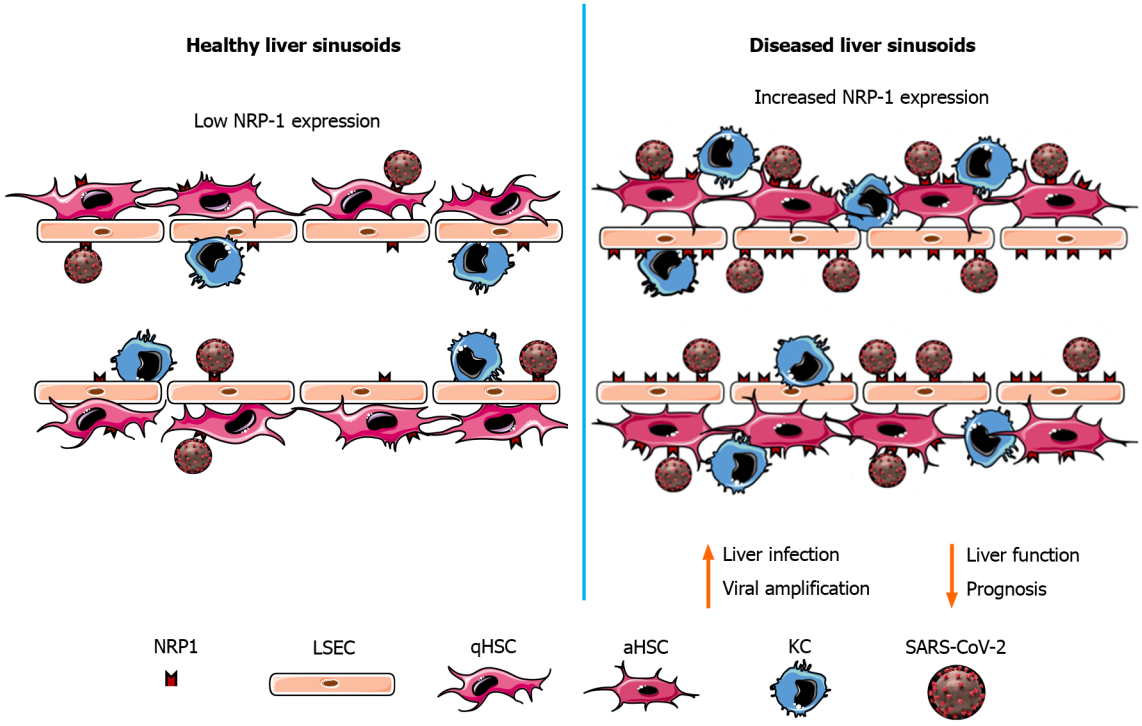


Figure 1 Neuropilin-1 expression in healthy and injured liver. Neuropilin-1 (NRP-1) is expressed at low levels in liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs) in healthy liver sinusoids. However, NRP-1 is upregulated in activated LSECs and HSCs present in several liver pathologies. The increase in NRP-1 during liver disease may facilitate and amplify infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), driving to liver failure and a worse prognosis because of the development of severe COVID-19 disease. SARS-CoV-2 image adapted from NIAID. aHSC: Activated hepatic stellate cell; KC: Kupffer cell; qHSC: Quiescent hepatic stellate cell.

LIVER FIBROTIC RESPONSE

Hepatic fibrosis is a scarring response involving various types of cells affected by liver diseases of different etiologies[70], including metabolic diseases, exacerbated immune responses, and viral infections. Hepatitis C virus (HCV) infection has some similarities to infections of the SARS-CoV-2 virus family[71]. In SARS, the defects observed in the liver are more the result of viral infection than other factors, such as drug toxicity or systemic inflammatory responses[71]. However, the cause of the liver disease observed in a high percentage of COVID-19 patients following SARS-CoV-2 infection is not clear, but steatosis and fibrosis are observed in liver biopsies.

Wang *et al*[72] pointed to direct infection of the liver by SARS-CoV-2 as the cause of the steatosis, lobular inflammation, endothelitis, and fibrosis in patients with COVID-19. Even though the ACE2 receptor has been identified as the main mediator of virus entry[73], the probability that SARS-CoV-2 infects the liver by that route is low given its low or null expression in hepatocytes, HSCs, and LSECs[74]. The development of the fibrotic response in COVID-19 patients may then depend on other routes. It is interesting to note that high levels of other molecules involved in the infectivity of SARS-CoV-2 such as furin, TMPRSS11a, and NRP-1 were detected in infected cells[75] with and without ACE2 receptors. Apart from its involvement in the entry of SARS-CoV-2, the role of NRP-1 as a signaling platform has been established. Cao *et al*[38] found that NRP-1 was a signaling element in HSCs during fibrotic processes caused by viral infections. NRP-1 could thus be the link between infection with SARS-CoV-2 and the development of hepatic steatosis with the presence of a fibrotic response.

HSCs play a central role during liver fibrosis. Following activation, HSCs undergo a change from a quiescent phenotype to a myofibroblastic phenotype characterized by increased proliferation, motility, and accumulation of extracellular matrix. They also contribute to angiogenesis and vascular remodeling processes[70]. HSC activation is initiated by the binding of PDGF-B to its receptor along with a temporal increase in NRP-1. NRP-1 also promotes signaling *via* other key growth factors involved the development of liver fibrosis, such as TGF-β and VEGF[38]. The overexpression of NRP-1 in patients with cirrhosis caused by HCV infection contributes to the progression of liver fibrosis either by influencing the angiogenic response or effects on the PDGF and TGF-β signaling pathways. NRP-1 regulates not only the motility of

HSCs but also collagen deposition, and the severity of fibrosis associated with steatohepatitis and HCV infection are related to the expression of NRP-1. To date, no studies have linked NRP-1 expression with liver involvement in COVID-19 patients. However, it is tempting to hypothesize that the fibrotic response is related to the expression of NRP-1 as an extracellular coreceptor. In other scenarios, such as acute lung SARS or Middle East respiratory syndrome (MERS) infection, cell entry is mediated by TGF-β [76], a mechanism possibly relevant to SARS-CoV-2 and its coreceptor, NRP-1. In the acute phase of COVID-19, the levels of TGF-β are directly related to the development of pulmonary fibrosis[77] and liver fibrosis in pathologies derived from different etiologies[78]. Currently, no studies have found a similar relationship of TGF-β to the fibrotic processes observed in the livers in patients with COVID-19.

Additionally, the mechanical ventilation required by many COVID-19 patients alters hepatic hemodynamics, with reduced portal flow that can result in acute liver damage and activation of HSCs[79]. Although it has not been possible to relate the presence of metabolic fatty liver diseases with the increased risk of hospitalization, nor to the outcomes of hospitalized patients with both diseases, Campos-Murguía *et al*[80] observed an increased risk of the need for mechanical ventilation with the development of liver fibrosis, among other symptoms. However, other studies observed an increase in the severity of the disease in the presence of a fibrotic development. Even today, it is not clear whether SARS-CoV-2 is solely responsible for the development of liver damage or if the damage is a consequence of systemic inflammation caused by the virus or its treatment[81]. Regardless of the cause of liver damage, conditions such as hypoxia, inflammation, and fibrotic responses that develop as a result of the viral infection are related to elevated levels of NRP-1 in both HSCs and LSECs. Indeed, in hypoxic states, such as those observed during COVID-19 progression, HSCs respond through an increase in VEGF. As a result, there is a concomitant increase in the expression of NRP-1 in LSECs, HSC motility, and TGF-β-dependent collagen production[47]. In the presence of TGF-β the glycome of activated HSCs favors the binding of galectins to NRP-1, which promotes migration and further activation[19,82]. It is interesting to note that the levels of both galectin-1 and -3 are increased in COVID-19 patients and have been significantly associated with the severity of the disease[83].

In addition to all the above, the activation of HSCs, which drives liver fibrosis, is induced by profibrotic and proinflammatory cytokines. The resulting inflammatory environment during the development and progression of COVID-19 may be one of the causes of the reported liver damage and a cause of HSC activation, with the consequent induction of the fibrotic response. In fact, during the development of liver fibrosis, infiltration of a subgroup of macrophages enriched in genes associated with tissue repair has been observed[84,85]. The genes encode coreceptors of NRP-1 and the inflammatory cytokines that regulate its expression[86]. One of those cytokines is IL-6, which results from activation of the immune system by SARS-CoV-2 in COVID-19 patients and is associated with altered levels of liver enzymes[87]. In various viral infections, IL-6 is associated with the development of liver fibrosis[88] and with an increase in the expression of NRP-1[89]. In addition, the level of galectin-3, which binds to NRP-1 and has a structure similar to a part of the SARS-CoV-2 spike protein [90], leads to dysregulated expression of proinflammatory cytokines[83,91]. Its expression is increased secondary to diverse types of injury mediated by viral diseases including hepatitis B, hepatitis C, and, SARS-CoV-2[92]. The findings suggest a nexus between systemic inflammation and subsequent liver fibrosis in patients with COVID-19 through NRP-1.

LIVER CANCER

Hepatocellular carcinoma

Viral infection of the liver is the leading cause of hepatocellular carcinoma (HCC) worldwide[93]. Changes in signaling pathways during viral infection promote inflammation that contributes to the development and progression of chronic liver disease beginning with hepatic cirrhosis and finally, HCC[94]. Interestingly, HCV elimination improves the overall survival of HCC patients, indicating that viral infections complicate the outcome[95]. Similarly, SARS-CoV-2 viral infection may facilitate HCC or complicate the outcome of the disease. In the context of the SARS-CoV-2 pandemic, several reports have linked the presence of cancer with an increase of a worse outcome in COVID-19 patients[96,97]. Furthermore, a prospective nationwide cohort study carried out in China revealed that 1% of COVID-19 patients had a history of cancer.

Those patients had an increased risk of complications, ICU admission, and a fatal outcome compared with patients without cancer. Interestingly, cancer survivors had more likely to suffer disease complications than healthy people but less likely than cancer patients[98]. Recent studies describe a possible link between SARS-CoV-2 receptor ACE2 in cancer tissues and increased risk of infection[99]. In liver cancer, Dai *et al*[100] found that upregulated expression of ACE2, the primary SARS-CoV-2 ligand in infected cells, was related to improved survival in HCC patients. However, an increase in ACE2 expression might make such patients more vulnerable to SARS-CoV-2 infection. The link between NRP-1 and liver cancer was discovered some years ago, with increased NRP-1 expression in hepatocellular carcinoma[39]. NRP-1 expression was higher in LSECs from HCC biopsies than from healthy liver biopsies. Hepatocyte expression of NRP-1 correlated with primary HCC and increased with tumor progression. Blocking NRP-1 protein led to impaired tumor growth and vascular remodeling[39]. Interestingly, NRP-1 expression stimulated the activation of HSCs [101], liver-resident myofibroblast-like cells that contribute to the malignant growth of liver metastasis[23,24,102]. The NRP-1-dependent activation boosted tumor proliferation, cell migration, and invasiveness[101]. Therefore, it is tempting to hypothesize that HCC patients with increased NRP-1 expression in LSECs, tumor cells, and HSCs may have an increased risk of SARS-CoV-2 infection.

Cholangiocarcinoma

Cholangiocarcinoma (CCA) is a relatively uncommon liver malignancy, accounting for about 10% of liver cancers[103]. The risk of CCA appears to be increased by both HCV and hepatitis B virus (HBV) infection[104], which may also account for increased SARS-CoV-2 risk. Little is known about the involvement of NRP-1 in CCA. There is evidence that NRP-1 expression is elevated in intrahepatic CCA tissue compared with normal biliary tissue. Moreover, the association of NRP-1 and CCA development has been confirmed by NRP-1 knockdown leading to impaired cancer cell proliferation, blocked cell cycle, reduced cell migration, and reduced focal adhesion kinase expression[105]. In line with that finding, NRP-1 overexpression in intrahepatic CCA cells that was associated with miR-320a downregulation boosted cell proliferation and epithelial to mesenchymal transition and stimulated tumor angiogenesis[106]. Recently, high NRP-1 expression was correlated with poor prognosis and reduced overall survival of intrahepatic CCA patients[107]. Intrahepatic CCA may favor development of an inflammatory milieu driving to vascular permeabilization and increased expression of NRP-1 upon activation of liver-resident cells. The inflammation may be mediated by IL-13, which significantly increased in CCA patients and is known to promote the expression of adhesion molecules in endothelial cells[108,109]. Therefore, CCA could indirectly promote NRP-1 expression in both LSECs and HSCs and facilitate liver damage by immune infiltration. Based on the data, SARS-CoV-2 infection may be facilitated by CCA through NRP-1 overexpression. HCV, HBV, or SARS-CoV-2 infection could drive to liver inflammation and LSEC and HSC activation. The process would lead to increased recruitment of inflammatory cells, NRP-1 overexpression, and assisted viral infection accompanied by liver injury.

CYTOKINE STORM MAY INCREASE LIVER DAMAGE AND NRP-1 EXPRES-SION

Cytokine storm is a potentially fatal consequence of SARS-CoV-2 infection. The release of inflammatory cytokines into the blood of infected individuals is a major turning point in the prognosis and survival of patients with severe disease[110]. The liver filters and detoxifies the blood supply coming from the portal vein and hepatic artery. During COVID-19, both amplifying viruses and cytokines released during the cytokine storm enter the liver and flow through the liver sinusoids and small diameter capillaries in contact with nonparenchymal liver cells, LSECs, KCs, and HSCs[51]. These small vessels have specific properties adapted for their function, such as fenestrations in the endothelial layer, tightly controlled immune tolerance, close contact with nonparenchymal-cell subsets and hepatocytes, and a wide array of cell adhesion molecules and receptors[111,112].

As mentioned previously, the cytokine storm is induced by the release of inflammatory mediators such as IL-6, IL-1β, IL-10, TNF-α, interferon-γ, macrophage inflammatory protein (MIP) 1α and 1β, and VEGF[67,113] and complicates the course of the disease, driving to multiorgan failure and coagulation. The role of these inflammatory mediators in the recruitment of immune and nonimmune cells into the infectious foci,

mainly the lungs, is widely recognized and well characterized. However, these soluble proteins have the intrinsic ability to switch on a wide spectrum of cellular responses in both immune cells and nonimmune cells in epithelial, endothelial, and other tissues [114]. Some of the soluble mediators released during the cytokine storm may increase liver permeability. The effects of TNF-α, IL-6, and IL-1β increase the expression of intercellular adhesion molecule 1 (ICAM-1) in endothelial cells[115-117], which may take place during SARS-CoV-2 infection. LSEC ICAM-1 mediates the adhesion and infiltration of immune cells in the liver[118], which may further increase the number of inflammatory cells and increase the extent of liver damage.

VEGF mediates the recruitment of several immune populations[119] and is considered the main stimulus for the proliferation and migration of endothelial cells [120]. It is also a ligand of NRP-1[53]. Interestingly, VEGF acts on endothelial cells not only as a proliferative and promigratory signal, but it can stimulate the expression of NRP-1[121,122], creating a feedback loop. Consequently, VEGF may facilitate SARS-CoV-2 infection in the liver through the upregulation of the local expression of NRP-1, the recently discovered SARS-CoV-2 receptor. It is tempting to hypothesize that this process may take place in other tissues such as the lung epithelium and that the involvement of IL-6 in promoting increased NRP-1 expression might be underestimated. Previous reports have described NRP-1 upregulation in pancreatic cancer[89, 123], which was stimulated by IL-6 and mediated by STAT3 transcription activity[89]. Interestingly, the IL-6/STAT3 pathway plays a significant role during HSC activation [124] and might boost the expression of NRP-1 in these liver-specific cells.

CONCLUSION

The severity of SARS-CoV-2 infection is increased in patients with previous pathologies. A high proportion of COVID-19 patients experience liver damage. NRP-1 is a recently discovered coreceptor for SARS-CoV-2 virus, and is overexpressed in the injured and pathologic liver. Patients with SARS-CoV-2 infection and liver diseases should be followed to monitor the liver response and overall health status. The increased expression in NRP-1 in the pathologic liver of patients suffering from COVID-19 may represent an amplifying pathway to further complicate the infection and worsen the prognosis and severity of the disease. There is evidence of a link between liver NRP-1 and SARS-CoV-2 infection, but further study is needed to determine whether previous liver disease and NRP-1 influence COVID-19 progression, severity and mortality. Even though the presence of liver disease can promote the increased severity of COVID-19 disease, direct infection and liver injury by SARS-COV-2 virus cannot be ruled out. Liver diseases, such as fibrosis and different types of liver cancers, share active mediators that are directly linked to NRP-1, indicating a feasible and direct relationship of NRP-1, liver disease with COVID-19.

REFERENCES

1 **Zhu N**, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, Wang D, Xu W, Wu G, Gao GF, Tan W; China Novel Coronavirus Investigating and Research Team. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med* 2020; **382**: 727-733 [PMID: 31978945 DOI: 10.1056/NEJMoa2001017]

2 **Zhou F**, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* 2020; **395**: 1054-1062 [PMID: 32171076 DOI: 10.1016/S0140-6736(20)30566-3]

3 **Belice T**, Demir I. The gender differences as a risk factor in diabetic patients with COVID-19. *Iran J Microbiol* 2020; **12**: 625-628 [PMID: 33613918 DOI: 10.18502/ijm.v12i6.5038]

4 **Abe T**, Egbuche O, Igwe J, Jegede O, Wagle B, Olanipekun T, Onwuanyi A. Cardiovascular complications in COVID-19 patients with or without diabetes mellitus. *Endocrinol Diabetes Metab* 2020; e00218 [PMID: 33614986 DOI: 10.1002/edm2.218]

5 **Al Heialy S**, Hachim MY, Hachim IY, Bin Naeem K, Hannawi H, Lakshmanan J, Al Salmi I, Hannawi S. Combination of obesity and co-morbidities leads to unfavorable outcomes in COVID-19 patients. *Saudi J Biol Sci* 2021; **28**: 1445-1450 [PMID: 33293887 DOI: 10.1016/j.sjbs.2020.11.081]

6 **Signes-Costa J**, Núñez-Gil IJ, Soriano JB, Arroyo-Espliguero R, Eid CM, Romero R, Uribarri A, Fernández-Rozas I, Aguado MG, Becerra-Muñoz VM, Huang J, Pepe M, Cerrato E, Raposeiras S, Gonzalez A, Franco-Leon F, Wang L, Alfonso E, Ugo F, García-Prieto JF, Feltes G, Abumayyaleh M, Espejo-Paeres C, Jativa J, Masjuan AL, Macaya C, Carbonell Asíns JA, Estrada V; HOPE COVID-19 investigators. Prevalence and 30-Day Mortality in Hospitalized Patients With Covid-19

and Prior Lung Diseases. *Arch Bronconeumol* 2021; **57** Suppl 2: 13-20 [PMID: 33423874 DOI: 10.1016/j.arbres.2020.11.012]

7 **Cai Q**, Huang D, Yu H, Zhu Z, Xia Z, Su Y, Li Z, Zhou G, Gou J, Qu J, Sun Y, Liu Y, He Q, Chen J, Liu L, Xu L. COVID-19: Abnormal liver function tests. *J Hepatol* 2020; **73**: 566-574 [PMID: 32298767 DOI: 10.1016/j.jhep.2020.04.006]

8 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280. e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]

9 **Daly JL**, Simonetti B, Klein K, Chen KE, Williamson MK, Antón-Plágaro C, Shoemark DK, Simón-Gracia L, Bauer M, Hollandi R, Greber UF, Horvath P, Sessions RB, Helenius A, Hiscox JA, Teesalu T, Matthews DA, Davidson AD, Collins BM, Cullen PJ, Yamauchi Y. Neuropilin-1 is a host factor for SARS-CoV-2 infection. *Science* 2020; **370**: 861-865 [PMID: 33082294 DOI: 10.1126/science.abd3072]

10 **Cantuti-Castelvetri L**, Ojha R, Pedro LD, Djannatian M, Franz J, Kuivanen S, van der Meer F, Kallio K, Kaya T, Anastasina M, Smura T, Levanov L, Szirovicza L, Tobi A, Kallio-Kokko H, Österlund P, Joensuu M, Meunier FA, Butcher SJ, Winkler MS, Mollenhauer B, Helenius A, Gokce O, Teesalu T, Hepojoki J, Vapalahti O, Stadelmann C, Balistreri G, Simons M. Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity. *Science* 2020; **370**: 856-860 [PMID: 33082293 DOI: 10.1126/science.abd2985]

11 **Schwarz Q**, Ruhrberg C. Neuropilin, you gotta let me know: should I stay or should I go? *Cell Adh Migr* 2010; **4**: 61-66 [PMID: 20026901 DOI: 10.4161/cam.4.1.10207]

12 **Parker MW**, Guo HF, Li X, Linkugel AD, Vander Kooi CW. Function of members of the neuropilin family as essential pleiotropic cell surface receptors. *Biochemistry* 2012; **51**: 9437-9446 [PMID: 23116416 DOI: 10.1021/bi3012143]

13 **Cerami E**, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2012; **2**: 401-404 [PMID: 22588877 DOI: 10.1158/2159-8290.CD-12-0095]

14 **Kawasaki T**, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, Fujisawa H. A requirement for neuropilin-1 in embryonic vessel formation. *Development* 1999; **126**: 4895-4902 [PMID: 10518505]

15 **Yuan L**, Moyon D, Pardanaud L, Bréant C, Karkkainen MJ, Alitalo K, Eichmann A. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 2002; **129**: 4797-4806 [PMID: 12361971]

16 **Soker S**, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998; **92**: 735-745 [PMID: 9529250 DOI: 10.1016/s0092-8674(00)81402-6]

17 **Kofler N**, Simons M. The expanding role of neuropilin: regulation of transforming growth factor-β and platelet-derived growth factor signaling in the vasculature. *Curr Opin Hematol* 2016; **23**: 260-267 [PMID: 26849476 DOI: 10.1097/MOH.0000000000000233]

18 **Mohseni N**, Roshan R, Naderi S, Behdani M, Kazemi-Lomedasht F. *In vitro* combination therapy of pathologic angiogenesis using anti-vascular endothelial growth factor and anti-neuropilin-1 nanobodies. *Iran J Basic Med Sci* 2020; **23**: 1335-1339 [PMID: 33149867 DOI: 10.22038/ijbms.2020.47782.11000]

19 **Wu MH**, Chen YL, Lee KH, Chang CC, Cheng TM, Wu SY, Tu CC, Tsui WL. Glycosylation-dependent galectin-1/neuropilin-1 interactions promote liver fibrosis through activation of TGF-β- and PDGF-like signals in hepatic stellate cells. *Sci Rep* 2017; **7**: 11006 [PMID: 28887481 DOI: 10.1038/s41598-017-11212-1]

20 **Wang L**, Feng Y, Xie X, Wu H, Su XN, Qi J, Xin W, Gao L, Zhang Y, Shah VH, Zhu Q. Neuropilin-1 aggravates liver cirrhosis by promoting angiogenesis *via* VEGFR2-dependent PI3K/Akt pathway in hepatic sinusoidal endothelial cells. *EBioMedicine* 2019; **43**: 525-536 [PMID: 31060904 DOI: 10.1016/j.ebiom.2019.04.050]

21 **De Vlaeminck Y**, Bonelli S, Awad RM, Dewilde M, Rizzolio S, Lecocq Q, Bolli E, Santos AR, Laoui D, Schoonooghe S, Tamagnone L, Goyvaerts C, Mazzone M, Breckpot K, Van Ginderachter JA. Targeting Neuropilin-1 with Nanobodies Reduces Colorectal Carcinoma Development. *Cancers (Basel)* 2020; **12** [PMID: 33266104 DOI: 10.3390/cancers12123582]

22 **Cao H**, Li Y, Huang L, Bai B, Xu Z. Clinicopathological Significance of Neuropilin 1 Expression in Gastric Cancer: A Meta-Analysis. *Dis Markers* 2020; **2020**: 4763492 [PMID: 33014187 DOI: 10.1155/2020/4763492]

23 **Benedicto A**, Herrero A, Romayor I, Marquez J, Smedsrød B, Olaso E, Arteta B. Liver sinusoidal endothelial cell ICAM-1 mediated tumor/endothelial crosstalk drives the development of liver metastasis by initiating inflammatory and angiogenic responses. *Sci Rep* 2019; **9**: 13111 [PMID: 31511625 DOI: 10.1038/s41598-019-49473-7]

24 **Herrero A**, Benedicto A, Romayor I, Olaso E, Arteta B. Inhibition of COX-2 Impairs Colon Cancer Liver Metastasis through Reduced Stromal Cell Reaction. *Biomol Ther (Seoul)* 2021 [PMID: 33455946 DOI: 10.4062/biomolther.2020.160]

25 **Okuno M**, Sato T, Kitamoto T, Imai S, Kawada N, Suzuki Y, Yoshimura H, Moriwaki H, Onuki K, Masushige S, Muto Y, Friedman SL, Kato S, Kojima S. Increased 9,13-di-cis-retinoic acid in rat hepatic fibrosis: implication for a potential link between retinoid loss and TGF-beta mediated

fibrogenesis in vivo. *J Hepatol* 1999; **30**: 1073-1080 [PMID: 10406186 DOI: 10.1016/s0168-8278(99)80262-1]

26 **Hong J**, Kim S, Lin PC. Interleukin-33 and ST2 Signaling in Tumor Microenvironment. *J Interferon Cytokine Res* 2019; **39**: 61-71 [PMID: 30256696 DOI: 10.1089/jir.2018.0044]

27 **Costa-Silva B**, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, Becker A, Hoshino A, Mark MT, Molina H, Xiang J, Zhang T, Theilen TM, García-Santos G, Williams C, Ararso Y, Huang Y, Rodrigues G, Shen TL, Labori KJ, Lothe IM, Kure EH, Hernandez J, Doussot A, Ebbesen SH, Grandgenett PM, Hollingsworth MA, Jain M, Mallya K, Batra SK, Jarnagin WR, Schwartz RE, Matei I, Peinado H, Stanger BZ, Bromberg J, Lyden D. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol* 2015; **17**: 816-826 [PMID: 25985394 DOI: 10.1038/ncb3169]

28 **Kocabayoglu P**, Lade A, Lee YA, Dragomir AC, Sun X, Fiel MI, Thung S, Aloman C, Soriano P, Hoshida Y, Friedman SL. β-PDGF receptor expressed by hepatic stellate cells regulates fibrosis in murine liver injury, but not carcinogenesis. *J Hepatol* 2015; **63**: 141-147 [PMID: 25678385 DOI: 10.1016/j.jhep.2015.01.036]

29 **Cadamuro M**, Brivio S, Mertens J, Vismara M, Moncsek A, Milani C, Fingas C, Cristina Malerba M, Nardo G, Dall'Olmo L, Milani E, Mariotti V, Stecca T, Massani M, Spirli C, Fiorotto R, Indraccolo S, Strazzabosco M, Fabris L. Platelet-derived growth factor-D enables liver myofibroblasts to promote tumor lymphangiogenesis in cholangiocarcinoma. *J Hepatol* 2019; **70**: 700-709 [PMID: 30553841 DOI: 10.1016/j.jhep.2018.12.004]

30 **Yuge R**, Kitadai Y, Shinagawa K, Onoyama M, Tanaka S, Yasui W, Chayama K. mTOR and PDGF pathway blockade inhibits liver metastasis of colorectal cancer by modulating the tumor microenvironment. *Am J Pathol* 2015; **185**: 399-408 [PMID: 25478811 DOI: 10.1016/j.ajpath.2014.10.014]

31 **Patel S**, Nanavati P, Sharma J, Chavda V, Savjani J. Functional Role of Novel Indomethacin Derivatives for the Treatment of Hepatocellular Carcinoma Through Inhibition of Platelet-Derived Growth Factor. *Arch Med Res* 2021 [PMID: 33551224 DOI: 10.1016/j.arcmed.2021.01.005]

32 **Blouin A**, Bolender RP, Weibel ER. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. *J Cell Biol* 1977; **72**: 441-455 [PMID: 833203 DOI: 10.1083/jcb.72.2.441]

33 **Smedsrød B**, De Bleser PJ, Braet F, Lovisetti P, Vanderkerken K, Wisse E, Geerts A. Cell biology of liver endothelial and Kupffer cells. *Gut* 1994; **35**: 1509-1516 [PMID: 7828963 DOI: 10.1136/gut.35.11.1509]

34 **Friedman SL**. Hepatic stellate cells. *Prog Liver Dis* 1996; **14**: 101-130 [PMID: 9055576]

35 **Freitas-Lopes MA**, Mafra K, David BA, Carvalho-Gontijo R, Menezes GB. Differential Location and Distribution of Hepatic Immune Cells. *Cells* 2017; **6** [PMID: 29215603 DOI: 10.3390/cells6040048]

36 **McNamara HA**, Cockburn IA. The three Rs: Recruitment, Retention and Residence of leukocytes in the liver. *Clin Transl Immunology* 2016; **5**: e123 [PMID: 28435674 DOI: 10.1038/cti.2016.84]

37 **Braet F**, Shleper M, Paizi M, Brodsky S, Kopeiko N, Resnick N, Spira G. Liver sinusoidal endothelial cell modulation upon resection and shear stress in vitro. *Comp Hepatol* 2004; **3**: 7 [PMID: 15341660 DOI: 10.1186/1476-5926-3-7]

38 **Cao S**, Yaqoob U, Das A, Shergill U, Jagavelu K, Huebert RC, Routray C, Abdelmoneim S, Vasdev M, Leof E, Charlton M, Watts RJ, Mukhopadhyay D, Shah VH. Neuropilin-1 promotes cirrhosis of the rodent and human liver by enhancing PDGF/TGF-beta signaling in hepatic stellate cells. *J Clin Invest* 2010; **120**: 2379-2394 [PMID: 20577048 DOI: 10.1172/JCI41203]

39 **Bergé M**, Allanic D, Bonnin P, de Montrion C, Richard J, Suc M, Boivin JF, Contrerès JO, Lockhart BP, Pocard M, Lévy BI, Tucker GC, Tobelem G, Merkulova-Rainon T. Neuropilin-1 is upregulated in hepatocellular carcinoma and contributes to tumour growth and vascular remodelling. *J Hepatol* 2011; **55**: 866-875 [PMID: 21338642 DOI: 10.1016/j.jhep.2011.01.033]

40 **Aung NY**, Ohe R, Meng H, Kabasawa T, Yang S, Kato T, Yamakawa M. Specific Neuropilins Expression in Alveolar Macrophages among Tissue-Specific Macrophages. *PLoS One* 2016; **11**: e0147358 [PMID: 26900851 DOI: 10.1371/journal.pone.0147358]

41 **Rantakari P**, Patten DA, Valtonen J, Karikoski M, Gerke H, Dawes H, Laurila J, Ohlmeier S, Elima K, Hübscher SG, Weston CJ, Jalkanen S, Adams DH, Salmi M, Shetty S. Stabilin-1 expression defines a subset of macrophages that mediate tissue homeostasis and prevent fibrosis in chronic liver injury. *Proc Natl Acad Sci USA* 2016; **113**: 9298-9303 [PMID: 27474165 DOI: 10.1073/pnas.1604780113]

42 **Sørensen KK**, Simon-Santamaria J, McCuskey RS, Smedsrød B. Liver Sinusoidal Endothelial Cells. *Compr Physiol* 2015; **5**: 1751-1774 [PMID: 26426467 DOI: 10.1002/cphy.c140078]

43 **Herrnberger L**, Hennig R, Kremer W, Hellerbrand C, Goepferich A, Kalbitzer HR, Tamm ER. Formation of fenestrae in murine liver sinusoids depends on plasmalemma vesicle-associated protein and is required for lipoprotein passage. *PLoS One* 2014; **9**: e115005 [PMID: 25541982 DOI: 10.1371/journal.pone.0115005]

44 **Auvinen K**, Lokka E, Mokkala E, Jäppinen N, Tyystjärvi S, Saine H, Peurla M, Shetty S, Elima K, Rantakari P, Salmi M. Fenestral diaphragms and PLVAP associations in liver sinusoidal endothelial cells are developmentally regulated. *Sci Rep* 2019; **9**: 15698 [PMID: 31666588 DOI: 10.1038/s41598-019-52068-x]

45 **Chen Y**, Pu Q, Ma Y, Zhang H, Ye T, Zhao C, Huang X, Ren Y, Qiao L, Liu HM, Esmon CT, Ding

BS, Cao Z. Aging Reprograms the Hematopoietic-Vascular Niche to Impede Regeneration and Promote Fibrosis. *Cell Metab* 2021; **33**: 395-410. e4 [PMID: 33357457 DOI: 10.1016/j.cmet.2020.11.019]

46 **LE Couteur DG**, Cogger VC, McCuskey RS, DE Cabo R, Smedsrød B, Sorensen KK, Warren A, Fraser R. Age-related changes in the liver sinusoidal endothelium: a mechanism for dyslipidemia. *Ann N Y Acad Sci* 2007; **1114**: 79-87 [PMID: 17804522 DOI: 10.1196/annals.1396.003]

47 **Elpek GÖ**. Neuropilins and liver. *World J Gastroenterol* 2015; **21**: 7065-7073 [PMID: 26109793 DOI: 10.3748/wjg.v21.i23.7065]

48 **Fleischer JR**, Jodszuweit CA, Ghadimi M, De Oliveira T, Conradi LC. Vascular Heterogeneity With a Special Focus on the Hepatic Microenvironment. *Front Physiol* 2020; **11**: 591901 [PMID: 33262705 DOI: 10.3389/fphys.2020.591901]

49 **Autiero M**, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Shibuya M, Collen D, Conway EM, Carmeliet P. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med* 2003; **9**: 936-943 [PMID: 12796773 DOI: 10.1038/nm884]

50 **Teran M**, Nugent MA. Synergistic Binding of Vascular Endothelial Growth Factor-A and Its Receptors to Heparin Selectively Modulates Complex Affinity. *J Biol Chem* 2015; **290**: 16451-16462 [PMID: 25979342 DOI: 10.1074/jbc.M114.627372]

51 **Oie CI**, Olsen R, Smedsrød B, Hansen JB. Liver sinusoidal endothelial cells are the principal site for elimination of unfractionated heparin from the circulation. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G520-G528 [PMID: 18063704 DOI: 10.1152/ajpgi.00489.2007]

52 **Vander Kooi CW**, Jusino MA, Perman B, Neau DB, Bellamy HD, Leahy DJ. Structural basis for ligand and heparin binding to neuropilin B domains. *Proc Natl Acad Sci USA* 2007; **104**: 6152-6157 [PMID: 17405859 DOI: 10.1073/pnas.0700043104]

53 **Klagsbrun M**, Takashima S, Mamluk R. The role of neuropilin in vascular and tumor biology. *Adv Exp Med Biol* 2002; **515**: 33-48 [PMID: 12613541 DOI: 10.1007/978-1-4615-0119-0_3]

54 **Mamluk R**, Gechtman Z, Kutcher ME, Gasiunas N, Gallagher J, Klagsbrun M. Neuropilin-1 binds vascular endothelial growth factor 165, placenta growth factor-2, and heparin *via* its b1b2 domain. *J Biol Chem* 2002; **277**: 24818-24825 [PMID: 11986311 DOI: 10.1074/jbc.M200730200]

55 **Roth S**, Gong W, Gressner AM. Expression of different isoforms of TGF-beta and the latent TGF-beta binding protein (LTBP) by rat Kupffer cells. *J Hepatol* 1998; **29**: 915-922 [PMID: 9875638 DOI: 10.1016/S0168-8278(98)80119-0]

56 **Dewidar B**, Meyer C, Dooley S, Meindl-Beinker AN. TGF-β in Hepatic Stellate Cell Activation and Liver Fibrogenesis-Updated 2019. *Cells* 2019; **8** [PMID: 31718044 DOI: 10.3390/cells8111419]

57 **Racanelli V**, Rehermann B. The liver as an immunological organ. *Hepatology* 2006; **43**: S54-S62 [PMID: 16447271 DOI: 10.1002/hep.21060]

58 **Prud'homme GJ**, Glinka Y. Neuropilins are multifunctional coreceptors involved in tumor initiation, growth, metastasis and immunity. *Oncotarget* 2012; **3**: 921-939 [PMID: 22948112 DOI: 10.18632/oncotarget.626]

59 **Machhi J**, Herskovitz J, Senan AM, Dutta D, Nath B, Oleynikov MD, Blomberg WR, Meigs DD, Hasan M, Patel M, Kline P, Chang RC, Chang L, Gendelman HE, Kevadiya BD. The Natural History, Pathobiology, and Clinical Manifestations of SARS-CoV-2 Infections. *J Neuroimmune Pharmacol* 2020; **15**: 359-386 [PMID: 32696264 DOI: 10.1007/s11481-020-09944-5]

60 **Xu L**, Liu J, Lu M, Yang D, Zheng X. Liver injury during highly pathogenic human coronavirus infections. *Liver Int* 2020; **40**: 998-1004 [PMID: 32170806 DOI: 10.1111/liv.14435]

61 **Wu Z**, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA* 2020; **323**: 1239-1242 [PMID: 32091533 DOI: 10.1001/jama.2020.2648]

62 **Guan WJ**, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY, Wang JL, Liang ZJ, Peng YX, Wei L, Liu Y, Hu YH, Peng P, Wang JM, Liu JY, Chen Z, Li G, Zheng ZJ, Qiu SQ, Luo J, Ye CJ, Zhu SY, Zhong NS; China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 2020; **382**: 1708-1720 [PMID: 32109013 DOI: 10.1056/NEJMoa2002032]

63 **Yang X**, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, Zou X, Yuan S, Shang Y. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med* 2020; **8**: 475-481 [PMID: 32105632 DOI: 10.1016/S2213-2600(20)30079-5]

64 **Chen N**, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020; **395**: 507-513 [PMID: 32007143 DOI: 10.1016/S0140-6736(20)30211-7]

65 **Zhang C**, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020; **5**: 428-430 [PMID: 32145190 DOI: 10.1016/S2468-1253(20)30057-1]

66 **Sun LJ**, Yu JW, Shi YG, Zhang XY, Shu MN, Chen MY. Hepatitis C virus core protein induces dysfunction of liver sinusoidal endothelial cell by down-regulation of silent information regulator 1.

J Med Virol 2018; **90**: 926-935 [PMID: 29350417 DOI: 10.1002/jmv.25034]

67 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

68 **Xu Z**, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med* 2020; **8**: 420-422 [PMID: 32085846 DOI: 10.1016/S2213-2600(20)30076-X]

69 **Wong SH**, Lui RN, Sung JJ. Covid-19 and the digestive system. *J Gastroenterol Hepatol* 2020; **35**: 744-748 [PMID: 32215956 DOI: 10.1111/jgh.15047]

70 **Friedman SL**. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; **134**: 1655-1669 [PMID: 18471545 DOI: 10.1053/j.gastro.2008.03.003]

71 **Humar A**, McGilvray I, Phillips MJ, Levy GA. Severe acute respiratory syndrome and the liver. *Hepatology* 2004; **39**: 291-294 [PMID: 14767979 DOI: 10.1002/hep.20069]

72 **Wang Y**, Liu S, Liu H, Li W, Lin F, Jiang L, Li X, Xu P, Zhang L, Zhao L, Cao Y, Kang J, Yang J, Li L, Liu X, Li Y, Nie R, Mu J, Lu F, Zhao S, Lu J, Zhao J. SARS-CoV-2 infection of the liver directly contributes to hepatic impairment in patients with COVID-19. *J Hepatol* 2020; **73**: 807-816 [PMID: 32437830 DOI: 10.1016/j.jhep.2020.05.002]

73 **Li W**, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003; **426**: 450-454 [PMID: 14647384 DOI: 10.1038/nature02145]

74 **Pirola CJ**, Sookoian S. SARS-CoV-2 virus and liver expression of host receptors: Putative mechanisms of liver involvement in COVID-19. *Liver Int* 2020; **40**: 2038-2040 [PMID: 32352224 DOI: 10.1111/liv.14500]

75 **Cantuti-Castelvetri L**, Ojha R, Pedro L, Djannatian M, Franz J, Kuivanen S, Kallio K, Kaya T, Anastasina M, Smura T, Levanov L, Szirovicza L, Tobi A, Kallio-Kokko H, Österlund P, Joensuu M, Meunier F, Butcher S, Winkler MS, Mollenhauer B, Helenius A, Gokce O, Teesalu T, Hepojoki J, Vapalahti O, Stadelmann C, Balistreri G, Simons M. Neuropilin-1 facilitates SARS-CoV-2 cell entry and provides a possible pathway into the central nervous system. 2020 Preprint. Available from: *bioRxiv*: 2020.06.07. 137802 [DOI: 10.1101/2020.06.07.137802]

76 **Evans RM**, Lippman SM. Shining Light on the COVID-19 Pandemic: A Vitamin D Receptor Checkpoint in Defense of Unregulated Wound Healing. *Cell Metab* 2020; **32**: 704-709 [PMID: 32941797 DOI: 10.1016/j.cmet.2020.09.007]

77 **Zhao X**, Nicholls JM, Chen YG. Severe acute respiratory syndrome-associated coronavirus nucleocapsid protein interacts with Smad3 and modulates transforming growth factor-beta signaling. *J Biol Chem* 2008; **283**: 3272-3280 [PMID: 18055455 DOI: 10.1074/jbc.M708033200]

78 **Caja L**, Dituri F, Mancarella S, Caballero-Diaz D, Moustakas A, Giannelli G, Fabregat I. TGF-β and the Tissue Microenvironment: Relevance in Fibrosis and Cancer. *Int J Mol Sci* 2018; **19** [PMID: 29701666 DOI: 10.3390/ijms19051294]

79 **Saviano A**, Wrensch F, Ghany MG, Baumert TF. Liver disease and COVID-19: from Pathogenesis to Clinical Care. *Hepatology* 2020 [PMID: 33332624 DOI: 10.1002/hep.31684]

80 **Campos-Murguía A**, Román-Calleja BM, Toledo-Coronado IV, González-Regueiro JA, Solís-Ortega AA, Kúsulas-Delint D, Cruz-Contreras M, Cruz-Yedra N, Cubero FJ, Nevzorova YA, Martínez-Cabrera CF, Moreno-Guillén P, Lozano-Cruz OA, Chapa-Ibargüengoitia M, Gulías-Herrero A, Aguilar-Salinas CA, Ruiz-Margáin A, Macías-Rodríguez RU. Liver fibrosis in patients with metabolic associated fatty liver disease is a risk factor for adverse outcomes in COVID-19. *Dig Liver Dis* 2021 [PMID: 33551355 DOI: 10.1016/j.dld.2021.01.019]

81 **Alqahtani SA**, Schattenberg JM. Liver injury in COVID-19: The current evidence. *United European Gastroenterol J* 2020; **8**: 509-519 [PMID: 32450787 DOI: 10.1177/2050640620924157]

82 **Henderson NC**, Mackinnon AC, Farnworth SL, Poirier F, Russo FP, Iredale JP, Haslett C, Simpson KJ, Sethi T. Galectin-3 regulates myofibroblast activation and hepatic fibrosis. *Proc Natl Acad Sci USA* 2006; **103**: 5060-5065 [PMID: 16549783 DOI: 10.1073/pnas.0511167103]

83 **Kazancioglu S**, Yilmaz FM, Bastug A, Ozbay BO, Aydos O, Yücel Ç, Bodur H, Yilmaz G. Assessment of Galectin-1, Galectin-3, and PGE2 Levels in Patients with COVID-19. *Jpn J Infect Dis* 2021 [PMID: 33790073 DOI: 10.7883/yoken.JJID.2021.020]

84 **Merad M**, Martin JC. Pathological inflammation in patients with COVID-19: a key role for monocytes and macrophages. *Nat Rev Immunol* 2020; **20**: 355-362 [PMID: 32376901 DOI: 10.1038/s41577-020-0331-4]

85 **Tacke F**, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. *J Hepatol* 2014; **60**: 1090-1096 [PMID: 24412603 DOI: 10.1016/j.jhep.2013.12.025]

86 **Levi M**, Nieuwdorp M, van der Poll T, Stroes E. Metabolic modulation of inflammation-induced activation of coagulation. *Semin Thromb Hemost* 2008; **34**: 26-32 [PMID: 18393140 DOI: 10.1055/s-2008-1066020]

87 **Da BL**, Kushner T, El Halabi M, Paka P, Khalid M, Uberoi A, Lee BT, Perumalswami PV, Rutledge SM, Schiano TD, Friedman S, Saberi B. Liver Injury in Hospitalized Patients with COVID-19 Correlates with Hyper Inflammatory Response and Elevated IL-6. *Hepatol Commun* 2020 [PMID: 33230491 DOI: 10.1002/hep4.1631]

88 **Fuster D**, Tsui JI, Cheng DM, Quinn EK, Armah KA, Nunes D, Freiberg MS, Samet JH. Interleukin-6 is associated with noninvasive markers of liver fibrosis in HIV-infected patients with alcohol problems. *AIDS Res Hum Retroviruses* 2013; **29**: 1110-1116 [PMID: 23601055 DOI: 10.1089/AID.2012.0348]

89 **Yang Y**, Yang L, Li Y. Neuropilin-1 (NRP-1) upregulated by IL-6/STAT3 signaling contributes to invasion in pancreatic neuroendocrine neoplasms. *Hum Pathol* 2018; **81**: 192-200 [PMID: 30420046 DOI: 10.1016/j.humpath.2018.06.030]

90 **Caniglia JL**, Guda MR, Asuthkar S, Tsung AJ, Velpula KK. A potential role for Galectin-3 inhibitors in the treatment of COVID-19. *PeerJ* 2020; **8**: e9392 [PMID: 32587806 DOI: 10.7717/peerj.9392]

91 **Colnot C**, Ripoche MA, Milon G, Montagutelli X, Crocker PR, Poirier F. Maintenance of granulocyte numbers during acute peritonitis is defective in galectin-3-null mutant mice. *Immunology* 1998; **94**: 290-296 [PMID: 9767409 DOI: 10.1046/j.1365-2567.1998.00517.x]

92 **Early Breast Cancer Trialists' Collaborative Group (EBCTCG)**, Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, McGale P, Pan HC, Taylor C, Wang YC, Dowsett M, Ingle J, Peto R. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* 2011; **378**: 771-784 [PMID: 21802721 DOI: 10.1016/S0140-6736(11)60993-8]

93 **El-Serag HB**. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012; **142**: 1264-1273. e1 [PMID: 22537432 DOI: 10.1053/j.gastro.2011.12.061]

94 **Wu JW**, Kao JH, Tseng TC. Three Heads are Better than Two: HBcrAg as a New Predictor of HBV-related HCC. *Clin Mol Hepatol* 2021 [PMID: 33618507 DOI: 10.3350/cmh.2021.0012]

95 **Nahon P**, Layese R, Cagnot C, Asselah T, Guyader D, Pol S, Pageaux GP, De Lédinghen V, Ouzan D, Zoulim F, Audureau E; ANRS CO12 CirVir group. HCV Eradication in Primary or Secondary Prevention Optimizes Hepatocellular Carcinoma Curative Management. *Cancer Prev Res (Phila)* 2021; **14**: 581-592 [PMID: 33608313 DOI: 10.1158/1940-6207.CAPR-20-0465]

96 **Deng G**, Yin M, Chen X, Zeng F. Clinical determinants for fatality of 44,672 patients with COVID-19. *Crit Care* 2020; **24**: 179 [PMID: 32345311 DOI: 10.1186/s13054-020-02902-w]

97 **Williamson EJ**, Walker AJ, Bhaskaran K, Bacon S, Bates C, Morton CE, Curtis HJ, Mehrkar A, Evans D, Inglesby P, Cockburn J, McDonald HI, MacKenna B, Tomlinson L, Douglas IJ, Rentsch CT, Mathur R, Wong AYS, Grieve R, Harrison D, Forbes H, Schultze A, Croker R, Parry J, Hester F, Harper S, Perera R, Evans SJW, Smeeth L, Goldacre B. Factors associated with COVID-19-related death using OpenSAFELY. *Nature* 2020; **584**: 430-436 [PMID: 32640463 DOI: 10.1038/s41586-020-2521-4]

98 **Liang W**, Guan W, Chen R, Wang W, Li J, Xu K, Li C, Ai Q, Lu W, Liang H, Li S, He J. Cancer patients in SARS-CoV-2 infection: a nationwide analysis in China. *Lancet Oncol* 2020; **21**: 335-337 [PMID: 32066541 DOI: 10.1016/S1470-2045(20)30096-6]

99 **Hoang T**, Nguyen TQ, Tran TTA. Genetic Susceptibility of ACE2 and TMPRSS2 in Six Common Cancers and Possible Impacts on COVID-19. *Cancer Res Treat* 2020 [PMID: 33421977 DOI: 10.4143/crt.2020.950]

100 **Dai YJ**, Hu F, Li H, Huang HY, Wang DW, Liang Y. A profiling analysis on the receptor ACE2 expression reveals the potential risk of different type of cancers vulnerable to SARS-CoV-2 infection. *Ann Transl Med* 2020; **8**: 481 [PMID: 32395525 DOI: 10.21037/atm.2020.03.61]

101 **Xu ZC**, Shen HX, Chen C, Ma L, Li WZ, Wang L, Geng ZM. Neuropilin-1 promotes primary liver cancer progression by potentiating the activity of hepatic stellate cells. *Oncol Lett* 2018; **15**: 2245-2251 [PMID: 29434931 DOI: 10.3892/ol.2017.7541]

102 **Romayor I**, Badiola I, Benedicto A, Márquez J, Herrero A, Arteta B, Olaso E. Silencing of sinusoidal DDR1 reduces murine liver metastasis by colon carcinoma. *Sci Rep* 2020; **10**: 18398 [PMID: 33110221 DOI: 10.1038/s41598-020-75395-w]

103 **Cardinale V**, Bragazzi MC, Carpino G, Torrice A, Fraveto A, Gentile R, Pasqualino V, Melandro F, Aliberti C, Bastianelli C, Brunelli R, Berloco PB, Gaudio E, Alvaro D. Cholangiocarcinoma: increasing burden of classifications. *Hepatobiliary Surg Nutr* 2013; **2**: 272-280 [PMID: 24570958 DOI: 10.3978/j.issn.2304-3881.2013.10.02]

104 **Tan JH**, Zhou WY, Zhou L, Cao RC, Zhang GW. Viral hepatitis B and C infections increase the risks of intrahepatic and extrahepatic cholangiocarcinoma: Evidence from a systematic review and meta-analysis. *Turk J Gastroenterol* 2020; **31**: 246-256 [PMID: 32343237 DOI: 10.5152/tjg.2020.19056]

105 **Zhu H**, Jiang X, Zhou X, Dong X, Xie K, Yang C, Jiang H, Sun X, Lu J. Neuropilin-1 regulated by miR-320 contributes to the growth and metastasis of cholangiocarcinoma cells. *Liver Int* 2018; **38**: 125-135 [PMID: 28618167 DOI: 10.1111/liv.13495]

106 **Zhu H**, Zhai B, He C, Li Z, Gao H, Niu Z, Jiang X, Lu J, Sun X. LncRNA TTN-AS1 promotes the progression of cholangiocarcinoma *via* the miR-320a/neuropilin-1 axis. *Cell Death Dis* 2020; **11**: 637 [PMID: 32801339 DOI: 10.1038/s41419-020-02896-x]

107 **Wu YN**, He LH, Bai ZT, Li X. NRP1 is a Prognostic Factor and Promotes the Growth and Migration of Cells in Intrahepatic Cholangiocarcinoma. *Cancer Manag Res* 2020; **12**: 7021-7032 [PMID: 32848461 DOI: 10.2147/CMAR.S260091]

108 **Raggi C**, Correnti M, Sica A, Andersen JB, Cardinale V, Alvaro D, Chiorino G, Forti E, Glaser S, Alpini G, Destro A, Sozio F, Di Tommaso L, Roncalli M, Banales JM, Coulouarn C, Bujanda L, Torzilli G, Invernizzi P. Cholangiocarcinoma stem-like subset shapes tumor-initiating niche by

educating associated macrophages. *J Hepatol* 2017; **66**: 102-115 [PMID: 27593106 DOI: 10.1016/j.jhep.2016.08.012]

109 **Bochner BS**, Klunk DA, Sterbinsky SA, Coffman RL, Schleimer RP. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J Immunol* 1995; **154**: 799-803 [PMID: 7529288]

110 **Kovalchuk A**, Wang B, Li D, Rodriguez-Juarez R, Ilnytskyy S, Kovalchuk I, Kovalchuk O. Fighting the storm: could novel anti-TNFα and anti-IL-6 *C. sativa* cultivars tame cytokine storm in COVID-19? *Aging (Albany NY)* 2021; **13**: 1571-1590 [PMID: 33465050 DOI: 10.18632/aging.202500]

111 **Shetty S**, Lalor PF, Adams DH. Liver sinusoidal endothelial cells - gatekeepers of hepatic immunity. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 555-567 [PMID: 29844586 DOI: 10.1038/s41575-018-0020-y]

112 **Knolle PA**, Gerken G. Local control of the immune response in the liver. *Immunol Rev* 2000; **174**: 21-34 [PMID: 10807504 DOI: 10.1034/j.1600-0528.2002.017408.x]

113 **Zhu Z**, Cai T, Fan L, Lou K, Hua X, Huang Z, Gao G. Clinical value of immune-inflammatory parameters to assess the severity of coronavirus disease 2019. *Int J Infect Dis* 2020; **95**: 332-339 [PMID: 32334118 DOI: 10.1016/j.ijid.2020.04.041]

114 **Schumacher N**, Rose-John S. ADAM17 Activity and IL-6 Trans-Signaling in Inflammation and Cancer. *Cancers (Basel)* 2019; **11** [PMID: 31694340 DOI: 10.3390/cancers11111736]

115 **Eaton KV**, Yang HL, Giachelli CM, Scatena M. Engineering macrophages to control the inflammatory response and angiogenesis. *Exp Cell Res* 2015; **339**: 300-309 [PMID: 26610863 DOI: 10.1016/j.yexcr.2015.11.021]

116 **Gangopadhyay A**, Lazure DA, Thomas P. Adhesion of colorectal carcinoma cells to the endothelium is mediated by cytokines from CEA stimulated Kupffer cells. *Clin Exp Metastasis* 1998; **16**: 703-712 [PMID: 10211983 DOI: 10.1023/a:1006576627429]

117 **McHale JF**, Harari OA, Marshall D, Haskard DO. Vascular endothelial cell expression of ICAM-1 and VCAM-1 at the onset of eliciting contact hypersensitivity in mice: evidence for a dominant role of TNF-alpha. *J Immunol* 1999; **162**: 1648-1655 [PMID: 9973425]

118 **Yang L**, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood* 2005; **106**: 584-592 [PMID: 15811956 DOI: 10.1182/blood-2004-12-4942]

119 **Rivera LB**, Bergers G. Intertwined regulation of angiogenesis and immunity by myeloid cells. *Trends Immunol* 2015; **36**: 240-249 [PMID: 25770923 DOI: 10.1016/j.it.2015.02.005]

120 **Djordjevic S**, Driscoll PC. Targeting VEGF signalling *via* the neuropilin co-receptor. *Drug Discov Today* 2013; **18**: 447-455 [PMID: 23228652 DOI: 10.1016/j.drudis.2012.11.013]

121 **Oh H**, Takagi H, Otani A, Koyama S, Kemmochi S, Uemura A, Honda Y. Selective induction of neuropilin-1 by vascular endothelial growth factor (VEGF): a mechanism contributing to VEGF-induced angiogenesis. *Proc Natl Acad Sci USA* 2002; **99**: 383-388 [PMID: 11756651 DOI: 10.1073/pnas.012074399]

122 **Mehta V**, Fields L, Evans IM, Yamaji M, Pellet-Many C, Jones T, Mahmoud M, Zachary I. VEGF (Vascular Endothelial Growth Factor) Induces NRP1 (Neuropilin-1) Cleavage *via* ADAMs (a Disintegrin and Metalloproteinase) 9 and 10 to Generate Novel Carboxy-Terminal NRP1 Fragments That Regulate Angiogenic Signaling. *Arterioscler Thromb Vasc Biol* 2018; **38**: 1845-1858 [PMID: 29880492 DOI: 10.1161/ATVBAHA.118.311118]

123 **Feurino LW**, Zhang Y, Bharadwaj U, Zhang R, Li F, Fisher WE, Brunicardi FC, Chen C, Yao Q, Min L. IL-6 stimulates Th2 type cytokine secretion and upregulates VEGF and NRP-1 expression in pancreatic cancer cells. *Cancer Biol Ther* 2007; **6**: 1096-1100 [PMID: 17568185 DOI: 10.4161/cbt.6.7.4328]

124 **Kagan P**, Sultan M, Tachlytski I, Safran M, Ben-Ari Z. Both MAPK and STAT3 signal transduction pathways are necessary for IL-6-dependent hepatic stellate cells activation. *PLoS One* 2017; **12**: e0176173 [PMID: 28472150 DOI: 10.1371/journal.pone.0176173]

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MINIREVIEWS

Hepatitis delta virus: From infection to new therapeutic strategies

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Abstract

The hepatitis delta virus (HDV) is a small RNA virus that encodes a single protein and which requires the hepatitis B virus (HBV)-encoded hepatitis B surface antigen (HBsAg) for its assembly and transmission. HBV/HDV co-infections exist worldwide and show a higher prevalence among selected groups of HBV-infected populations, specifically intravenous drug users, practitioners of high-risk sexual behaviours, and patients with cirrhosis and hepatocellular carcinoma. The chronic form of HDV-related hepatitis is usually severe and rapidly progressive. Patterns of the viral infection itself, including the status of co-infection or super-infection, virus genotypes (both for HBV and HDV), and persistence of the virus' replication, influence the outcome of the accompanying and manifested liver disease. Unfortunately, disease severity is burdened by the lack of an effective cure for either virus type. For decades, the main treatment option has been interferon, administered as mono-therapy or in combination with nucleos(t)ide analogues. While its efficacy has been reported for different doses, durations and courses, only a minority of patients achieve a sustained response, which is the foundation of eventual improvement in related liver fibrosis. The need for an efficient therapeutic alternative remains. Research efforts towards this end have led to new treatment options that target specific steps in the HDV life cycle; the most promising among these are myrcludex B, which inhibits virus entry into hepatocytes, lonafarnib, which inhibits farnesylation of the viral-encoded L-HDAg large hepatitis D antigen, and REP-2139, which interferes with HBsAg release and assembly.

Key Words: Hepatitis delta virus; Hepatitis B virus; Myrcludex; Lonafarnib; REP 2139

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Core Tip: The hepatitis delta virus (HDV) is a small defective virus, of interest to scientists for its replicative reliance on and ability to inhibit the common hepatitis B virus (HBV). HDV infection occurs worldwide but shows some geographic variation and its prevalence is generally underestimated. HDV/HBV co-infection causes severe liver disease and the persistence of viral replication is associated with poor prognosis, although interferon is effective in around a quarter of patients. Improved understanding of the HDV life cycle has led to identification of specific antiviral targets and new drugs, including inhibitors of the sodium tauro-cholate cotransporting polypeptide receptor and farnesyltransferase.

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INTRODUCTION

The hepatitis delta virus (HDV) is a defective virus that survives in the host by enveloping itself with the hepatitis B surface antigen (HBsAg) provided by the hepatitis B virus (HBV)[1]. This unique virus was discovered in 1977 by Rizzetto *et al* [2] following description of the delta antigen in HBsAg carriers. The subsequent research uncovering HDV modes of replication and its interactions with HBV have provided information with clinical implications[3]. HDV is highly infectious and induces a heterogeneous liver disease that rapidly progresses to cirrhosis; the outcome itself is influenced by several viral and host factors, including virus genotypes and level of viremia (for both HDV and HBV) as well as the presence of any concomitant causes of liver damage[3]. According to recent reports, 10.58% of general HBV-positive individuals have co-infection with HDV, excluding the intravenous drug users (IVDUs) and practitioners of high-risk sexual behaviours (HRSBs)[4].

Collectively, these data highlight the need for HDV screening and control strategies, which must be developed rationally. In the industrialized world, the decline of new infections has regrettably reduced awareness and testing for HDV[1,5]. Moreover, there is a significant lacuna in the literature, with limited data reported from the Americas, Southern Africa and Asia[4], although reliable data from across the globe are required to overcome this pathogenic threat. Once HDV infection is diagnosed, the greatest challenge to clinical care is related to the treatment of associated liver disease. Interferon (IFN) treatment has been the only pharmacological option for decades but its efficacy remains unsatisfactory[6]. The perseverance of HDV researchers is beginning to pay off, however, with enhanced knowledge on the viral life cycle leading to development of drugs targeting specific steps in such, several of which are now under advanced clinical evaluation[6-8].

VIROLOGY

HDV is similar to viroids and virusoids in its structure and replication process but it presents a distinctive identity. Accordingly, HDV was classified as the sole member of the *Deltaviridae* genus[1,9]. The virus contains about 1700 nucleotides and has a circular structure that is formed by base-pairing involving 74% of its genomic RNA. It encodes a single protein - the delta antigen (HDAg) — which combines with the HDV genome to form a ribonucleoprotein complex (referred to as an RNP). The RNP becomes enveloped upon interaction with HBV, which serves as a helper virus, providing the HBsAg component. The latter is necessary for HDV assembly, release and transmission, but it is not required for any of the other steps in replication[3,10]. HDAg is present in two isoforms: small (S-HDAg) and large (L-HDAg), each carrying out a specific role in the HDV life cycle. S-HDAg is composed of 195 amino-acids and promotes RNA replication, while L-HDAg is composed of 214 amino-acids and is essential for the virion packaging. The isoforms differ by 19 amino-acids located in the C-terminal region of L-HDAg exclusively, the presence of which results from an

editing process that is regulated by the adenosine deaminase enzyme ADAR1[11]. HDAg modification occurs at the post-transcriptional level, similar to the well-known processes of phosphorylation, acetylation, methylation and isoprenylation, all of which regulate the hepatitis viral life cycles (Figure 1).

HDV replicates exclusively in the liver, inside hepatocyte nuclei. The virus uses the same entry mechanism as HBV, being enveloped by HBsAg, differentiated in the small (S-HBsAg), large (L-HBsAg) and medium (also known as M-HBsAg) forms. After ligating the hepatocyte's surface heparan sulphate proteoglycans (commonly referred to as HSPGs), the pre-S1 domain of L-HBsAg binds to the receptor for HBV, identified as the sodium tauro-cholate cotransporting polypeptide (NTCP)[12]. The viral RNP then translocates into the nucleus, where its genomic RNA is transcribed first into the complementary antigenomic RNA, then into new genomic RNA and mRNA by the host's RNA polymerase II enzyme. The fact that HDV does not encode its own RNA polymerase enzyme but redirects the host's is a further peculiarity of this hepatitis virus.

Once linear polymers are produced in the HDV-infected hepatocyte, they self-cleave *via* a ribozyme activity of the virus and then circularize. The viral mRNA then migrates from the nucleus to the cytoplasm, for subsequent synthesis of either S-HDAg or L-HDAg. The protein products associate with the viral genomic RNA to form new ribonucleoproteins, each of approximately 20 nm in diameter. This complex relies on HBV surface antigens for complete formation and subsequent secretion[13]. The latter process occurs only after a farnesylation process[14], which makes molecules more lipophilic, whereby L-HDAg interacts with HBsAg at the endoplasmic reticulum.

While at first evaluation this entire process appears rudimentary, HDV has evolved a complex and unusual replicative mechanism, intriguing for researchers but proving a challenge to fully elucidate. It has been proposed, in the field of innovative research, that viruses other than HBV, such as flavivirus and hepacivirus, could package the HDV ribonucleoprotein[15]. This unconventional transmission of HDV certainly requires confirmation in a human system; however, the newest knowledge on its interaction with the NTCP receptor[12] and the farnesylation process of L-HDAg[14] has led to new therapeutic proposals.

EPIDEMIOLOGY

Sero-epidemiological studies performed between the 1980s and 1990s estimated the HDV prevalence among HBsAg-positive patients to be 5%, equating to about 20 million people worldwide[3,16]. Regional prevalence of HDV infection was higher in South Europe, Middle East, East Africa and Asia, with relatively lower rates in Northern Europe, South Africa and North America[17]. During the more recent 2000s and 2010s, the introduction of HBV vaccination, institution of preventive measures and improvements in hygiene habits have led to decreases in HDV prevalence rates in multiple regions. In Taiwan, for example, HDV prevalence among HBsAg-positive subjects declined substantially, from 24% in 1983 to 4% in 1995[18], while in Italy, it declined from 23% in 1987 to 14% in 1992 to 8% in 1997[19]. However, in parallel with the decreased prevalence rates in previously endemic areas, other HDV hot zones emerged, including South Eastern Russia, Northern India, Vietnam and Albania.

Moreover, starting in the early 2000s, immigration activities have prompted a new rise in HDV prevalence in some European States, following a large influx of immigrants from endemic areas, such as Romania, the ex-Soviet Union and North Africa[20]. France also experienced a remarkable increase in cases, reaching a rate of 6.5% in 2010 after remaining stable at around 1% from 1997 to 2005[21]. Similarly, Germany showed an initial decline during the 1990s (from 19% to 7%) but then experienced an upswing trend, with rates fluctuating between 8% and 14%, in the 2000s[22].

Nowadays, HDV infection maintains its worldwide distribution. According to two recent systematic reviews, global prevalence is around 0.16%-0.98% among the general population and 4.5%-14.6% among HBsAg-positive subjects[4,23]. Geographic variations are still present, considering both the overall population and HBV carriers. Among the general population worldwide, Mongolia has reported the highest HDV prevalence[4], with primacy among HBsAg-positive people (prevalence rate of 36.9%). However, prevalence rates greater than 10% are also reported by Moldova and Western and Middle African countries[23]. According to demographical characteristics, men are slightly more affected than women[4] and the elderly are at greater risk

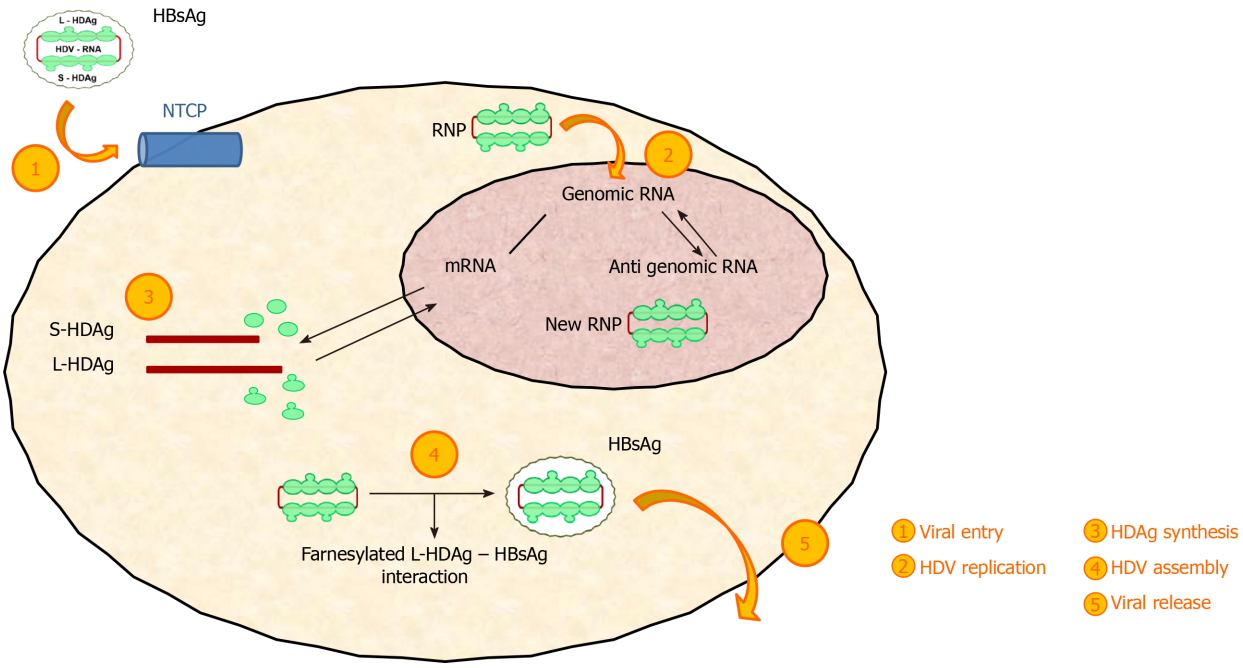


Figure 1 Schematic representation of the hepatitis D virus life cycle. New drugs interfere with the hepatitis B surface antigen at the viral entry or viral assembly level. HBsAg: Hepatitis B surface antigen; HDAg: Hepatitis D antigen; HDV: Hepatitis delta virus; L-HDAg: Large hepatitis D antigen; NTCP: Sodium taurocholate cotransporting polypeptide; RNP: Ribonucleoprotein complex; S-HDAg: Small hepatitis D antigen.

than the young (80% among those ≥ 50-years-old *vs* 3% among those < 30-years-old) [24].

Among the eight recognized HDV genotypes (identified as 1-8), genotype 1 is the most common and it is present worldwide[23,25]. The other seven genotypes are more localized, with genotypes 2 and 4 mainly confined to Asia, genotype 3 to Latin America and genotypes 5-8 to Africa[23].

As exposure to blood of infected subjects is the predominant route of HDV transmission, some groups of high-risk people have been identified. Most HDV-infected patients are IVDUs, who had been infected upon needle-stick injury or use of contaminated syringes[26]. The reported prevalence rates among this subgroup range from 21%[27] to 36%[28], with a pooled odds ratio (OR) of 19.0[23]. Another group of high-risk individuals are practitioners of HRSBs, including commercial sex workers and promiscuous homosexuals[26], among whom the reported prevalence reaches up to 11%[29], with a pooled OR of 18.7[23]. The risk of infection is also higher among the human immunodeficiency virus-positive population (pooled OR: 6.6) and haemodialytic patients (pooled OR: 3.4)[23]. Nowadays, nosocomial infection and transfusion transmission occur less frequently than in the past. However, some cultural practices, such as tattoos and piercing, are quickly becoming an appreciable route of transmission[30].

Taken together, these data show that the global presence of HDV infection has not decreased and it is probably still underestimated.

CLINICAL MANIFESTATIONS AND DIAGNOSIS

Viremia, genotypes and liver disease

The replicative activity of HDV can influence the course of liver disease[31].

A multicentre study found that subjects with persistent HDV replication had worse prognosis in terms of liver failure, need for liver transplantation and/or death than those with undetectable HDV-RNA[32]. On the other hand, the few patients in whom HDV-RNA had become undetectable during their follow-up were determined to be less likely to develop liver cirrhosis than those with persistently positive HDV-RNA levels[32].

To date, little is known about the determinants of spontaneous clearance of HDV-RNA, despite the early clearance of HDV infection being one of the main parameters determining prognosis of the related liver disease[10,33].

Although HBV replication is usually suppressed in the presence of HDV infection, high levels of HBV viremia are associated with more severe liver damage[34]. In addition, HBV genotypes may also play a role in the course of chronic hepatitis D (CHD), which is considered the most severe and rapidly progressive form of chronic viral hepatitis[3].

Besides host-related factors, other viral factors might be involved in clearance of the virus. One such factor is genotypic variability. Among the eight known genotypes, HDV-1 has the highest pathogenic potential[31,33,35], while HDV-2 and HDV-4 are associated with milder forms of the disease[35]. According to a study conducted in Taiwan, patients infected with HDV-2 develop liver failure less frequently than those with genotype 1[34]. HDV-3 has been associated with an increased risk of fulminant hepatitis[3,36], whereas HDV-5 has a better prognosis than HDV-1[33]. The pathogenic properties of HDV 5-8 are not well characterized[7,36]. Among the spectrum of host and viral factors that may underlie the different outcomes of infection with these various genotypes, we can include variability in virus replication and virion assembly efficacy, both of which contribute to the rate of HDV virion secretion, and such host factors as race or presence of single-nucleotide polymorphisms[35].

Clinical manifestation and outcomes of the hepatitis D disease differ according to the HDV acquisition modality, itself depending on HBsAg status of the infected individual. In HBV/HDV co-infections, the presence of both viruses causes wide hepatic necrosis, bringing about severe or occasionally fulminant hepatitis[37]. Acute hepatitis can occur either with a single peak of disease (mono-phasic) or with two distinct peaks (biphasic). In the latter case, the first peak matches to an initial HBV spread, and the second peak to HDV propagation[7,37]. The HBV/HDV co-infection usually leads to a self-limited acute hepatitis; indeed, only 2% of patients with co-infection progress to cirrhosis[3,38-41]. The diagnosis of co-infection is confirmed by the simultaneous presence of serological markers for primary HBV and HDV infections[37]. Generally, the first of these to appear are the host's antibody to hepatitis B core protein (anti-HBc IgM), while that to hepatitis D (anti-HD IgM) appears within 2 wk from clinical onset, usually remaining detectable for up to 5-6 wk afterwards. The anti-HD IgG antibody reaches a detectable level after the IgM antibodies disappear, and may persist for months or even years. A failure in anti-HD IgM clearance predicts the chronicity of hepatitis[26,35,42].

Conversely, in HDV super-infection, the virus infects individuals with chronic HBV infection, and it typically leads to HDV persistence with development of cirrhosis[35]. It may present either as an exacerbation of a known chronic hepatitis B status accompanied by hepatic decompensation, or as a new acute hepatitis status in asymptomatic HBsAg carriers[35]. Super-infection usually leads to a chronic infection (in > 90% of cases), with rapid progression to cirrhosis[23,38-40]. Diagnosis of the super-infection is based upon a positive test for anti-HD IgM and a negative test for anti-HBc IgM in HBsAg carriers[35,37]. The super-infection itself is characterized by early presence of HDV-RNA and HDV antigen, followed by increase in anti-HD IgM that remains persistently detectable, in parallel with development of chronicity. In addition, during the acute phase, there is a suppression of HBV replication[35].

CHD

Among chronic viral hepatitis, CHD is distinguished by its severity and higher risk of evolution. It involves a 3-fold increase in the risk of cirrhosis and a 2-fold increase in the risk of death, as compared to HBV and hepatitis C virus infection alone[3,7,35,39,43]. Half of the patients with CHD have experienced a previous acute hepatitis attack, which often represents the time of super-infection with HDV. Clinically, CHD can range from asymptomatic forms, which are discovered incidentally, to symptomatic ones, manifested as fatigue, malaise, and anorexia[3,35]. Diagnosis is achieved upon detection of high titres of anti-HD IgG and IgM in serum, whereas the HD antigen remains persistently detectable in the liver[35]. Typically, alanine aminotransferase (ALT) levels remain stably elevated or fluctuate, reflecting the destruction of hepatic cells. The lowering of such levels is usually meaningful for tracking disease progression to cirrhosis[35,44].

Cirrhosis

The main complication of chronic hepatitis is the development of cirrhosis. Cirrhosis occurs in 70% of cases within 5 to 10 years after hepatitis development, but in 15% of patients it may occur within 1 year to 2 years[7,37]. While the precise mechanism of this progression remains unknown, it has been proposed that the long form of HDAg may stimulate liver fibrosis through interaction with transforming growth factor-β-induced signal activation[35].

Despite liver biopsy remaining the gold standard procedure for diagnosis and staging of cirrhosis, there are validated (and less invasive) predictive scoring systems applicable to patients with HDV disease. The Delta-4 fibrosis score uses gamma-glutamyl transferase (commonly referred to as GGT), ALT, platelet count, and liver stiffness as parameters[45]. A similar score called the "delta Fibrosis Score" considers GGT along with age, albumin and serum cholinesterase[46]. Abbas *et al*[40] proposed to simply use spleen size and platelet count as the predictive parameters of cirrhosis. Other non-invasive markers of fibrosis that are in use clinically include components of the extracellular matrix, namely procollagen III N-peptide, collagen IV, and hyaluronic acid; however, these factors have lower diagnostic accuracy than in other forms of chronic hepatitis[35].

Once HDV cirrhosis is established, it can remain asymptomatic or induce non-specific symptoms, such as muscle weakness and jaundice. In the advanced stage, it can also evolve complications[37]. In half of the cases, these complications occur within 8 years and include portal hypertension, abdominal ascites, gastrointestinal bleeding and hepatic encephalopathy[31,44]. On average, the annual incidence of liver decompensation in cirrhosis ranges from 2.6% to 3.6%, being more than doubled compared to HDV-negative hepatitis B cases[3,44,47]. Among HDV-positive patients, mortality from cirrhosis and hepatocellular carcinoma (HCC) is higher than that for patients with HBV infection alone[23,44,48].

HCC

HCC is a frequent cause of death from cancer worldwide, and its incidence and mortality rates remain on an upward trajectory. The estimated annual incidence of HCC ranges from 2.6% to 2.8%[26,47]. Chronic viral hepatitis, particularly related to hepatitis B, C and D viruses, is responsible for more than 80% of all cases of HCC[43,49]. While both HBV and hepatitis C virus have already been classified by the World Health Organization as oncogenic viruses, the role of HDV infection in HCC development is still under debate[43,48].

Previous studies have shown that the incidence of HCC was similar among patients with HDV and HBV chronic infections, suggesting that HDV is not a contributory factor, *per se*, to the carcinogenic process. Contrariwise, recent lines of evidence from cohort studies have shown a significantly increased risk of HCC among patients with HDV liver disease, which suggests a greater extent of correlation to HDV than to HBV replication[26,43,48,50,51].

Some studies have estimated that between 16.7% and 20% of cirrhosis or HCC cases among people with hepatitis B worldwide are attributable to HDV infection[23]. Nevertheless, the mechanisms of HCC development in HDV-infected patients remain debated[37]. Bockmann *et al*[48] suggest that the high rate of HCC was caused by the rapid progression to liver cirrhosis and not by viral activity at baseline. In addition, experiments in transgenic mice expressing HDAg proteins seem to rule out a direct carcinogenic role of HDV[3]; however, the L-HDAg may promote oxidative stress through activation of signal transducer and transcription-3 and nuclear factor-kappa B, factors vital to cancer cell communication and carcinogenesis. Moreover, it may also promote cancer cell survival through epigenetic mechanisms, such as DNA methylation of the tumour suppressor gene and acetylation of histone H3 of the clustering promoter[40].

TREATMENT

HDV infection with related liver disease is difficult to treat. Drugs for HBV cure are generally not effective on HDV, and the absence of its own polymerase for the latter complicates attempts to identify suitable therapeutic targets. The ever popular IFN-based therapy was introduced about 35 years ago. Pegylated (Peg)-IFN-α is now preferred over the standard formulation, according to its comparatively improved efficacy and safety. This underlies the biochemical and virological responses that are achieved in about 25% of treated patients[47], with response usually being evaluated clinically at 6 mo post-treatment. Guidelines from the European Association for Study of the Liver recommend Peg-IFN for 48 wk (Table 1) in patients with compensated liver disease and nucleos(t)ide analogues for HBV/HDV patients if HBV replication is above 2000 IU/mL[52]. In the last decade, articles published on the HDV have addressed numerous questions related to the role of combination therapy, to the optimal dose and duration of PEG-IFN administration, and to the real end-points of HDV treatment as well as the clinical benefit.

Table 1 Established and undergoing drugs in the management of hepatitis delta virus chronic hepatitis

Molecule	Drug	Mechanism of action	Drug administration	Duration of therapy	Current therapy/clinical trials	Most frequent side effects
Interferon	Pegylated Interferon-α	Immunomodulatory and antiviral activity	Subcutaneously Weekly	Recommended at least 48 wk	Established Therapy; Long-term follow up, after discontinuation of therapy, available	Flu-like symptoms; Headache; Myalgias; Arthralgias; Anemia; Leukopenia; Thrombocytopenia
Myristoylated Lipopeptide	Myrcludex B; Bulevirtide	Interference with HDV viral entry through NTCP	SubcutaneouslyDaily; ± Peg-IFN; ± Tenofovir	At present maximum 48 wk	Ongoing Phase 3 clinical trial	Thromocytopenia; Neutropenia; Lymphopenia; Eosinophilia; Bile Acids elevation
Farnesyl-transferase Inhibitor	Lonafarnib	Inhibition of HDV viral assembly	Orally; Daily; ± Ritonavir; ± Peg- IFN	At present maximum 48 wk	Ongoing Phase 3 clinical trial	Nausea; Diarrhea; Loss of appetite; Weight loss; Abdominal Bloating Increased ALT levels
Phosphorothioate nucleic acid polymer	Rep 2139	Post-entry inhibition of HBsAg secretion; Possible interference with viral entry	Intravenously; Weekly; ± Peg-IFN	At present maximum 30 wk	Phase 2 clinical trial	Thromocytopenia; Neutropenia; Anaemia; Increased ALT levels

HDV: Hepatitis delta virus; NTCP: Sodium tauro-cholate cotransporting polypeptide; Peg-IFN: Pegylated interferon; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen.

Strategies to improve the efficacy of Peg-IFN-α

In a large randomized controlled study - the Hep-Net-International Delta Hepatitis Intervention Trial (also referred to as the HIDIT-1) - performed by the German Network, 90 patients were assigned to receive weekly treatment with either 180 μg of Peg-IFN-α plus 10 mg of adefovir (31 patients), 180 μg of Peg-IFN-α plus placebo (29 patients), or 10 mg of adefovir alone (30 patients) for 48 wk[53]. Twenty-four weeks after the treatment initiation, a sustained HDV-RNA clearance was observed in 28% of the patients who had been treated with Peg-IFN-α either as mono-therapy or in combination with adefovir; no therapeutic effect was observed in the group treated with adefovir alone. The HIDIT-2 study[54] embodied a second attempt to clarify if combination therapy was superior to Peg-IFN-α alone in treatment of HDV patients, but this time examining tenofovir use. In that trial[54], 120 patients with HDV-RNA positivity were randomized to receive either Peg-IFN-α plus tenofovir or Peg-IFN-α plus placebo for 96 wk. At the end of therapy, viremia was negative in 48% of patients on combination therapy and in 33% of those on the Peg-IFN-α mono-therapy[54]. At week 24 post-therapy, virological response was observed in less than 30% of patients. The combination therapy with tenofovir did not provide benefits in HDV patients with low HBV-DNA levels, not even when 96 wk of the Peg-IFN-α regimen was compared to 48 wk. However, more side-effects were associated to the prolonged therapy, having consequence for the most advanced form of liver disease.

While other studies have also suggested no clear benefit in extending Peg-IFN-α treatment, positive results were obtained from a retrospective study that included 99 patients, with a median treatment duration of 24 mo and post-treatment follow-up of 55 mo[55]. Altogether, however, the previous studies have recognized the efficacy of IFN in about one-third of treated patients[56]. Yet, the optimal treatment duration remains to be defined and the addition of nucleos(t)ide analogues does not seem to improve the result[57,58].

Predictors of long-term response to IFN

Fifty-six percent of patients showing negativity for HDV-RNA (*i.e.* sustained viral response) in the HIDIT-1 trial experienced a late relapse[59]. These data prompted questions regarding the role of the 6-mo post-therapy negative viremia, considering its potential roles as a surrogate marker of HDV cure and sustained viral response[60]. New suggestions arose from a further sub-analysis of the HIDIT-1 trial to investigate positive and negative predictors of response[60]; in particular, the negative level of HDV-RNA at week 24 of treatment or at the end of treatment was found to identify responders with a prediction value of 71% or 100% respectively. Moreover, HBsAg kinetic parameters during treatment were proposed for monitoring response to

therapy, in association to the HDV-RNA. According to an Italian study, a cut-off of HBsAg < 1000 at month 6 of Peg-IFN therapy is able to discriminate responders and partial responders from non-responders[61]. Furthermore, the association of HBsAg reduction (0.105 Log) and HDV-RNA decrease (1.610 Log) from baseline to month 6 was found to be predictive of HBsAg clearance.

Clinical impact of IFN-based therapy

An important end-point of HDV therapy is HDV-RNA clearance, as determined by sensitive assays[62]. Unfortunately, the presence of minimal residual virus that falls below the threshold of detection of an assay could explain reactivation in the presence of HBsAg. The ideal goal of therapy in HBV/HDV co-infection is the clearance of HBsAg; although, this is rarely achieved[63]. In any case, IFN treatment was reported effective in altering the disease progression-associated events[64]. The loss of HDV-RNA during follow-up was a more frequent occurrence among the IFN-α treated patients and it was linked to long-term survival without complications[55,65]. Based on this observation, a durable undetectability of HDV viremia was proposed as a surrogate and more realistic end-point[64].

New therapeutic targets in HDV

Some new promising drugs have advanced in phase II clinical trials. Specifically, these represent inhibitors of the NTCP receptor[66] and farnesyltransferase[67] and the group of nucleic acid polymers[68] (Table 1).

Myrcludex B: Myrcludex B (MyrB, subsequently named bulevirtide) is a myristoylated synthetic peptide, containing 47 amino acids of the preS1 domain of the HBV L-surface protein. It is able to bind to the NTCP, which has been identified as a receptor for HBV/HDV entry (Figure 1)[12]. The concentration of this drug required to inhibit the virus is 100 times lower than that required for bile salt transport inhibition[69].

After a preliminary study[70], Bogomolov *et al*[71] assessed the efficacy and safety of MyrB in a total of 24 patients. The study design assigned 7 patients to receive the 48-wk Peg-IFN-α treatment, 7 patients to receive 2 mg MyrB administered by subcutaneous injection for 24 wk followed by Peg-IFN for 48 wk, and 7 patients to receive MyrB and Peg-IFN for 24 wk plus an additional 24 wk of Peg-IFN. Notably, the HDV-RNA levels decreased by at least one log in the MyrB cohort at week 24 and ALT normalized in 6 patients. While the decline of viremia was observed in all three treatment groups, HBsAg level showed no change in any.

A subsequent phase II trial enrolled 120 patients with CHD, and randomized them into four treatment groups. Three groups were treated with three different doses of MyrB (2 mg, 5 mg and 10 mg) in combination with tenofovir (245 mg/d), while one group was administered tenofovir alone[72]. At the end of treatment, the best result (a -2.7 Log decline of HDV-RNA) was found in patients who received the higher dose of MyrB plus tenofovir. However, for all groups, about two-thirds of the patients who were responders experienced relapse during the follow-up.

A new phase II study was designed to evaluate the efficacy of 2 mg and 5 mg MyrB combination therapy with Peg-IFN-α, and compared to Peg-IFN-α and MyrB mono-therapies[73]. The MyrB and Peg-IFN combination yielded high rates of off-treatment HDV-RNA suppression and reduction in HBsAg. Recently, MyrB was authorized as an orphan medicine by the European Medicine Agency, making it available for usage in patients with HDV compensated liver disease and active viral replication.

Lonafarnib: Lonafarnib (LNF) is a farnesyl-transferase inhibitor, originally developed in cancer treatment. In the HDV setting, the drug inhibits the farnesylation of L-HDAg, which is essential for interaction with HBsAg in the assembly of new viral particles. In a first, short-term controlled study, patients received oral LNF at 100 mg or 200 mg twice daily. While the drug was effective in reducing virus levels, it was mostly associated with gastro-intestinal adverse events and weight loss[74].

Four different subsequent trials were designed and identified as 'LOWR-HDV'. In studies 1 and 2, the LNF was administered at different doses, either alone or in combination with ritonavir or Peg-IFN. Overall, it induced a significant viral decline [75], with a mean log reduction of -5.57 Log^{10} U/mL achieved in the triple combination group[76]. In study 3, patients on nucleos(t)ide analogues were treated with LNF at 50 mg/d, 75 mg/d, 100 mg/d plus ritonavir for 12 wk or 24 wk[77]. The regimen of LNF 50 mg plus ritonavir once daily was superior, when compared to the higher doses of the drug. Finally, the regimen of dose escalation was tested in study 4, the LOWR-HDV-4[78].

Altogether, the findings from these different LOWR-HDV studies supported the antiviral effect of LNF. Moreover, its combination with ritonavir allowed for the use of lower doses and produced more manageable side effects. At present, a phase 3 clinical study (D-LIVR) is ongoing. This is a randomized placebo-controlled trial comparing 50 mg LNF plus ritonavir twice per day with or without Peg-IFN-α in patients maintained on nucleos(t)ide analogues.

Nucleic acid polymers: Nucleic acid polymers are phosphorothioate oligonucleotides with antimicrobial activity, some of which have shown more specific HBV inhibiting properties (*e.g.*, REP 2139). The antiviral effect seems to be related to various mechanisms, including the inhibition of HBsAg release and assembly and the interaction with both S-HDAg and L-HDAg[79,80]. REP 2139 was tested in 12 patients with CHD at a dose of 500 mg administered intravenously once a week for 15 wk, followed by a 250 mg dose combined with subcutaneous injection of 180 μg Peg-IFN for 15 wk and finally Peg-IFN mono-therapy given for 33 wk[81]. Nine patients, who became HDV-RNA-negative during treatment, remained non-viremic at the end of treatment and showed a mean HDV-RNA decline of 5.34 Log^{10} IU/L. REP 2139 induced a remarkable HBsAg decline (3.5 Log^{10} IU/mL) compared to baseline. The most common side effects experienced by patients during treatment were thrombocytopenia, neutropenia and increased ALT levels. Eleven participants were followed for 3.5 years in the REP 301-LTF study[82] and they showed a long-term safety profile as well as persistent virological control and functional cure.

CONCLUSION

HDV remains an important health problem worldwide. Although the infection map is subject to change due to diffusion of the HBV vaccine, some geographic areas are currently a virus reservoir. Moreover, the real burden of infection appears to be underestimated, both in the general and at-risk populations. It is known that HDV-related liver disease is influenced by replicative activity of the virus, which is crucial in inducing cirrhosis and promoting hepatic decompensation. For this reason, the research for new therapeutic strategies has focused on molecules interfering with the replication cycle of HDV.

These novel drugs have demonstrated antiviral efficacy and tolerability, but there remain open questions to be answered and clarified in long-term treatment and follow-up. MyrB is administered *via* subcutaneous injection. Although it induced a decrease in viral loads, it did not reduce the HBsAg level. On the other hand, MyrB showed a good safety profile, even in some patients with compensated cirrhosis. LNF, on the other hand, has the advantage of oral intake. Moreover, when administered in combination with ritonavir, it was the determinant for reduction of gastrointestinal side-effects. For both drugs, viral reactivation and ALT flares were reported at the end of treatment. REP 2139 requires an intravenous administration. It produced a rapid reduction in HBsAg levels, and not only in HDV viremia, in a lasting way; however, this effect needs to be tested on larger numbers of patients.

In conclusion, MyrB, LNF and REP 2139 represent expected and promising therapeutic options for HDV infection. Further studies are needed to define the utility of combination therapy with IFN.

REFERENCES

1 **Rizzetto M**. The adventure of delta. *Liver Int* 2016; **36** Suppl 1: 135-140 [PMID: 26725911 DOI: 10.1111/liv.13018]

2 **Rizzetto M**, Canese MG, Aricò S, Crivelli O, Trepo C, Bonino F, Verme G. Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and in serum of HBsAg carriers. *Gut* 1977; **18**: 997-1003 [PMID: 75123 DOI: 10.1136/gut.18.12.997]

3 **Sureau C**, Negro F. The hepatitis delta virus: Replication and pathogenesis. *J Hepatol* 2016; **64**: S102-S116 [PMID: 27084031 DOI: 10.1016/j.jhep.2016.02.013]

4 **Chen HY**, Shen DT, Ji DZ, Han PC, Zhang WM, Ma JF, Chen WS, Goyal H, Pan S, Xu HG. Prevalence and burden of hepatitis D virus infection in the global population: a systematic review and meta-analysis. *Gut* 2019; **68**: 512-521 [PMID: 30228220 DOI: 10.1136/gutjnl-2018-316601]

5 **Kushner T**, Serper M, Kaplan DE. Delta hepatitis within the Veterans Affairs medical system in the United States: Prevalence, risk factors, and outcomes. *J Hepatol* 2015; **63**: 586-592 [PMID: 25962883 DOI: 10.1016/j.jhep.2015.04.025]

6 **Caviglia GP**, Rizzetto M. Treatment of hepatitis D: an unmet medical need. *Clin Microbiol Infect*

2020; **26**: 824-827 [PMID: 32120043 DOI: 10.1016/j.cmi.2020.02.031]
7 **Mentha N**, Clément S, Negro F, Alfaiate D. A review on hepatitis D: From virology to new therapies. *J Adv Res* 2019; **17**: 3-15 [PMID: 31193285 DOI: 10.1016/j.jare.2019.03.009]
8 **Gilman C**, Heller T, Koh C. Chronic hepatitis delta: A state-of-the-art review and new therapies. *World J Gastroenterol* 2019; **25**: 4580-4597 [PMID: 31528088 DOI: 10.3748/wjg.v25.i32.4580]
9 **Mason WS**, Burrell CJ, Casey J. Deltavirus. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. Virus Taxonomy, VIIIth Report of International Committee on Taxonomy of Viruses. 1st ed. London: Elsevier Academic Press, 2005: 735-738
10 **Botelho-Souza LF**, Vasconcelos MPA, Dos Santos AO, Salcedo JMV, Vieira DS. Hepatitis delta: virological and clinical aspects. *Virol J* 2017; **14**: 177 [PMID: 28903779 DOI: 10.1186/s12985-017-0845-y]
11 **Casey JL**. Control of ADAR1 editing of hepatitis delta virus RNAs. *Curr Top Microbiol Immunol* 2012; **353**: 123-143 [PMID: 21732238 DOI: 10.1007/82_2011_146]
12 **Yan H**, Peng B, Liu Y, Xu G, He W, Ren B, Jing Z, Sui J, Li W. Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide. *J Virol* 2014; **88**: 3273-3284 [PMID: 24390325 DOI: 10.1128/JVI.03478-13]
13 **Shih HH**, Jeng KS, Syu WJ, Huang YH, Su CW, Peng WL, Sheen IJ, Wu JC. Hepatitis B surface antigen levels and sequences of natural hepatitis B virus variants influence the assembly and secretion of hepatitis d virus. *J Virol* 2008; **82**: 2250-2264 [PMID: 18094179 DOI: 10.1128/JVI.02155-07]
14 **Glenn JS**, Watson JA, Havel CM, White JM. Identification of a prenylation site in delta virus large antigen. *Science* 1992; **256**: 1331-1333 [PMID: 1598578 DOI: 10.1126/science.1598578]
15 **Perez-Vargas J**, Amirache F, Boson B, Mialon C, Freitas N, Sureau C, Fusil F, Cosset FL. Enveloped viruses distinct from HBV induce dissemination of hepatitis D virus in vivo. *Nat Commun* 2019; **10**: 2098 [PMID: 31068585 DOI: 10.1038/s41467-019-10117-z]
16 **Lempp FA**, Ni Y, Urban S. Hepatitis delta virus: insights into a peculiar pathogen and novel treatment options. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 580-589 [PMID: 27534692 DOI: 10.1038/nrgastro.2016.126]
17 **Taylor J**, Purcell RH, Farci P. Hepatitis D (delta) virus. In: Knipe DM, Howley PM. Fields Virology. Philadelphia: Lippincott, Williams and Wilkins, 2013: 2222–2241
18 **Huo TI**, Wu JC, Lin RY, Sheng WY, Chang FY, Lee SD. Decreasing hepatitis D virus infection in Taiwan: an analysis of contributory factors. *J Gastroenterol Hepatol* 1997; **12**: 747-751 [PMID: 9430041 DOI: 10.1111/j.1440-1746.1997.tb00364.x]
19 **Gaeta GB**, Stroffolini T, Chiaramonte M, Ascione T, Stornaiuolo G, Lobello S, Sagnelli E, Brunetto MR, Rizzetto M. Chronic hepatitis D: a vanishing Disease? *Hepatology* 2000; **32**: 824-827 [PMID: 11003629 DOI: 10.1053/jhep.2000.17711]
20 **Rizzetto M**, Alavian SM. Hepatitis delta: the rediscovery. *Clin Liver Dis* 2013; **17**: 475-487 [PMID: 23905817 DOI: 10.1016/j.cld.2013.05.007]
21 **Servant-Delmas A**, Le Gal F, Gallian P, Gordien E, Laperche S. Increasing prevalence of HDV/HBV infection over 15 years in France. *J Clin Virol* 2014; **59**: 126-128 [PMID: 24365475 DOI: 10.1016/j.jcv.2013.11.016]
22 **Wedemeyer H**, Heidrich B, Manns MP. Hepatitis D virus infection--not a vanishing disease in Europe! *Hepatology* 2007; **45**: 1331-2; author reply 1332 [PMID: 17464980 DOI: 10.1002/hep.21590]
23 **Stockdale AJ**, Kreuels B, Henrion MYR, Giorgi E, Kyomuhangi I, de Martel C, Hutin Y, Geretti AM. The global prevalence of hepatitis D virus infection: Systematic review and meta-analysis. *J Hepatol* 2020; **73**: 523-532 [PMID: 32335166 DOI: 10.1016/j.jhep.2020.04.008]
24 **Stroffolini T**, Sagnelli E, Sagnelli C, Russello M, De Luca M, Rosina F, Cacopardo B, Brancaccio G, Furlan C, Gaeta GB, Licata A, Almasio PL; behalf of EPACRON study group. Hepatitis delta infection in Italian patients: towards the end of the story? *Infection* 2017; **45**: 277-281 [PMID: 27817147 DOI: 10.1007/s15010-016-0956-1]
25 **Le Gal F**, Brichler S, Drugan T, Alloui C, Roulot D, Pawlotsky JM, Dény P, Gordien E. Genetic diversity and worldwide distribution of the deltavirus genus: A study of 2,152 clinical strains. *Hepatology* 2017; **66**: 1826-1841 [PMID: 28992360 DOI: 10.1002/hep.29574]
26 **Hercun J**, Koh C, Heller T. Hepatitis Delta: Prevalence, Natural History, and Treatment Options. *Gastroenterol Clin North Am* 2020; **49**: 239-252 [PMID: 32389361 DOI: 10.1016/j.gtc.2020.01.004]
27 **Chen F**, Zhang J, Guo F, Wen B, Luo S, Yuan D, Lin Y, Ou W, Tang P, Dai G, Li F, Liu W, Qu X. Hepatitis B, C, and D virus infection showing distinct patterns between injection drug users and the general population. *J Gastroenterol Hepatol* 2017; **32**: 515-520 [PMID: 27248508 DOI: 10.1111/jgh.13460]
28 **Mahale P**, Aka PV, Chen X, Liu P, Fram BJ, Wang AS, Simenel S, Tseng FC, Chen S, Edlin BR, Glenn JS, O'Brien TR. Hepatitis D Viremia Among Injection Drug Users in San Francisco. *J Infect Dis* 2018; **217**: 1902-1906 [PMID: 29800369 DOI: 10.1093/infdis/jiy157]
29 **Lin HH**, Lee SS, Yu ML, Chang TT, Su CW, Hu BS, Chen YS, Huang CK, Lai CH, Lin JN, Wu JC. Changing hepatitis D virus epidemiology in a hepatitis B virus endemic area with a national vaccination program. *Hepatology* 2015; **61**: 1870-1879 [PMID: 25677884 DOI: 10.1002/hep.27742]
30 **Gheorghe L**, Csiki IE, Iacob S, Gheorghe C, Trifan A, Grigorescu M, Motoc A, Suceveanu A, Curescu M, Caruntu F, Sporea I, Brisc C, Rogoveanu I, Cerban R, Tugui L, Alexandrescu A. Hepatitis Delta Virus Infection in Romania: Prevalence and Risk Factors. *J Gastrointestin Liver Dis*

2015; **24**: 413-421 [PMID: 26697566 DOI: 10.15403/jgld.2014.1121.244.dtv]
31 **Niro GA**, Smedile A, Ippolito AM, Ciancio A, Fontana R, Olivero A, Valvano MR, Abate ML, Gioffreda D, Caviglia GP, Rizzetto M, Andriulli A. Outcome of chronic delta hepatitis in Italy: a long-term cohort study. *J Hepatol* 2010; **53**: 834-840 [PMID: 20800919 DOI: 10.1016/j.jhep.2010.06.008]
32 **Palom A**, Rodríguez-Tajes S, Navascués CA, García-Samaniego J, Riveiro-Barciela M, Lens S, Rodríguez M, Esteban R, Buti M. Long-term clinical outcomes in patients with chronic hepatitis delta: the role of persistent viraemia. *Aliment Pharmacol Ther* 2020; **51**: 158-166 [PMID: 31721254 DOI: 10.1111/apt.15521]
33 **Spaan M**, Carey I, Bruce M, Shang D, Horner M, Dusheiko G, Agarwal K. Hepatitis delta genotype 5 is associated with favourable disease outcome and better response to treatment compared to genotype 1. *J Hepatol* 2020; **72**: 1097-1104 [PMID: 31981726 DOI: 10.1016/j.jhep.2019.12.028]
34 **Su CW**, Huang YH, Huo TI, Shih HH, Sheen IJ, Chen SW, Lee PC, Lee SD, Wu JC. Genotypes and viremia of hepatitis B and D viruses are associated with outcomes of chronic hepatitis D patients. *Gastroenterology* 2006; **130**: 1625-1635 [PMID: 16697726 DOI: 10.1053/j.gastro.2006.01.035]
35 **Farci P**, Niro GA. Hepatitis D. In: Dooley JS, Lok ASF, Garcia-Tsao G, Pinzani M. Sherlock's Diseases of the Liver and Biliary System. New York: John Wiley and Sons, 2018: 421-432
36 **Usman Z**, Velkov S, Protzer U, Roggendorf M, Frishman D, Karimzadeh H. HDVdb: A Comprehensive Hepatitis D Virus Database. *Viruses* 2020; **12** [PMID: 32422927 DOI: 10.3390/v12050538]
37 **Farci P**, Niro GA. Clinical features of hepatitis D. *Semin Liver Dis* 2012; **32**: 228-236 [PMID: 22932971 DOI: 10.1055/s-0032-1323628]
38 **Ferrante ND**, Lo Re V 3rd. Epidemiology, Natural History, and Treatment of Hepatitis Delta Virus Infection in HIV/Hepatitis B Virus Coinfection. *Curr HIV/AIDS Rep* 2020; **17**: 405-414 [PMID: 32607773 DOI: 10.1007/s11904-020-00508-z]
39 **Miao Z**, Zhang S, Ou X, Li S, Ma Z, Wang W, Peppelenbosch MP, Liu J, Pan Q. Estimating the Global Prevalence, Disease Progression, and Clinical Outcome of Hepatitis Delta Virus Infection. *J Infect Dis* 2020; **221**: 1677-1687 [PMID: 31778167 DOI: 10.1093/infdis/jiz633]
40 **Abbas Z**, Qadeer MA, Mandviwalla HA, Abbas M. The Severity of Hepatitis D in Young Adults of Age 18-25 Years. *Cureus* 2020; **12**: e10855 [PMID: 33052263 DOI: 10.7759/cureus.10855]
41 **Buti M**, Homs M, Rodriguez-Frias F, Funalleras G, Jardí R, Sauleda S, Tabernero D, Schaper M, Esteban R. Clinical outcome of acute and chronic hepatitis delta over time: a long-term follow-up study. *J Viral Hepat* 2011; **18**: 434-442 [PMID: 20546496 DOI: 10.1111/j.1365-2893.2010.01324.x]
42 **Olivero A**, Smedile A. Hepatitis delta virus diagnosis. *Semin Liver Dis* 2012; **32**: 220-227 [PMID: 22932970 DOI: 10.1055/s-0032-1323627]
43 **Alfaiate D**, Clément S, Gomes D, Goossens N, Negro F. Chronic hepatitis D and hepatocellular carcinoma: A systematic review and meta-analysis of observational studies. *J Hepatol* 2020; **73**: 533-539 [PMID: 32151618 DOI: 10.1016/j.jhep.2020.02.030]
44 **Gu XH**, Chen Z, Dai RY, Zhang ML, Tang HM, Chen LB, Dong B. Analysis on the clinical features of 507 HDV-infected patients. *Cell Biochem Biophys* 2014; **70**: 1829-1832 [PMID: 25085619 DOI: 10.1007/s12013-014-0137-8]
45 **Da BL**, Surana P, Kleiner DE, Heller T, Koh C. The Delta-4 fibrosis score (D4FS): A novel fibrosis score in chronic hepatitis D. *Antiviral Res* 2020; **174**: 104691 [PMID: 31837393 DOI: 10.1016/j.antiviral.2019.104691]
46 **Lutterkort GL**, Wranke A, Yurdaydin C, Budde E, Westphal M, Lichtinghagen R, Stift J, Bremer B, Hardtke S, Keskin O, Idilman R, Koch A, Manns MP, Dienes HP, Wedemeyer H, Heidrich B. Non-invasive fibrosis score for hepatitis delta. *Liver Int* 2017; **37**: 196-204 [PMID: 27428078 DOI: 10.1111/liv.13205]
47 **Farci P**, Anna Niro G. Current and Future Management of Chronic Hepatitis D. *Gastroenterol Hepatol (N Y)* 2018; **14**: 342-351 [PMID: 30166948]
48 **Bockmann JH**, Grube M, Hamed V, von Felden J, Landahl J, Wehmeyer M, Giersch K, Hall MT, Murray JM, Dandri M, Lüth S, Lohse AW, Lütgehetmann M, Schulze Zur Wiesch J. High rates of cirrhosis and severe clinical events in patients with HBV/HDV co-infection: longitudinal analysis of a German cohort. *BMC Gastroenterol* 2020; **20**: 24 [PMID: 32000689 DOI: 10.1186/s12876-020-1168-9]
49 **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]
50 **Brancaccio G**, Fasano M, Grossi A, Santantonio TA, Gaeta GB. Clinical outcomes in patients with hepatitis D, cirrhosis and persistent hepatitis B virus replication, and receiving long-term tenofovir or entecavir. *Aliment Pharmacol Ther* 2019; **49**: 1071-1076 [PMID: 30793345 DOI: 10.1111/apt.15188]
51 **Ji J**, Sundquist K, Sundquist J. A population-based study of hepatitis D virus as potential risk factor for hepatocellular carcinoma. *J Natl Cancer Inst* 2012; **104**: 790-792 [PMID: 22423008 DOI: 10.1093/jnci/djs168]
52 **European Association for the Study of the Liver**. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]
53 **Wedemeyer H**, Yurdaydìn C, Dalekos GN, Erhardt A, Çakaloğlu Y, Değertekin H, Gürel S, Zeuzem S, Zachou K, Bozkaya H, Koch A, Bock T, Dienes HP, Manns MP; HIDIT Study Group.

Peginterferon plus adefovir versus either drug alone for hepatitis delta. *N Engl J Med* 2011; **364**: 322-331 [PMID: 21268724 DOI: 10.1056/NEJMoa0912696]

54 **Wedemeyer H**, Yurdaydin C, Hardtke S, Caruntu FA, Curescu MG, Yalcin K, Akarca US, Gürel S, Zeuzem S, Erhardt A, Lüth S, Papatheodoridis GV, Keskin O, Port K, Radu M, Celen MK, Idilman R, Weber K, Stift J, Wittkop U, Heidrich B, Mederacke I, von der Leyen H, Dienes HP, Cornberg M, Koch A, Manns MP; HIDIT-II study team. Peginterferon alfa-2a plus tenofovir disoproxil fumarate for hepatitis D (HIDIT-II): a randomised, placebo controlled, phase 2 trial. *Lancet Infect Dis* 2019; **19**: 275-286 [PMID: 30833068 DOI: 10.1016/S1473-3099(18)30663-7]

55 **Yurdaydin C**, Keskin O, Kalkan Ç, Karakaya F, Çaliskan A, Kabaçam G, Önder FO, Karatayli S, Karatayli E, Deda X, Bozkaya H, Bozdayi AM, Idilman R. Interferon Treatment Duration in Patients With Chronic Delta Hepatitis and its Effect on the Natural Course of the Disease. *J Infect Dis* 2018; **217**: 1184-1192 [PMID: 29425306 DOI: 10.1093/infdis/jix656]

56 **Abdrakhman A**, Ashimkhanova A, Almawi WY. Effectiveness of pegylated interferon monotherapy in the treatment of chronic hepatitis D virus infection: A meta-analysis. *Antiviral Res* 2021; **185**: 104995 [PMID: 33321155 DOI: 10.1016/j.antiviral.2020.104995]

57 **Niro GA**, Ciancio A, Tillman HL, Lagget M, Olivero A, Perri F, Fontana R, Little N, Campbell F, Smedile A, Manns MP, Andriulli A, Rizzetto M. Lamivudine therapy in chronic delta hepatitis: a multicentre randomized-controlled pilot study. *Aliment Pharmacol Ther* 2005; **22**: 227-232 [PMID: 16091060 DOI: 10.1111/j.1365-2036.2005.02542.x]

58 **Abbas Z**, Abbas M. Management of hepatitis delta: Need for novel therapeutic options. *World J Gastroenterol* 2015; **21**: 9461-9465 [PMID: 26327754 DOI: 10.3748/wjg.v21.i32.9461]

59 **Heidrich B**, Yurdaydın C, Kabaçam G, Ratsch BA, Zachou K, Bremer B, Dalekos GN, Erhardt A, Tabak F, Yalcin K, Gürel S, Zeuzem S, Cornberg M, Bock CT, Manns MP, Wedemeyer H; HIDIT-1 Study Group. Late HDV RNA relapse after peginterferon alpha-based therapy of chronic hepatitis delta. *Hepatology* 2014; **60**: 87-97 [PMID: 24585488 DOI: 10.1002/hep.27102]

60 **Keskin O**, Wedemeyer H, Tüzün A, Zachou K, Deda X, Dalekos GN, Heidrich B, Pehlivan S, Zeuzem S, Yalçın K, Gürel S, Tabak F, Idilman R, Bozkaya H, Manns M, Yurdaydin C. Association Between Level of Hepatitis D Virus RNA at Week 24 of Pegylated Interferon Therapy and Outcome. *Clin Gastroenterol Hepatol* 2015; **13**: 2342-49. e1-2 [PMID: 26044319 DOI: 10.1016/j.cgh.2015.05.029]

61 **Niro GA**, Smedile A, Fontana R, Olivero A, Ciancio A, Valvano MR, Pittaluga F, Coppola N, Wedemeyer H, Zachou K, Marrone A, Fasano M, Lotti G, Andreone P, Iacobellis A, Andriulli A, Rizzetto M. HBsAg kinetics in chronic hepatitis D during interferon therapy: on-treatment prediction of response. *Aliment Pharmacol Ther* 2016; **44**: 620-628 [PMID: 27443972 DOI: 10.1111/apt.13734]

62 **Katsoulidou A**, Manesis E, Rokka C, Issaris C, Pagoni A, Sypsa V, Hatzakis A. Development and assessment of a novel real-time PCR assay for quantitation of hepatitis D virus RNA to study viral kinetics in chronic hepatitis D. *J Viral Hepat* 2013; **20**: 256-262 [PMID: 23490370 DOI: 10.1111/jvh.12000]

63 **Rizzetto M**, Smedile A. Pegylated interferon therapy of chronic hepatitis D: in need of revision. *Hepatology* 2015; **61**: 1109-1111 [PMID: 25348580 DOI: 10.1002/hep.27585]

64 **Manesis EK**, Vourli G, Dalekos G, Vasiliadis T, Manolaki N, Hounta A, Koutsounas S, Vafiadis I, Nikolopoulou G, Giannoulis G, Germanidis G, Papatheodoridis G, Touloumi G. Prevalence and clinical course of hepatitis delta infection in Greece: a 13-year prospective study. *J Hepatol* 2013; **59**: 949-956 [PMID: 23850875 DOI: 10.1016/j.jhep.2013.07.005]

65 **Wranke A**, Serrano BC, Heidrich B, Kirschner J, Bremer B, Lehmann P, Hardtke S, Deterding K, Port K, Westphal M, Manns MP, Cornberg M, Wedemeyer H. Antiviral treatment and liver-related complications in hepatitis delta. *Hepatology* 2017; **65**: 414-425 [PMID: 27770553 DOI: 10.1002/hep.28876]

66 **Fukano K**, Tsukuda S, Watashi K, Wakita T. Concept of Viral Inhibitors *via* NTCP. *Semin Liver Dis* 2019; **39**: 78-85 [PMID: 30809790 DOI: 10.1055/s-0038-1676804]

67 **Bordier BB**, Ohkanda J, Liu P, Lee SY, Salazar FH, Marion PL, Ohashi K, Meuse L, Kay MA, Casey JL, Sebti SM, Hamilton AD, Glenn JS. In vivo antiviral efficacy of prenylation inhibitors against hepatitis delta virus. *J Clin Invest* 2003; **112**: 407-414 [PMID: 12897208 DOI: 10.1172/JCI17704]

68 **Vaillant A**. Nucleic acid polymers: Broad spectrum antiviral activity, antiviral mechanisms and optimization for the treatment of hepatitis B and hepatitis D infection. *Antiviral Res* 2016; **133**: 32-40 [PMID: 27400989 DOI: 10.1016/j.antiviral.2016.07.004]

69 **Slijepcevic D**, Kaufman C, Wichers CG, Gilglioni EH, Lempp FA, Duijst S, de Waart DR, Elferink RP, Mier W, Stieger B, Beuers U, Urban S, van de Graaf SF. Impaired uptake of conjugated bile acids and hepatitis b virus pres1-binding in na(+) -taurocholate cotransporting polypeptide knockout mice. *Hepatology* 2015; **62**: 207-219 [PMID: 25641256 DOI: 10.1002/hep.27694]

70 **Blank A**, Markert C, Hohmann N, Carls A, Mikus G, Lehr T, Alexandrov A, Haag M, Schwab M, Urban S, Haefeli WE. First-in-human application of the novel hepatitis B and hepatitis D virus entry inhibitor myrcludex B. *J Hepatol* 2016; **65**: 483-489 [PMID: 27132172 DOI: 10.1016/j.jhep.2016.04.013]

71 **Bogomolov P**, Alexandrov A, Voronkova N, Macievich M, Kokina K, Petrachenkova M, Lehr T, Lempp FA, Wedemeyer H, Haag M, Schwab M, Haefeli WE, Blank A, Urban S. Treatment of chronic hepatitis D with the entry inhibitor myrcludex B: First results of a phase Ib/IIa study. *J Hepatol* 2016; **65**: 490-498 [PMID: 27132170 DOI: 10.1016/j.jhep.2016.04.016]

72 **Wedemeyer H**, Bogomolov P, Blank A, Allweiss L, Dandri-Petersen M, Bremer B, Voronkova N,

Schöneweis K, Pathil A, Burhenne J, Haag M, Schwab M, Haefeli WE, Wiesch JSZ, Alexandrov A, Urban S. Final results of a multicenter, open-label phase 2b clinical trial to assess safety and efficacy of Myrcludex in combination with Tenofovir in patients with chronic HBV/HDV co-infection. *J Hepatol* 2018; **68**: S3 [DOI: 10.1016/S0168-8278(18)30224-1]

73 **Wedemeyer H**, Schöneweis K, Bogomolov PO, Voronkova N, Chulanov VP, Stepanova T, Bremer B, Allweiss L, Dandri M, Burhenne J, Haefeli WE, Ciesek S, Dittmer U, Alexandrov A, Urban S. Final results of a multicenter, open-label phase 2 clinical trial (MYR203) to assess safety and efficacy of myrcludex B with Peg-Interferon Alpha 2a in patients with chronic HBV/HDV co-infection. *J Hepatol* 2019; **70**: e81-e132

74 **Koh C**, Canini L, Dahari H, Zhao X, Uprichard SL, Haynes-Williams V, Winters MA, Subramanya G, Cooper SL, Pinto P, Wolff EF, Bishop R, Ai Thanda Han M, Cotler SJ, Kleiner DE, Keskin O, Idilman R, Yurdaydin C, Glenn JS, Heller T. Oral prenylation inhibition with lonafarnib in chronic hepatitis D infection: a proof-of-concept randomised, double-blind, placebo-controlled phase 2A trial. *Lancet Infect Dis* 2015; **15**: 1167-1174 [PMID: 26189433 DOI: 10.1016/S1473-3099(15)00074-2]

75 **Yurdaydin C**, Keskin O, Kalkan Ç, Karakaya F, Çalişkan A, Karatayli E, Karatayli S, Bozdayi AM, Koh C, Heller T, Idilman R, Glenn JS. Optimizing lonafarnib treatment for the management of chronic delta hepatitis: The LOWR HDV-1 study. *Hepatology* 2018; **67**: 1224-1236 [PMID: 29152762 DOI: 10.1002/hep.29658]

76 **Yurdaydin C**, Idilman R, Keskin O, Kalkan Ç, Karakaya MF, Caliskan A, Yurdcu E, Karatayli S, Bozdayi M, Koh C, Heller T, Glenn JS. A phase 2 dose-optimization study of lonafarnib with ritonavir for the treatment of chronic delta hepatitis end of treatment results from the LOWR HDV-2 study. *J Hepatol* 2017; **66**: S33- S34

77 **Koh C**, Surana P, Han T, Pryzek N, Kapuria D, Etzion O, Takyar V, Rotman Y, Canales R, Dahari H, Yurdaydin C, Glenn JS, Heller T. A phase 2 study exploring once daily dosing of ritonavir boosted lonafarnib for the treatment of chronic delta hepatitis-end of study results from the LOWR HD-3 study. *J Hepatol* 2017; **66**: S101-102

78 **Wedemeyer H**, Port K, Deterding K, Wranke A, Kirschner J, Bruno B, Martins B, Glenn JS, Kornberg M, Manns MP. A phase 2 dose-escalation study of lonafarnib plus ritonavir in patients with chronic hepatitis D: final results from the Lonafarnib with ritonavir in HDV-4 (LOWR HDV-4) study. *J Hepatol* 2017; **66**: S24 [DOI: 10.1016/S0168-8278(17)30310-0]

79 **Noordeen F**, Scougall CA, Grosse A, Qiao Q, Ajilian BB, Reaiche-Miller G, Finnie J, Werner M, Broering R, Schlaak JF, Vaillant A, Jilbert AR. Therapeutic Antiviral Effect of the Nucleic Acid Polymer REP 2055 against Persistent Duck Hepatitis B Virus Infection. *PLoS One* 2015; **10**: e0140909 [PMID: 26560490 DOI: 10.1371/journal.pone.0140909]

80 **Blanchet M**, Sinnathamby V, Vaillant A, Labonté P. Inhibition of HBsAg secretion by nucleic acid polymers in HepG2.2.15 cells. *Antiviral Res* 2019; **164**: 97-105 [PMID: 30771404 DOI: 10.1016/j.antiviral.2019.02.009]

81 **Bazinet M**, Pântea V, Cebotarescu V, Cojuhari L, Jimbei P, Albrecht J, Schmid P, Le Gal F, Gordien E, Krawczyk A, Mijočević H, Karimzadeh H, Roggendorf M, Vaillant A. Safety and efficacy of REP 2139 and pegylated interferon alfa-2a for treatment-naive patients with chronic hepatitis B virus and hepatitis D virus co-infection (REP 301 and REP 301-LTF): a non-randomised, open-label, phase 2 trial. *Lancet Gastroenterol Hepatol* 2017; **2**: 877-889 [PMID: 28964701 DOI: 10.1016/S2468-1253(17)30288-1]

82 **Bazinet M**, Pântea V, Cebotarescu V, Cojuhari L, Jimbei P, Anderson M, Gersch J, Holzmayer V, Elsner C, Krawczyk A, Kuhns MC, Cloherty G, Dittmer U, Vaillant A. Persistent control of Hepatitis B virus and Hepatitis Delta Virus infection following REP 2139-Ca and Pegylated Interferon therapy in chronic hepatitis B virus/hepatitis delta virus coinfection. *Hepatol Commun* 2020; **4**: 1-14 [DOI: 10.1002/hep4.1633]

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MINIREVIEWS

Usefulness of artificial intelligence in gastric neoplasms

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Abstract

Recently, studies in many medical fields have reported that image analysis based on artificial intelligence (AI) can be used to analyze structures or features that are difficult to identify with human eyes. To diagnose early gastric cancer, related efforts such as narrow-band imaging technology are on-going. However, diagnosis is often difficult. Therefore, a diagnostic method based on AI for endoscopic imaging was developed and its effectiveness was confirmed in many studies. The gastric cancer diagnostic program based on AI showed relatively high diagnostic accuracy and could differentially diagnose non-neoplastic lesions including benign gastric ulcers and dysplasia. An AI system has also been developed that helps to predict the invasion depth of gastric cancer through endoscopic images and observe the stomach during endoscopy without blind spots. Therefore, if AI is used in the field of endoscopy, it is expected to aid in the diagnosis of gastric neoplasms and determine the application of endoscopic therapy by predicting the invasion depth.

Key Words: Artificial intelligence; Convolutional neural network; Gastric neoplasm; Esophagogastroduodenoscopy; Diagnosis; Invasion depth

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Core Tip: Recently, image analysis based on artificial intelligence (AI) has been applied in the field of diagnostic endoscopy in gastroenterology, and active research is also being conducted on gastric neoplasms. Several studies reported that AI-based early gastric cancer diagnosis and the prediction of invasion depth showed excellent performance and that the differential diagnosis from non-neoplastic lesions including benign gastric ulcers was possible. Therefore, if AI is used in clinical practice, it can be expected to help diagnose gastric neoplasms and determine treatment methods.

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Grade B (Very good): B, B
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Grade D (Fair): 0
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INTRODUCTION

Gastric cancer is the fifth most common malignant neoplasm in the world and the third most common cause of cancer-related death[1,2]. Although advanced gastric cancer (AGC) is associated with poor outcomes, the detection of early gastric cancer (EGC) can improve survival up to 90%[1,3]. Endoscopy is the most important tool for detecting and diagnosing gastric cancer. However, the accuracy of detection relies upon the expertise and experience of the endoscopist and complex factors of the gastrointestinal (GI) tract. Accordingly, endoscopy techniques and related fields such as image-enhanced endoscopy have been developed to improve the diagnosis of EGC. Since its introduction in the 1950s, artificial intelligence (AI) such as deep learning (DL) has experienced remarkable progress in the last decade, and many researchers have studied the application of AI not only in the field of medical imaging but also in predicting patient prognosis based on medical records[4,5]. Many studies have utilized AI in endoscopic diagnosis. The application of AI in colonoscopy has significantly improved the adenoma detection rate (29.1% *vs* 20.3%, $P < 0.001$), and can even differentiate whether a detected polyp is non-neoplastic or neoplastic[6,7]. Based on such advancements, companies have already adapted AI for use in colonoscopy. Medtronic developed the GI Genius™ Intelligent Endoscopy Module that utilizes AI for the detection of colon polyps in real-time colonoscopy, while Olympus developed the EndoBRAIN-EYE[8]. In addition, Pentax and Fuji released the PENTAX medical Discovery™ and computer-assisted diagnosis (CAD)-EYE, respectively. Many studies have also been conducted in the field of AI in esophagogastroduodenoscopy (EGD). Thus, this article aimed to review recent developments and the use of AI in gastric neoplasms focusing on EGC, which has its unique characteristics among various GI diseases.

AI TECHNOLOGY

AI refers to machines that can do complex tasks like humans by imitating the cognitive functions of human intelligence such as learning and problem-solving (Figure 1). It was first introduced in 1955 and has been rapidly integrated into modern technologies and medicine[9]. Five subfields are included in AI, machine learning (ML), artificial neural network (ANN), natural language processing, DL, and computer vision[10]. ML is a field of AI where large amounts of data and algorithms are incorporated into the machine, and the machine automatically learns the input data by analyzing its patterns. Although the machine is capable of learning data patterns, the process still requires a certain amount of human instruction. DL is an important technique among many methods of ML, which is a process where the machine collects, analyzes, and processes data without receiving human instructions. Using massive amounts of data, the machine creates a learning model by extracting the key features of the given data. ANNs are the core technology of DL, and just as the human brain structure is formed by groups of neurons, the learning model of ML connects several computational nodes into several layers composed of an input layer, an output layer, and one or more hidden layers between them (Figure 2). The simplest type of neural network is called a perceptron, which consists of one input layer and one output node. The weight is a concept that gives a certain amount of importance to each input. The perceptron creates an output using inputs and weights. When an input is received, a weighted sum is calculated according to the weight, and when the value satisfies a specific criterion (activation function), the result is returned as 1 or 0. Convolutional neural network (CNN) is a kind of ANN, an algorithm that automatically learns features from the data, used mainly for image recognition[11]. It is an advanced ML model designed to think similarly to the human brain using large image datasets to learn patterns in correlating images. CNN is typically composed of three types of layers that extract features of the image and those that classify the data[12]. The convolution and pooling layers extract features of the image, while the fully connected layer is responsible for

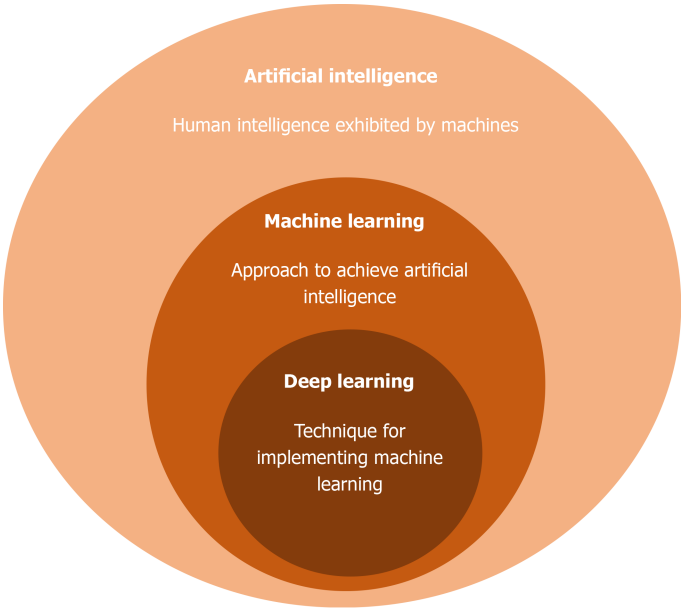


Figure 1 Overview of artificial intelligence, machine learning, and deep learning. Artificial intelligence refers to machines that can do complex tasks like humans by imitating human intelligence. One of the most important ways to achieve artificial intelligence is machine learning. Machine can learn by itself from the data provided to make accurate decisions. Deep learning is an important technique among many methods of machine learning. It is a kind of artificial neural network and learns data through an information input/output layer similar to neurons in the brain.

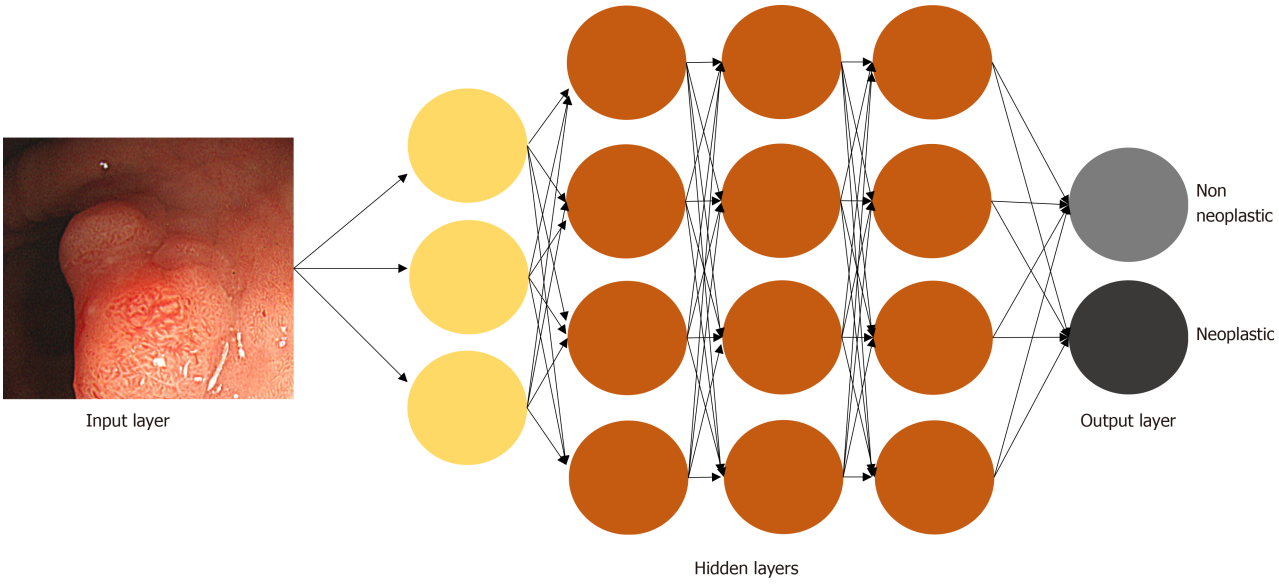


Figure 2 Illustrative model of artificial neural network. Once endoscopic image is selected as input layer, hidden layers are connected to next layer. Through this network, the input image is classified into output layer.

mapping them into output. The convolutional layer is a key in CNN, typically composed of a filter and an activation function. Using the image as input data, the filter extracts features of the image, and the activation function converts the value to a non-linear value. The CNN has multiple network layers of consecutive convolutional layers after pooling layers, and many filters are used as the input image is processed into consecutive convolutional layers. The extracted features are accumulated and become more complex to determine the characteristics of the input image. Subsequently, classification is performed through the fully connected layers, which are the last layers of CNN (Figure 3). As terms appearing in CNNs, one epoch refers to one forward and backward passes of the entire dataset to update the weight. The batch size is the number of training examples processed at one forward and backward pass, and iteration refers to the number of batches to complete one epoch.

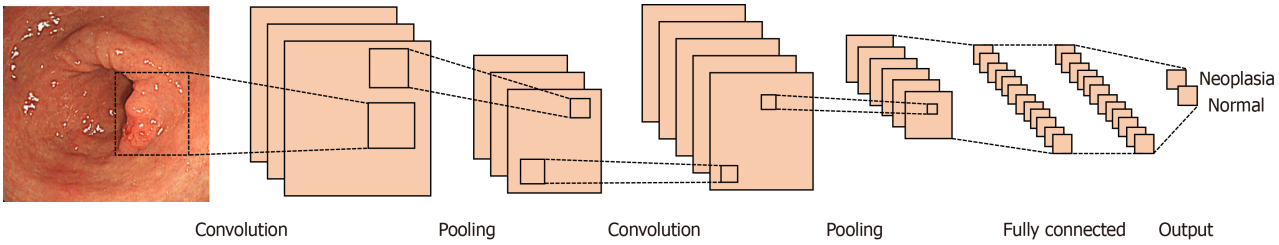


Figure 3 Overview of convolutional neural network. It is composed of stacks of convolutional layers, pooling layers, and fully connected layers. Convolutional and pooling layer extract features of input images, while fully connected layers make output based on classification.

Most examinations and diagnosis of GI tract diseases are performed through endoscopy and endoscopic imaging is one of the most effective applications of AI-based analytics in the field of medicine[9]. The use of CNN is ideal for endoscopic image recognition to detect and localize GI neoplasms. An AI algorithm learns what a neoplasm looks like in an endoscopic image using an image labeled by an endoscopist. After training, the CNN is tested on non-labeled new images to which it has not been previously exposed to and it is validated that the model can correctly identify previously unseen neoplasms. As a result, the algorithm can identify what it believes is a neoplasm in a real-time endoscopic video feed.

AI IN GASTRIC NEOPLASMS

Detection of gastric neoplasms

The detection of early-stage stomach cancer and precancerous lesions is essential to improving survival. Endoscopy is the most important and widely used detection tool for gastric cancer screening but since it is a manual procedure performed by an endoscopist, it is prone to technical and cognitive errors depending upon the endoscopist. EGC lesions usually show subtle changes of mucosa, such as elevation, depression, and redness. Moreover, they are surrounded by chronic inflammation or intestinal metaplasia. Therefore, there is a possibility of missing the subtle changes seen in the early forms of gastric cancer, especially in countries where the incidence of gastric cancer is low and where training is limited. Previous studies reported false-negative rates for detecting gastric cancer ranging between 4.6% and 25.8%[13-17]. A method to improve diagnostic accuracy involves the use of image-enhanced endoscopy such as narrow-band imaging (NBI) and blue laser imaging, which are more effective than conventional white light imaging alone[18,19]. However, such an optical diagnosis requires substantial expertise and experience[20], hindering its general use in gastroscopy. The 5-year survival rate of gastric cancer patients is highly correlated with the stage of gastric cancer at the time of diagnosis. Thus, it is paramount to improve the detection rates of EGC. Many groups have already started integrating AI into their routine practice to improve the overall detection rates of gastric cancer. AI-assisted evaluation can provide a better objective approach to improving diagnostic accuracy and avoiding unnecessary biopsies[10]. A list of studies using AI in gastric neoplasms is summarized in Table 1.

To evaluate the diagnostic accuracy of AI in the detection of gastric cancer, Hirasawa *et al*[21] used a CNN-based algorithm called the Single Shot MultiBox Detector to train using 13584 endoscopic images of gastric cancer, then tested using 2296 images (714 with confirmed gastric cancer) from 69 patients[21]. The overall sensitivity was 92.2% in the detection of gastric cancer, and the process took 47 s to analyze 2296 test images. The CNN accurately detected all invasive gastric cancer images. The detection rate for lesions larger than 6 mm was 98.6% while invasive cancers were all identified by AI. However, in the case of minute cancers that are less than 5 mm, 1 out of 6 (16.7 %) was detected, and 161 non-neoplastic lesions were included in the total 232 lesions that were machine-identified as gastric cancer, which produced a lower positive predictive value (PPV) of 30.6%[21]. The most common cause of false-positive lesions was gastritis with a change in color tone or irregular mucosal surface, which are sometimes difficult to distinguish even by endoscopists, and the next most common cause was normal structures such as cardia, pylorus, and angle. Ishioka *et al*[22] applied the same algorithm to video images collected from 62 patients who underwent endoscopic submucosal dissection (ESD) for EGC[22]. When

Table 1 Recently published articles on application of artificial intelligence in gastric neoplasms

Ref.	Purpose	AI type	Endoscopy type	Subjects	Outcomes
Detection of gastric neoplasms					
Hirasawa *et al* [21], 2018	Detect EGC	CNN (SSD)	Conventional endoscopy	Training: 13584 images; Test: 2296 images from 69 patients.	Sensitivity 92.2%, PPV 30.6%
Ishioka *et al*[22], 2019	Real time detection of EGC	CNN (SSD)	Conventional endoscopy	Live video of 62 patients	Accuracy 94.1%, median time 1 s (range: 0-44 s)
Sakai *et al*[23], 2018	Detect EGC	CNN	Conventional endoscopy	Training: 348943 images; Test: 9650 images	Accuracy 82.8%
Kanesaka *et al* [24], 2018	Detect EGC	SVM	M-NBI	Training: 126 images; Test: 81 images	Accuracy 96.3%, sensitivity 96.7%, specificity 95%
Li *et al*[25], 2020	Detect EGC	CNN (Inception-v3)	M-NBI	Training: 2088 images; Test: 341 images	Accuracy 91.2%, sensitivity 90.6%, specificity 90.9%
Horiuchi *et al* [26], 2020	Classifying EGC from gastritis	CNN (GoogLeNet)	M-NBI	Training: 2570 images; Test: 258 images.	Accuracy 85.3%, sensitivity 95.4%, specificity 71.0%, test speed 51.83 images/s (0.02 s/image)
Horiuchi *et al* [27], 2020	Detect EGC	CNN (GoogLeNet)	M-NBI	174 videos	Accuracy 85.1%, AUC 0.8684, sensitivity 87.4%, specificity 82.8%, PPV 83.5%, NPV 86.7%
Luo *et al*[28], 2019	Real time detection of EGC	GRAIDS	Conventional endoscopy	1036496 images from 84424 patients	Sensitivity (0.942) similar to the expert (0.945), superior to the competent (0.858) and the trainee (0.722) endoscopist
Ikenoyama *et al* [29], 2021	Detect EGC	CNN (SSD)	WLI, NBI chromoendoscopy	Training: 13584 images; Test: 2940 images.	Sensitivity 58.4%, specificity 87.3%, PPV 26.0%, NPV 96.5%
Classification of gastric neoplasms					
Sun *et al*[30], 2018	Classify ulcers	DCNN	Conventional endoscopy	854 images	Accuracy 86.6%, sensitivity 90.8%, specificity 83.5%
Lee *et al*[31], 2019	Detect EGC and benign ulcer	CNN (ResNet50, Inception-v3, VGG16)	Conventional endoscopy	Training: 717 images; Test: 70 images	AUC 0.95, 0.97, and 0.85 in Inception, ResNet50, and VGG16
Cho *et al*[32], 2019	Detect AGC, EGC, dysplasia	CNN (Inception-v4, ResNet152, Inception-Resnet-v2)	Conventional endoscopy	5217 images from 1469 patients	Gastric cancer: accuracy 81.9%, AUC 0.877; Gastric neoplasm: accuracy 85.5%, AUC 0.927
Kim *et al*[33], 2020	Classify gastric mesenchymal tumors	CNN	Endoscopic ultrasonography	Training: 905 images; Test: 212 images.	Accuracy 79.2%, sensitivity 83.0%. specificity 75.5%
Prediction of invasion depth					
Kubota *et al*[39], 2012	Predict invasion depth	Back propagation	Conventional endoscopy	Training: 800 images; Test: 90 images	Accuracy 77.9%, 29.1%, 51.0% and 55.3% in T1, T2, T3, and T4 stage; Accuracy 68.9% and 63.6% in T1a and T1b stage
Zhu *et al*[40], 2019	Predict invasion depth	CNN (ResNet50)	Conventional endoscopy	Training: 790 images; Test: 203 images	AUC 0.94, overall accuracy 89.2%, sensitivity 76.5%, specificity 95.6%
Yoon *et al*[41], 2019	Detect cancer, and predict invasion depth	CNN (VGG16, Grad-CAM)	Conventional endoscopy	11539 images	Detection AUC 0.981, depth prediction AUC 0.851 (undifferentiated type histology with a lower accuracy)
Cho *et al*[43], 2020	Predict invasion depth	CNN (Inception-ResNet-v2, DenseNet-161)	Conventional endoscopy	Training: 2899 images, test: 206 images	Internal validation: accuracy 84.1%, AUC 0.887; External validation: accuracy 77.3%, AUC 0.887
Nagao *et al*[44], 2020	Predict invasion depth	CNN (ResNet50)	WLI, NBI, indigo-carmine	16557 images from 1084 cases of gastric cancer	WLI: AUC 0.9590, sensitivity 89.2%, specificity 98.7%, accuracy 94.4%, PPV 98.3%, NPV 91.7%; NBI: AUC

					0.9048; Indigo-carmine: AUC 0.9191
Blind-spot monitoring					
Wu *et al*[48], 2019	Detect blind spot	DCNN	Conventional endoscopy	34513 images	Accuracy of detecting blind spot: 90.0%; Blind spot rate: 5.9%
Wu *et al*[49], 2019	Detect EGC and blind spot	DCNN	Conventional endoscopy	24549 images	Accuracy 92.5%, sensitivity 94.0%, specificity 91.0%, PPV 91.3%, NPV 93.8%
Chen *et al*[50], 2020	Detect blind spot	DCNN	Conventional endoscopy, U-TOE	Live video of 437 patients	Blind spot rate with AI: Sedated C-EGD, 3.4%; unsedated U-TOE, 21.8%; unsedated C- EGD, 31.2%

AI: Artificial intelligence; EGC: Early gastric cancer; CNN: Convolutional neural network; SSD: Single Shot MultiBox Detector; PPV: Positive predict value; SVM: Support vector machine; M-NBI: Magnified narrow-band imaging; AUC: Area under curve; GRAIDS: Gastrointestinal Artificial Intelligence Diagnostic System; WLI: White light imaging; NPV: Negative predict value; DCNN: Deep convolutional neural network; VGG: Visual Geometry Group; AGC: Advanced gastric cancer; Grad-CAM: Gradient-weighted class activation mapping; U-TOE: ultrathin transoral endoscopy; C-EGD: conventional esophagogastroduodenoscopy.

applied to the live video images, the diagnostic accuracy was 94.1%, and the median time for lesion detection was one second. Although the accuracy was low in minute cancers, AI showed great performance in lesions larger than 6 mm which looked very promising. In another study, Sakai *et al*[23] trained a CNN-based system with 348943 images (with data augmentation) obtained from 58 patients and tested 9650 images [23]. The accuracy of detecting gastric cancer by AI was 82.8%, and the image processing time was 4 ms *per* image.

Gastric cancer has many visual features that are challenging for endoscopists to describe. To improve diagnostic accuracy during endoscopy, several techniques have been developed to assist the gastroenterologist. Magnified NBI (M-NBI) has been shown to have higher detection rates for EGC, however, many endoscopists are not trained to confidently use M-NBI. To facilitate detection using M-NBI, Kanesaka *et al* [24] developed a CAD system to help diagnose EGC using only M-NBI images[24]. They used support vector machine to train with 66 EGC images and 60 non-cancer images, then tested detection and delineation of gastric cancer with 61 EGC and 20 non-cancer images. They reported an accuracy of 96.3%, a PPV of 98.3%, a sensitivity of 96.7%, and a specificity of 95%. Their CAD processed each image in 0.41 s[24]. In a related study, Li *et al*[25] used 386 non-cancerous M-NBI images and 1702 M-NBI images of EGC to train the Inception-v3 CNN model and tested 341 endoscopic images [25]. The sensitivity, specificity, and accuracy for the detection of EGC were 91.2%, 90.6%, and 90.9% respectively[25]. In another study by Horiuchi *et al*[26], the 22-layer GoogLeNet CNN model was trained using 1492 M-NBI images of EGC and 1078 M-NBI images of gastritis, then tested on 258 images (151 images of EGC)[26]. Further, the authors tried to determine if the differentiation between gastritis and cancer was possible. The reported accuracy for the detection of cancer was 85.3%. The sensitivity was 95.4%, the specificity was 71.0%, the PPV was 82.3%, and the negative predictive value (NPV) was 91.7%. The CNN falsely diagnosed 31 gastritis images as cancers, which were reported to have localized atrophy, atrophy of the fundic gland, and intestinal metaplasia[26]. The diagnostic performance of the same model was evaluated using 174 endoscopic videos (87 cancers and 87 non-cancers)[27]. The area under the curve (AUC) was 0.8684 and the accuracy, sensitivity, specificity, PPV, and NPV were 85.1%, 87.4%, 82.8%, 83.5%, and 86.7%, respectively. When compared to 11 experts, CAD was significantly more accurate than two experts, and not significantly different from eight experts[27].

As other studies were single-center results, or limited in the number of included cases, Luo *et al*[28] conducted a multi-center, case-controlled study of real-world endoscopic imaging to evaluate the accurate diagnosis of upper GI cancer with a CNN [28]. Using 157207 images obtained from 18765 participants from one university cancer center, the authors developed and validated the Gastrointestinal AI Diagnostic System (GRAIDS) algorithm through training, intrinsic verification, and internal validation. Then, they tested the performance of GRAIDS using a prospective validation dataset and additional external validation datasets obtained from five other hospitals, which included 879289 images from 65659 participants. The AUC in the external validation of the five participating hospitals ranged from 0.966 (95%CI: 0.965-0.967) to 0.990 (95%CI: 0.990-0.991)[28]. When compared to the diagnostic accuracy of the endoscopists, the diagnostic accuracy of the GRAIDS was 0.928 (95%CI: 0.919-0.937), which was

significantly lower than the diagnostic accuracy of 0.967 (95%CI: 0.961-0.973; $P < 0.0001$) of the expert endoscopist (professor with more than 10 years of endoscopic experience) and 0.956 (95%CI: 0.949-0.963; $P < 0.0001$) of the competent endoscopist (attending doctor with more than five years of endoscopic experience), but significantly higher than the diagnostic accuracy of 0.886 (95%CI: 0.875-0.897; $P < 0.0001$) of the trainee endoscopist (resident with two years of endoscopic experience). The sensitivity of the GRAIDS was not significantly different from the expert [0.942 (95%CI: 0.924-0.957) *vs* 0.945 (95%CI: 0.927-0.959); $P = 0.692$]. When compared to the competent expert [0.858 (95%CI: 0.832-0.880), $P < 0.0001$] and the trainee endoscopist [0.722 (95%CI: 0.691-0.752), $P < 0.0001$], the sensitivity of the GRAIDS was confirmed to be superior. The PPV of the GRAIDS was 0.814 (95%CI: 0.788-0.838), the expert endoscopist was 0.932 (95%CI: 0.913-0.948), the competent endoscopist was 0.974 (95%CI: 0.960-0.984), and the trainee endoscopist was 0.824 (95%CI: 0.795-0.850). The PPV of the GRAIDS was lower than that of the expert and the competent endoscopist but was similar to that of the trainee. These problems are mainly because the GRAIDS misinterprets normal structures (the pylorus, angle, mucus, gastric wall elevation during peristalsis, *etc.*) as lesions, and validation was conducted with data that had a low prevalence (3.8%-9.5%) in upper GI cancer. However, it seems that normal structures can be easily distinguished by the endoscopist and confirmed as false positives. This was a notable study that used more than one million images obtained from more than 80000 patients from different centers in China. A study by Ikenoyama *et al*[29] also compared the diagnostic accuracy of AI to that of endoscopists[29]. The AI model from the previous study by Hirasawa *et al*[21]. was tested on images obtained from 75 patients with gastric cancer [66 with mucosal cancer (T1a), and nine with submucosal cancer], and the diagnostic accuracy was compared to that of 67 endoscopists (33 board-certified endoscopists with more than 18 years of experience, and 34 uncertified endoscopists with about eight years of experience). The sensitivity, specificity, PPV, and NPV of the CNN were 58.4%, 87.3%, 26.0%, and 96.5% respectively. Compared to the CNN, the endoscopists showed a sensitivity, specificity, PPV, and NPV of 31.9%, 97.2%, 46.2%, and 94.9%, respectively, which showed that the CNN had significantly higher sensitivity than the endoscopists. Also, the average time it took for the CNN to evaluate an image was 45.5 ± 1.8 s, which was much faster than the 173.0 ± 66.0 min taken by an endoscopist and suggested that AI accurately diagnosed EGC at a much higher speed[29].

Classification of gastric neoplasms

While many studies have tested the diagnostic accuracy of AI in differentiating cancerous lesions from normal mucosa, several attempts at classifying other non-cancerous lesions have been made (Table 1). Sun *et al*[30] created a network-based model that could classify ulcers into different types (benign ulcers or malignant ulcers) with a performance comparable to that of endoscopists[30]. The study reported that the DL model was able to identify and classify ulcers with a total accuracy of 86.6%, which was comparable to that of the endoscopist with the highest accuracy (86.3%) and higher than that of the endoscopist with the lowest accuracy (62.5%)[30]. Lee *et al*[31] developed a model that could distinguish gastric ulcers and malignancy[31]. Using the Inception-v3 network, ResNet50 and the Visual Geometry Group (VGG) Net to classify normal *vs* cancer, normal *vs* benign ulcers, and cancer *vs* benign ulcers, 180 normal images, 200 ulcer images, and 337 cancer images were used for training. When tested on 20 normal, 20 ulcers, and 30 cancer images, the best performance was observed in ResNet50, with a diagnostic accuracy of 0.9649 for differentiating between normal *vs* cancer, 0.9262 for differentiating between normal *vs* ulcers, and 0.7712 for differentiating between cancer *vs* ulcers. Based on such findings, AI was proposed as an efficient means for the classification of endoscopic images[23]. Cho *et al*[32] made a novel attempt at developing a DL model that could automatically classify gastric neoplasms using conventional endoscopic images[32]. Using 5017 images from 1269 participants, three CNN architectures (Inception-v4, ResNet-152, and Inception-ResNet-v2) were used to train and validate the classification of conventional endoscopic images. The images were classified into two categories from two perspectives, which were cancer *vs* non-cancer, and neoplasm *vs* non-neoplasm. All images were grouped into five categories, AGC, EGC, high-grade dysplasia (HGD), low-grade dysplasia (LGD), and non-neoplasm. To compare the diagnostic accuracy, six endoscopists with experience with more than 6000 endoscopies also viewed and classified the endoscopic images. The Inception-ResNet-v2 model was reported to have the best performance at classifying the images into the five categories, with an accuracy of 84.6% (95%CI: 83.69-85.5) and a mean classification time of 0.0264 s. The AUC was highest for the detection of AGC (range: 0.802-0.855) and the lowest for

HGD (range: 0.491-0.522). In prospective validation, the performance of Inception-ResNet-v2 was not significantly inferior to that of the endoscopist with the worst performance. However, the endoscopist with the highest performance showed significantly better performance with a diagnostic accuracy of 87.6% (95%CI: 84.3-90.9) compared to 76.4% (95%CI: 72.1-80.7) for Inception-ResNet-v2. This suggested that AI could have the potential for classifying endoscopic lesions into several categories[32]. A recent study by Kim *et al*[33] assessed the ability of the CNN model to classify gastric mesenchymal tumors using endoscopic ultrasonography (EUS) images[33]. Using 905 EUS images from gastric mesenchymal tumors that were histologically confirmed by either resection or EUS-guided fine-needle biopsy, the CNN-CAD system was developed and validation was performed with 212 EUS images. The reported accuracy for detecting gastrointestinal stromal tumors was 79.2% using the CNN-CAD system, with a sensitivity and specificity of 83.0% and 75.5%, respectively. The performance was compared to that of six endoscopists (three experienced endoscopists who performed more than 500 EUS examinations, and three junior endoscopists who performed less than 200 EUS examinations). When compared to the diagnostic accuracy of the endoscopists, the sensitivity of CNN-CAD system was not significantly different from that of any of the endoscopists. The specificity and diagnostic accuracy of CNN-CAD system were significantly higher than that of two experienced endoscopists and one junior endoscopist[33], which suggested the potential application of AI in the classification of EUS images as well.

Prediction of invasion depth

The prediction of the invasion depth of gastric cancer (T-staging) is very important as it is an essential factor in determining the treatment method and prognosis of EGC. Tumors in the early stages that do not involve lymphovascular invasion and have an invasion depth no deeper than 500 μm of submucosa can be treated by endoscopic resection alone[34]. The gross findings of the tumor seen on endoscopy or EUS are used to determine the invasion depth of EGC. Some studies have reported that conventional endoscopy was comparable to EUS in predicting the invasion depth of EGC[35,36]. The reported overall accuracy of invasion depth using conventional endoscopy ranged between 69% and 79%[35,37]. In a study of depth prediction scores for differentiated EGCs, tumor sizes more than 30 mm, marked redness, an uneven surface, and marginal elevation were associated with deeper submucosal cancers[38]. However, gastric cancer depth can be difficult to determine by endoscopy alone and some patients may undergo surgery when endoscopic resection could have been an effective method of treatment. To overcome such problems, the utilization of AI to determine the depth of invasion has been studied (Table 1). Kubota *et al*[39] used retrospectively collected 902 conventional endoscopic gastric cancer images from 344 patients who underwent surgery or endoscopic resection to train and validate with a backpropagation algorithm for determining the depth of invasion[39]. The overall accuracy for detecting the depth of invasion was 64.7%, with 77.2% at the T1 stage (68.9% for T1a and 63.6% for T1b), 49.1% at the T2 stage, 51.0% at the T3 stage, and 55.3% at the T4 stage. This computer-aided system suggested a novel approach of using AI to determine cancer invasion depth by endoscopy[39]. Zhu *et al*[40] used 790 images from gastric cancer patients to train and another 203 images to validate ResNet50. The overall accuracy was reported to be 89.2%, which was significantly higher than the overall accuracy of 77.5% of the experienced endoscopists. The AUC for AI was 0.94 (95%CI: 0.90-0.97), and the sensitivity, specificity, PPV, and NPV were 76.5%, 95.6%, 89.7%, and 89.0%, respectively[40]. To test AI in the diagnostic accuracy for EGC stages, Yoon *et al*[41,42] included 800 patients, 428 patients with T1a and 372 patients with T1b histology-proven EGC, and selected 11539 images (896 T1a images, 809 T1b-EGC images, and 9834 non-cancer images) to train and validate the lesion-based VGG16-network and gradient-weighted class activation mapping (Grad-CAM) [41,42]. The overall AUC for EGC detection and invasion depth prediction was 0.981 and 0.851, respectively. Interestingly, the study also analyzed the factors affecting the AI prediction of invasion depth. The images of undifferentiated-type histology were associated with inaccurate predictions of invasion depth, especially in T1b cases[41]. As previous studies used already diagnosed gastric cancer images for training and testing, Cho *et al*[43] used Inception-ResNet-v2 and DenseNet-161 models to test the diagnostic accuracy of gastric neoplasms and invasion depth[43]. The authors used 2899 conventional endoscopic images obtained from 846 patients with confirmed pathology including LGD, HGD, EGC, and AGC. The AUC and diagnostic accuracy for determining the invasion depth were 0.887 and 77.3%, respectively, in the external validation set for the DenseNet-161 model. When applied to clinical simulation, the AI misdiagnosed only two cases that had submucosa invasion (misdiagnosed as mucosal

lesions), which were also misdiagnosed by the endoscopists. In 89 patients who underwent surgery, 11 cases were actually mucosal-confined lesions, among which AI correctly classified six cases as mucosal lesions. The authors developed an algorithm with substantial performance in predicting invasion depth from the endoscopic images of neoplasms[43]. As other studies used images obtained from conventional endoscopy, Nagao *et al*[44] retrospectively collected 16557 gastric cancer images from 1084 cases to train and validate ResNet50 for predicting invasion depth by conventional white light, non-magnifying NBI, and indigo-carmine stained images[44]. The AUC using white light imaging, NBI, and indigo-carmine stain imaging were reported to be 0.9590, 0.9048, and 0.9481 respectively, and the lesion-based accuracy for predicting invasion depth using white light imaging, NBI, and indigo-carmine were 94.5%, 94.3%, and 95.5%, respectively[44].

Blind-spot monitoring

Observing the whole stomach is a basic prerequisite for the diagnosis of gastric cancer at an early stage. To avoid blind spots, standardized procedures and guidelines have been made to map the entire stomach during gastroscopy. The European Society of Gastrointestinal Endoscopy published a protocol including 10 images of the stomach while the systematic screening protocol for the stomach published by Japanese researchers suggested 22 standard images of the stomach to avoid missing suspicious cancerous lesions[45,46]. However, insufficient supervision and the lack of practical tools make it difficult to follow protocols, which is related to the quality of endoscopic examinations[47]. To localize blind spots during EGD that may have been missed by an endoscopist, Wu *et al*[48] developed the WISENSE system, a real-time CNN to detect blind spots (Table 1)[48]. As the scope was inserted into the stomach, the deep CNN (DCNN) captured images and filled them into the corresponding part of the model, which enabled the endoscopist to identify the blind spots. These blind spots of the gastric mucosa, such as the lesser curvature of the antrum and the fundus, are areas that may hide lesions. If blind spots are not viewed during endoscopy, lesions could be missed. Trained on 34513 images of gastric locations agreed upon by at least four endoscopists, WISENSE was able to detect blind spots with an accuracy of 90.0% by identifying anatomic landmarks in EGD. In a single-center randomized control trial, 153 patients had their blind spots detected by WISENSE *vs* 150 in the control group without AI. The blind spot rate, defined as the proportion of the number of unobserved sites in 26 sites, was 5.9% in the WISENSE group which was significantly less than 22.5% in the control group ($P < 0.001$), suggesting that AI can also be used to improve the quality of EGD by identifying blind spots[48]. In another study by Wu *et al*[49], a DCNN was used for detecting gastric cancer and identifying blind spots. There were 24549 images used for training, and a grid model for the stomach was developed to generate a virtual stomach model[49]. The study reported a diagnostic accuracy of 92.5% for detecting malignancy, which was significantly higher than that of six experts. The reported sensitivity, specificity, PPV, and NPV were 94.0%, 91.0%, 91.3%, and 93.8%, respectively. The DCNN correctly identified the EGD images into 10 parts with an accuracy of 90.0% and into 26 parts with an accuracy of 65.9%, which was not significantly different from those of the endoscopists. When the model was tested on endoscopic videos, the DCNN accurately presented the covered parts synchronized with the process of EGD to verify that the entire stomach was mapped [49]. A related study by Chen *et al*[50] used ENDOANGEL (developed from WISENSE) to compare blind-spot monitoring in sedated conventional EGD (C-EGD), unsedated ultrathin transoral endoscopy (U-TOE), and unsedated C-EGD[50]. This prospective, 3-parallel-group, randomized study reported that the blind-spot rate with AI was significantly lower in sedated C-EGD compared with unsedated U-TOE and unsedated C-EGD (sedated C-EGD *vs* unsedated U-TOE *vs* unsedated CEGD: 3.4% *vs* 21.8% *vs* 31.2%, $P < 0.05$). Although the number of studies is limited, the application of AI in monitoring blind spots is very promising.

Prediction of curative endoscopic resection

Expanded indications for ESD in EGC include the undifferentiated type that is less than 2 cm and does not have ulcerations. However, observational studies have reported conflicting results. Thus, ESD in such groups has been considered an investigational treatment[34]. A meta-analysis of curative resection for EGC with undifferentiated type histology reported a rate of 61.4%, suggesting ESD as a feasible treatment for undifferentiated-type EGC[51]. To aid in the accurate prediction of curative resection in such cases, Bang *et al*[52] selected ML models that could predict curative resection in undifferentiated-type EGC. The XGBoost classifier presented the best performance with an accuracy of 81.5% in the first external validation and 89.8% in the

second external validation[52]. The size of the lesion was the most important feature that could be explained by AI analysis. As such, AI could aid in decisions for therapeutic management.

FUTURE PERSPECTIVES OF AI

The real-time application of AI in the field of medicine is within reach. Endoscopic models that automatically detect colon polyps or gastric cancers during endoscopy sessions and highlight them using segmentation box have already received approval for use in Europe, Japan, and other countries, while many systems are currently under development[8]. Many software codes have been provided as open-source codes, which can be freely utilized in research or actual practice. Architectures can be modified by fine-tuning an already established pre-trained model by adjusting layers of the ANN, increasing the learning epoch, adjusting the batch size, adjusting the iteration, or modifying hyperparameters such as the optimizer. Aside from adjusting the complex algorithms to optimize the model, recent developments have enabled the automatic optimization of hyperparameters in ML (*i.e.*, AutoGluon) that makes AI more user-friendly and easier to use for clinicians unfamiliar with AI[52]. Most research on AI for gastroenterology has focused on developing algorithms for the detection of lesions, the classification of images to improve diagnostic accuracy, predicting prognosis, and to improve the quality of screening endoscopy. In the near future, AI will most likely be applied to therapeutic management. Recently, AI-based treatment methods have been developed using technologies such as microendoscopy, decision support system-based treatment modalities, robot-assisted treatment, application, and digital therapeutics[53]. However, such development comes with social issues other than technology, such as patient safety, ethics, legal responsibility, government approval, and cost-effectiveness, which need to be addressed as well. Although studies have shown that accuracy of detecting gastric cancer by AI is comparable to some doctors, experienced doctors with expertise have shown better performance than AI. This means that there is limitation to relying solely on AI alone. However, beneficial factors from application of AI, such as improved efficacy and time spent on repetitive task, must be acknowledged as well. Accordingly, the most applicable field of AI would be medical image data processing that could aid in improved diagnostic performance of trainees and non-expert doctors. The AI algorithm, especially DL, is comparable to a black box that learns from training data. Using the patterns learned from the training data, the output values can be predicted from newly input data. This means that efficacy and accuracy are highly dependent upon the quality and quantity of the training data. Like any other clinical research, the quality and quantity of the usable data are undeniably essential in proving the quality of the evidence and the outcome. It is important to gather high-quality clinical data, while developing a model that accurately tests the data is equally important. To effectively utilize such an AI algorithm in clinical practice, further studies and discussions on the usefulness, profitability, possible risks, medicolegal responsibility, and regulatory measures of AI are needed.

CONCLUSION

AI in the field of endoscopy was first applied for the detection of colon polyps. As described in this review article, many studies have already been published as stepping-stones toward the application of AI in detecting gastric neoplasms such as EGC. As there is a lack of such prospective studies in the detection of EGC, randomized controlled studies are needed to advance the technique. It is expected that the application of AI would not only provide guidelines for the endoscopic treatment of EGC or avoid unnecessary surgery by predicting the invasion depth but also help improve the overall prognosis of patients with EGC. There is no doubt that the development of AI-based endoscopy would also help to alleviate physical fatigue that can be a burden to endoscopists. Such achievements can only be done when the application of AI can improve the quality of imaging diagnosis beyond that of human capability, and optical biopsy is possible. This is possible by improving AI performance using the specific characteristics of different organs and diseases. AI is being studied and developed by scientists all over the world in various fields with hopes of providing accuracy and convenience. In the field of medicine, medical records and imaging are becoming digitalized and a new phase in the history of

medicine is expected within five to 10 years. Accordingly, clinicians and researchers need to carefully approach and evaluate the results of further clinical studies using AI-based technology with great interest.

REFERENCES

1 **Sano T**, Coit DG, Kim HH, Roviello F, Kassab P, Wittekind C, Yamamoto Y, Ohashi Y. Proposal of a new stage grouping of gastric cancer for TNM classification: International Gastric Cancer Association staging project. *Gastric Cancer* 2017; **20**: 217-225 [PMID: 26897166 DOI: 10.1007/s10120-016-0601-9]

2 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

3 **Amin MB**, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, Meyer L, Gress DM, Byrd DR, Winchester DP. The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin* 2017; **67**: 93-99 [PMID: 28094848 DOI: 10.3322/caac.21388]

4 **Erickson BJ**, Korfiatis P, Akkus Z, Kline TL. Machine Learning for Medical Imaging. *Radiographics* 2017; **37**: 505-515 [PMID: 28212054 DOI: 10.1148/rg.2017160130]

5 **Heo J**, Yoon JG, Park H, Kim YD, Nam HS, Heo JH. Machine Learning-Based Model for Prediction of Outcomes in Acute Stroke. *Stroke* 2019; **50**: 1263-1265 [PMID: 30890116 DOI: 10.1161/STROKEAHA.118.024293]

6 **Song EM**, Park B, Ha CA, Hwang SW, Park SH, Yang DH, Ye BD, Myung SJ, Yang SK, Kim N, Byeon JS. Endoscopic diagnosis and treatment planning for colorectal polyps using a deep-learning model. *Sci Rep* 2020; **10**: 30 [PMID: 31913337 DOI: 10.1038/s41598-019-56697-0]

7 **Wang P**, Berzin TM, Glissen Brown JR, Bharadwaj S, Becq A, Xiao X, Liu P, Li L, Song Y, Zhang D, Li Y, Xu G, Tu M, Liu X. Real-time automatic detection system increases colonoscopic polyp and adenoma detection rates: a prospective randomised controlled study. *Gut* 2019; **68**: 1813-1819 [PMID: 30814121 DOI: 10.1136/gutjnl-2018-317500]

8 **Attardo S**, Chandrasekar VT, Spadaccini M, Maselli R, Patel HK, Desai M, Capogreco A, Badalamenti M, Galtieri PA, Pellegatta G, Fugazza A, Carrara S, Anderloni A, Occhipinti P, Hassan C, Sharma P, Repici A. Artificial intelligence technologies for the detection of colorectal lesions: The future is now. *World J Gastroenterol* 2020; **26**: 5606-5616 [PMID: 33088155 DOI: 10.3748/wjg.v26.i37.5606]

9 **Abadir AP**, Ali MF, Karnes W, Samarasena JB. Artificial Intelligence in Gastrointestinal Endoscopy. *Clin Endosc* 2020; **53**: 132-141 [PMID: 32252506 DOI: 10.5946/ce.2020.038]

10 **Jin P**, Ji X, Kang W, Li Y, Liu H, Ma F, Ma S, Hu H, Li W, Tian Y. Artificial intelligence in gastric cancer: a systematic review. *J Cancer Res Clin Oncol* 2020; **146**: 2339-2350 [PMID: 32613386 DOI: 10.1007/s00432-020-03304-9]

11 **Ebigbo A**, Palm C, Probst A, Mendel R, Manzeneder J, Prinz F, de Souza LA, Papa JP, Siersema P, Messmann H. A technical review of artificial intelligence as applied to gastrointestinal endoscopy: clarifying the terminology. *Endosc Int Open* 2019; **7**: E1616-E1623 [PMID: 31788542 DOI: 10.1055/a-1010-5705]

12 **Yamashita R**, Nishio M, Do RKG, Togashi K. Convolutional neural networks: an overview and application in radiology. *Insights Imaging* 2018; **9**: 611-629 [PMID: 29934920 DOI: 10.1007/s13244-018-0639-9]

13 **Amin A**, Gilmour H, Graham L, Paterson-Brown S, Terrace J, Crofts TJ. Gastric adenocarcinoma missed at endoscopy. *J R Coll Surg Edinb* 2002; **47**: 681-684 [PMID: 12463707]

14 **Hosokawa O**, Hattori M, Douden K, Hayashi H, Ohta K, Kaizaki Y. Difference in accuracy between gastroscopy and colonoscopy for detection of cancer. *Hepatogastroenterology* 2007; **54**: 442-444 [PMID: 17523293]

15 **Menon S**, Trudgill N. How commonly is upper gastrointestinal cancer missed at endoscopy? *Endosc Int Open* 2014; **2**: E46-E50 [PMID: 26135259 DOI: 10.1055/s-0034-1365524]

16 **Voutilainen ME**, Juhola MT. Evaluation of the diagnostic accuracy of gastroscopy to detect gastric tumours: clinicopathological features and prognosis of patients with gastric cancer missed on endoscopy. *Eur J Gastroenterol Hepatol* 2005; **17**: 1345-1349 [PMID: 16292088 DOI: 10.1097/00042737-200512000-00013]

17 **Yalamarthi S**, Witherspoon P, McCole D, Auld CD. Missed diagnoses in patients with upper gastrointestinal cancers. *Endoscopy* 2004; **36**: 874-879 [PMID: 15452783 DOI: 10.1055/s-2004-825853]

18 **Diao W**, Huang X, Shen L, Zeng Z. Diagnostic ability of blue laser imaging combined with magnifying endoscopy for early esophageal cancer. *Dig Liver Dis* 2018; **50**: 1035-1040 [PMID: 29685806 DOI: 10.1016/j.dld.2018.03.027]

19 **Dutta AK**, Sajith KG, Pulimood AB, Chacko A. Narrow band imaging *vs* white light gastroscopy in detecting potentially premalignant gastric lesions: a randomized prospective crossover study. *Indian J Gastroenterol* 2013; **32**: 37-42 [PMID: 22983839 DOI: 10.1007/s12664-012-0246-5]

20 **Ishihara R**, Takeuchi Y, Chatani R, Kidu T, Inoue T, Hanaoka N, Yamamoto S, Higashino K, Uedo

N, Iishi H, Tatsuta M, Tomita Y, Ishiguro S. Prospective evaluation of narrow-band imaging endoscopy for screening of esophageal squamous mucosal high-grade neoplasia in experienced and less experienced endoscopists. *Dis Esophagus* 2010; **23**: 480-486 [PMID: 20095991 DOI: 10.1111/j.1442-2050.2009.01039.x]

21 **Hirasawa T**, Aoyama K, Tanimoto T, Ishihara S, Shichijo S, Ozawa T, Ohnishi T, Fujishiro M, Matsuo K, Fujisaki J, Tada T. Application of artificial intelligence using a convolutional neural network for detecting gastric cancer in endoscopic images. *Gastric Cancer* 2018; **21**: 653-660 [PMID: 29335825 DOI: 10.1007/s10120-018-0793-2]

22 **Ishioka M**, Hirasawa T, Tada T. Detecting gastric cancer from video images using convolutional neural networks. *Dig Endosc* 2019; **31**: e34-e35 [PMID: 30449050 DOI: 10.1111/den.13306]

23 **Sakai Y**, Takemoto S, Hori K, Nishimura M, Ikematsu H, Yano T, Yokota H. Automatic detection of early gastric cancer in endoscopic images using a transferring convolutional neural network. *Annu Int Conf IEEE Eng Med Biol Soc* 2018; **2018**: 4138-4141 [PMID: 30441266 DOI: 10.1109/EMBC.2018.8513274]

24 **Kanesaka T**, Lee TC, Uedo N, Lin KP, Chen HZ, Lee JY, Wang HP, Chang HT. Computer-aided diagnosis for identifying and delineating early gastric cancers in magnifying narrow-band imaging. *Gastrointest Endosc* 2018; **87**: 1339-1344 [PMID: 29225083 DOI: 10.1016/j.gie.2017.11.029]

25 **Li L**, Chen Y, Shen Z, Zhang X, Sang J, Ding Y, Yang X, Li J, Chen M, Jin C, Chen C, Yu C. Convolutional neural network for the diagnosis of early gastric cancer based on magnifying narrow band imaging. *Gastric Cancer* 2020; **23**: 126-132 [PMID: 31332619 DOI: 10.1007/s10120-019-00992-2]

26 **Horiuchi Y**, Aoyama K, Tokai Y, Hirasawa T, Yoshimizu S, Ishiyama A, Yoshio T, Tsuchida T, Fujisaki J, Tada T. Convolutional Neural Network for Differentiating Gastric Cancer from Gastritis Using Magnified Endoscopy with Narrow Band Imaging. *Dig Dis Sci* 2020; **65**: 1355-1363 [PMID: 31584138 DOI: 10.1007/s10620-019-05862-6]

27 **Horiuchi Y**, Hirasawa T, Ishizuka N, Tokai Y, Namikawa K, Yoshimizu S, Ishiyama A, Yoshio T, Tsuchida T, Fujisaki J, Tada T. Performance of a computer-aided diagnosis system in diagnosing early gastric cancer using magnifying endoscopy videos with narrow-band imaging (with videos). *Gastrointest Endosc* 2020; **92**: 856-865. e1 [PMID: 32422155 DOI: 10.1016/j.gie.2020.04.079]

28 **Luo H**, Xu G, Li C, He L, Luo L, Wang Z, Jing B, Deng Y, Jin Y, Li Y, Li B, Tan W, He C, Seeruttun SR, Wu Q, Huang J, Huang DW, Chen B, Lin SB, Chen QM, Yuan CM, Chen HX, Pu HY, Zhou F, He Y, Xu RH. Real-time artificial intelligence for detection of upper gastrointestinal cancer by endoscopy: a multicentre, case-control, diagnostic study. *Lancet Oncol* 2019; **20**: 1645-1654 [PMID: 31591062 DOI: 10.1016/S1470-2045(19)30637-0]

29 **Ikenoyama Y**, Hirasawa T, Ishioka M, Namikawa K, Yoshimizu S, Horiuchi Y, Ishiyama A, Yoshio T, Tsuchida T, Takeuchi Y, Shichijo S, Katayama N, Fujisaki J, Tada T. Detecting early gastric cancer: Comparison between the diagnostic ability of convolutional neural networks and endoscopists. *Dig Endosc* 2021; **33**: 141-150 [PMID: 32282110 DOI: 10.1111/den.13688]

30 **Sun JY**, Sang WL, Kang MC, Kim SW, Ko SJ. A Novel Gastric Ulcer Differentiation System Using Convolutional Neural Networks. Proceedings of the 2018 IEEE 31st International Symposium on Computer-Based Medical Systems (CBMS); 2018 June 1; IEEE, 2018: 351-356

31 **Lee JH**, Kim YJ, Kim YW, Park S, Choi YI, Park DK, Kim KG, Chung JW. Spotting malignancies from gastric endoscopic images using deep learning. *Surg Endosc* 2019; **33**: 3790-3797 [PMID: 30719560 DOI: 10.1007/s00464-019-06677-2]

32 **Cho BJ**, Bang CS, Park SW, Yang YJ, Seo SI, Lim H, Shin WG, Hong JT, Yoo YT, Hong SH, Choi JH, Lee JJ, Baik GH. Automated classification of gastric neoplasms in endoscopic images using a convolutional neural network. *Endoscopy* 2019; **51**: 1121-1129 [PMID: 31443108 DOI: 10.1055/a-0981-6133]

33 **Kim YH**, Kim GH, Kim KB, Lee MW, Lee BE, Baek DH, Kim DH, Park JC. Application of A Convolutional Neural Network in The Diagnosis of Gastric Mesenchymal Tumors on Endoscopic Ultrasonography Images. *J Clin Med* 2020; **9** [PMID: 33003602 DOI: 10.3390/jcm9103162]

34 **Japanese Gastric Cancer Association**. Japanese gastric cancer treatment guidelines 2014 (ver. 4). *Gastric Cancer* 2017; **20**: 1-19 [PMID: 27342689 DOI: 10.1007/s10120-016-0622-4]

35 **Choi J**, Kim SG, Im JP, Kim JS, Jung HC, Song IS. Comparison of endoscopic ultrasonography and conventional endoscopy for prediction of depth of tumor invasion in early gastric cancer. *Endoscopy* 2010; **42**: 705-713 [PMID: 20652857 DOI: 10.1055/s-0030-1255617]

36 **Pei Q**, Wang L, Pan J, Ling T, Lv Y, Zou X. Endoscopic ultrasonography for staging depth of invasion in early gastric cancer: A meta-analysis. *J Gastroenterol Hepatol* 2015; **30**: 1566-1573 [PMID: 26094975 DOI: 10.1111/jgh.13014]

37 **Sano T**, Okuyama Y, Kobori O, Shimizu T, Morioka Y. Early gastric cancer. Endoscopic diagnosis of depth of invasion. *Dig Dis Sci* 1990; **35**: 1340-1344 [PMID: 2226095 DOI: 10.1007/BF01536738]

38 **Abe S**, Oda I, Shimazu T, Kinjo T, Tada K, Sakamoto T, Kusano C, Gotoda T. Depth-predicting score for differentiated early gastric cancer. *Gastric Cancer* 2011; **14**: 35-40 [PMID: 21327924 DOI: 10.1007/s10120-011-0002-z]

39 **Kubota K**, Kuroda J, Yoshida M, Ohta K, Kitajima M. Medical image analysis: computer-aided diagnosis of gastric cancer invasion on endoscopic images. *Surg Endosc* 2012; **26**: 1485-1489 [PMID: 22083334 DOI: 10.1007/s00464-011-2036-z]

40 **Zhu Y**, Wang QC, Xu MD, Zhang Z, Cheng J, Zhong YS, Zhang YQ, Chen WF, Yao LQ, Zhou PH, Li QL. Application of convolutional neural network in the diagnosis of the invasion depth of gastric

cancer based on conventional endoscopy. *Gastrointest Endosc* 2019; **89**: 806-815. e1 [PMID: 30452913 DOI: 10.1016/j.gie.2018.11.011]

41 **Yoon HJ**, Kim S, Kim JH, Keum JS, Oh SI, Jo J, Chun J, Youn YH, Park H, Kwon IG, Choi SH, Noh SH. A Lesion-Based Convolutional Neural Network Improves Endoscopic Detection and Depth Prediction of Early Gastric Cancer. *J Clin Med* 2019; **8** [PMID: 31454949 DOI: 10.3390/jcm8091310]

42 **Yoon HJ**, Kim JH. Lesion-Based Convolutional Neural Network in Diagnosis of Early Gastric Cancer. *Clin Endosc* 2020; **53**: 127-131 [PMID: 32252505 DOI: 10.5946/ce.2020.046]

43 **Cho BJ**, Bang CS, Lee JJ, Seo CW, Kim JH. Prediction of Submucosal Invasion for Gastric Neoplasms in Endoscopic Images Using Deep-Learning. *J Clin Med* 2020; **9** [PMID: 32549190 DOI: 10.3390/jcm9061858]

44 **Nagao S**, Tsuji Y, Sakaguchi Y, Takahashi Y, Minatsuki C, Niimi K, Yamashita H, Yamamichi N, Seto Y, Tada T, Koike K. Highly accurate artificial intelligence systems to predict the invasion depth of gastric cancer: efficacy of conventional white-light imaging, nonmagnifying narrow-band imaging, and indigo-carmine dye contrast imaging. *Gastrointest Endosc* 2020; **92**: 866-873. e1 [PMID: 32592776 DOI: 10.1016/j.gie.2020.06.047]

45 **Bisschops R**, Areia M, Coron E, Dobru D, Kaskas B, Kuvaev R, Pech O, Ragunath K, Weusten B, Familiari P, Domagk D, Valori R, Kaminski MF, Spada C, Bretthauer M, Bennett C, Senore C, Dinis-Ribeiro M, Rutter MD. Performance measures for upper gastrointestinal endoscopy: a European Society of Gastrointestinal Endoscopy (ESGE) Quality Improvement Initiative. *Endoscopy* 2016; **48**: 843-864 [PMID: 27548885 DOI: 10.1055/s-0042-113128]

46 **Yao K**. The endoscopic diagnosis of early gastric cancer. *Ann Gastroenterol* 2013; **26**: 11-22 [PMID: 24714327]

47 **Rutter MD**, Rees CJ. Quality in gastrointestinal endoscopy. *Endoscopy* 2014; **46**: 526-528 [PMID: 24788539 DOI: 10.1055/s-0034-1365738]

48 **Wu L**, Zhang J, Zhou W, An P, Shen L, Liu J, Jiang X, Huang X, Mu G, Wan X, Lv X, Gao J, Cui N, Hu S, Chen Y, Hu X, Li J, Chen D, Gong D, He X, Ding Q, Zhu X, Li S, Wei X, Li X, Wang X, Zhou J, Zhang M, Yu HG. Randomised controlled trial of WISENSE, a real-time quality improving system for monitoring blind spots during esophagogastroduodenoscopy. *Gut* 2019; **68**: 2161-2169 [PMID: 30858305 DOI: 10.1136/gutjnl-2018-317366]

49 **Wu L**, Zhou W, Wan X, Zhang J, Shen L, Hu S, Ding Q, Mu G, Yin A, Huang X, Liu J, Jiang X, Wang Z, Deng Y, Liu M, Lin R, Ling T, Li P, Wu Q, Jin P, Chen J, Yu H. A deep neural network improves endoscopic detection of early gastric cancer without blind spots. *Endoscopy* 2019; **51**: 522-531 [PMID: 30861533 DOI: 10.1055/a-0855-3532]

50 **Chen D**, Wu L, Li Y, Zhang J, Liu J, Huang L, Jiang X, Huang X, Mu G, Hu S, Hu X, Gong D, He X, Yu H. Comparing blind spots of unsedated ultrafine, sedated, and unsedated conventional gastroscopy with and without artificial intelligence: a prospective, single-blind, 3-parallel-group, randomized, single-center trial. *Gastrointest Endosc* 2020; **91**: 332-339. e3 [PMID: 31541626 DOI: 10.1016/j.gie.2019.09.016]

51 **Bang CS**, Baik GH, Shin IS, Kim JB, Suk KT, Yoon JH, Kim YS, Kim DJ, Shin WG, Kim KH, Kim HY, Lim H, Kang HS, Kim JH, Jung SW, Kae SH, Jang HJ, Choi MH. Endoscopic submucosal dissection for early gastric cancer with undifferentiated-type histology: A meta-analysis. *World J Gastroenterol* 2015; **21**: 6032-6043 [PMID: 26019470 DOI: 10.3748/wjg.v21.i19.6032]

52 **Bang CS**, Ahn JY, Kim JH, Kim YI, Choi IJ, Shin WG. Establishing Machine Learning Models to Predict Curative Resection in Early Gastric Cancer with Undifferentiated Histology: Development and Usability Study. *J Med Internet Res* 2021; **23**: e25053 [PMID: 33856358 DOI: 10.2196/25053]

53 **Bang CS**. Deep Learning in Upper Gastrointestinal Disorders: Status and Future Perspectives. *Korean J Gastroenterol* 2020; **75**: 120-131 [PMID: 32209800 DOI: 10.4166/kjg.2020.75.3.120]

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MINIREVIEWS

Current impact of viral hepatitis on liver cancer development: The challenge remains

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Abstract

Chronic infections due to hepatitis B and hepatitis C viruses are responsible for most cases of hepatocellular carcinoma (HCC) worldwide, and this association is likely to remain during the next decade. Moreover, viral hepatitis-related HCC imposes an important burden on public health in terms of disability-adjusted life years. In order to reduce such a burden, some major challenges must be faced. Universal vaccination against hepatitis B virus, especially in the neonatal period, is probably the most relevant primary preventive measure against the develo-

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pment of HCC. Moreover, considering the large adult population already infected with hepatitis B and C viruses, it is also imperative to identify these individuals to ensure their access to treatment. Both hepatitis B and C currently have highly effective therapies, which are able to diminish the risk of development of liver cancer. Finally, it is essential for individuals at high-risk of HCC to be included in surveillance programs, so that tumors are detected at an early stage. Patients with hepatitis B or C and advanced liver fibrosis or cirrhosis benefit from being followed in a surveillance program. As hepatitis B virus is oncogenic and capable of leading to liver cancer even in individuals with early stages of liver fibrosis, other high-risk groups of patients with hepatitis B are also candidates for surveillance. Considerable effort is required concerning these strategies in order to decrease the incidence and the mortality of viral hepatitis-related HCC.

Key Words: Hepatitis B virus; Hepatitis C virus; Hepatocellular carcinoma; Epidemiology; Vaccination; Surveillance

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Core Tip: Hepatitis B and C are associated with most cases of hepatocellular carcinoma, and it is estimated that this scenario will remain for the next decade. This review highlights the impact of viral hepatitis on the development of liver cancer, the characteristics of viral hepatitis-related hepatocellular carcinoma, and the challenges that must be faced in order to reduce their burden.

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INTRODUCTION

Viral infections are the leading cause of hepatitis, with hepatitis B virus (HBV) and hepatitis C virus (HCV) being the most important causes of chronic viral hepatitis worldwide. It was estimated that nearly 292 million individuals had chronic HBV infection in 2016 (prevalence of 3.9%) and 71 million people had chronic HCV infection in 2015 (prevalence of 1%)[1-3]. Hepatitis D virus (HDV) is a defective virus, requiring HBV coexistence in order to replicate. It was estimated that HDV caused chronic infection in approximately 12 million individuals in 2016[4]. Most HBV, HCV and HDV infections are concentrated in low- and lower middle-income countries[3].

The incidence of liver cancer is the sixth highest of all malignant neoplasms (905677 new cases in 2020, an incidence of 11.6/100000 inhabitants). Moreover, it is the second cause of cancer-related death in the world (830180 deaths in 2020, mortality of 10.7/100000 inhabitants)[5]. Hepatocellular carcinoma (HCC) accounts for approximately 75%-85% of primary liver cancers[6,7]. HCC frequently develops in patients with cirrhosis, with an annual incidence of 1%-4%[8]. HBV and HCV are currently the two most important risk factors for HCC development worldwide. This is reflected by the geographical distribution of liver cancer, which coincides with those of HBV and HCV, predominating in transitioning countries, particularly in Asia and Africa[6,7,9]. In a systematic review of 260 studies involving 119006 patients with HCC, the seroprevalence of HBV and/or HCV was over 60% in most of the 50 countries which contributed with data[10]. Furthermore, it is noteworthy that, according to the Global Burden of Disease Study, HBV-related liver cancer was associated with 5.80 million disability-adjusted life years, and HCV-related liver cancer, with 2.88 million disability-adjusted life years in 2019[11]. The impact of HBV may be further increased by the fact that HCC usually develops at an earlier age in patients infected with this virus[12].

Despite vaccination against HBV and effective treatments for HBV and HCV, it is estimated that their impact on liver health will remain for the next decade at least. Using data from 195 countries or territories between 1990 and 2017, a recent study proposed Bayesian models to project primary liver cancer incidence through 2030. The authors estimated that the age-standardized incidence rate of liver cancer will increase globally from 11.80 in 2017 to 14.08 per 100000 inhabitants in 2030. According to the etiology of liver disease, HBV was responsible for 46.5% of cases of liver cancer in 1990 and 42.4% in 2017, and it is estimated that it will be associated with 40.7% of cases in 2030. With regard to HCV, it was associated with 25.2% of cases of primary liver cancer in 1990 and 27.0% in 2017, and it is projected that it will be responsible for 26.8% of cases in 2030[13]. The aim of this article is to review the current impact of viral hepatitis on the development of liver cancer, the characteristics of viral hepatitis-related HCC and the challenges to reduce their burden (Figure 1).

IMMUNE ASPECTS OF VIRAL HEPATITIS IN HEPATOCARCINOGENESIS

In chronic viral hepatitis, the development of fibrosis, cirrhosis and HCC is linked to the activity of the immune response in the infected liver. Both HBV and HCV uniquely infect hepatocytes and replicate in a non-cytopathic manner. The ensuing liver damage that is observed during the acute and the chronic phase of infection is the consequence of virus-specific as well as non-specific immune activity within the inflamed liver. Histologically, active phases of chronic HBV and HCV infections are characterized by extensive infiltration around the portal tract areas consisting predominantly of CD4+ and CD8+ T cells, but these cells are incapable of clearing these viruses. Multiple mechanisms have been described that explain the weak activity of CD4+ and CD8+ T cells, and that are the main reasons for persistence of HBV and HCV in the liver, and the slowly progressing liver disease[14-16]. The mechanisms include impairment of dendritic cells and natural killer (NK) cells, increased production of immunosuppressive cytokines and an increase in the numbers of regulatory T cells. In addition, the continuous exposure of immune cells to high levels of viral antigens for many years further contributes and maintains the persistent infection due to exhaustion of HBV and HCV-specific T cells, which renders these cells functionally impaired mediated *via* triggering of exhaustion markers, such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and others[17].

In the liver, the balance between immune tolerance and immune activation is normally tightly controlled to ensure efficient elimination of pathogen products and transformed cells. Dysregulation of this balance due to viral infection has severe consequences for the process of immune surveillance that is highly efficient in the detection and elimination of transformed cells, and contributes to HCC development [18]. Also, alterations in lipid metabolism and the release of reactive oxygen species contribute to tumor initiation[19].

Interestingly, the status of tumor-infiltrating immune cells in HCC has a high degree of resemblance to that observed in an HBV or HCV inflamed liver: an increased T cell infiltrate is observed in HCC, but the CD8+ T cells are dysfunctional with low production of the cytolytic enzymes granzyme and perforin, low overall cytokine production, and relatively high expression of the exhaustion markers PD-1 and CTLA-4[20,21]. In addition, increased numbers of regulatory T cells and reduced numbers and functionality of NK cells have been reported by many groups[18,22,23]. These immune dysfunctions are generally more pronounced in the advanced HCC stages.

The importance of the immune system in controlling the development and progression to advanced stages of HCC is highlighted by observations that increased T cell infiltrates are associated with improved overall survival in HCC and lower tumor recurrence following resection[24-26]. Furthermore, the enrichment of exhausted T cells in HCC is associated with poorer survival, while higher numbers of infiltrating NK cells are associated with better survival. Using cytometry by time of flight and RNA sequencing, a recent study defined different immune subsets and showed that the tumor microenvironment in HBV-related HCC consisted of a more immunosuppressive and exhausted phenotype with an enrichment of regulatory T cells and resident memory T cells that differs from the non-viral-related HCC environment. The study further demonstrated that regulatory T cells are associated with poor prognosis, and memory T cells, with good prognosis[27].

The crucial importance of the patient's immune activity against the tumor has initiated the development of numerous immunotherapeutic approaches in recent years, all aimed at boosting anti-tumor immunity[28]. The approaches are diverse and

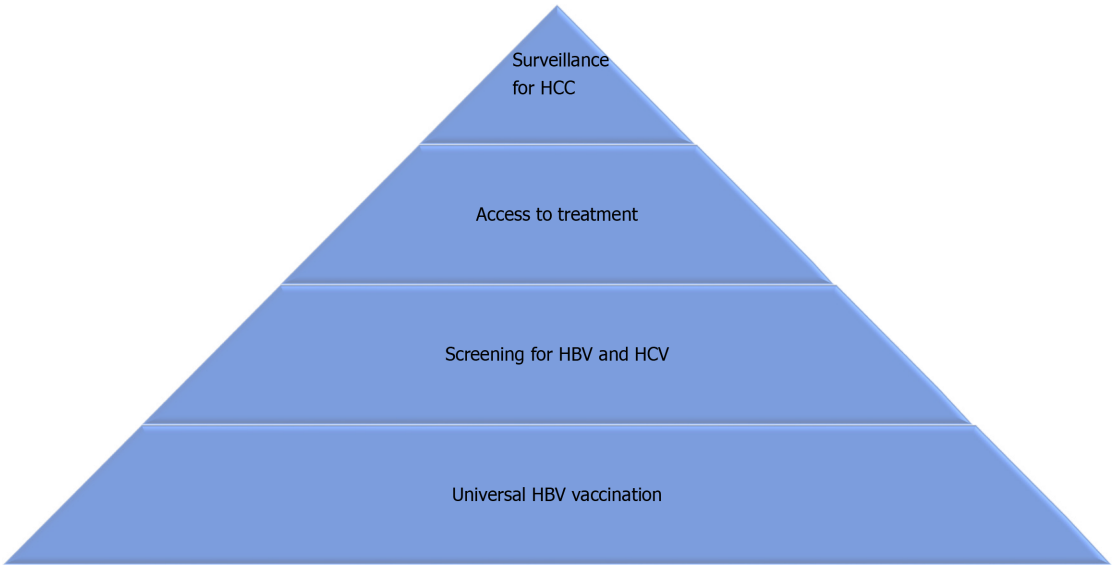


Figure 1 Major challenges to reduce the burden of viral hepatitis-related hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

include strategies using peptide vaccines and cell-based therapies. Moreover, therapies aimed at neutralizing exhaustion markers using immune checkpoint inhibitors have shown promising results and PD-1 blockade has been approved as therapy for HCC. However, many studies are ongoing to improve therapy success by examining the therapeutic outcomes using different combinations of checkpoint inhibitors.

HEPATITIS B AND HCC

Implications on HCC

Advances in next-generation sequencing technology and integrative studies combining multiomic approaches, encompassing genomic and epigenomic characterization and transcriptome, proteomic, and metabolomic analysis of HCCs have shown that HCC is heterogeneous at the histomolecular level, with variable molecular features and clinical outcomes[29-31]. While ongoing hepatic inflammation, increased cell turnover, repeated cycles of cell death and regeneration, DNA instability leading to an alteration in DNA and dysregulation of CpG island methylation are common pathways leading to HCC regardless of the underlying etiology of liver disease[32], there are HBV specific pathogenesis pathways in HCC development. HBV promotes HCC through viral DNA integration into host genes that can provide a growth advantage to the host cell. HBV has been found to be integrated into or near cancer-related genes[33,34]. For example, promoter mutation of telomerase reverse transcriptase, which is often integrated by HBV DNA, is the most frequent genetic event in HBV-associated HCC. The translated protein products of the viral HBV genome have also been implicated in promoting carcinogenesis. For instance, HBx protein, a regulatory protein required for viral replication, has been shown to regulate hepatocyte proliferation, regeneration and apoptosis by modulating the PI3K/Akt-mTOR, STAT3/Nanog, and Wnt/β-catenin signaling pathways to promote the progression of HCC[35-37].

Occult HBV infection

Occult HBV infection is a condition where HBV DNA replication occurs in the liver in patients with negative serum HBsAg[38]. While detection of anti-HBc (hepatitis B core antibody) in the blood indicates prior HBV infection, 20% of patients with occult HBV infection are seronegative for anti-HBc. HBV DNA analysis should be further pursued to confirm the diagnosis[39]. Occult HBV infection increases the risk of HCC development in patients with or without chronic liver disease, which is thought to be mediated by HBV DNA integration into the host genome, the production of pro-oncogenic proteins such as HBx protein, and persistent low-grade necroinflammation leading to fibrosis progression[39]. Currently, HCC surveillance is not routinely

recommended in patients with occult HBV infection in the absence of advanced fibrosis/cirrhosis.

HCC surveillance in hepatitis B

HCC continues to be one of the leading causes of cancer-related death throughout the world and it frequently develops within the context of cirrhosis[40]. This has also led to the development of guidelines by all the major liver societies for HCC surveillance in patients with cirrhosis given its potential for early-stage cancer detection, which likely links to curative treatment[41,42]. Given its high oncogenic potential and its tendency to develop HCC in the absence of cirrhosis, screening for HCC is indicated in a broader population of HBV patients. All HBV patients with HBV cirrhosis (except for patients with severe liver dysfunction and not eligible for liver transplantation) are recommended to undergo HCC surveillance. Surveillance is also recommended in a selected high-risk population of non-cirrhotic HBV patients, as demonstrated in Table 1[43].

Eradication of hepatitis B through vaccination

HBV vaccination is an effective primary prevention method for HCC. The effectiveness of HBV vaccination in reducing HCC incidence was reported in a nationwide population-based study from Taiwan. The study used data from 2 Taiwanese HCC registry systems and showed that HCC incidence was four-fold higher in the HBV unvaccinated cohort than in the vaccinated at birth cohorts. Among the 1509 patients who were 6-26 years old diagnosed with HCC from 1983 through 2011, 1343 were born before, and 166 were born after the HBV vaccination program began. Moreover, the relative risks for HCC in patients vaccinated at 6-9 years old, 10-14 years old, 15-19 years old, and 20-26 years old in comparison to those who were not vaccinated were 0.26 [95% confidence interval (CI): 0.17-0.40], 0.34 (95%CI: 0.25-0.48), 0.37 (95%CI: 0.25-0.51), and 0.42 (95%CI: 0.32-0.56), respectively[44]. Although neonatal HBV vaccination is recommended in most countries, vaccine coverage is still less than 70% in most African countries where the incidence rates of HCC remain high[45], providing a clear window of opportunity for improvement in primary prevention of HCC[40].

Major challenges

Aside from identifying infected patients, offering effective treatment against HBV and surveying those at high risk for HCC, the major challenge in order to reduce HBV-related HCC burden is widening the vaccination coverage against the virus. Despite the increase in vaccination coverage between 2010 and 2018 in 97% of the 194 member states of the World Health Organization (WHO), with an increment from 73% to 84% in the completion of the three-dose vaccination schedule and with an increase from 28% to 42% in the neonatal vaccination coverage in the 108 countries in which newborns are vaccinated, improvements are still necessary[46]. For instance, vaccination coverage requires improvement in countries such as Nigeria, India and Indonesia, which together are responsible for 50% of HBV infections in children under 5 years of age[3].

HEPATITIS C AND HCC

Implications on HCC

HCV is one of the most important risk factors for HCC. As previously mentioned, HCV is a non-cytopathic virus, which leads to liver damage through immune-mediated mechanisms. However, the virus itself also plays a role in the development of HCC, which becomes clear when considering, for instance, that HCV genotype 3 is associated with a higher risk of HCC than other genotypes[47]. The mechanism through which HCV genotype 3 is involved in hepatocarcinogenesis is not yet completely understood, but the down-regulation of the phosphatase and tensin homolog (*PTEN*) gene, which has a tumor suppressor role, might be implicated[48].

Direct-acting antiviral (DAA) therapy in HCV-infected patients has been shown to reduce all-cause mortality (including liver-related and unrelated causes), as well as the incidence of HCC in patients with cirrhosis[49]. However, in the subgroup of patients with advanced fibrosis or cirrhosis, the risk of developing HCC after achieving sustained virologic response (SVR) remains high, ranging from 0.3% to 1.8% per year [50]. For this reason, different guidelines recommend lifelong surveillance for patients with advanced fibrosis or cirrhosis even after SVR[51]. Interestingly, recent studies have attempted to differentiate subgroups of patients with cirrhosis at the highest risk

Table 1 Indications for surveillance of hepatocellular carcinoma in patients with hepatitis B without cirrhosis

Indications for surveillance
Asian males > 40 yr
Asian females > 50 yr
African ancestry
First degree relative with HCC
HDV co-infection

HCC: Hepatocellular carcinoma; HDV: Hepatitis D virus.

for HCC after SVR. In an Italian study, the combination of clinical predictors (male sex and diabetes), albumin, and genetics identified patients at high risk for HCC[52]. Furthermore, it is suggested that changes in liver stiffness measured by transient elastography at 1 and 3 years after the end of HCV treatment could identify patients at low risk for HCC[53].

Population screening for hepatitis C

In 2017, the WHO Global Hepatitis Report stated that 71 million people worldwide were infected by HCV[54]. About 3.5 million to 5.0 million of these individuals are children and adolescents, and 2.3 million are co-infected with the human immunodeficiency virus (HIV)[51,54]. HCV infection causes approximately 400000 deaths annually, mainly from cirrhosis-related complications and HCC[54]. Despite the current availability of DAA therapies with high rates of SVR, the prevalence of HCV infection has not changed. One of the reasons that explain this situation is that only 20% of individuals with HCV infection worldwide are aware of their diagnosis. Another reason is that access to DAA treatments continues to be limited mainly by costs in several countries[54].

The WHO Global Health Sector Strategy on Viral Hepatitis recommended that an effort should be made in order to increase the HCV diagnosis rate to 30% by 2020 and to 90% by 2030[55]. Overall, HCV screening consists in a universal, one-time, and opt-out screening strategy in adults 18 years of age and older with an upper age limit of 79 years. However, periodic screening tests should be offered to individuals with behaviors, conditions or circumstances associated with an increased risk of exposure to HCV[51].

Vertical transmission could be present in about 5% of deliveries from mothers with HCV and accounts for the majority of HCV infections in the pediatric population[56]. Universal prenatal screening for HCV facilitates better identification of at-risk infants who require HCV testing[57]. This evaluation results in better detection of HCV infection in the pediatric population and enables early therapeutic interventions.

Eradication of hepatitis C

DAA regimens have a high cure rate for HCV infection[51]. Furthermore, new formulations have simplified the duration and administration of the treatment. However, in 2015, only 7% of people diagnosed with HCV had started antiviral treatment with DAA[54]. According to the strategy suggested by the WHO, access to DAA should reach 80% of eligible people by 2030[55]. After this declaration, access to treatment has improved in different countries. An example is the strategy in Australia, where more than 80% of the HCV-infected population was diagnosed during the last two decades and where there is currently an unrestricted DAA access program that permits prescriptions by any registered medical practitioner. This allowed for the initiation of DAA treatment in Australia for approximately 70% of the total population with HCV-related cirrhosis between 2014 and 2017[58].

Simultaneously with screening and therapy access, different preventive measures must be carried out in people capable of transmitting HCV (*e.g.*, people who inject drugs) in order to avoid new infections or reinfections in those who have been previously treated successfully. Global coverage of harm reduction programs for people who inject drugs, including needle and syringe programs, is currently less than 10%[54].

Major challenges

Currently, the major challenge in order to reduce HCV-related HCC burden is identifying infected patients. The silent course of the early stages of this disease makes this challenge even harder to overcome. Therefore, efforts should be made in order to improve awareness of the population and health care workers regarding the underdiagnosis of HCV[54]. It is also of great importance to shorten the pathway leading from the diagnosis to the extremely effective treatments against HCV, which could even allow for subgroups of patients to be treated in primary care[59].

Yet another challenge relates to the coronavirus disease 2019 pandemic. It is estimated that the impact of the pandemic on the global efforts towards HCV eradication could lead to an excess of 44800 cases of HCC[60].

CO-INFECTIONS AND HCC

As previously mentioned, HBV and HCV infections are the most common risk factors for HCC worldwide[61]. Individuals living with HIV are a high-risk population for developing HCC, mostly as a consequence of HCV and HBV co-infection[62,63]. Indeed, HCC has become a major clinical problem in HIV. Studies from Europe suggest that the incidence of HCC has risen in HIV-infected patients over the last 20 years, and in Spain alone HCC is the second cause of death in HIV-HCV co-infected patients with cirrhosis[64]. Studies from both the United States and Europe suggest that HIV may hasten the evolution of HBV-related HCC, resulting in an earlier age of HCC presentation in HIV co-infected patients[65,66].

Although the implementation of new treatments for HCV with DAA agents is expected to decrease HCC incidence, new cases will continue to emerge in the near-medium term[67,68]. Moreover, a recent study that reported an HCC risk increase of 1% every year in HIV-HCV co-infected individuals in the HEPAVIR cohort in Europe included in their cohort 61% of individuals with cured HCV, indicating that this population remains at risk for HCC[63].

In HIV-HBV co-infection, a recent study from the EuroSIDA cohort showed a stable incidence of HCC in non-cirrhotic individuals under HBV therapy (tenofovir specifically), while increasing rates of HCC were seen over time in those not on tenofovir. This suggests a potential threshold to not survey the former group for HCC. However, the same study did show a continued increase in HCC risk among those that had cirrhosis, indicating the importance of surveillance in this population[69].

Most of the data reported on HIV and HCC originate in resource-rich settings. These dynamics related to poor outcomes are likely to be increased in resource-limited settings, where most of HIV infections occur. A small African study of 60 patients found that those infected with HIV developed HCC at a younger age (32 years) compared to those without HIV (49 years)[70], and a more recent study from South Africa showed the age of HCC development to be lower in those co-infected with HBV and HIV (mean age of 36 years *vs* 46 years in HIV-uninfected). Interestingly, this study showed that females were more impacted by HCC when co-infected with HIV, and the age of HCC presentation was much lower in females with HIV (mean age of 36 years *vs* 50 years in HIV-uninfected)[71]. In South America and Asia overall, data are scarce on the interrelation between HCC and HIV infection. The specific mechanisms underlying younger HCC occurrence in co-infected individuals with viral hepatitis and HIV is unclear. However, a large Swiss study found a direct association between CD4+ T cells and the risk of developing HCC, suggesting that impairment of the immune system could be implicated[72].

Surveillance for HCC represents a major challenge in co-infected populations not only due to the complex nature of dual disease, but also because most guidelines are tailored towards mono-infected individuals at risk for HCC. In addition, recent studies show that ultrasound surveillance has a low performance for HCC in HBV or HCV individuals co-infected with HIV, with a suboptimal 43% rate of early-stage diagnosis, compared to 63%-71% found in studies on HIV-uninfected cohorts[73,74].

Triple co-infection of HIV, HBV and HDV is rather uncommon, ranging from 1% to 20% depending on the geographical area[75]. However, studies performed in small cohorts have shown that the main impact of HDV on HBV-HIV disease is an accelerated pace to cirrhosis and hepatic decompensation[76]. In this context, a Swiss HIV cohort reported a 9-fold increase in HCC in those triple co-infected with HIV-HBV-HDV compared to HDV-negative individuals[77].

CONCLUSION

Despite the advances in prevention and treatment of viral hepatitis, it is clear that many challenges remain in order to reduce the burden of viral hepatitis-related HCC. Universal neonatal vaccination against HBV, as well as vaccination of at-risk adult populations, screening for HBV and HCV, and access to highly-effective treatments against both viruses are instrumental measures that must be pursued with the objective of diminishing their impact on global health and particularly on the development of HCC. Finally, effective surveillance for HCC must be offered to patients who already have advanced fibrosis or cirrhosis and to high-risk HBV-infected individuals, so that HCC is detected at earlier stages, allowing for curative treatments and longer survival.

REFERENCES

1 **Polaris Observatory HCV Collaborators**. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol Hepatol* 2017; **2**: 161-176 [PMID: 28404132 DOI: 10.1016/S2468-1253(16)30181-9]

2 **Polaris Observatory Collaborators**. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol* 2018; **3**: 383-403 [PMID: 29599078 DOI: 10.1016/S2468-1253(18)30056-6]

3 **Razavi H**. Global Epidemiology of Viral Hepatitis. *Gastroenterol Clin North Am* 2020; **49**: 179-189 [PMID: 32389357 DOI: 10.1016/j.gtc.2020.01.001]

4 **Stockdale AJ**, Kreuels B, Henrion MYR, Giorgi E, Kyomuhangi I, de Martel C, Hutin Y, Geretti AM. The global prevalence of hepatitis D virus infection: Systematic review and meta-analysis. *J Hepatol* 2020; **73**: 523-532 [PMID: 32335166 DOI: 10.1016/j.jhep.2020.04.008]

5 **World Health Organization**. Global Cancer Observatory - GLOBOCAN 2020. [cited 17 January 2021]. In: World Health Organization [Internet]. Available from: https://gco.iarc.fr/

6 **McGlynn KA**, Petrick JL, El-Serag HB. Epidemiology of Hepatocellular Carcinoma. *Hepatology* 2021; **73** Suppl 1: 4-13 [PMID: 32319693 DOI: 10.1002/hep.31288]

7 **Arnold M**, Abnet CC, Neale RE, Vignat J, Giovannucci EL, McGlynn KA, Bray F. Global Burden of 5 Major Types of Gastrointestinal Cancer. *Gastroenterology* 2020; **159**: 335-349. e15 [PMID: 32247694 DOI: 10.1053/j.gastro.2020.02.068]

8 **Moon AM**, Singal AG, Tapper EB. Contemporary Epidemiology of Chronic Liver Disease and Cirrhosis. *Clin Gastroenterol Hepatol* 2020; **18**: 2650-2666 [PMID: 31401364 DOI: 10.1016/j.cgh.2019.07.060]

9 **Debes JD**, Chan AJ, Balderramo D, Kikuchi L, Gonzalez Ballerga E, Prieto JE, Tapias M, Idrovo V, Davalos MB, Cairo F, Barreyro FJ, Paredes S, Hernandez N, Avendaño K, Diaz Ferrer J, Yang JD, Carrera E, Garcia JA, Mattos AZ, Hirsch BS, Gonçalves PT, Carrilho FJ, Roberts LR. Hepatocellular carcinoma in South America: Evaluation of risk factors, demographics and therapy. *Liver Int* 2018; **38**: 136-143 [PMID: 28640517 DOI: 10.1111/liv.13502]

10 **de Martel C**, Maucort-Boulch D, Plummer M, Franceschi S. World-wide relative contribution of hepatitis B and C viruses in hepatocellular carcinoma. *Hepatology* 2015; **62**: 1190-1200 [PMID: 26146815 DOI: 10.1002/hep.27969]

11 **GBD 2019 Diseases and Injuries Collaborators**. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet* 2020; **396**: 1204-1222 [PMID: 33069326 DOI: 10.1016/S0140-6736(20)30925-9]

12 **Chan AJ**, Balderramo D, Kikuchi L, Ballerga EG, Prieto JE, Tapias M, Idrovo V, Davalos MB, Cairo F, Barreyro FJ, Paredes S, Hernandez N, Avendaño K, Ferrer JD, Yang JD, Carrera E, Mattos AZ, Hirsch BS, Gonçalves PT, Carrilho FJ, Roberts LR, Debes JD. Early Age Hepatocellular Carcinoma Associated With Hepatitis B Infection in South America. *Clin Gastroenterol Hepatol* 2017; **15**: 1631-1632 [PMID: 28532694 DOI: 10.1016/j.cgh.2017.05.015]

13 **Liu Z**, Xu K, Jiang Y, Cai N, Fan J, Mao X, Suo C, Jin L, Zhang T, Chen X. Global trend of aetiology-based primary liver cancer incidence from 1990 to 2030: a modelling study. *Int J Epidemiol* 2021; **50**: 128-142 [PMID: 33349860 DOI: 10.1093/ije/dyaa196]

14 **Park SH**, Rehermann B. Immune responses to HCV and other hepatitis viruses. *Immunity* 2014; **40**: 13-24 [PMID: 24439265 DOI: 10.1016/j.immuni.2013.12.010]

15 **Bertoletti A**, Ferrari C. Adaptive immunity in HBV infection. *J Hepatol* 2016; **64**: S71-S83 [PMID: 27084039 DOI: 10.1016/j.jhep.2016.01.026]

16 **Boeijen LL**, Hoogeveen RC, Boonstra A, Lauer GM. Hepatitis B virus infection and the immune response: The big questions. *Best Pract Res Clin Gastroenterol* 2017; **31**: 265-272 [PMID: 28774408 DOI: 10.1016/j.bpg.2017.05.003]

17 **Wieland D**, Hofmann M, Thimme R. Overcoming CD8+ T-Cell Exhaustion in Viral Hepatitis: Lessons from the Mouse Model and Clinical Perspectives. *Dig Dis* 2017; **35**: 334-338 [PMID: 28468011 DOI: 10.1159/000456584]

18 **Ringelhan M**, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of

hepatocellular carcinoma. *Nat Immunol* 2018; **19**: 222-232 [PMID: 29379119 DOI: 10.1038/s41590-018-0044-z]

19 **Farinati F**, Piciocchi M, Lavezzo E, Bortolami M, Cardin R. Oxidative stress and inducible nitric oxide synthase induction in carcinogenesis. *Dig Dis* 2010; **28**: 579-584 [PMID: 21088405 DOI: 10.1159/000320052]

20 **Unitt E**, Rushbrook SM, Marshall A, Davies S, Gibbs P, Morris LS, Coleman N, Alexander GJ. Compromised lymphocytes infiltrate hepatocellular carcinoma: the role of T-regulatory cells. *Hepatology* 2005; **41**: 722-730 [PMID: 15791620 DOI: 10.1002/hep.20644]

21 **Zhou G**, Sprengers D, Boor PPC, Doukas M, Schutz H, Mancham S, Pedroza-Gonzalez A, Polak WG, de Jonge J, Gaspersz M, Dong H, Thielemans K, Pan Q, IJzermans JNM, Bruno MJ, Kwekkeboom J. Antibodies Against Immune Checkpoint Molecules Restore Functions of Tumor-Infiltrating T Cells in Hepatocellular Carcinomas. *Gastroenterology* 2017; **153**: 1107-1119. e10 [PMID: 28648905 DOI: 10.1053/j.gastro.2017.06.017]

22 **Fu J**, Xu D, Liu Z, Shi M, Zhao P, Fu B, Zhang Z, Yang H, Zhang H, Zhou C, Yao J, Jin L, Wang H, Yang Y, Fu YX, Wang FS. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 2007; **132**: 2328-2339 [PMID: 17570208 DOI: 10.1053/j.gastro.2007.03.102]

23 **Tian Z**, Chen Y, Gao B. Natural killer cells in liver disease. *Hepatology* 2013; **57**: 1654-1662 [PMID: 23111952 DOI: 10.1002/hep.26115]

24 **Unitt E**, Marshall A, Gelson W, Rushbrook SM, Davies S, Vowler SL, Morris LS, Coleman N, Alexander GJ. Tumour lymphocytic infiltrate and recurrence of hepatocellular carcinoma following liver transplantation. *J Hepatol* 2006; **45**: 246-253 [PMID: 16580084 DOI: 10.1016/j.jhep.2005.12.027]

25 **Flecken T**, Schmidt N, Hild S, Gostick E, Drognitz O, Zeiser R, Schemmer P, Bruns H, Eiermann T, Price DA, Blum HE, Neumann-Haefelin C, Thimme R. Immunodominance and functional alterations of tumor-associated antigen-specific CD8+ T-cell responses in hepatocellular carcinoma. *Hepatology* 2014; **59**: 1415-1426 [PMID: 24002931 DOI: 10.1002/hep.26731]

26 **Wu Y**, Kuang DM, Pan WD, Wan YL, Lao XM, Wang D, Li XF, Zheng L. Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions. *Hepatology* 2013; **57**: 1107-1116 [PMID: 23225218 DOI: 10.1002/hep.26192]

27 **Lim CJ**, Lee YH, Pan L, Lai L, Chua C, Wasser M, Lim TKH, Yeong J, Toh HC, Lee SY, Chan CY, Goh BK, Chung A, Heikenwälder M, Ng IO, Chow P, Albani S, Chew V. Multidimensional analyses reveal distinct immune microenvironment in hepatitis B virus-related hepatocellular carcinoma. *Gut* 2019; **68**: 916-927 [PMID: 29970455 DOI: 10.1136/gutjnl-2018-316510]

28 **Pinato DJ**, Guerra N, Fessas P, Murphy R, Mineo T, Mauri FA, Mukherjee SK, Thursz M, Wong CN, Sharma R, Rimassa L. Immune-based therapies for hepatocellular carcinoma. *Oncogene* 2020; **39**: 3620-3637 [PMID: 32157213 DOI: 10.1038/s41388-020-1249-9]

29 **Cancer Genome Atlas Research Network**. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341. e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]

30 **Schulze K**, Imbeaud S, Letouzé E, Alexandrov LB, Calderaro J, Rebouissou S, Couchy G, Meiller C, Shinde J, Soysouvanh F, Calatayud AL, Pinyol R, Pelletier L, Balabaud C, Laurent A, Blanc JF, Mazzaferro V, Calvo F, Villanueva A, Nault JC, Bioulac-Sage P, Stratton MR, Llovet JM, Zucman-Rossi J. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet* 2015; **47**: 505-511 [PMID: 25822088 DOI: 10.1038/ng.3252]

31 **Hoshida Y**, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, Villanueva A, Newell P, Ikeda K, Hashimoto M, Watanabe G, Gabriel S, Friedman SL, Kumada H, Llovet JM, Golub TR. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res* 2009; **69**: 7385-7392 [PMID: 19723656 DOI: 10.1158/0008-5472.CAN-09-1089]

32 **Chisari FV**. Rous-Whipple Award Lecture. Viruses, immunity, and cancer: lessons from hepatitis B. *Am J Pathol* 2000; **156**: 1117-1132 [PMID: 10751335 DOI: 10.1016/s0002-9440(10)64980-2]

33 **Sung WK**, Zheng H, Li S, Chen R, Liu X, Li Y, Lee NP, Lee WH, Ariyaratne PN, Tennakoon C, Mulawadi FH, Wong KF, Liu AM, Poon RT, Fan ST, Chan KL, Gong Z, Hu Y, Lin Z, Wang G, Zhang Q, Barber TD, Chou WC, Aggarwal A, Hao K, Zhou W, Zhang C, Hardwick J, Buser C, Xu J, Kan Z, Dai H, Mao M, Reinhard C, Wang J, Luk JM. Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. *Nat Genet* 2012; **44**: 765-769 [PMID: 22634754 DOI: 10.1038/ng.2295]

34 **Zhao LH**, Liu X, Yan HX, Li WY, Zeng X, Yang Y, Zhao J, Liu SP, Zhuang XH, Lin C, Qin CJ, Zhao Y, Pan ZY, Huang G, Liu H, Zhang J, Wang RY, Wen W, Lv GS, Zhang HL, Wu H, Huang S, Wang MD, Tang L, Cao HZ, Wang L, Lee TL, Jiang H, Tan YX, Yuan SX, Hou GJ, Tao QF, Xu QG, Zhang XQ, Wu MC, Xu X, Wang J, Yang HM, Zhou WP, Wang HY. Genomic and oncogenic preference of HBV integration in hepatocellular carcinoma. *Nat Commun* 2016; **7**: 12992 [PMID: 27703150 DOI: 10.1038/ncomms12992]

35 **Wang P**, Guo QS, Wang ZW, Qian HX. HBx induces HepG-2 cells autophagy through PI3K/Akt-mTOR pathway. *Mol Cell Biochem* 2013; **372**: 161-168 [PMID: 23001846 DOI: 10.1007/s11010-012-1457-x]

36 **Ching RHH**, Sze KMF, Lau EYT, Chiu YT, Lee JMF, Ng IOL, Lee TKW. C-terminal truncated hepatitis B virus X protein regulates tumorigenicity, self-renewal and drug resistance *via*

STAT3/Nanog signaling pathway. *Oncotarget* 2017; **8**: 23507-23516 [PMID: 28186991 DOI: 10.18632/oncotarget.15183]

37 **Chen Z**, Tang J, Cai X, Huang Y, Gao Q, Liang L, Tian L, Yang Y, Zheng Y, Hu Y, Tang N. HBx mutations promote hepatoma cell migration through the Wnt/β-catenin signaling pathway. *Cancer Sci* 2016; **107**: 1380-1389 [PMID: 27420729 DOI: 10.1111/cas.13014]

38 **Raimondo G**, Locarnini S, Pollicino T, Levrero M, Zoulim F, Lok AS; Taormina Workshop on Occult HBV Infection Faculty Members. Update of the statements on biology and clinical impact of occult hepatitis B virus infection. *J Hepatol* 2019; **71**: 397-408 [PMID: 31004683 DOI: 10.1016/j.jhep.2019.03.034]

39 **Mak LY**, Wong DK, Pollicino T, Raimondo G, Hollinger FB, Yuen MF. Occult hepatitis B infection and hepatocellular carcinoma: Epidemiology, virology, hepatocarcinogenesis and clinical significance. *J Hepatol* 2020; **73**: 952-964 [PMID: 32504662 DOI: 10.1016/j.jhep.2020.05.042]

40 **Yang JD**, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 589-604 [PMID: 31439937 DOI: 10.1038/s41575-019-0186-y]

41 **Marrero JA**, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; **68**: 723-750 [PMID: 29624699 DOI: 10.1002/hep.29913]

42 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

43 **Terrault NA**, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM, Brown RS Jr, Bzowej NH, Wong JB. Update on Prevention, Diagnosis, and Treatment of Chronic Hepatitis B: AASLD 2018 Hepatitis B Guidance. *Clin Liver Dis (Hoboken)* 2018; **12**: 33-34 [PMID: 30988907 DOI: 10.1002/cld.728]

44 **Chang MH**, You SL, Chen CJ, Liu CJ, Lai MW, Wu TC, Wu SF, Lee CM, Yang SS, Chu HC, Wang TE, Chen BW, Chuang WL, Soon MS, Lin CY, Chiou ST, Kuo HS, Chen DS; Taiwan Hepatoma Study Group. Long-term Effects of Hepatitis B Immunization of Infants in Preventing Liver Cancer. *Gastroenterology* 2016; **151**: 472-480. e1 [PMID: 27269245 DOI: 10.1053/j.gastro.2016.05.048]

45 **World Health Organization**. WHO-UNICEF estimates of HepB3 coverage. [cited 17 January 2021]. In: World Health Organization [Internet]. Available from: http://apps.who.int/immunization_monitoring/globalsummary/timeseries/tswucoveragehepb3.html

46 **Peck M**, Gacic-Dobo M, Diallo MS, Nedelec Y, Sodha SV, Wallace AS. Global Routine Vaccination Coverage, 2018. *MMWR Morb Mortal Wkly Rep* 2019; **68**: 937-942 [PMID: 31647786 DOI: 10.15585/mmwr.mm6842a1]

47 **Kanwal F**, Kramer JR, Ilyas J, Duan Z, El-Serag HB. HCV genotype 3 is associated with an increased risk of cirrhosis and hepatocellular cancer in a national sample of U.S. Veterans with HCV. *Hepatology* 2014; **60**: 98-105 [PMID: 24615981 DOI: 10.1002/hep.27095]

48 **Goossens N**, Negro F. Is genotype 3 of the hepatitis C virus the new villain? *Hepatology* 2014; **59**: 2403-2412 [PMID: 24155107 DOI: 10.1002/hep.26905]

49 **Carrat F**, Fontaine H, Dorival C, Simony M, Diallo A, Hezode C, De Ledinghen V, Larrey D, Haour G, Bronowicki JP, Zoulim F, Asselah T, Marcellin P, Thabut D, Leroy V, Tran A, Habersetzer F, Samuel D, Guyader D, Chazouilleres O, Mathurin P, Metivier S, Alric L, Riachi G, Gournay J, Abergel A, Cales P, Ganne N, Loustaud-Ratti V, D'Alteroche L, Causse X, Geist C, Minello A, Rosa I, Gelu-Simeon M, Portal I, Raffi F, Bourliere M, Pol S; French ANRS CO22 Hepather cohort. Clinical outcomes in patients with chronic hepatitis C after direct-acting antiviral treatment: a prospective cohort study. *Lancet* 2019; **393**: 1453-1464 [PMID: 30765123 DOI: 10.1016/S0140-6736(18)32111-1]

50 **Kanwal F**, Kramer J, Asch SM, Chayanupatkul M, Cao Y, El-Serag HB. Risk of Hepatocellular Cancer in HCV Patients Treated With Direct-Acting Antiviral Agents. *Gastroenterology* 2017; **153**: 996-1005. e1 [PMID: 28642197 DOI: 10.1053/j.gastro.2017.06.012]

51 **Ghany MG**, Morgan TR; AASLD-IDSA Hepatitis C Guidance Panel. Hepatitis C Guidance 2019 Update: American Association for the Study of Liver Diseases-Infectious Diseases Society of America Recommendations for Testing, Managing, and Treating Hepatitis C Virus Infection. *Hepatology* 2020; **71**: 686-721 [PMID: 31816111 DOI: 10.1002/hep.31060]

52 **Degasperi E**, Galmozzi E, Pelusi S, D'Ambrosio R, Soffredini R, Borghi M, Perbellini R, Facchetti F, Iavarone M, Sangiovanni A, Valenti L, Lampertico P. Hepatic Fat-Genetic Risk Score Predicts Hepatocellular Carcinoma in Patients With Cirrhotic HCV Treated With DAAs. *Hepatology* 2020; **72**: 1912-1923 [PMID: 32762045 DOI: 10.1002/hep.31500]

53 **Alonso López S**, Manzano ML, Gea F, Gutiérrez ML, Ahumada AM, Devesa MJ, Olveira A, Polo BA, Márquez L, Fernández I, Cobo JCR, Rayón L, Riado D, Izquierdo S, Usón C, Real Y, Rincón D, Fernández-Rodríguez CM, Bañares R. A Model Based on Noninvasive Markers Predicts Very Low Hepatocellular Carcinoma Risk After Viral Response in Hepatitis C Virus-Advanced Fibrosis. *Hepatology* 2020; **72**: 1924-1934 [PMID: 33022803 DOI: 10.1002/hep.31588]

54 **Centre for Disease Analysis**. Web Annex B. WHO estimates of the prevalence and incidence of hepatitis C virus infection by World Health Organization region, 2015. Geneva: World Health Organization, 2018: 1-68

55 **World Health Organization**. Global Health Sector Strategy on Viral Hepatitis 2016-2021. Towards

Ending Viral Hepatitis. [cited 17 January 2021]. In: World Health Organization [Internet]. Available from: https://apps.who.int/iris/bitstream/handle/10665/246177/WHO-HIV-2016.06-eng.pdf?sequence=1

56 **Indolfi G**, Azzari C, Resti M. Perinatal transmission of hepatitis C virus. *J Pediatr* 2013; **163**: 1549-1552. e1 [PMID: 23919905 DOI: 10.1016/j.jpeds.2013.06.077]

57 **Epstein RL**, Sabharwal V, Wachman EM, Saia KA, Vellozzi C, Hariri S, Linas BP. Perinatal Transmission of Hepatitis C Virus: Defining the Cascade of Care. *J Pediatr* 2018; **203**: 34-40. e1 [PMID: 30170857 DOI: 10.1016/j.jpeds.2018.07.006]

58 **Dore GJ**, Hajarizadeh B. Elimination of Hepatitis C Virus in Australia: Laying the Foundation. *Infect Dis Clin North Am* 2018; **32**: 269-279 [PMID: 29778255 DOI: 10.1016/j.idc.2018.02.006]

59 **Pawlotsky JM**, Ramers CB, Dillon JF, Feld JJ, Lazarus JV. Simplification of Care for Chronic Hepatitis C Virus Infection. *Semin Liver Dis* 2020; **40**: 392-402 [PMID: 32725611 DOI: 10.1055/s-0040-1713657]

60 **Blach S**, Kondili LA, Aghemo A, Cai Z, Dugan E, Estes C, Gamkrelidze I, Ma S, Pawlotsky JM, Razavi-Shearer D, Razavi H, Waked I, Zeuzem S, Craxi A. Impact of COVID-19 on global HCV elimination efforts. *J Hepatol* 2021; **74**: 31-36 [PMID: 32777322 DOI: 10.1016/j.jhep.2020.07.042]

61 **Debes JD**, de Knegt RJ, Boonstra A. The Path to Cancer and Back: Immune Modulation During Hepatitis C Virus Infection, Progression to Fibrosis and Cancer, and Unexpected Roles of New Antivirals. *Transplantation* 2017; **101**: 910-915 [PMID: 28045877 DOI: 10.1097/TP.0000000000001623]

62 **Merchante N**, Merino E, Rodríguez-Arrondo F, Tural C, Muñoz J, Delgado-Fernández M, Jover F, Galindo MJ, Rivero A, López-Aldeguer J, Aguirrebengoa K, Romero-Palacios A, Martínez E, Pineda JA. HIV/hepatitis C virus-coinfected patients who achieved sustained virological response are still at risk of developing hepatocellular carcinoma. *AIDS* 2014; **28**: 41-47 [PMID: 24056067 DOI: 10.1097/QAD.0000000000000005]

63 **Merchante N**, Rodríguez-Arrondo F, Revollo B, Merino E, Ibarra S, Galindo MJ, Montero M, García-Deltoro M, Rivero-Juárez A, Téllez F, Delgado-Fernández M, Ríos-Villegas MJ, García MA, Vera-Méndez FJ, Ojeda-Burgos G, López-Ruz MA, Metola L, Omar M, Alemán-Valls MR, Aguirrebengoa K, Portu J, Raffo M, Macías J, Pineda JA; GEHEP-002 Study Group. Hepatocellular carcinoma after sustained virological response with interferon-free regimens in HIV/hepatitis C virus-coinfected patients. *AIDS* 2018; **32**: 1423-1430 [PMID: 29596108 DOI: 10.1097/QAD.0000000000001809]

64 **Merchante N**, Merino E, López-Aldeguer J, Jover F, Delgado-Fernández M, Galindo MJ, Ortega E, Rivero A, Mínguez C, Romero-Palacios A, Padilla S, Márquez-Solero M, Amador C, Ríos-Villegas MJ, Téllez F, Portilla J, Pineda JA. Increasing incidence of hepatocellular carcinoma in HIV-infected patients in Spain. *Clin Infect Dis* 2013; **56**: 143-150 [PMID: 22955438 DOI: 10.1093/cid/cis777]

65 **Sahasrabuddhe VV**, Shiels MS, McGlynn KA, Engels EA. The risk of hepatocellular carcinoma among individuals with acquired immunodeficiency syndrome in the United States. *Cancer* 2012; **118**: 6226-6233 [PMID: 22736272 DOI: 10.1002/cncr.27694]

66 **Bräu N**, Fox RK, Xiao P, Marks K, Naqvi Z, Taylor LE, Trikha A, Sherman M, Sulkowski MS, Dieterich DT, Rigsby MO, Wright TL, Hernandez MD, Jain MK, Khatri GK, Sterling RK, Bonacini M, Martyn CA, Aytaman A, Llovet JM, Brown ST, Bini EJ; North American Liver Cancer in HIV Study Group. Presentation and outcome of hepatocellular carcinoma in HIV-infected patients: a U.S.-Canadian multicenter study. *J Hepatol* 2007; **47**: 527-537 [PMID: 17692986 DOI: 10.1016/j.jhep.2007.06.010]

67 **Yu ML**, Lin SM, Chuang WL, Dai CY, Wang JH, Lu SN, Sheen IS, Chang WY, Lee CM, Liaw YF. A sustained virological response to interferon or interferon/ribavirin reduces hepatocellular carcinoma and improves survival in chronic hepatitis C: a nationwide, multicentre study in Taiwan. *Antivir Ther* 2006; **11**: 985-994 [PMID: 17302368]

68 **Merchante N**, Rodríguez-Fernández M, Pineda JA. Screening for Hepatocellular Carcinoma in HIV-Infected Patients: Current Evidence and Controversies. *Curr HIV/AIDS Rep* 2020; **17**: 6-17 [PMID: 31933273 DOI: 10.1007/s11904-019-00475-0]

69 **Wandeler G**, Mauron E, Atkinson A, Dufour JF, Kraus D, Reiss P, Peters L, Dabis F, Fehr J, Bernasconi E, van der Valk M, Smit C, Gjærde LK, Rockstroh J, Neau D, Bonnet F, Rauch A; Swiss HIV Cohort Study, Athena Observational Cohort Study, EuroSIDA, ANRS CO3 Aquitaine Cohort. Incidence of hepatocellular carcinoma in HIV/HBV-coinfected patients on tenofovir therapy: Relevance for screening strategies. *J Hepatol* 2019; **71**: 274-280 [PMID: 30965070 DOI: 10.1016/j.jhep.2019.03.032]

70 **Tanon A**, Jaquet A, Ekouevi DK, Akakpo J, Adoubi I, Diomande I, Houngbe F, Zannou MD, Sasco AJ, Eholie SP, Dabis F, Bissagnene E; IeDEA West Africa Collaboration. The spectrum of cancers in West Africa: associations with human immunodeficiency virus. *PLoS One* 2012; **7**: e48108 [PMID: 23144732 DOI: 10.1371/journal.pone.0048108]

71 **Maponga TG**, Glashoff RH, Vermeulen H, Robertson B, Burmeister S, Bernon M, Omoshoro-Jones J, Ruff P, Neugut AI, Jacobson JS, Preiser W, Andersson MI. Hepatitis B virus-associated hepatocellular carcinoma in South Africa in the era of HIV. *BMC Gastroenterol* 2020; **20**: 226 [PMID: 32660431 DOI: 10.1186/s12876-020-01372-2]

72 **Clifford GM**, Rickenbach M, Polesel J, Dal Maso L, Steffen I, Ledergerber B, Rauch A, Probst-Hensch NM, Bouchardy C, Levi F, Franceschi S; Swiss HIV Cohort. Influence of HIV-related immunodeficiency on the risk of hepatocellular carcinoma. *AIDS* 2008; **22**: 2135-2141 [PMID:

18832877 DOI: 10.1097/QAD.0b013e32831103ad]

73 **Singal AG**, Pillai A, Tiro J. Early detection, curative treatment, and survival rates for hepatocellular carcinoma surveillance in patients with cirrhosis: a meta-analysis. *PLoS Med* 2014; **11**: e1001624 [PMID: 24691105 DOI: 10.1371/journal.pmed.1001624]

74 **Rodríguez de Lope C**, Reig M, Matilla A, Ferrer MT, Dueñas E, Mínguez B, F Castroagudín J, Ortiz I, Pascual S, Lledó JL, Gallego A, Arenas JI, Aracil C, Forne M, Muñoz C, Pons F, Sala M, Iñarrairaegui M, Martin-Llahi M, Andreu V, Garre C, Rendón P, Fuentes J, Crespo J, Rodríguez M, Bruix J, Varela M; en representación del Grupo de Estudio de Cáncer Hepático (GECH). Clinical characteristics of hepatocellular carcinoma in Spain. Comparison with the 2008-2009 period and analysis of the causes of diagnosis out of screening programs. Analysis of 686 cases in 73 centers. *Med Clin (Barc)* 2017; **149**: 61-71 [PMID: 28279536 DOI: 10.1016/j.medcli.2016.12.048]

75 **Ferrante ND**, Lo Re V 3rd. Epidemiology, Natural History, and Treatment of Hepatitis Delta Virus Infection in HIV/Hepatitis B Virus Coinfection. *Curr HIV/AIDS Rep* 2020; **17**: 405-414 [PMID: 32607773 DOI: 10.1007/s11904-020-00508-z]

76 **Soriano V**, Grint D, d'Arminio Monforte A, Horban A, Leen C, Poveda E, Antunes F, de Wit S, Lundgren J, Rockstroh J, Peters L. Hepatitis delta in HIV-infected individuals in Europe. *AIDS* 2011; **25**: 1987-1992 [PMID: 21857493 DOI: 10.1097/QAD.0b013e32834babb3]

77 **Béguelin C**, Moradpour D, Sahli R, Suter-Riniker F, Lüthi A, Cavassini M, Günthard HF, Battegay M, Bernasconi E, Schmid P, Calmy A, Braun DL, Furrer H, Rauch A, Wandeler G; Swiss HIV Cohort Study. Hepatitis delta-associated mortality in HIV/HBV-coinfected patients. *J Hepatol* 2017; **66**: 297-303 [PMID: 27746337 DOI: 10.1016/j.jhep.2016.10.007]

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MINIREVIEWS

Room for improvement in the treatment of pancreatic cancer: Novel opportunities from gene targeted therapy

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Abstract

Pancreatic cancer is one of the highest and in fact, unchanged mortality-associated tumor, with an exceptionally low survival rate due to its challenging diagnostic approach. So far, its treatment is based on a combination of approaches (such as surgical resection with or rarely without chemotherapeutic agents), but with finite limits. Thus, looking for additional space to improve pancreatic tumorigenesis therapeutic approach, research has focused on gene therapy with unexpectedly growing horizons not only for the treatment of inoperable pancreatic disease, but also for its early stages. *In vivo* gene delivery viral vectors, despite few disadvantages (possible immunogenicity, toxicity, mutagenicity, or high cost), could be one of the most efficient cancer gene therapeutic strategies for clinical application due to their superiority compared with other systems (*ex vivo* delivery strategies). Their dominance consists of simple preparation, easy operation and a wide range of functions. Adenoviruses are one of the most common used vectors, inducing strong immune as well as inflammatory reactions. Oncolytic virotherapy, using the above mentioned *in vivo* viral vectors, is one of the most promising non-pathogenic, highly-selective cytotoxic anti-cancer therapy using anti-cancer agents with high anti-tumor potency and strong oncolytic effect. There have been a variety of targeted therapeutic and pre-clinical strategies tested for gene therapy in pancreatic cancer such as gene-editing systems (*e.g.*, clustered regularly interspaced palindromic repeats-Cas9), RNA interference technology (*e.g.*, microRNAs, short hairpin RNA or small interfering RNA), adoptive immunotherapy and vaccination (*e.g.*, chimeric antigen receptor T-cell therapy) with

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Core Tip: Pancreatic cancer still remains one of the leading causes of cancer deaths worldwide. While there are various therapeutic approaches established, there is still a crucial need for improvement of conventional treatments and establishment of novel therapies to increase efficacy. This review exhibits an overview of the most promising present and future prospects regarding gene therapy which offers a new favorable opportunity not only to tackle with an inoperable pancreatic cancer, but also, to treat effectively its early stages.

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INTRODUCTION

Pancreatic cancer is an ever-worsening healthcare issue, predicted to ascend to the second place of highest mortality-associated neoplasias. The conundrum it represents for patients and physicians alike is aggravated by its challenging diagnosis, dismal prognosis, less than satisfactory treatment, and low survivability. Epidemiologically, the rate of new cases is approximately 13.1/100000 per year and the mean age of onset is 71 years for men and 75 for women, with the former slightly more affected[1]. The estimated 5-year survival is up to 8% and a mere 9.7% of patients are metastasis-free at diagnosis[2]. A mainstay of its globally increasing incidence is possibly the unequivocal relationship between its risk factors, mainly obesity, smoking, and alcohol, as well as the Western lifestyle[3]. Other contributing factors are age, gender, genetic and chronic inflammatory conditions.

There are numerous classifications of pancreatic cancer, such as macroscopic, pathological, molecular, and surgical, depending on the characteristic being observed. The most frequently recurring form of pancreatic neoplasia is the adenocarcinoma, a tumour of solid type and ductal origin. The most commonly affected site is the head of the pancreas, whereas roughly a fifth of the cancers concern the organ diffusely[4]. Pathophysiologically, there is a well-established sequelae, starting from a precursor lesion and culminating to proper neoplasia, through a series of oncogene and tumour-suppressive gene mutations, such as *KRAS*, *p53*, *p16*, *SMAD4* and *CDNK27*[5]. Precancerous lesions are further divided in pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm and intraductal papillary mucinous neoplasm, with PanIN being the most frequent[4]. The two-tiered categorization of PanIN (low and high grade) bears great clinical significance, since the probability and duration of progression between low and high grade have been established and can be predicted [6,7].

Clinical presentation is non-specific and usually includes weight loss, abdominal pain, jaundice, malabsorption, and diabetes mellitus. This fact, together with the relative rarity of the disease, make screening ineffective, apart from patients with a positive family history of pancreatic neoplasia where screening is considered rationale [4,8]. Diagnostic process includes clinical check-up, routine blood work-up and imaging, along with the search for signs of malabsorption, diabetes, and hypoalbuminemia. Specifically, a combination of two imaging modalities, preferably endoscopic ultrasound and computerized tomography or magnetic resonance imaging/magnetic resonance cholangiopancreatography scan, is favored by the International Cancer of the Pancreas Screening Consortium[9,10].

As far as treatment is concerned, surgical resection and adjuvant or neoadjuvant chemotherapy is the best therapy so far[11]. The observed response to treatment is dependent on a plethora of factors, such as tumour stage at diagnosis, age, and comorbidities. However, 5-year survival remains low, ranging from 8%-27%, with the latter concerning complete resection of local non-metastatic disease. The surgical therapies of choice are pancreatoduodenectomy (Whipple's), distal or total pancreatectomy, coupled with potent chemotherapeutic agents, where applicable[12-14]. The most used regimens contain gemcitabine, nab-paclitaxel and mFOLFIRINOX[15,16].

METHODS AND RESULTS

We conducted a literature search in PubMed database, including most of the published studies in English language. The following keywords were used for literature search: "gene therapy", "gene therapy AND pancreatic cancer", "pancreatic cancer", "anti-angiogenesis AND pancreatic cancer", "viral vectors AND gene editing, "miRNA", "siRNA", "oncolytic virotherapy". Three and seven hundred forty-seven thousands publications fulfilled search requirements. Of these, 85 articles were chosen due to the appropriate content and the English language criteria . From the 85 articles, 35 were original articles and 50 were review articles, which included case series, case reports, clinical trials and clinical cohort studies. We reviewed all the abstracts and found 69 full-text articles appropriate for the current study (Figure 1). The three authors (DA, GM and GF) independently reviewed the abstracts and the full text of the related articles.

IS THERE ROOM FOR ANY IMPROVEMENT ON PANCREATIC CANCER TREATMENT?

As the mortality of pancreatic cancer has been unchanged the last decades, the pressure of discovery for a new therapeutic armamentarium is more pivotal than ever. Additionally, the fact that pancreatic ductal adenocarcinoma (PDAC) is expected to be levelled off at the second place of the most lethal neoplasias worldwide pushes the healthcare systems to investigate new treatment protocols. Over the last 20 years, promising results have been stated by the induction of gene-based therapies, shifting from the therapy of inherited diseases to acquired disorders, but most importantly cancer[17]. Gene therapy delivers genetic material to cells for the treatment of a particular disorder. Gene editing or gene transfer might be conducted *ex vivo* in isolated cells or *in vivo*, in cells located within their corresponding tissue environment [18]. The primary principles of gene therapy are to induce anti-tumor immune effects intervening with various signaling pathways. There are various types of gene-based therapy approaches that could be classified into three groups: (1) gene editing or addition; (2) genetically engineered cell therapy; and (3) oncolytic virotherapy (Figure 2)[19]. Gene delivery systems also play an important role in gene therapy affecting both the safety and efficiency of gene transfer. Furthermore, improved targeting relies upon the development of viral and non-viral methodologies of the therapeutic gene specific to the tumour[19].

GENE DELIVERY METHODS

Ex vivo delivery

In this particular system, sampling cells extracted from the patient (bone marrow or target tissue), are genetically modified (*in vitro* culture or proliferation) with a vector including the therapeutic gene of choice and subsequently reintroduced in the same individual after gene transfer[20]. In cancer treatment, cells explanted from cancer patients can be also cultured and genetically engineered *in vitro*, but normally they are utilised for the secretion of cytokines or vaccination[21]. In case of a tumor like PDAC characterized by invasive nature and accelerated growth, the *ex vivo* strategy is not well adjusted and a "ready to use" gene therapy product could be theoretically a better option[20].

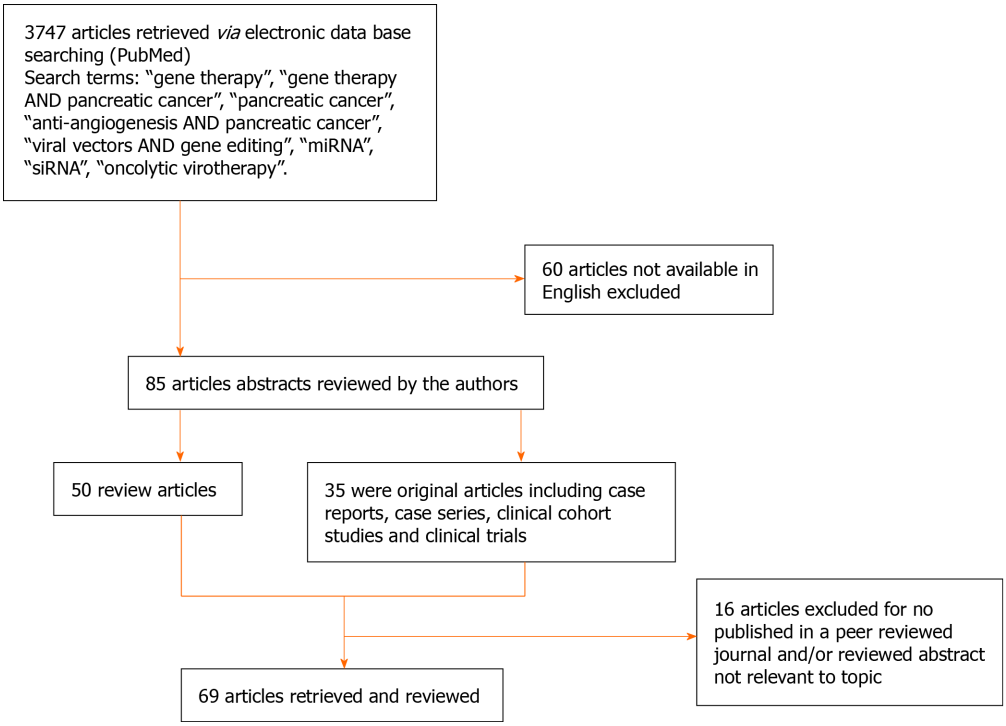


Figure 1 Decision tree for literature research strategy.

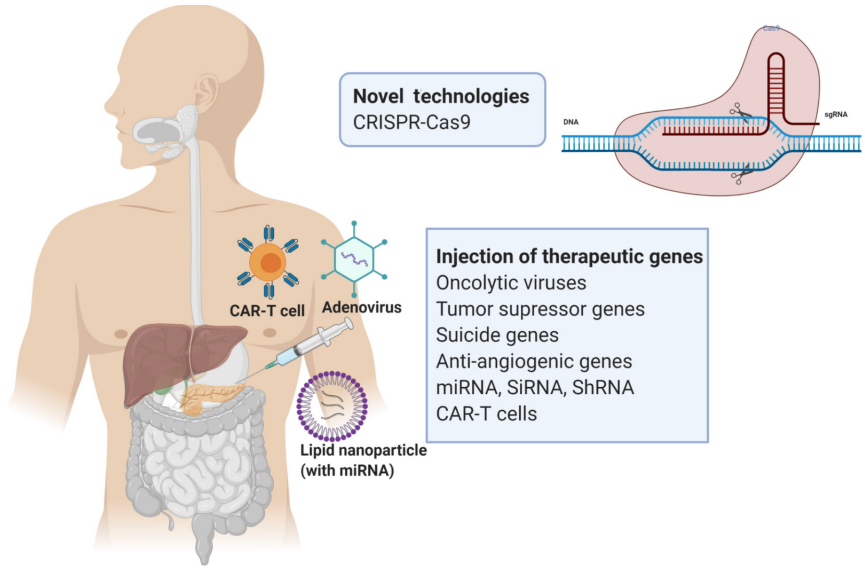


Figure 2 Summary of the therapeutic strategies of gene therapy for pancreatic cancer. The above diagram was created with Biorender.com. CAR-T: Chimeric antigen receptor T-cell; CRISPR-Cas9: Clustered regularly interspaced palindromic repeats-Cas9.

In vivo delivery

The second system of gene delivery is the *in vivo* delivery which includes injecting the gene vectors carrying the therapeutic genes into the bloodstream or the target tissue (tumor). In situ injections are used with imaging technologies and are tested in cases such as myopathy, cystic fibrosis and most importantly cancer[20]. Nowadays, the majority of clinical trials are being established following the methodology of intratumoral injection induced by ultrasound or computed tomography, gene-eluting stent implantation and vascular perfusion of tumor[21]. *In vivo* delivery could be one of the most efficient strategies for clinical application due to its superiority compared with other systems, based on its simple preparation, easy operation and broad range of functions. However, there still exist some disadvantages such as the lack of specificity on particular targets, short-term curative effect and immunologic complications[22].

GENE DELIVERY VECTORS

Presently there are two categories of gene delivery vectors: viral and non-viral vector systems[19]. The most widely used viral vectors are those that are mainly based on adenoviruses (ADs), retroviruses (RVs) and adeno-associated viruses (AAVs)[23]. Non-viral vector systems comprise of biological or chemical vectors, as well as physical approaches of gene delivery to present RNA molecules, naked DNA (plasmid DNA form) or oligonucleotides into the targeted cells[19,21]. Physical delivery methods primarily, consist of electroporation and microinjection. Nowadays, viral vectors represent the most efficient gene transfer method and thus, the most widely used gene delivery systems for cancer gene therapy[21,23].

Viral vectors

It is estimated that viral therapies represent more than two-thirds of clinical trials on gene therapy. More specifically, these viruses are capable of infecting and entering the cell *via* different mechanisms and transfer DNA into recipient cells without the need of any chemical or physical preparation. The gene of choice will then enter the nucleus resulting in host gene assimilation and expression[21].

The most common viral vectors in cancer gene therapy as previously described include RV, AD, AAV, herpes simplex virus (HSV), lentivirus, pox virus, Epstein-Bar virus and Newcastle disease virus[23]. Viral vectors usually offer long-term gene expression and better insertion capacity and efficiency, but might be linked with immunogenicity, toxicity, mutagenicity and high cost[24,25]. Thus, for the appropriate selection of viral vectors both advantages and disadvantages should be considered.

One of the most common class of viral vectors are ADs. ADs could infect a large variety of cells and for this reason, have been used for a long time for preclinical and clinical gene therapeutic strategies for PDAC. These vectors induce strong immune and inflammatory reactions. Nevertheless, no major incident has been observed during clinical trials with adenoviral vectors. Third generation AD vectors (no viral sequence) haven been developed to solve some issues of toxicity[22,25]. Another important class of viral vectors are AAV. They are capable of effectively transducing cells from liver and brain but have, rarely been examined in pancreatic cells[19,21,25]. The retroviridae family includes viral vectors such as lentiviral (derived from HIV-1) and retroviral (derived from murine leukemia virus) vectors. They are small RNA vectors with the advantage of low immunogenicity and ability of transducing many cells with high efficiency. However, these viral vectors have a high risk of immunogenicity. They are commonly used in *ex vivo* strategies but also in *in situ* administration, as they can transduce proliferating cells such as cancer cells[20,26]. A new class of lentiviral and retroviral known as self-activating vectors have been developed with specific modification allowing them to bypass the transactivation of oncogenes during their integration into the host cells[27]. Finally, many studies have been conducted for another class of viral vectors known as the HSVs. These viruses are highly immunogenic and can carry and incorporate huge amounts of DNA; therefore, compared with other viruses, they include a complex genome making it difficult to transfer genetic information to the target cell[20].

Oncolytic virotherapy

Oncolytic virotherapy is one of the most promising anti-cancer therapies using agents with high anti-tumor potency and strong oncolytic effect[2]. Pancreatic ductal carcinoma development is linked with a sequence of changes helping cancer cells to grow more rapidly. Nevertheless, these particular changes such as high metabolic activity, lack of interferon response and uncontrolled cell division, cause cancer cells to be more sensitive to viral infection[28]. These viruses are non-pathogenic and have a high selectivity and cytotoxicity to cancer cells[29]. In addition, they are able to stimulate their anticancer activity *via* various mechanisms such as targeting of specific pathways (dysfunction pathway TP53) and stimulation of specific and non-specific anti-tumor immunity[30].

Over the years, ADs have been assessed for their potential in PDAC therapy by different research programs. AV5, also known as Oncorine H101 was the first adenovirus to be approved for the treatment of nasopharyngeal cancer[29]. Oncorine H101 was chemically engineered to replicate and kill tumor cells demonstrating *p53* mutations. This specific oncovirus is closely related to the adenovirus Onyx-15[2]. Onyx-15, which replicates in cancer cells harbouring *p53* mutations, was not effective as a monotherapy. During phase II clinical trials it was demonstrated that combination of Onyx-15 with the anticancer drug gemcitabine was initially well tolerated in

pancreatic patients, but some issues appeared such as high titer neutralizing antibodies growth and low replication of the virus[29,31]. There has been a vast number of attempts to enhanced the efficacy of potential ADs for PDAC therapy *via* genetic engineering of the virus to induce the development of variants particular to the disease characteristics. One example is the adenovirus AxE1AdB-UPRT expressing uracil phosphoribosyltransferase, importing 5-FU resistance[32].

After the success of talimogene laherparepvec (T-VEC) approved for the treatment of nasopharyngeal malignancy, HSVs have been representing promising oncoviruses for cancer therapy[2,29]. T-VEC is currently being assessed in phase I clinical trials as monotherapy or in combination with radiotherapy for the treatment of Merkel cell carcinoma and melanoma[33]. Furthermore, it has also been assessed in clinical phase I trials for PDAC as monotherapy and displayed no clinical efficacy in 17 pancreatic cancer patients[31]. Another HSV with promising anti-cancer activity is Myb34.5, which has been assessed preclinically in pancreatic cancer models. The intratumoral injection of Myb34.5 variant induced apoptosis and inhibition of pancreatic tumors growth. This effect was further enhanced with the combination of gemcitabine[34].

TARGETED THERAPEUTIC AND PRE-CLINICAL STRATEGIES

The basic overall strategy differs upon the final objective either through introducing a therapeutic gene which is down-expressed or missing (*e.g.*, suicide gene) or through reintroducing a deficient gene (*e.g.*, tumor suppressor gene) or through inhibiting gene expression (*e.g.*, oncogene). There exist many barriers to the overall gene therapy delivery that could be overcome by intranuclear and intracellular penetration, protein and mature mRNA expression and entry to tumor cells[20]. There have been a variety of pre-clinical approaches for PDAC gene therapy and non-viral gene therapy applications such as gene-editing systems (*e.g.*, CRISP-CAS) and RNA interference technology (Table 1)[20,21].

Anti-angiogenic genes

Gene therapy serves as an effective strategy for therapeutic intervention to angiogenesis based on safety, specific targeting, and cost-effectiveness. Many strategies have been tested *in vitro* or *in vivo* by transfer of gene encoding anti-angiogenic molecules such as vasostatin, angiostatin, vascular endothelial growth factor (VEGF) and soluble fibroblast growth factor receptor[20,21]. Angiogenesis plays an important role in tumor growth. VEGF is a glycoprotein essential in angiogenesis, which is overexpressed in 90% of pancreatic cancers and is responsible for increased tumor progression and microvessel density, as well as poor prognosis. Clinical studies have demonstrated that serum levels of VEGF are much higher in metastatic prostate cancer patients than non-metastatic ones[35]. Various anti-VEGF therapies with molecular targeted agents and monoclonal antibodies have been developed for the therapy of different cancers. However, not substantial benefit and survival has been observed in pancreatic cancer clinical trials phase III with the use of anti-angiogenic agents, considering their high treatment costs as well[21,35,36].

Tumor suppressor genes

The main goal of this strategy is to induce the activation of a specific tumor suppressor gene, which is not expressed anymore during tumor growth[20]. The *p53* gene is one of the widely known tumor suppressor genes, which encodes TP53. TP53 protein is a particularly important tumor suppressor having a critical role in cancer development and therapy. It provides fundamental functions in cellular responses in various stressors and is responsible for the control of apoptotic cells entry (mutated in 50% of human tumors)[20,37]. Strategies based on gene transfer of TP53 gene have been utilized for therapy of various cancers (*e.g.* colon and liver cancer)[20]. Experimental studies have demonstrated that *p53* gene transfer *via* an AD vector, suppresses human pancreatic cancer cells *in vitro*[38]. Furthermore, intraperitoneal administration of the p53 retroviral vector in nude mice, inhibited significantly pancreatic tumor growth compared with the control group[39].

Suicide genes

Suicide gene therapy is based on the transduction of tumor cells with a gene encoding specific enzymes capable of converting nontoxic prodrugs into toxic metabolites[21]. One of the most-well known suicide gene strategies is the herpes simplex virus thymidine kinase gene (*HSV-TK* gene). This gene codes the thymidine kinase enzyme

Table 1 Targeted therapeutic and pre-clinical strategies tested for gene therapy in pancreatic cancer

Therapeutic strategy	Gene examples
Gene transfer	Anti-angiogenic genes (*VEGF*, *angiostatin*, *endostatin*, *thrombostatin etc.*)
	Tumor suppressor genes (*p53*)
	Suicide genes (*HSV-TK*, *Cytochrome P40*)
RNA therapy	miRNA (miR-21), ShRNA, siRNA and antisense oligonucleotides (ISIS-2503 and AEG35156)
Gene editing technology	CRISPR-Cas9
Active immunotherapy	Cytokine expression
	Interleukin expression
Adoptive immunotherapy	Peptide, pulsed dendritic cells DNA, bacteria and engineered cells
Vaccination	PLD-1 and CTLA-4 inhibitors
	CAR-T cells (targeting MUC-1)

VEGF: Vascular endothelial growth factor; *HSV-TK*: Herpes simplex virus thymidine kinase gene; CRISPR-Cas9: Clustered regularly interspaced palindromic repeats-Cas9; PLD-1: Phospholipase D1; CTLA-4: Cytotoxic T-lymphocyte antigen 4; CAR-T: chimeric antigen receptor T- cell; MUC-1: Mucin 1, cell surface associated.

responsible for the conversion of ganciclovir an antiviral drug with no antitumor toxicity to a toxic metabolite capable of interfering DNA replication and inhibiting DNA synthesis resulting in cell death[40,41]. Its therapeutic effect is based on a "by-standard effect" in which tumor cells transduced with HSV-TK causing toxicity to neighbouring tumor cells (unmodified)[21]. HSV-TK delivery *via* adenovirus and retrovirus have shown great anti-tumor efficiency in pancreatic cells both *in vitro* and *in vivo*[41,42]. Moreover, experimental studies have shown that the combination of retrovirus and adenovirus delivery of HSV-TK is more effective in tumor growth inhibition compared to single delivery *in vivo*[43].

Other examples of suicide gene strategies successfully tested *in vivo* (PDAC models) include: the nitroreductase gene responsible for the transformation of CB1954 into 4-hydroxylamine, cytosine deaminase gene responsible for the transformation 5-flurocytosine into 5-fluoroucacil and *cytochrome P40* gene responsible for the transformation of ifosfamide into acrolein[20,21,44]. The cytochrome P450/ifosfamide system proof of concept from pre-clinical studies has been used to conduct phase I and II clinical trials in pancreatic cancer patients with phenomenal success (improvement of 1-year survival by 3-fold and median survival doubled)[20,45].

RNA THERAPY

Another strategy besides the gene transfer with vectors is the RNA interference, which is based on post-transcriptional inhibition of gene expression *via* two groups of small non-coding RNAs: miRNAs and interfering RNAs including shRNAs and siRNAS[2,20]. miRNAs are a family of single stranded RNAs consisting of 21-24 ribonucleotides, which are usually transcribed *via* RNA polymerase II and act by repressing protein production by translational silencing[46]. Loss of their expression may result in important dysfunctions during carcinogenesis. Studies of mRNA expression in a large scale (microRNome) helped to better understand their role in cancer development and their respective mechanisms responsible for these dysfunctions. These miRNAs known as oncoMIR are able to be regulated through different gene therapy approaches (Figure 3)[20]. An experimental study has shown that targeting the oncogenic miRNA-21 could suppress tumor growth in pancreatic cancer *in vitro* and *in vivo*[47]. Many miRNAs have a critical role in PDAC growth process *via* the regulation of important pathways, such as targeting Kirsten rat sarcoma viral oncogene homolog mutation. However, no miRNA therapeutics have been tested clinically for pancreatic cancer treatment[48].

On the contrary to miRNAs, siRNAs are commonly used in a variety of clinical applications because of their lack of genomic integration and more simplistic sequencing. They are double-stranded RNA molecules (20-30 nucleotides in length),

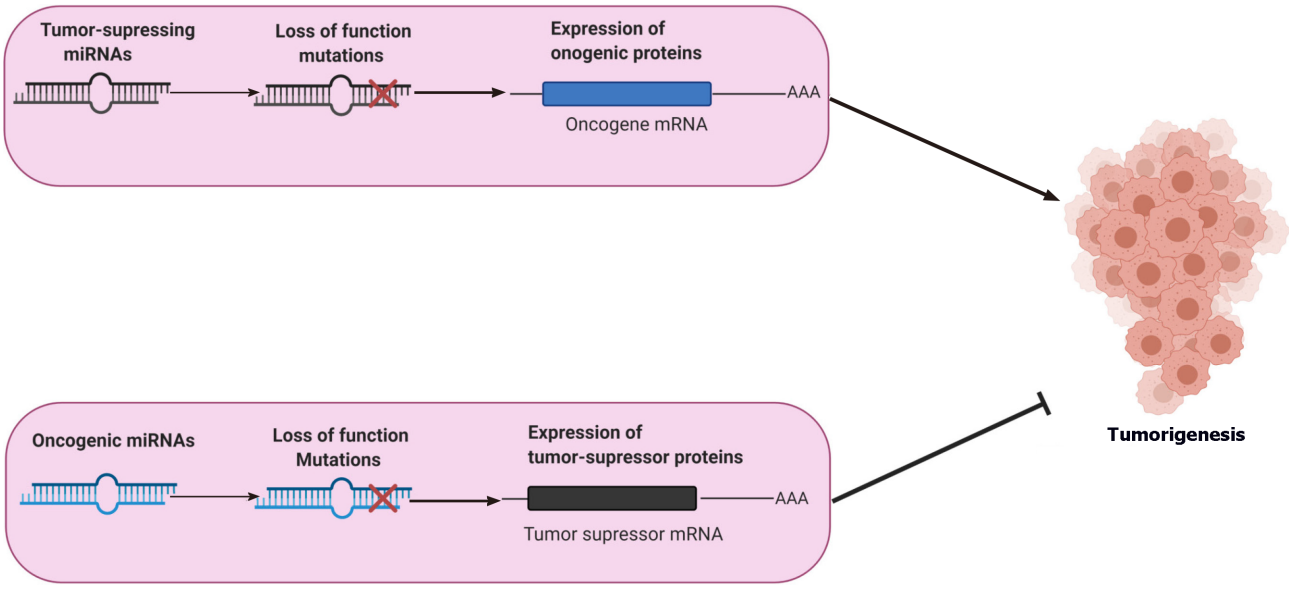


Figure 3 Roles of miRNAs in the regulation of tumorigenesis. miRNAs are essential for the regulation of tumorigenesis. The primary role of tumor-suppressing miRNAs is to suppress the expression of oncogenes, thus inhibiting tumorigenesis whereas oncogenic miRNAs (oncoMIR) block translation of tumor suppressor genes inducing tumorigenesis and, thus tumor formation. Loss of function (mutations) in tumor-suppressing miRNAs induce tumorigenesis (increased expression of oncogenic proteins). However, loss of function (mutations) in oncogenic miRNAs (increased expression of tumor-suppressor mRNA) results in the suppression of tumorigenesis. The above diagram was created with Biorender.com.

which like miRNAs inhibit translation resulting in gene silencing[49]. One of the most essential features of RNA interference (RNAi) technology is the small size of payload increasing the number of drugs delivered to the tumor of choice[50]. In an experimental study based on antitumor activity of drug-loaded polymeric micelles in pancreatic cancer, it was demonstrated that particles with a size of 30 nm or less, were able to penetrate poorly permeable pancreatic tumors, resulting in high antitumor activity[51]. In addition, conjugate siRNAs studies in pancreatic cancer have shown promising results in the clinic. One example is the liposomal conjugate of the drug Atu027 (siRNA-targeting protein kinase 3) which have enhanced tumor penetration [52,53].

In the same category of interfering RNA is the antisense strategy. This strategy is based on the synthesis of nucleic acid strands capable of interacting with the mRNA of the expressed target gene resulting in its inactivation. Furthermore, it is possible to affect the pre-RNA splicing, thus disrupting the mRNA exon content[20]. More specifically, antisense oligonucleotides (ASOs) are single-stranded nucleotide sequences that have been modified at their phosphodiester backbone allowing to protect them from degradation, thus facilitating cell entry[54]. ASOs have been tested clinically in targeting pancreatic cancer. One example is the ISIS-2503 and AEG35156 oligonucleotides. ISIS-2503 is an ASO targeting h-ras (important for pancreatic tumor progression) and AEG35156 synthesized for targeting X-linked inhibitor of apoptosis protein, an anti-apoptic protein overexpressed in pancreatic cancer. Both of these ASOs have been tested in clinical phase trials but with no real success due to their low clinical benefit[55-57].

GENE EDITING TECHNOLOGY

A recent and more sophisticated strategy is genome editing technology including a few techniques for genome manipulation by "rewriting genetic material". Clustered regularly interspaced palindromic repeats-Cas9 (CRISPR-Cas9) system has been given a lot of attention over the years due to its broad range of therapeutic applications. This system offers the ability to induce the cleavage of specific double-stranded DNA segments from the genome *via* the introduction of CRISPR sequences, marking cleavage sites through Cas9 protein endonucleases[58]. This strategy has been used for the therapeutic approach of pulmonary cancer, as well as HIV infection. Gene transfer of this system has also been investigated in pancreatic cancer models *in vivo*[59,60]. Current research is focused on utilizing CRISPR-Cas9 for screening of new gene

targets and to investigate different mechanisms of known genetic aberrations[61]. CRISPR-Cas9 cell therapies are currently tested in the clinical settings for various cancers and immunologic syndromes. CRISPR-Cas9 approach have been demonstrated to be also applicable to inflammatory conditions such as lupus nephritis [62]. However, significant challenges remain, including its efficiency of transduction, compared for example with oncolytic viruses[63].

IMMUNOTHERAPY AND VACCINATION

Tumor cells are able of escaping the recognition and elimination from the immune system resulting in aberrant tumor development and aggressiveness in certain cancers [20,21]. Even though immune cells are found in large numbers within the tumor stroma, they mostly correspond to immunosuppressive subsets such as T-helper 17 cells, regulatory T cells and tumor-associated macrophages (TAMs). Effectors T-cells located in the tumor are quite rare in respect with other tumors and express immune checkpoint receptors such as PD-1, at extremely high levels. PDAC induces a pro-tumoral inflammation and immunosuppressive environment. Furthermore, PDAC cells have a particular role due to paracrine signaling induced by cytokines such as IL-6 and GM-CSF[64,65]. Cancer cells also express checkpoint molecules such as programmed cell death 1 Ligand 1 (PD-L1). A small number of studies have been conducted in pancreatic cancer targeting these immune checkpoint inhibitors. Both PD-L1 and cytotoxic T-lymphocyte associated protein 4 inhibitors have been investigated in patients with metastatic or locally advanced pancreatic cancer in two clinical trials. However, the clinical outcomes were not as expected, with no clinical benefit[66, 67].

Cancer immunotherapy has been developed over the years to improve immune intolerance using active and passive immunity. The first approach is based on the stimulation of immune response to tumor-associated antigens through cancer vaccines. The second approach is based on administering activated effector cells, cytokines or monoclonal antibodies targeting specific tumor cells[21]. To prevent any side effects gene therapies have been evolved to promote local cytokine or interleukin either by *ex-vivo* approach or by the tumor cells themselves (Figure 4). Another promising approach for pancreatic cancer therapy is chimeric antigen receptor T-cell therapy. This approach uses patient's own T cells to target cancer cells *via* genetic engineering. The remarkable results observed in haematological malignancies have moved the attention further to solid tumors like pancreatic cancer[68]. In pancreatic cancer, antigens which are widely expressed are mesothelin and mucin 1 that are cell surface associated. Vaccine experiments have been conducted *in vivo* in various pancreatic cancer models using antigen-pulsed dendritic cells[69].

CONCLUSION

It is more than clear that pancreatic cancer is considered more than a challenging diagnosis, given the fact that the current treatment protocols provide a slight survival expectation. Nevertheless, research on gene therapy has been taking place unexpectedly increasingly, offering a new promising opportunity not only to tackle with an inoperable pancreatic disease, but also, to treat effectively early stages of PDAC. Pancreatic cancer gene therapy with oncolytic virotherapy and adoptive immunotherapy seem to be the cutting-edge therapeutic technology approach with exciting new breakthroughs in recent years. Focusing on vector development and methods to enhance the selectivity of either gene delivery or gene expression in combination with conventional chemotherapeutic agents (*e.g.* gemcitabine) would enhance the therapeutic benefit in human, either metastatic or non, pancreatic cancer model despite its high degree of complexity. Eventually, it seems that there is enough room for improvement in the treatment of pancreatic cancer. However, further clinical trials are considered of crucial importance testing the efficacy, efficiency, and safety of these new gene therapies.

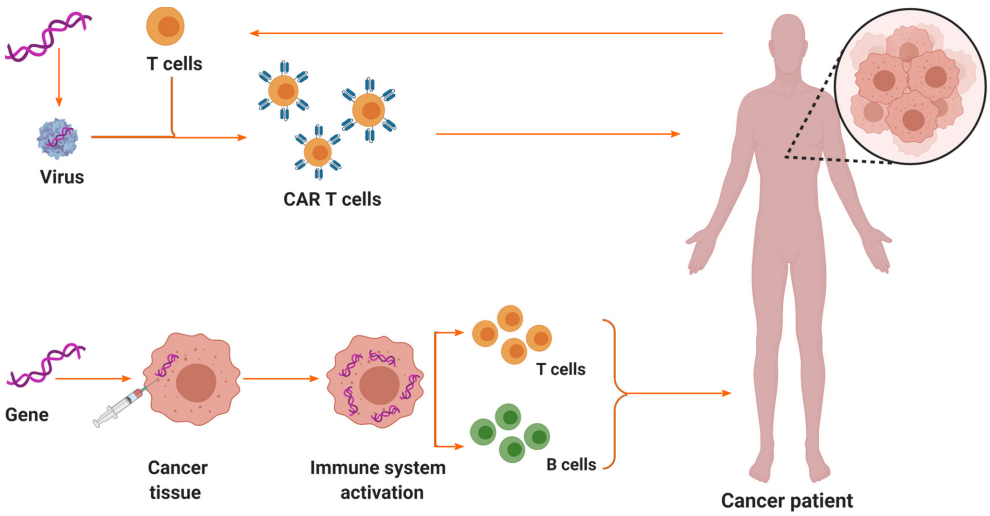


Figure 4 Schematic diagrams of two examples of immunotherapy for pancreatic cancer: chimeric antigen receptor T-cell therapy and immunogenic therapy (*in vivo* gene transfer). The above diagram was created with Biorender.com. CAR T-cell: Chimeric antigen receptor T-cell.

REFERENCES

1 **Capasso M**, Franceschi M, Rodriguez-Castro KI, Crafa P, Cambiè G, Miraglia C, Barchi A, Nouvenne A, Leandro G, Meschi T, De' Angelis GL, Di Mario F. Epidemiology and risk factors of pancreatic cancer. *Acta Biomed* 2018; **89**: 141-146 [PMID: 30561407 DOI: 10.23750/abm.v89i9-S.7923]

2 **Sato-Dahlman M**, Wirth K, Yamamoto M. Role of Gene Therapy in Pancreatic Cancer-A Review. *Cancers (Basel)* 2018; **10** [PMID: 29614005 DOI: 10.3390/cancers10040103]

3 **Christenson ES**, Jaffee E, Azad NS. Current and emerging therapies for patients with advanced pancreatic ductal adenocarcinoma: a bright future. *Lancet Oncol* 2020; **21**: e135-e145 [PMID: 32135117 DOI: 10.1016/S1470-2045(19)30795-8]

4 **McGuigan A**, Kelly P, Turkington RC, Jones C, Coleman HG, McCain RS. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J Gastroenterol* 2018; **24**: 4846-4861 [PMID: 30487695 DOI: 10.3748/wjg.v24.i43.4846]

5 **Goral V**. Pancreatic Cancer: Pathogenesis and Diagnosis. *Asian Pac J Cancer Prev* 2015; **16**: 5619-5624 [PMID: 26320426 DOI: 10.7314/APJCP.2015.16.14.5619]

6 **Peters MLB**, Eckel A, Mueller PP, Tramontano AC, Weaver DT, Lietz A, Hur C, Kong CY, Pandharipande PV. Progression to pancreatic ductal adenocarcinoma from pancreatic intraepithelial neoplasia: Results of a simulation model. *Pancreatology* 2018; **18**: 928-934 [PMID: 30143405 DOI: 10.1016/j.pan.2018.07.009]

7 **Basturk O**, Hong SM, Wood LD, Adsay NV, Albores-Saavedra J, Biankin AV, Brosens LA, Fukushima N, Goggins M, Hruban RH, Kato Y, Klimstra DS, Klöppel G, Krasinskas A, Longnecker DS, Matthaei H, Offerhaus GJ, Shimizu M, Takaori K, Terris B, Yachida S, Esposito I, Furukawa T; Baltimore Consensus Meeting. A Revised Classification System and Recommendations From the Baltimore Consensus Meeting for Neoplastic Precursor Lesions in the Pancreas. *Am J Surg Pathol* 2015; **39**: 1730-1741 [PMID: 26559377 DOI: 10.1097/PAS.0000000000000533]

8 **Aslanian HR**, Lee JH, Canto MI. AGA Clinical Practice Update on Pancreas Cancer Screening in High-Risk Individuals: Expert Review. *Gastroenterology* 2020; **159**: 358-362 [PMID: 32416142 DOI: 10.1053/j.gastro.2020.03.088]

9 **Costache MI**, Costache CA, Dumitrescu CI, Tica AA, Popescu M, Baluta EA, Anghel AC, Saftoiu A, Dumitrescu D. Which is the Best Imaging Method in Pancreatic Adenocarcinoma Diagnosis and Staging - CT, MRI or EUS? *Curr Health Sci J* 2017; **43**: 132-136 [PMID: 30595868 DOI: 10.12865/CHSJ.43.02.05]

10 **Unger K**, Mehta KY, Kaur P, Wang Y, Menon SS, Jain SK, Moonjelly RA, Suman S, Datta K, Singh R, Fogel P, Cheema AK. Metabolomics based predictive classifier for early detection of pancreatic ductal adenocarcinoma. *Oncotarget* 2018; **9**: 23078-23090 [PMID: 29796173 DOI: 10.18632/oncotarget.25212]

11 **Ducreux M**, Cuhna AS, Caramella C, Hollebecque A, Burtin P, Goéré D, Seufferlein T, Haustermans K, Van Laethem JL, Conroy T, Arnold D; ESMO Guidelines Committee. Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2015; **26** Suppl 5: v56-v68 [PMID: 26314780 DOI: 10.1093/annonc/mdv295]

12 **Kim KS**, Kwon J, Kim K, Chie EK. Impact of Resection Margin Distance on Survival of Pancreatic Cancer: A Systematic Review and Meta-Analysis. *Cancer Res Treat* 2017; **49**: 824-833 [PMID: 27561314 DOI: 10.4143/crt.2016.336]

13 **Pericleous S**, Middleton N, McKay SC, Bowers KA, Hutchins RR. Systematic review and meta-

analysis of case-matched studies comparing open and laparoscopic distal pancreatectomy: is it a safe procedure? *Pancreas* 2012; **41**: 993-1000 [PMID: 22836858 DOI: 10.1097/MPA.0b013e31824f3669]

14 **Venkat R**, Edil BH, Schulick RD, Lidor AO, Makary MA, Wolfgang CL. Laparoscopic distal pancreatectomy is associated with significantly less overall morbidity compared to the open technique: a systematic review and meta-analysis. *Ann Surg* 2012; **255**: 1048-1059 [PMID: 22511003 DOI: 10.1097/SLA.0b013e318251ee09]

15 **Altorki N**, Harrison S. What is the role of neoadjuvant chemotherapy, radiation, and adjuvant treatment in resectable esophageal cancer? *Ann Cardiothorac Surg* 2017; **6**: 167-174 [PMID: 28447006 DOI: 10.21037/acs.2017.03.16]

16 **Ghosn M**, Kourie HR, El Rassy E, Haddad FG, Hanna C, El Karak F, Nasr D. Where does chemotherapy stands in the treatment of ampullary carcinoma? *World J Gastrointest Oncol* 2016; **8**: 745-750 [PMID: 27795814 DOI: 10.4251/wjgo.v8.i10.745]

17 **Ginn SL**, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012 - an update. *J Gene Med* 2013; **15**: 65-77 [PMID: 23355455 DOI: 10.1002/jgm.2698]

18 **Singh HM**, Ungerechts G, Tsimberidou AM. Gene and cell therapy for pancreatic cancer. *Expert Opin Biol Ther* 2015; **15**: 505-516 [PMID: 25582170 DOI: 10.1517/14712598.2015.1001734]

19 **Halloran CM**, Ghaneh P, Neoptolemos JP, Costello E. Gene therapy for pancreatic cancer--current and prospective strategies. *Surg Oncol* 2000; **9**: 181-191 [PMID: 11476989 DOI: 10.1016/s0960-7404(01)00011-1]

20 **Rouanet M**, Lebrin M, Gross F, Bournet B, Cordelier P, Buscail L. Gene Therapy for Pancreatic Cancer: Specificity, Issues and Hopes. *Int J Mol Sci* 2017; **18** [PMID: 28594388 DOI: 10.3390/ijms18061231]

21 **Liu SX**, Xia ZS, Zhong YQ. Gene therapy in pancreatic cancer. *World J Gastroenterol* 2014; **20**: 13343-13368 [PMID: 25309069 DOI: 10.3748/wjg.v20.i37.13343]

22 **Nayerossadat N**, Maedeh T, Ali PA. Viral and nonviral delivery systems for gene delivery. *Adv Biomed Res* 2012; **1**: 27 [PMID: 23210086 DOI: 10.4103/2277-9175.98152]

23 **Kamimura K**, Yokoo T, Terai S. Gene Therapy for Pancreatic Diseases: Current Status. *Int J Mol Sci* 2018; **19** [PMID: 30384450 DOI: 10.3390/ijms19113415]

24 **Wang W**, Li W, Ma N, Steinhoff G. Non-viral gene delivery methods. *Curr Pharm Biotechnol* 2013; **14**: 46-60 [PMID: 23437936 DOI: 10.2174/1389201011314010008]

25 **Touchefeu Y**, Harrington KJ, Galmiche JP, Vassaux G. Review article: gene therapy, recent developments and future prospects in gastrointestinal oncology. *Aliment Pharmacol Ther* 2010; **32**: 953-968 [PMID: 20937041 DOI: 10.1111/j.1365-2036.2010.04424.x]

26 **Hacein-Bey-Abina S**, Hauer J, Lim A, Picard C, Wang GP, Berry CC, Martinache C, Rieux-Laucat F, Latour S, Belohradsky BH, Leiva L, Sorensen R, Debré M, Casanova JL, Blanche S, Durandy A, Bushman FD, Fischer A, Cavazzana-Calvo M. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2010; **363**: 355-364 [PMID: 20660403 DOI: 10.1056/NEJMoa1000164]

27 **Hanoun N**, Gayral M, Pointreau A, Buscail L, Cordelier P. Initial Characterization of Integrase-Defective Lentiviral Vectors for Pancreatic Cancer Gene Therapy. *Hum Gene Ther* 2016; **27**: 184-192 [PMID: 26731312 DOI: 10.1089/hum.2015.151]

28 **Pol J**, Bloy N, Obrist F, Eggermont A, Galon J, Cremer I, Erbs P, Limacher JM, Preville X, Zitvogel L, Kroemer G, Galluzzi L. Trial Watch:: Oncolytic viruses for cancer therapy. *Oncoimmunology* 2014; **3**: e28694 [PMID: 25097804 DOI: 10.4161/onci.28694]

29 **Haller SD**, Monaco ML, Essani K. The Present Status of Immuno-Oncolytic Viruses in the Treatment of Pancreatic Cancer. *Viruses* 2020; **12** [PMID: 33213031 DOI: 10.3390/v12111318]

30 **Huang F**, Wang BR, Wu YQ, Wang FC, Zhang J, Wang YG. Oncolytic viruses against cancer stem cells: A promising approach for gastrointestinal cancer. *World J Gastroenterol* 2016; **22**: 7999-8009 [PMID: 27672294 DOI: 10.3748/wjg.v22.i35.7999]

31 **Eissa IR**, Bustos-Villalobos I, Ichinose T, Matsumura S, Naoe Y, Miyajima N, Morimoto D, Mukoyama N, Zhiwen W, Tanaka M, Hasegawa H, Sumigama S, Aleksic B, Kodera Y, Kasuya H. The Current Status and Future Prospects of Oncolytic Viruses in Clinical Trials against Melanoma, Glioma, Pancreatic, and Breast Cancers. *Cancers (Basel)* 2018; **10** [PMID: 30261620 DOI: 10.3390/cancers10100356]

32 **Yokoda RT**, Nagalo BM, Borad MJ. Oncolytic Adenoviruses in Gastrointestinal Cancers. *Biomedicines* 2018; **6** [PMID: 29534501 DOI: 10.3390/biomedicines6010033]

33 **Andtbacka RH**, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J, Delman KA, Spitler LE, Puzanov I, Agarwala SS, Milhem M, Cranmer L, Curti B, Lewis K, Ross M, Guthrie T, Linette GP, Daniels GA, Harrington K, Middleton MR, Miller WH Jr, Zager JS, Ye Y, Yao B, Li A, Doleman S, VanderWalde A, Gansert J, Coffin RS. Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. *J Clin Oncol* 2015; **33**: 2780-2788 [PMID: 26014293 DOI: 10.1200/JCO.2014.58.3377]

34 **Yokoda R**, Nagalo BM, Arora M, Egan JB, Bogenberger JM, DeLeon TT, Zhou Y, Ahn DH, Borad MJ. Oncolytic virotherapy in upper gastrointestinal tract cancers. *Oncolytic Virother* 2017; **7**: 13-24 [PMID: 29616200 DOI: 10.2147/OV.S161397]

35 **Bilusic M**, Wong YN. Anti-angiogenesis in prostate cancer: knocked down but not out. *Asian J Androl* 2014; **16**: 372-377 [PMID: 24759579 DOI: 10.4103/1008-682X.125903]

36 **Melegh Z**, Oltean S. Targeting Angiogenesis in Prostate Cancer. *Int J Mol Sci* 2019; **20** [PMID: 31151317 DOI: 10.3390/ijms20112676]

37 **Aubrey BJ**, Strasser A, Kelly GL. Tumor-Suppressor Functions of the TP53 Pathway. *Cold Spring Harb Perspect Med* 2016; **6** [PMID: 27141080 DOI: 10.1101/cshperspect.a026062]

38 **Bouvet M**, Bold RJ, Lee J, Evans DB, Abbruzzese JL, Chiao PJ, McConkey DJ, Chandra J, Chada S, Fang B, Roth JA. Adenovirus-mediated wild-type p53 tumor suppressor gene therapy induces apoptosis and suppresses growth of human pancreatic cancer [seecomments]. *Ann Surg Oncol* 1998; **5**: 681-688 [PMID: 9869513 DOI: 10.1007/bf02303477]

39 **Hwang RF**, Gordon EM, Anderson WF, Parekh D. Gene therapy for primary and metastatic pancreatic cancer with intraperitoneal retroviral vector bearing the wild-type p53 gene. *Surgery* 1998; **124**: 143-50; discussion 150 [PMID: 9706132 DOI: 10.1016/S0039-6060(98)70114-X]

40 **Fogar P**, Greco E, Basso D, Habeler W, Navaglia F, Zambon CF, Tormen D, Gallo N, Cecchetto A, Plebani M, Pedrazzoli S. Suicide gene therapy with HSV-TK in pancreatic cancer has no effect in vivo in a mouse model. *Eur J Surg Oncol* 2003; **29**: 721-730 [PMID: 14602490 DOI: 10.1016/j.ejso.2003.08.001]

41 **Wang J**, Lu XX, Chen DZ, Li SF, Zhang LS. Herpes simplex virus thymidine kinase and ganciclovir suicide gene therapy for human pancreatic cancer. *World J Gastroenterol* 2004; **10**: 400-403 [PMID: 14760766 DOI: 10.3748/wjg.v10.i3.400]

42 **Mäkinen K**, Loimas S, Wahlfors J, Alhava E, Jänne J. Evaluation of herpes simplex thymidine kinase mediated gene therapy in experimental pancreatic cancer. *J Gene Med* 2000; **2**: 361-367 [PMID: 11045430 DOI: 10.1002/1521-2254(200009/10)2:5<361::AID-JGM125>3.0.CO;2-7]

43 **Carrió M**, Romagosa A, Mercadé E, Mazo A, Nadal M, Gómez-Foix AM, Fillat C. Enhanced pancreatic tumor regression by a combination of adenovirus and retrovirus-mediated delivery of the herpes simplex virus thymidine kinase gene. *Gene Ther* 1999; **6**: 547-553 [PMID: 10476214 DOI: 10.1038/sj.gt.3300846]

44 **Fogar P**, Navaglia F, Basso D, Greco E, Zambon CF, Fadi E, Falda A, Stranges A, Vannozzi F, Danesi R, Pedrazzoli S, Plebani M. Suicide gene therapy with the yeast fusion gene cytosine deaminase/uracil phosphoribosyltransferase is not enough for pancreatic cancer. *Pancreas* 2007; **35**: 224-231 [PMID: 17895842 DOI: 10.1097/mpa.0b013e3180622519]

45 **Löhr M**, Hoffmeyer A, Kröger J, Freund M, Hain J, Holle A, Karle P, Knöfel WT, Liebe S, Müller P, Nizze H, Renner M, Saller RM, Wagner T, Hauenstein K, Günzburg WH, Salmons B. Microencapsulated cell-mediated treatment of inoperable pancreatic carcinoma. *Lancet* 2001; **357**: 1591-1592 [PMID: 11377651 DOI: 10.1016/s0140-6736(00)04749-8]

46 **He L**, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004; **5**: 522-531 [PMID: 15211354 DOI: 10.1038/nrg1379]

47 **Sicard F**, Gayral M, Lulka H, Buscail L, Cordelier P. Targeting miR-21 for the therapy of pancreatic cancer. *Mol Ther* 2013; **21**: 986-994 [PMID: 23481326 DOI: 10.1038/mt.2013.35]

48 **Merhautova J**, Demlova R, Slaby O. MicroRNA-Based Therapy in Animal Models of Selected Gastrointestinal Cancers. *Front Pharmacol* 2016; **7**: 329 [PMID: 27729862 DOI: 10.3389/fphar.2016.00329]

49 **Wang T**, Shigdar S, Shamaileh HA, Gantier MP, Yin W, Xiang D, Wang L, Zhou SF, Hou Y, Wang P, Zhang W, Pu C, Duan W. Challenges and opportunities for siRNA-based cancer treatment. *Cancer Lett* 2017; **387**: 77-83 [PMID: 27045474 DOI: 10.1016/j.canlet.2016.03.045]

50 **Ying H**, Dey P, Yao W, Kimmelman AC, Draetta GF, Maitra A, DePinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev* 2016; **30**: 355-385 [PMID: 26883357 DOI: 10.1101/gad.275776.115]

51 **Cabral H**, Matsumoto Y, Mizuno K, Chen Q, Murakami M, Kimura M, Terada Y, Kano MR, Miyazono K, Uesaka M, Nishiyama N, Kataoka K. Accumulation of sub-100 nm polymeric micelles in poorly permeable tumours depends on size. *Nat Nanotechnol* 2011; **6**: 815-823 [PMID: 22020122 DOI: 10.1038/nnano.2011.166]

52 **Golan T**, Khvalevsky EZ, Hubert A, Gabai RM, Hen N, Segal A, Domb A, Harari G, David EB, Raskin S, Goldes Y, Goldin E, Eliakim R, Lahav M, Kopleman Y, Dancour A, Shemi A, Galun E. RNAi therapy targeting KRAS in combination with chemotherapy for locally advanced pancreatic cancer patients. *Oncotarget* 2015; **6**: 24560-24570 [PMID: 26009994 DOI: 10.18632/oncotarget.4183]

53 **Schultheis B**, Strumberg D, Santel A, Vank C, Gebhardt F, Keil O, Lange C, Giese K, Kaufmann J, Khan M, Drevs J. First-in-human phase I study of the liposomal RNA interference therapeutic Atu027 in patients with advanced solid tumors. *J Clin Oncol* 2014; **32**: 4141-4148 [PMID: 25403217 DOI: 10.1200/JCO.2013.55.0376]

54 **Dowdy SF**. Overcoming cellular barriers for RNA therapeutics. *Nat Biotechnol* 2017; **35**: 222-229 [PMID: 28244992 DOI: 10.1038/nbt.3802]

55 **Mahadevan D**, Chalasani P, Rensvold D, Kurtin S, Pretzinger C, Jolivet J, Ramanathan RK, Von Hoff DD, Weiss GJ. Phase I trial of AEG35156 an antisense oligonucleotide to XIAP plus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma. *Am J Clin Oncol* 2013; **36**: 239-243 [PMID: 22441342 DOI: 10.1097/COC.0b013e3182467a13]

56 **Adjei AA**, Dy GK, Erlichman C, Reid JM, Sloan JA, Pitot HC, Alberts SR, Goldberg RM, Hanson LJ, Atherton PJ, Watanabe T, Geary RS, Holmlund J, Dorr FA. A phase I trial of ISIS 2503, an antisense inhibitor of H-ras, in combination with gemcitabine in patients with advanced cancer. *Clin Cancer Res* 2003; **9**: 115-123 [PMID: 12538459]

57 **Alberts SR**, Schroeder M, Erlichman C, Steen PD, Foster NR, Moore DF Jr, Rowland KM Jr, Nair S, Tschetter LK, Fitch TR. Gemcitabine and ISIS-2503 for patients with locally advanced or metastatic

pancreatic adenocarcinoma: a North Central Cancer Treatment Group phase II trial. *J Clin Oncol* 2004; **22**: 4944-4950 [PMID: 15611509 DOI: 10.1200/JCO.2004.05.034]

58 **Deltcheva E**, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 2011; **471**: 602-607 [PMID: 21455174 DOI: 10.1038/nature09886]

59 **Chiou SH**, Winters IP, Wang J, Naranjo S, Dudgeon C, Tamburini FB, Brady JJ, Yang D, Grüner BM, Chuang CH, Caswell DR, Zeng H, Chu P, Kim GE, Carpizo DR, Kim SK, Winslow MM. Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing. *Genes Dev* 2015; **29**: 1576-1585 [PMID: 26178787 DOI: 10.1101/gad.264861.115]

60 **Huang Z**, Tomitaka A, Raymond A, Nair M. Current application of CRISPR/Cas9 gene-editing technique to eradication of HIV/AIDS. *Gene Ther* 2017; **24**: 377-384 [PMID: 28471431 DOI: 10.1038/gt.2017.35]

61 **Maresch R**, Mueller S, Veltkamp C, Öllinger R, Friedrich M, Heid I, Steiger K, Weber J, Engleitner T, Barenboim M, Klein S, Louzada S, Banerjee R, Strong A, Stauber T, Gross N, Geumann U, Lange S, Ringelhan M, Varela I, Unger K, Yang F, Schmid RM, Vassiliou GS, Braren R, Schneider G, Heikenwalder M, Bradley A, Saur D, Rad R. Multiplexed pancreatic genome engineering and cancer induction by transfection-based CRISPR/Cas9 delivery in mice. *Nat Commun* 2016; **7**: 10770 [PMID: 26916719 DOI: 10.1038/ncomms10770]

62 **Cruz NM**, Freedman BS. CRISPR Gene Editing in the Kidney. *Am J Kidney Dis* 2018; **71**: 874-883 [PMID: 29606501 DOI: 10.1053/j.ajkd.2018.02.347]

63 **Glass Z**, Lee M, Li Y, Xu Q. Engineering the Delivery System for CRISPR-Based Genome Editing. *Trends Biotechnol* 2018; **36**: 173-185 [PMID: 29305085 DOI: 10.1016/j.tibtech.2017.11.006]

64 **Seo YD**, Pillarisetty VG. T-cell programming in pancreatic adenocarcinoma: a review. *Cancer Gene Ther* 2017; **24**: 106-113 [PMID: 27910859 DOI: 10.1038/cgt.2016.66]

65 **Sideras K**, Braat H, Kwekkeboom J, van Eijck CH, Peppelenbosch MP, Sleijfer S, Bruno M. Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies. *Cancer Treat Rev* 2014; **40**: 513-522 [PMID: 24315741 DOI: 10.1016/j.ctrv.2013.11.005]

66 **Johansson H**, Andersson R, Bauden M, Hammes S, Holdenrieder S, Ansari D. Immune checkpoint therapy for pancreatic cancer. *World J Gastroenterol* 2016; **22**: 9457-9476 [PMID: 27920468 DOI: 10.3748/wjg.v22.i43.9457]

67 **Johnson BA 3rd**, Yarchoan M, Lee V, Laheru DA, Jaffee EM. Strategies for Increasing Pancreatic Tumor Immunogenicity. *Clin Cancer Res* 2017; **23**: 1656-1669 [PMID: 28373364 DOI: 10.1158/1078-0432.CCR-16-2318]

68 **DeSelm CJ**, Tano ZE, Varghese AM, Adusumilli PS. CAR T-cell therapy for pancreatic cancer. *J Surg Oncol* 2017; **116**: 63-74 [PMID: 28346697 DOI: 10.1002/jso.24627]

69 **Mohammed S**, Sukumaran S, Bajgain P, Watanabe N, Heslop HE, Rooney CM, Brenner MK, Fisher WE, Leen AM, Vera JF. Improving Chimeric Antigen Receptor-Modified T Cell Function by Reversing the Immunosuppressive Tumor Microenvironment of Pancreatic Cancer. *Mol Ther* 2017; **25**: 249-258 [PMID: 28129119 DOI: 10.1016/j.ymthe.2016.10.016]

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Basic Study

Fasudil prevents liver fibrosis *via* activating natural killer cells and suppressing hepatic stellate cells

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Abstract

BACKGROUND

Fasudil, as a Ras homology family member A (RhoA) kinase inhibitor, is used to improve brain microcirculation and promote nerve regeneration clinically. Increasing evidence shows that Rho-kinase inhibition could improve liver fibrosis.

AIM

To evaluate the anti-fibrotic effects of Fasudil in a mouse model of liver fibrosis induced by thioacetamide (TAA).

METHODS

C57BL/6 mice were administered TAA once every 3 d for 12 times. At 1 wk after induction with TAA, Fasudil was intraperitoneally injected once a day for 3 wk, followed by hematoxylin and eosin staining, sirius red staining, western blotting, and quantitative polymerase chain reaction (qPCR), and immune cell activation was assayed by fluorescence-activated cell sorting. Furthermore, the effects of Fasudil on hepatic stellate cells and natural killer (NK) cells were assayed *in vitro*.

RESULTS

First, we found that TAA-induced liver injury was protected, and the positive area of sirius red staining and type I collagen deposition were significantly decreased by Fasudil treatment. Furthermore, western blot and qPCR assays showed that the levels of alpha smooth muscle actin (α-SMA), matrix metalloproteinase 2 (MMP-2), MMP-9, and transforming growth factor beta 1 (TGF-β1) were inhibited by Fasudil. Moreover, flow cytometry analysis revealed that NK cells were activated by Fasudil treatment in *vivo* and *in vitro*. Furthermore, Fasudil directly promoted the apoptosis and inhibited the proliferation of hepatic stellate

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cells by decreasing α-SMA and TGF-β1.

CONCLUSION
Fasudil inhibits liver fibrosis by activating NK cells and blocking hepatic stellate cell activation, thereby providing a feasible solution for the clinical treatment of liver fibrosis.

Key Words: Liver fibrosis; Natural killer cells; Fasudil; Hepatic stellate cells

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Core Tip: Liver fibrosis is caused by inflammation and characterized by accumulation of the extracellular matrix; there is no clinically safe and efficient drug to treat this condition. Fasudil treatment inhibited liver injury and liver fibrosis *in vivo*, and prevented liver fibrosis *via* activating natural killer cells but suppressing hepatic stellate cells in a thioacetamide-induced model. As a drug used clinically, these results provide a feasible solution for the clinical treatment of liver fibrosis.

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INTRODUCTION

Liver fibrosis is caused by inflammation and characterized by the accumulation of extracellular matrix (ECM), which are major characteristics of chronic liver diseases such as cirrhosis and primary hepatic carcinoma[1]. The traditional view is that liver fibrosis is irreversible, but recent studies have shown that liver fibrosis can be partially reversed in experimental models of liver fibrosis[2]. Unfortunately, to date, there is no clinically safe and efficient drug to treat liver fibrosis in humans.

The mechanisms underlying hepatic fibrosis are inflammatory reaction, hepatic stellate cell (HSC) activation, and the biological function of various cytokines[3]. HSCs, as hepatic lipid-storing cells, are sensitive to hepatic injury and participate in the pathogenesis of liver fibrosis[4]. During chronic diseases, agents from damaged hepatocytes or other cells can promote the activation of HSCs. Then these activated HSCs become highly proliferative myofibroblast-like cells, exhibiting a migratory phenotype and producing large amounts of extracellular matrix proteins such as collagens and fibronectin, thereby leading to the development of hepatic fibrosis[5,6]. Therefore, HSC-specific targeted strategies are required for the effective treatment of liver fibrosis.

Immune cells in the liver exert different roles during the pathogenesis of liver fibrosis. Hepatic macrophages promote fibrosis by perpetuating an inflammatory phase, which leads to the release of pro-inflammatory cytokines and chemokines, and then the activation of HSCs[7]. T helper type 2 (Th2) cells can produce cytokines, including interleukin (IL)-13, IL-4, and IL-5, which are shown to promote fibrosis[8]. Natural killer (NK) cells suppress the pathogenesis of liver fibrosis by killing HSCs and producing interferon-γ (IFN-γ). Also, NK cells can induce apoptosis of HSCs and help clear senescent-activated HSCs, thereby facilitating the resolution of fibrosis, while the exact mechanisms remain unknown. NK cells are suppressed during the advanced stage of liver fibrosis in mice and patients[9,10]. Thus, properly regulating the activation of intrahepatic immune cells is essential for treating liver fibrosis. Practically, the restoration and promotion of NK cell activity might be an attractive strategy for the regression of liver fibrosis.

Ras homology family member A (RhoA) and its major downstream effector Rho-associated protein kinase (ROCK) play important roles in several downstream effects of the small GTP-binding protein Rho. Several cellular events including adhesion, motility, and contractility are regulated by Rho kinase[11]. Furthermore, increasing evidence has demonstrated that Rho kinase inhibition can improve liver fibrosis. The

Rho kinase, Y27632, decreases fibrotic parameters in models of liver fibrosis, and inhibits the activation status of the primary HSCs[12]. Additionally, RhoA-ROCK signaling pathway also plays a key role in regulating the function of immune cells. For example, the RhoA-ROCK pathway promotes the activation of downstream chemokine receptors. Therefore, T-cell activation, polarization, and migration are promoted by chemokines[13]. Fasudil, as a first-generation selective Rho/ROCK inhibitor in the clinic, is clinically used to improve brain microcirculation and promote nerve regeneration[14]. More importantly, Fasudil can prevent lung and skin fibroses in hypochlorous acid-injected mice[15]. Recently, Fasudil was shown to exert anti-inflammatory effects and markedly reduce the accumulation of ECM in type 1 diabetic rats[16]. However, the viability of Fasudil for liver fibrosis therapy and the associated mechanisms are still unclear. The regulatory effects of Fasudil on NK cells in liver fibrosis have not been fully explained.

In this study, we determined the effects of Fasudil on the progression of liver fibrosis and clarified the related mechanisms. We found that Fasudil performed anti-proliferative effects in a mouse model of thioacetamide (TAA)-induced liver fibrosis, providing a feasible solution for the clinical treatment of liver fibrosis.

MATERIALS AND METHODS

Animal model

C57BL/6 mice (male, 4-6 wk old) were provided by HuaFuKang Biological Technology Co., Ltd. (Beijing, China). The animals were caged under specific pathogen-free conditions, housed under a controlled temperature 23 ± 1°C and relative humidity 45%. The animal model of hepatic fibrosis was induced by administration of TAA at 200 mg/kg (T104039; Aladdin, Shanghai, China) once every 3 d for 12 times [17]. At 1 wk after induction with TAA, Fasudil (10 mg/kg) was intraperitoneally injected once a day for 3 wk. The procedures were approved by the Research Ethics Committee of Shandong University (Jinan, China).

Cell line and reagents

LX-2, an immortalized human hepatic stellate cell line preserved in our laboratory, was grown in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Inc., Waltham, MA, United States). This cell line carries a typical phenotype and biochemical properties of activated HSCs, and was checked by referring to the International Cell Line Authentication Committee and National Center for Biotechnology Information databases[18]. Fasudil was purchased from Tianjin Chase Sun Pharmaceutical Co. Ltd. (Tianjin Shi, China).

Hematoxylin and eosin staining

In a TAA-induced hepatic fibrosis model, the mice were sacrificed under mild ether anesthesia after Fasudil treatment. Then mouse liver specimens were fixed overnight in 4% paraformaldehyde/phosphate-buffered saline (PBS) and embedded in paraffin. Then liver sections were examined and photographed under a light microscope after hematoxylin and eosin (Beyotime, Shanghai, China) staining. The criteria used for scoring fibrosis and inflammation were as follows: Score 0, normal (no visible fibrosis and inflammation); score 1, fibrosis and inflammation present (5%-30%); score 2, mild fibrosis and inflammation (31%-50%); and score 3, severe fibrosis and inflammation (51%-75%).

Sirius red staining

Liver specimens were stained with 0.4% sirius red in saturated picric acid for 0.5 h. The positively stained area was selected by threshold adjustment on a gray scale picture using Image J software (NIH, Bethesda, MD, United States) and the ratio of positively stained area/total area was then calculated. At least five different fields on each slide were measured. The stained area were determined using Image-pro Plus 6.0 software.

RNA extraction and real-time reverse transcription-polymerase chain reaction

The treated cells were added to TRIzol (Invitrogen, Thermo Fisher Scientific, Inc.), and the total RNA was extracted from cells following the manufacturer's instructions. Then cDNA was obtained and synthesized using M-MLV Reverse Transcriptase (Invitrogen; Thermo Fisher scientific, Inc.). The mRNA levels of matrix metalloproteinase 2 (MMP-

2), MMP-9, transforming growth factor beta (TGF-β), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and Ki67 were detected according to the SYBR Green Master Mix kit instructions (Roche Diagnostics, Indianapolis, IN, United States). The primer sequences for quantitative polymerase chain reaction (qPCR) amplification are listed in Table 1. PCR was conducted using the iCycleriQ real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, United States).

Cell cycle analysis and apoptosis assay

After treatment, the cell cycle distribution of HSCs seeded was assayed by flow cytometry. Briefly, HSCs (1.5×10^5) were plated in 12-well plates and treated with Fasudil at the indicated concentrations (5 and 10 mM). After 24 h, cells were harvested and washed with ice-cold PBS. Then they were fixed in cold 70% ethanol and stored at 4°C overnight, and the fixed cells were washed and re-suspended in 1 mL staining solution containing 50 mg/mL propidium iodide (PI) (Beijing Solarbio Science & Technology, Co., Ltd.) and 100 mg/mL RNase for 30 min at 37°C. Then cell cycle analysis was conducted using a flow cytometer (BD Calibur).

FITC-Annexin V/PI (eBioscience; Thermo Fisher Scientific, Inc.) was utilized to determine rates of apoptosis. LX2 cells were plated and treated with Fasudil (5 mM, 10 mM) for 24 h. Cells were harvested and stained following the manufacturer's instructions. Finally, the flow cytometry data were analyzed using WinMDI.

Western blotting

Protein was extracted from LX-2 cells, NK-92 cells, or liver tissues with RIPA protein lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) (Beyotime, Shanghai, China). The protein concentration of each sample was determined by the BCA assay. Proteins (30 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel, and then transferred to PVDF membranes (Millipore, Billerica, MA, United States). The following primary antibodies were used: Anti-phosphorylated extracellular signal-related kinase (p-ERK) (#14227S; Cell Signaling Technology, Danvers, MA, USA), anti-ERK (#4348S, Cell signaling Technology), alpha smooth muscle actin (α-SMA) (#4668S; Cell signaling Technology), Collagen I (WL008; Wanleibio Co., Ltd., Shenyang, China), and β-actin (#3700; Cell signaling Technology). For ECL detection, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (#A0208; Beyotime, Shanghai, China) at a dilution of 1:10000 for 1 h at 37°C. ECL detection was performed according to the manufacturer's protocol (Millipore). Analysis of blots was performed using Image J.

Isolation of liver mononuclear cells and flow cytometry

Mice were euthanized and the liver tissues were harvested, and then intrahepatic liver mononuclear cells were isolated as indicated previously[19]. Antibodies used for fluorescence-activated cell sorting (FACS) analysis were PerCP-Cy5.5 anti-cluster of differentiation 3 (CD3) (#35-0031-82), FITC anti-NK1.1 (#11-5941-82), PE anti-CD69 (#12-0691-82), PE anti-NKG2A (#12-5897-81), PE anti-NKG2D (#12-5882-82), or isotype-matched controls (eBioscience, San Diego, CA, United States). Cells were analyzed on a FACSCalibur flow cytometer, and then analyzed using WinMDI analysis software.

Cytolysis assay

The cytolytic activity of NK-92 cells against LX2 was determined using the MTT assay. Briefly, 1×10^4 target cells (LX2 cells) were seeded into 96-well plates. NK-92 cells, pretreated with Fasudil (10 mM) overnight, were added to target cells at effector/target ratios of 1:20, 1:10, or 1:5. Then the effector and target cell were co-incubated for 12 h, and then 20 μL MTT (5 mg/mL) were added. After 4 h, the absorbance (A) at 490 nm was determined using a scanning multi-well spectrophotometer (Bio-Rad). The percent specific lysis was calculated using the following formula: % specific lysis $=1-(OD_{E+T}-OD_E)/OD_T \times 100\%$.

Statistical analyses

Data are shown as mean ± SD of three independent experiments. Significant differences were analyzed using the Student's *t*-test or one-way analysis of variance. Statistical analyses were conducted using GraphPad Prism (version 5.0a). $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

Table 1 Primers used in this study

Species	Gene	Primer (5' to 3')
Mouse	β-actin	F: ATCATGTTTGAGACCTTCAACA
		R: CATCTCTTGCTCGAAGTCCA
Mouse	MMP2	F: CTCCTGGTATGAGATAGCAAA
		R: TGCACCACCAACTGCTTAGC
Mouse	MMP9	F: TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC
		R: TGGACCTGTGGGTTGTTGACCTCAAACTTGGC
Mouse	α-SMA	F: CTGACAGAGGCACCACTGAA
		R: GAAGGAATAGCCACGCTCAG
Human	α-SMA	F: GATCTCAGTGCAGAGGCTCG
		R: TTTGCTTGTCCAGGTGGTCC
Human	Ki67	F: TCCTCCAGGGATCCAACGA
		R: GGCAGGCGGGAGGTCTT
Human	Bcl-2	F: CTTCCCTCATCCTCCTGCTAC
		R: ACAAACTGGGTAAAGGTGATGG
Human	Bax	F: CTCCTGGTATGAGATAGCAAA
		R: TGCACCACCAACTGCTTAGC
Human	Rho	F: TCCTCCAGGGATCCAACGA
		R: GGCAGGCGGGAGGTCTT
Human	β-actin	F: CACTGTGTTGGCGTACAGGT
		R: TCATCACCATTGGCAATGAG

Note: α-SMA, alpha smooth muscle actin; β-actin, beta actin; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; MMP, matrix metalloproteinase; Rho, Ras homology family member

RESULTS

Fasudil protects TAA-induced liver injury

To clarify whether Fasudil influences hepatic fibrosis, a fibrotic mouse model was induced by intraperitoneal injection of TAA (200 mg/mL) once time every 3 d[11]. At 7 d after induction with TAA, Fasudil (10 mg/kg) was injected once a day for 3 wk, and then mice were sacrificed (Figure 1A). As shown in Figure 1B, compared with the normal gross features of the livers observed in the control group, the liver surface from the TAA-treated group showed several spots of nodules, while the livers from the TAA + Fasudil group had much fewer nodular spots. There was no significant difference in liver/body ratio among these three groups. Meanwhile, the liver of the TAA-treated group exhibited a lobular structure destruction and inflammatory cell infiltration. Also, livers from TAA-treated mice gained loss of structural integrity, while TAA + Fasudil-treated mice revealed structural integrity of liver with no significant inflammatory cell infiltration (Figure 1C). Fasudil significantly lowered the indices of liver (Table 2). Furthermore, we observed a significant reduction of alanine aminotransferase and aspartate aminotransferase in serum after Fasudil treatment compared with TAA-treated mice ($P < 0.05$; Figure 1D). These data suggest that Fasudil might blunt TAA-induced liver fibrosis by preventing liver injury.

Fasudil prevents TAA-induced liver fibrosis in vivo

Hepatic collagen I deposition is an important characteristic of liver fibrosis level in mice[20]. As shown in Figure 2A, sirius red staining exhibited a significant increase in perisinusoidal collagen fibers in the TAA-treated group, which was significantly decreased by Fasudil treatment ($P < 0.05$). Furthermore, fibrosis-related genes such as α-SMA, MMP-2/9, and TGF-β1[21] were analyzed to evaluate the impact of Fasudil on hepatic fibrosis. As shown in Figure 2B, the expression of α-SMA and collagen I in

Table 2 Effect of Fasudil on the pathologic grading of hepatic fibrosis mice induce by thioacetamide

Group	Pathologic grading of hepatic fibrosis			
	0	I	II	III
Ctrl (n = 5)	15	0	0	0
TAA (n = 5)	0	0	6	7
TAA + Fasudil	0	7	6	2

Results are 15 fields of vision from five mice per group (three fields were chosen from one liver section). TAA: Thioacetamide.

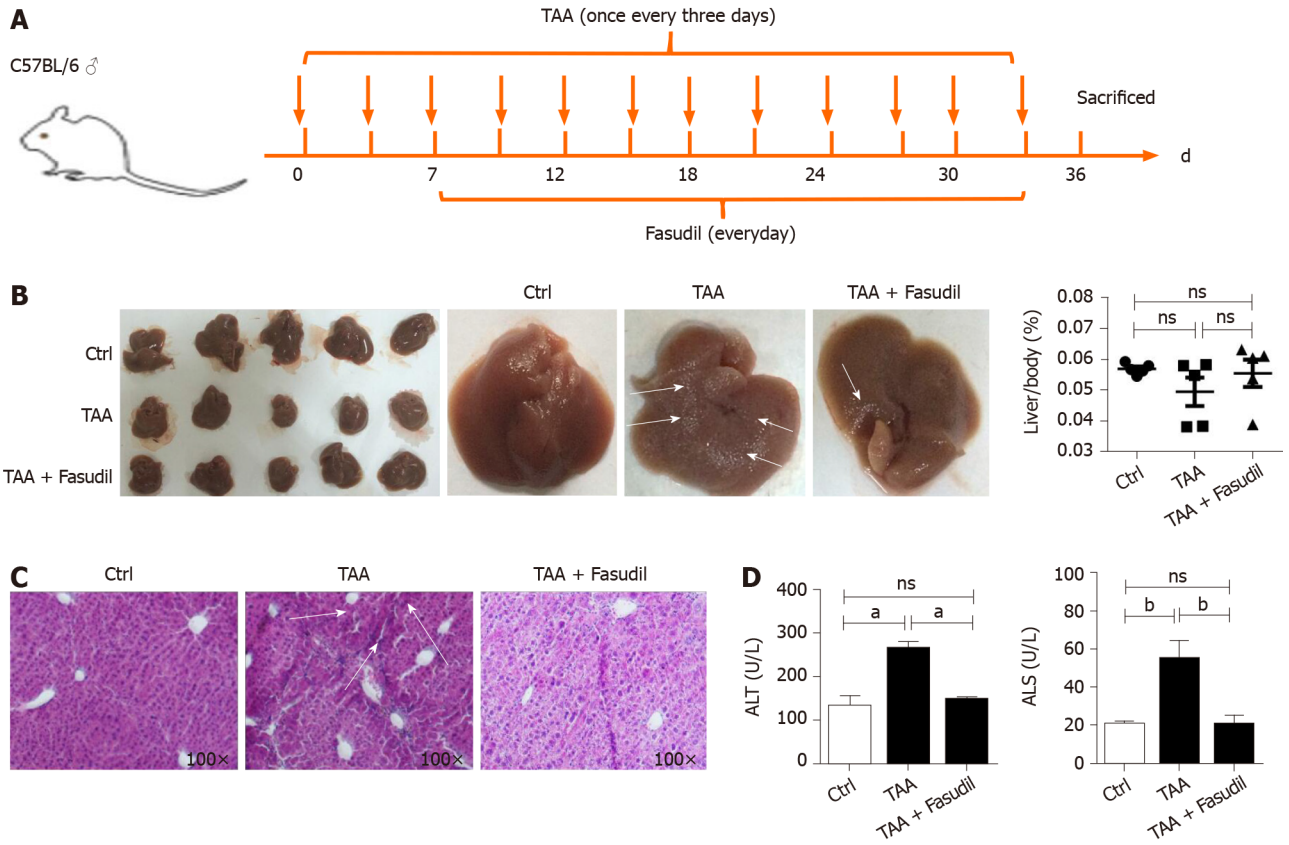


Figure 1 Fasudil protects thioacetamide-induced liver injury. A: Establishment of a liver fibrosis mouse model; B: Representative detection of liver images is shown. Gross pathological examination of liver taken from normal control, thioacetamide (TAA)-treated, and TAA + Fasudil-treated groups, and the liver/body ratio was assayed among these three groups; C: Histological observation of liver fibrosis by hematoxylin and eosin staining under light-field microscope with × 100 magnification; D: Enzyme-linked immunoassay was used to assess the level of serum alanine aminotransferase and aspartate aminotransferase. Data represent the mean ± SD from at least three independent experiments (n = 5/group). $^{a}P < 0.05$, $^{b}P < 0.01$.

TAA + Fasudil-treated mice was significantly lower than that in TAA-treated group. Meanwhile, mRNA levels of MMP-2 and MMP-9 were also downregulated by Fasudil treatment (Figure 2C). TGF-β1 induced by TAA was significantly decreased at both the mRNA and protein levels after Fasudil treatment ($P < 0.05$; Figure 2D). These data clearly demonstrate that TAA-induced liver fibrogenesis is markedly inhibited by Fasudil treatment.

NK cells are activated by Fasudil in a TAA-induced fibrotic mouse model

Immune cells in the liver play critical roles during the development of liver fibrosis [22]. As shown in Figure 3A, TAA treatment increased the total number of mononuclear cells in the liver compared to the normal control group, while no significant differences were observed between TAA-treated and TAA + Fasudil-treated groups. NK cells suppress liver fibrosis *via* strong cytolysis against HSCs[23, 24], and impaired NK cell may contribute to accelerated liver fibrosis progression in a TAA-induced fibrotic mouse model. Here, the proportion of NK cells was not

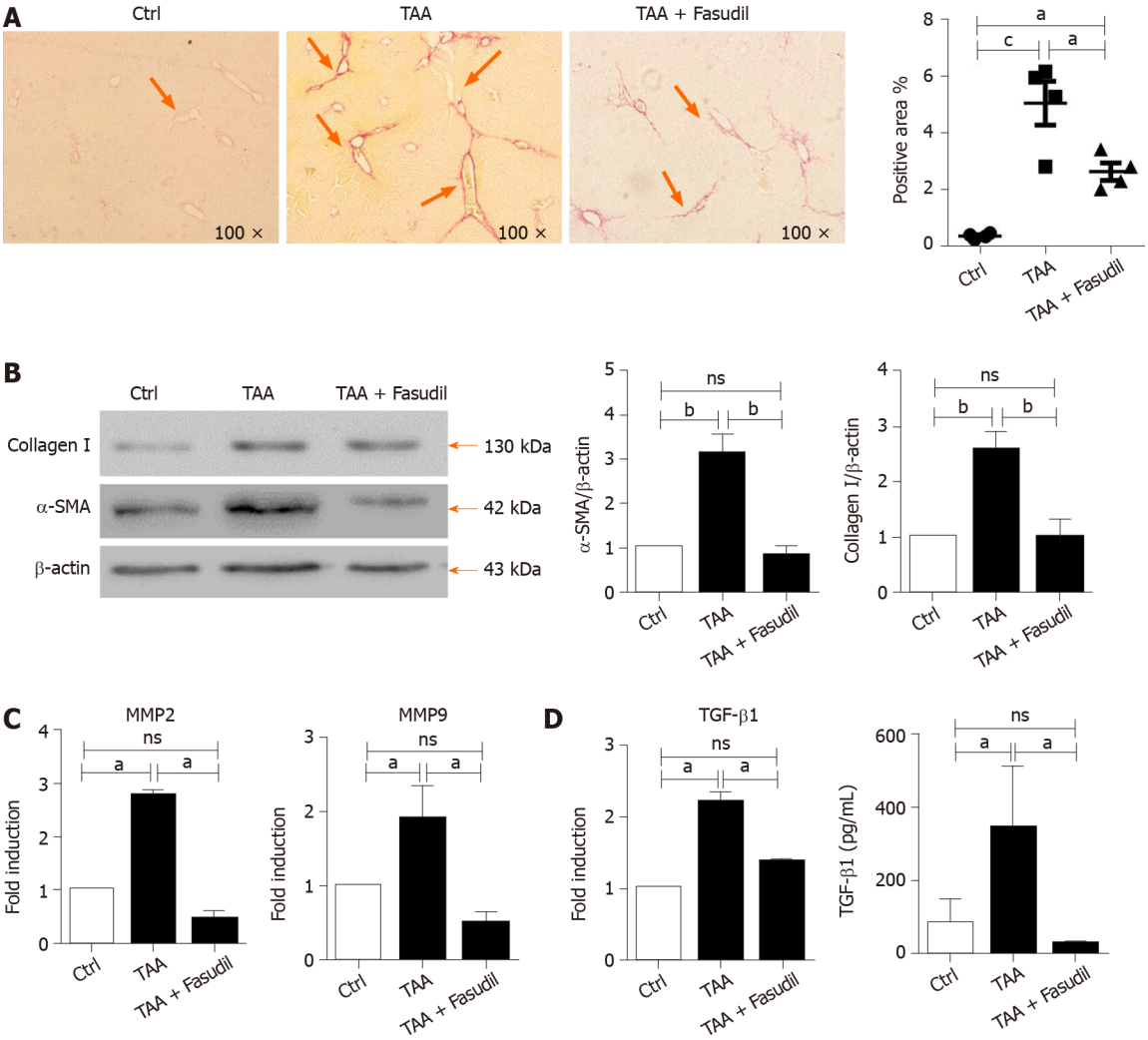


Figure 2 Fasudil prevents thioacetamide-induced liver fibrosis. A: Histological observation of collagen deposition by sirius red staining under a light-field microscope with × 100 magnification (left). Quantitative analysis of sirius red staining of each group (right); B: Expression of alpha smooth muscle actin (a-SMA) and collagen I in liver tissues was assayed by western blotting. Right panel shows the quantification of α-SMA and collagen I protein levels; C: Levels of matrix metalloproteinase 2 (MMP2) and MMP9 mRNA in liver tissues were detected by real time-reverse transcription polymerase chain reaction (RT-PCR); D: Hepatic expression of transforming growth factor beta 1 (TGF-β1) mRNA was detected by real time-RT-PCR, the secretion level of TGF-β1 in serum was assayed by enzyme-linked immunoassay. Data represent the mean ± SD from at least three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$.

significantly affected after Fasudil treatment (Figure 3B), while the level of the activation marker CD69 was markedly increased in NK cells from TAA + Fasudil-treated mice than that from TAA-treated mice ($P < 0.05$; Figure 3C), accompanied by the elevation of activation receptor NKG2D (Figure 3D). Meanwhile, the proportion of $CD69^{+}CD8^{+}$T cells (Figure 3E) and $CD69^{+}CD4^{+}$T cells (Figure 3F) in the liver was not significantly affected by Fasudil treatment. These data suggest that NK cells might be activated by Fasudil and exhibit anti-liver fibrosis effects in a TAA-induced fibrotic mouse model.

Fasudil promotes NK cell activation via ERK and nuclear factor kappa B signaling pathways

To investigate the mechanism by which Fasudil treatment suppresses the progression of liver fibrosis by activating NK cells, human NK-92 cells were treated with different concentrations of Fasudil (5 mM, 10 mM) for 24 h *in vitro*, and the concentration of 10 mM was equivalent to *in vivo* experiments. Then the levels of ERK, nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) were examined by western blotting. The levels of p-ERK and p-NF-kB were significantly increased in Fasudil-treated NK-92 cells, while activation of the STAT3 signaling pathway was not affected by Fasudil (Figure 4A). To determine the effects of NK cells activated by Fasudil on HSCs, we assayed the lysis of LX2 cells (human HSC cell line) by NK cells at different effector: target ratios. First, we treated NK-92 cells with Fasudil

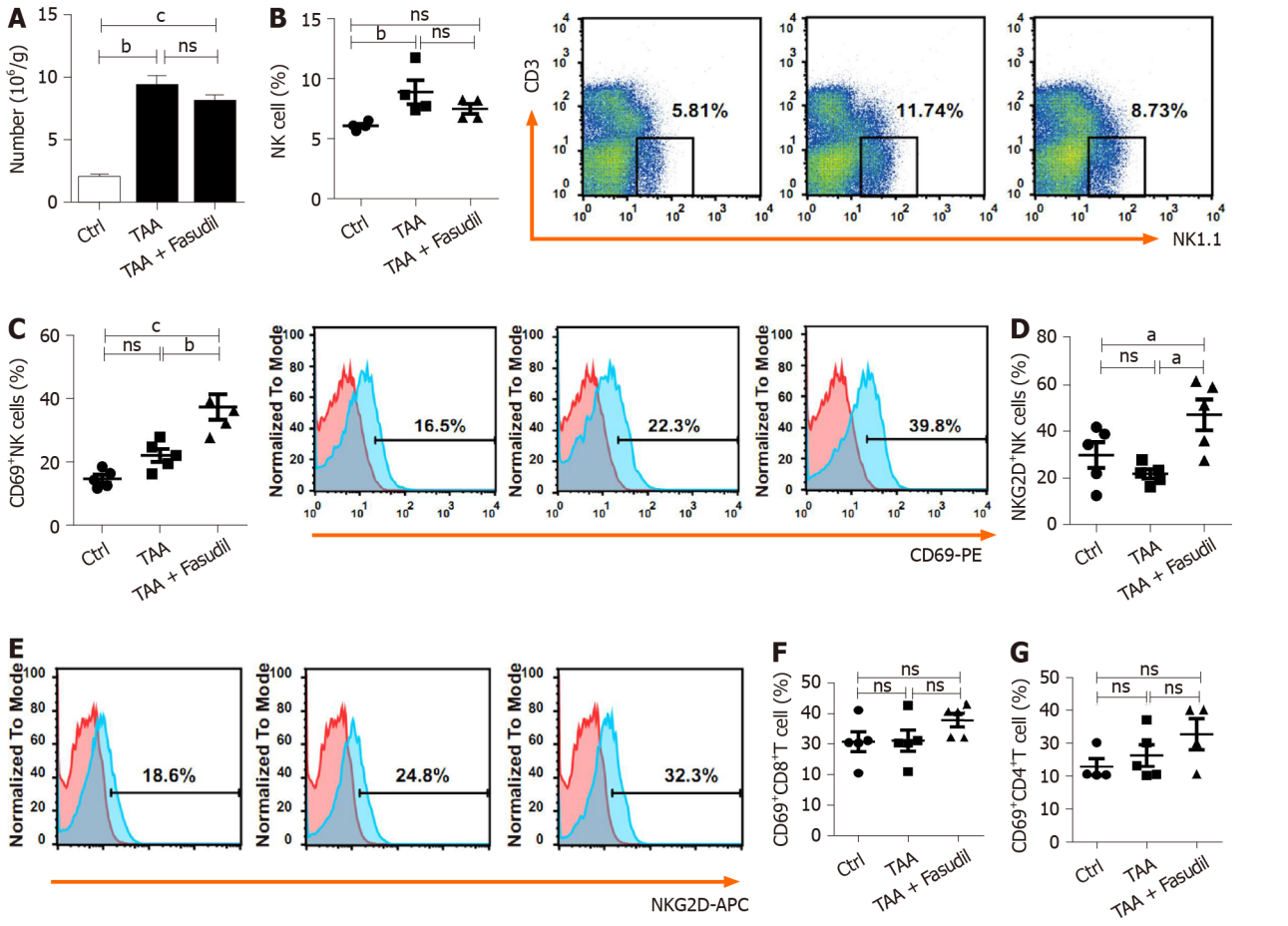


Figure 3 Natural killer cells are activated in a Fasudil-treated liver fibrotic mouse model. After the last treatment, the mice were sacrificed and the intrahepatic lymphocytes were isolated. A: Absolute number of mononuclear cells in the liver from the normal control, thioacetamide (TAA)-induced, and TAA + Fasudil groups was assessed; B-G: Then the percentages and representative fluorescence-activated cell sorting (FACS) plots of natural killer (NK) cells among lymphocytes (B), $CD69^+$ NK cells and representative FACS plots (C), quantification of $NKG2D^+$ NK cells (D), representative FACS plots of NKG2D on NK cells (E), $CD69^+CD8^+$ T cells (F) and $CD69^+CD4^+$ T cells (G) in liver from the above three groups were analyzed by FACS. Data represent the mean ± SD from at least three independent experiments (n = 5/group). $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

(10 mM) for 24 h, and assayed the cytotoxicity against cultured LX2 cells. We found that Fasudil treatment increased the lysis activity of NK cells against cultured LX2 cells (Figure 4B). Then, liver mononuclear cells from normal mice were isolated and treated with different concentrations of Fasudil for 24 h *in vitro*. FACS analysis showed the frequency of $CD69^+$ NK cells increased after Fasudil treatment (Figure 4C). Furthermore, the activation receptor NKG2D was enhanced in NK cells, while the inhibitory receptor NKG2A was not significantly influenced by Fasudil treatment (Figure 4D). In addition, the proportion of IFN-γ^+ NK cells was increased after Fasudil treatment (Figure 4E).

Taken together, these results demonstrate that Fasudil can promote NK cell activation and cytotoxicity by activating the ERK and NF-κB signaling pathways.

Fasudil inhibits proliferation but promotes the apoptosis of HSCs

The proliferation and activation of HSCs are one of the main triggers of liver fibrosis [25]. To observe the direct effects of Fasudil on HSCs, LX2 cells were treated with different concentrations of Fasudil (5 mM, 10 mM) for 24 h *in vitro*, and then the survival of these HSCs was observed. As shown in Figure 5A, the percentage of apoptotic HSCs was promoted by Fasudil treatment. Meanwhile, the level of the anti-apoptotic gene Bcl-2 was decreased, while the level of the pro-apoptotic gene Bax was induced in LX2 cells treated with Fasudil (Figure 5B), accompanied by an increase in p-ERK level (Figure 5C). Furthermore, Fasudil arrested the HSC cell cycle in the G1 phase (Figure 5D). Accordingly, the proliferation-related gene Ki67 in LX2 cells was downregulated (Figure 5E). Previous studies have reported that RhoA is involved in expression of the fibrosis-associated protein α-SMA[26]. Fasudil is a RhoA kinase inhibitor; we found that Fasudil decreased the expression of α-SMA (Figure 5E and F)

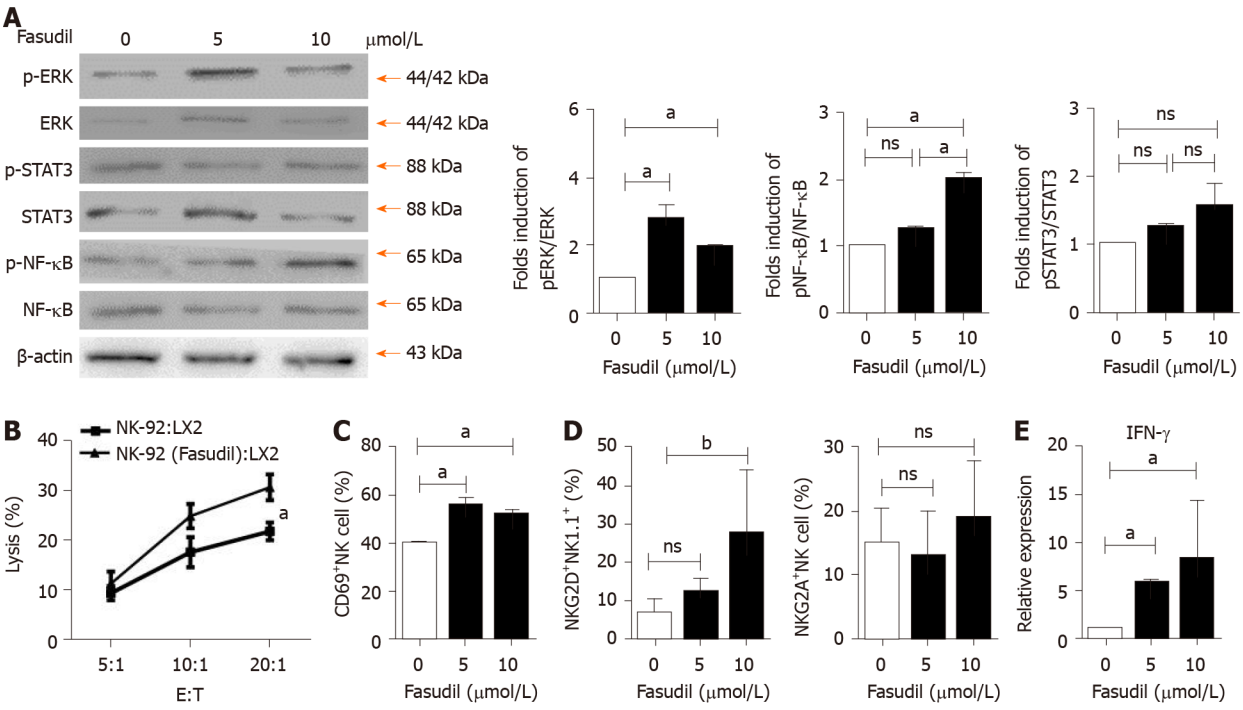


Figure 4 Fasudil can promote natural killer cell activation *via* extracellular signal-related kinase and nuclear factor kappa B signaling pathways. A: Natural killer (NK)-92 cells were treated with different concentrations of Fasudil (5 mM, 10 mM) for 24 h *in vitro*, and extracellular signal-related kinase (ERK), nuclear factor kappa B (NF-kB), and signal transducer and activator of transcription 3 (STAT3) levels were detected by western blotting. Then mononuclear cells from the livers of normal mice were isolated and then treated with Fasudil (5 mM, 10 mM) for 24 h *in vitro*; B: 1 × 10^4 LX2 cells were seeded into 96-well plates. NK-92 cells pretreated with Fasudil (10 mM) were added at different ratios (1:20, 1:10, or 1:5) for 12 h. Then the cytotoxicity was analyzed; C-E: Frequencies of $CD69^+$ NK cells (C), NKG2D NK cells, and NKG2A NK cells (D), as well as the proportion of IFN-γ-producing NK cells (D) were analyzed by FACS (E). Data represent the mean ± SD from at least three independent experiments. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

accompanied by the downregulation of RhoA (Figure 5G). These results indicate that Fasudil has a direct inhibitory effect on HSCs.

DISCUSSION

The current therapeutic interventions for liver fibrosis include removing the injurious stimuli, decreasing hepatic inflammation, suppressing HSC activation, and accelerating matrix degradation[27]. Repurposing old drugs for new clinical applications is a superior strategy for drug development. Recently, Rilpivirine, a widely used anti-HIV drug, was found to ameliorate liver fibrosis through selective STAT1-dependent induction of HSC apoptosis[28]. So, repositioning existing drugs might be an effective strategy for obtaining therapeutics against liver fibrosis.

Fasudil, a RhoA kinase inhibitor, is clinically applied for improving brain microcirculation and promoting nerve regeneration. Xie *et al*[16] found that RhoA/ROCK signaling pathway activation plays an important role in the development of diabetic hepatic fibrosis. Interestingly, the RhoA/ROCK-2 signaling pathway is necessary for activated HSC; the administration of RhoA/ROCK inhibitor can exert anti-inflammatory effects, markedly decrease collagen synthesis, and delay hepatic fibrosis[16, 29]. In accordance with this, we found that liver injury and liver fibrosis induced by repeated injection of TAA were prevented by Fasudil treatment (Figures 1 and 2). In addition, several studies have proved that RhoA/ROCK pathway activation promotes TGF-β1 secretion in several models[30]. As a RhoA/ROCK inhibitor, Fasudil inhibited the induction of TGF-β1 secretion in TAA-induced liver fibrosis, consistent with previous studies.

The immune response plays important roles in the development and treatment of liver fibrosis[31]. NK cells can promote the secretion of IFN-γ through the Janus kinase-STAT signaling pathway, thereby promoting the killing of HSCs[32]. The inhibition of NK cells cytotoxicity promotes the accumulation of LX2 cells[33]. In this study, with the elevation of CD69 and NKG2D, hepatic NK cell activation was promoted by Fasudil treatment (Figure 3). Additionally, NK cells were activated in

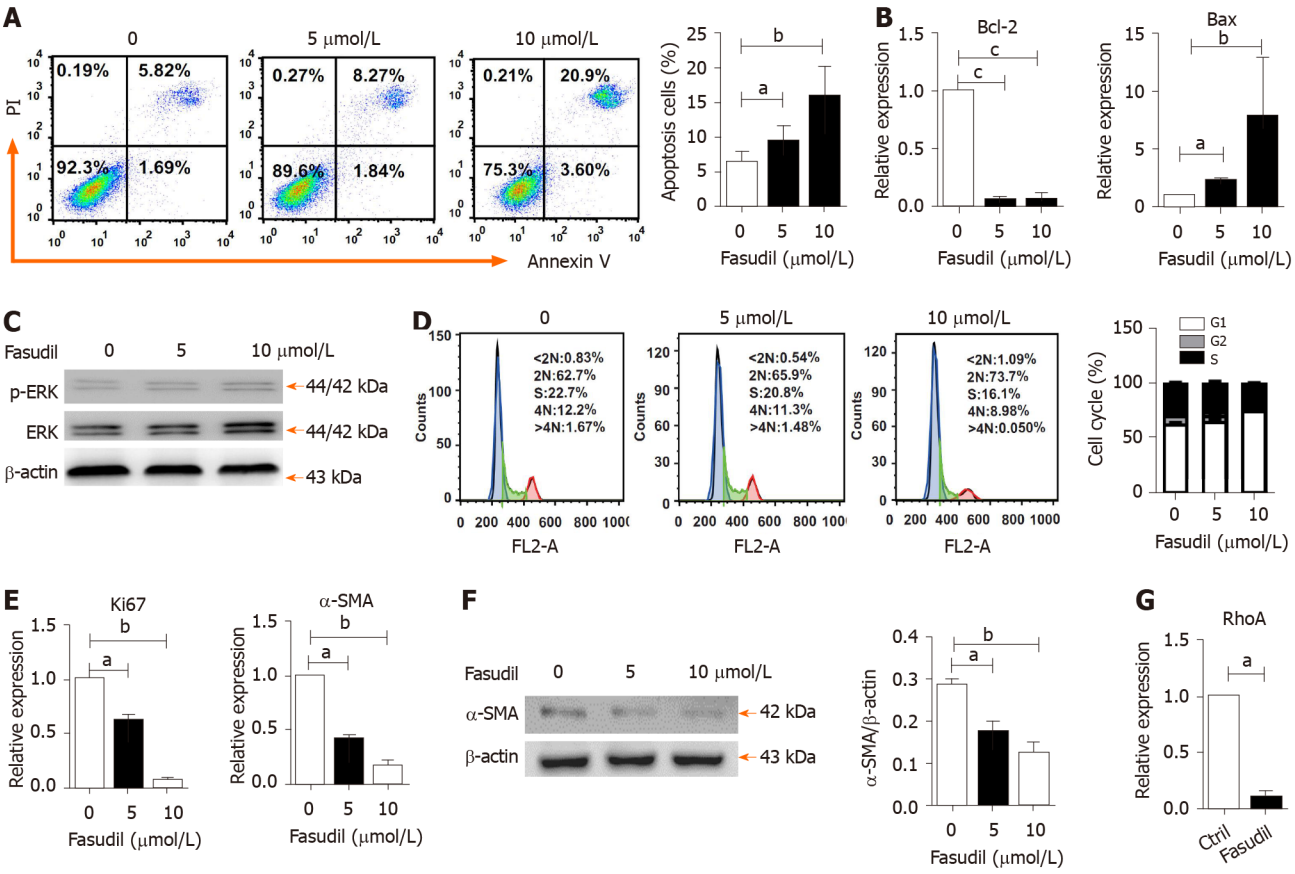


Figure 5 Fasudil inhibits the proliferation but promotes the apoptosis of hepatic stellate cells. A: LX2 cells were treated with different concentrations of Fasudil (5 mM, 10 mM) for 24 h *in vitro*; B: The apoptosis of LX2 cells was analyzed by fluorescence-activated cell sorting (FACS). mRNA levels of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) in LX2 cells were detected by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR); C: The phosphorylated extracellular signal-related kinase (ERK)/ERK levels in LX2 cells were detected by western blotting. The cell cycle of LX2 cells was analyzed by FACS; D: Left panel shows the representative FACS plots of cell cycle, and right panel shows the quantification of percentage of cells in G1, S and G2 phase; E: Relative expression level of Ki67 and alpha smooth muscle actin (α-SMA) mRNA in LX2 cells was analyzed by qRT-PCR; F: α-SMA level in LX2 cells was analyzed by western blotting; G: mRNA levels of Ras homology family member A in LX2 cells were analyzed by qRT-PCR. Data represent the mean ± SD from at least three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

spleen from mice with Fasudil treatment (data not shown). ERK and NF-κB signals are critical for NK cell-mediated cytolysis of target cells[34]. Here, we found that Fasudil drove ERK and NF-κB activation, and augmented the cytolysis activity of NK cells against HSCs. In addition, Fasudil promoted the secretion of IFN-γ in NK cells (Figure 4). Although STAT3 activation is essential for NK cells to exert anti-tumor effects[35], it was not significantly influenced by Fasudil treatment (Figure 4). These results demonstrate the impact of Fasudil on NK cell function in fibrotic mouse model.

In terms of T cells, different T-cell subpopulations play different roles in the process of liver fibrosis. Previous studies have reported that T-cell impairment generally does not alter fibrogenesis, while Th2 and Th17 $CD4^{+}$T cells are involved in the inflammatory process, promoting cytokine production and HSC activation[36]. Here, the ratio of $CD4^{+}$ T cells and $CD8^{+}$ T cells was upregulated in the liver and spleen (data not shown), but the activation of these T cells was not significantly affected by Fasudil treatment (Figure 3). The exact roles of T cell subtypes in Fasudil-treated liver fibrosis need to be further investigated.

HSCs play a vital role in the pathogenesis of liver fibrosis. They can secret fibrogenic factors and then promote several kinds of cell types including portal fibrocytes, fibroblasts, and bone marrow-derived myofibroblasts to produce collagen and thereby propagate fibrosis[4,37]. Interestingly, we found that Fasudil treatment significantly increased apoptosis and inhibited the proliferation of LX2 cells, indicating the direct effect of Fasudil on HSCs (Figure 5), consistent with previous studies[38]. Actually, the effect of ROCK signal pathway on apoptosis is complicated. For example, inhibition of the RhoA/ROCK signaling pathway promotes the apoptosis of human urethral scar fibroblasts[38], cardiac fibroblasts[39], and neutrophils[40]. However, the decrease of apoptosis by RhoA/ROCK inhibition is also found in several cell types, such as

nitrergic neurons[41] and progenitor cells[42]. RhoA is positively correlated with the expression of α-SMA, a gene involved in liver fibrosis[43]. Indeed, Fasudil, as an inhibitor targeting RhoA kinases, significantly inhibited both the expression of RhoA and α-SMA in HSCs. In addition, ERK is an important mediator of signal transduction in HSCs, which is involved in the growth, differentiation, survival, and apoptosis of HSCs[44]. In line with previous studies, we observed an increase in the p-ERK level by Fasudil treatment, which would promote HSC apoptosis. Thus, Fasudil directly disrupts HSC activation and survival in TAA-induced liver fibrosis.

CONCLUSION

In conclusion, our findings demonstrated that Fasudil prevented TAA-induced liver fibrosis by directly suppressing the cell cycle and activation of HSCs, and activating NK cells. Because Fasudil is a drug that has been used clinically, it has high safety and reliability and can provide a feasible solution for clinical treatment of liver fibrosis.

ARTICLE HIGHLIGHTS

Research background

Rho kinase inhibition reportedly improves liver fibrosis. Fasudil, as a RhoA kinase inhibitor, is used to improve brain microcirculation and promote nerve regeneration clinically. The viability of Fasudil in liver fibrosis is still unknown.

Research motivation

Repositioning existing drugs *e.g.,* Fasudil might be an effective strategy for obtaining therapeutics against liver fibrosis.

Research objectives

To evaluate the anti-fibrotic effects of Fasudil *in vitro* and in a mouse model of thioacetamide (TAA)-induced liver fibrosis.

Research methods

The anti-fibrotic effect of Fasudil was investigated in a TAA-induced mouse model. At 1 wk after induction with TAA, Fasudil was intraperitoneally injected once a day for 3 wk, hepatic pathological changes, liver fibrosis and immune cell activation were determined using hematoxylin and eosin staining, sirius red staining, western blotting and quantitative polymerase chain reaction and fluorescence-activated cell sorting. Furthermore, the effect of Fasudil on hepatic stellate cell (HSC) and natural killer (NK) cells was assayed *in vitro*.

Research results

Treatment with Fasudil alleviated hepatic pathological changes and reversed hepatic fibrosis in the TAA-chronic models with decreased deposition of collagen fibers, reduced expression of HSC activation marker (alpha smooth muscle actin), and reduced secretion of transforming growth factor beta 1 (TGF-β1), matrix metalloproteinase 2 (MMP-2), and MMP-9. Fasudil treatment increased NK cell activation and cytotoxicity by activating the extracellular signal-related kinase and nuclear factor kappa B signaling pathways. Fasudil directly promoted the apoptosis and inhibited the proliferation of HSC by decreasing α-SMA and TGF-β1.

Research conclusions

Fasudil inhibited liver fibrosis by activating NK cells and blocking HSC activation.

Research perspectives

Fasudil treatment prevents liver fibrosis *via* activating NK cells but suppressing HSCs. As a drug used clinically, these results provide a feasible solution for the clinical treatment of liver fibrosis.

REFERENCES

1 **Gaba RC**, Mendoza-Elias N, Regan DP, Garcia KD, Lokken RP, Schwind RM, Eichner M, Thomas FM, Rund LA, Schook LB, Schachtschneider KM. Characterization of an Inducible Alcoholic Liver Fibrosis Model for Hepatocellular Carcinoma Investigation in a Transgenic Porcine Tumorigenic Platform. *J Vasc Interv Radiol* 2018; **29**: 1194-1202. e1 [PMID: 29887183 DOI: 10.1016/j.jvir.2018.03.007]

2 **Bansal MB**, Chamroonkul N. Antifibrotics in liver disease: are we getting closer to clinical use? *Hepatol Int* 2019; **13**: 25-39 [PMID: 30302735 DOI: 10.1007/s12072-018-9897-3]

3 **Trivedi P**, Wang S, Friedman SL. The Power of Plasticity-Metabolic Regulation of Hepatic Stellate Cells. *Cell Metab* 2021; **33**: 242-257 [PMID: 33232666 DOI: 10.1016/j.cmet.2020.10.026]

4 **Gandhi CR**. Hepatic stellate cell activation and pro-fibrogenic signals. *J Hepatol* 2017; **67**: 1104-1105 [PMID: 28939135 DOI: 10.1016/j.jhep.2017.06.001]

5 **Yuan X**, Gong Z, Wang B, Guo X, Yang L, Li D, Zhang Y. Astragaloside Inhibits Hepatic Fibrosis by Modulation of TGF-*β*1/Smad Signaling Pathway. *Evid Based Complement Alternat Med* 2018; **2018**: 3231647 [PMID: 29853950 DOI: 10.1155/2018/3231647]

6 **Tan Z**, Liu Q, Jiang R, Lv L, Shoto SS, Maillet I, Quesniaux V, Tang J, Zhang W, Sun B, Ryffel B. Interleukin-33 drives hepatic fibrosis through activation of hepatic stellate cells. *Cell Mol Immunol* 2018; **15**: 388-398 [PMID: 28194023 DOI: 10.1038/cmi.2016.63]

7 **Sun YY**, Li XF, Meng XM, Huang C, Zhang L, Li J. Macrophage Phenotype in Liver Injury and Repair. *Scand J Immunol* 2017; **85**: 166-174 [PMID: 27491503 DOI: 10.1111/sji.12468]

8 **Gieseck RL 3rd**, Wilson MS, Wynn TA. Type 2 immunity in tissue repair and fibrosis. *Nat Rev Immunol* 2018; **18**: 62-76 [PMID: 28853443 DOI: 10.1038/nri.2017.90]

9 **Li X**, Zhang M, Liu J, Huang Z, Zhao Q, Huang Y, Li X, Gao Z. Intrahepatic NK cells function suppressed in advanced liver fibrosis. *Eur J Clin Invest* 2016; **46**: 864-872 [PMID: 27555302 DOI: 10.1111/eci.12669]

10 **Amer J**, Salhab A, Doron S, Morali G, Safadi R. A novel flow cytometry tool for fibrosis scoring through hepatic stellate cell differentiation. *Cytometry A* 2018; **93**: 427-435 [PMID: 29517852 DOI: 10.1002/cyto.a.23202]

11 **Abedi F**, Hayes AW, Reiter R, Karimi G. Acute lung injury: The therapeutic role of Rho kinase inhibitors. *Pharmacol Res* 2020; **155**: 104736 [PMID: 32135249 DOI: 10.1016/j.phrs.2020.104736]

12 **Görtzen J**, Trebicka J. Rho-kinase inhibition is beneficial in fibrosis. *Hepatology* 2017; **65**: 1780-1781 [PMID: 28027585 DOI: 10.1002/hep.29024]

13 **Pan W**, Nagpal K, Suárez-Fueyo A, Ferretti A, Yoshida N, Tsokos MG, Tsokos GC. The Regulatory Subunit PPP2R2A of PP2A Enhances Th1 and Th17 Differentiation through Activation of the GEF-H1/RhoA/ROCK Signaling Pathway. *J Immunol* 2021; **206**: 1719-1728 [PMID: 33762326 DOI: 10.4049/jimmunol.2001266]

14 **Han J**, Zhao Y, Zhang Y, Li C, Yi Y, Pan C, Tian J, Yang Y, Cui H, Wang L, Liu S, Liu J, Deng N, Liang A. RhoA/ROCK Signaling Pathway Mediates Shuanghuanglian Injection-Induced Pseudo-allergic Reactions. *Front Pharmacol* 2018; **9**: 87 [PMID: 29487527 DOI: 10.3389/fphar.2018.00087]

15 **Bei Y**, Hua-Huy T, Nicco C, Duong-Quy S, Le-Dong NN, Tiev KP, Chéreau C, Batteux F, Dinh-Xuan AT. RhoA/Rho-kinase activation promotes lung fibrosis in an animal model of systemic sclerosis. *Exp Lung Res* 2016; **42**: 44-55 [PMID: 26873329 DOI: 10.3109/01902148.2016.1141263]

16 **Xie Y**, Song T, Huo M, Zhang Y, Zhang YY, Ma ZH, Wang N, Zhang JP, Chu L. Fasudil alleviates hepatic fibrosis in type 1 diabetic rats: involvement of the inflammation and RhoA/ROCK pathway. *Eur Rev Med Pharmacol Sci* 2018; **22**: 5665-5677 [PMID: 30229844 DOI: 10.26355/eurrev_201809_15834]

17 **Boyer-Diaz Z**, Aristu-Zabalza P, Andrés-Rozas M, Robert C, Ortega-Ribera M, Fernández-Iglesias A, Broqua P, Junien JL, Wettstein G, Bosch J, Gracia-Sancho J. Pan-PPAR agonist lanifibranor improves portal hypertension and hepatic fibrosis in experimental advanced chronic liver disease. *J Hepatol* 2021; **74**: 1188-1199 [PMID: 33278455 DOI: 10.1016/j.jhep.2020.11.045]

18 **Chen L**, Li J, Zhang J, Dai C, Liu X, Wang J, Gao Z, Guo H, Wang R, Lu S, Wang F, Zhang H, Chen H, Fan X, Wang S, Qin Z. S100A4 promotes liver fibrosis *via* activation of hepatic stellate cells. *J Hepatol* 2015; **62**: 156-164 [PMID: 25111176 DOI: 10.1016/j.jhep.2014.07.035]

19 **Han Q**, Hou Z, Yin C, Zhang C, Zhang J. 5'-triphosphate siRNA targeting HBx elicits a potent anti-HBV immune response in pAAV-HBV transfected mice. *Antiviral Res* 2019; **161**: 36-45 [PMID: 30448255 DOI: 10.1016/j.antiviral.2018.11.006]

20 **Saha D**, Martuza RL, Rabkin SD. Macrophage Polarization Contributes to Glioblastoma Eradication by Combination Immunovirotherapy and Immune Checkpoint Blockade. *Cancer Cell* 2017; **32**: 253-267. e5 [PMID: 28810147 DOI: 10.1016/j.ccell.2017.07.006]

21 **Vicente A**, Byström B, Lindström M, Stenevi U, Pedrosa Domellöf F. Aniridia-related keratopathy: Structural changes in naïve and transplanted corneal buttons. *PLoS One* 2018; **13**: e0198822 [PMID: 29889891 DOI: 10.1371/journal.pone.0198822]

22 **Nakano R**, Ohira M, Yano T, Imaoka Y, Tanaka Y, Ohdan H. Hepatic irradiation persistently eliminates liver resident NK cells. *PLoS One* 2018; **13**: e0198904 [PMID: 29897952 DOI: 10.1371/journal.pone.0198904]

23 **Fan Y**, Zhang W, Wei H, Sun R, Tian Z, Chen Y. Hepatic NK cells attenuate fibrosis progression of non-alcoholic steatohepatitis in dependent of CXCL10-mediated recruitment. *Liver Int* 2020; **40**: 598-608 [PMID: 31758647 DOI: 10.1111/liv.14307]

24 **Li T**, Yang Y, Song H, Li H, Cui A, Liu Y, Su L, Crispe IN, Tu Z. Activated NK cells kill hepatic stellate cells *via* p38/PI3K signaling in a TRAIL-involved degranulation manner. *J Leukoc Biol* 2019; **105**: 695-704 [PMID: 30748035 DOI: 10.1002/JLB.2A0118-031RR]

25 **Ma L**, Yang X, Wei R, Ye T, Zhou JK, Wen M, Men R, Li P, Dong B, Liu L, Fu X, Xu H, Aqeilan RI, Wei YQ, Yang L, Peng Y. MicroRNA-214 promotes hepatic stellate cell activation and liver fibrosis by suppressing Sufu expression. *Cell Death Dis* 2018; **9**: 718 [PMID: 29915227 DOI: 10.1038/s41419-018-0752-1]

26 **Kim J**, Kang W, Kang SH, Park SH, Kim JY, Yang S, Ha SY, Paik YH. Proline-rich tyrosine kinase 2 mediates transforming growth factor-beta-induced hepatic stellate cell activation and liver fibrosis. *Sci Rep* 2020; **10**: 21018 [PMID: 33273492 DOI: 10.1038/s41598-020-78056-0]

27 **Kisseleva T**, Brenner D. Molecular and cellular mechanisms of liver fibrosis and its regression. *Nat Rev Gastroenterol Hepatol* 2021; **18**: 151-166 [PMID: 33128017 DOI: 10.1038/s41575-020-00372-7]

28 **Martí-Rodrigo A**, Alegre F, Moragrega ÁB, García-García F, Martí-Rodrigo P, Fernández-Iglesias A, Gracia-Sancho J, Apostolova N, Esplugues JV, Blas-García A. Rilpivirine attenuates liver fibrosis through selective STAT1-mediated apoptosis in hepatic stellate cells. *Gut* 2020; **69**: 920-932 [PMID: 31530714 DOI: 10.1136/gutjnl-2019-318372]

29 **Lai SS**, Fu X, Cheng Q, Yu ZH, Jiang EZ, Zhao DD, Yu DC, Qiu YD, Gao X, Ju HX, Wang W, Jiang Q, Zhu MS, Li CJ, Xue B. HSC-specific knockdown of GGPPS alleviated CCl_4-induced chronic liver fibrosis through mediating RhoA/Rock pathway. *Am J Transl Res* 2019; **11**: 2382-2392 [PMID: 31105844]

30 **Tian Y**, Han YX, Guo HF, Jin HT, Sun C, Qi X, Ma LY, Bo SW. Upregulated microRNA-485 suppresses apoptosis of renal tubular epithelial cells in mice with lupus nephritis *via* regulating the TGF-β-MAPK signaling pathway by inhibiting RhoA expression. *J Cell Biochem* 2018; **119**: 9154-9167 [PMID: 30145800 DOI: 10.1002/jcb.27178]

31 **Shi Y**, Wang Y, Li Q, Liu K, Hou J, Shao C. Immunoregulatory mechanisms of mesenchymal stem and stromal cells in inflammatory diseases. *Nat Rev Nephrol* 2018; **14**: 493-507 [PMID: 29895977 DOI: 10.1038/s41581-018-0023-5]

32 **Dufva O**, Kankainen M, Kelkka T, Sekiguchi N, Awad SA, Eldfors S, Yadav B, Kuusanmäki H, Malani D, Andersson EI, Pietarinen P, Saikko L, Kovanen PE, Ojala T, Lee DA, Loughran TP Jr, Nakazawa H, Suzumiya J, Suzuki R, Ko YH, Kim WS, Chuang SS, Aittokallio T, Chan WC, Ohshima K, Ishida F, Mustjoki S. Aggressive natural killer-cell leukemia mutational landscape and drug profiling highlight JAK-STAT signaling as therapeutic target. *Nat Commun* 2018; **9**: 1567 [PMID: 29674644 DOI: 10.1038/s41467-018-03987-2]

33 **Jin H**, Jia Y, Yao Z, Huang J, Hao M, Yao S, Lian N, Zhang F, Zhang C, Chen X, Bian M, Shao J, Wu L, Chen A, Zheng S. Hepatic stellate cell interferes with NK cell regulation of fibrogenesis *via* curcumin induced senescence of hepatic stellate cell. *Cell Signal* 2017; **33**: 79-85 [PMID: 28188823 DOI: 10.1016/j.cellsig.2017.02.006]

34 **Niogret C**, Miah SMS, Rota G, Fonta NP, Wang H, Held W, Birchmeier W, Sexl V, Yang W, Vivier E, Ho PC, Brossay L, Guarda G. Shp-2 is critical for ERK and metabolic engagement downstream of IL-15 receptor in NK cells. *Nat Commun* 2019; **10**: 1444 [PMID: 30926899 DOI: 10.1038/s41467-019-09431-3]

35 **Verdeil G**, Lawrence T, Schmitt-Verhulst AM, Auphan-Anezin N. Targeting STAT3 and STAT5 in Tumor-Associated Immune Cells to Improve Immunotherapy. *Cancers (Basel)* 2019; **11** [PMID: 31766350 DOI: 10.3390/cancers11121832]

36 **Lacoste B**, Raymond VA, Lapierre P, Bilodeau M. Protection against Acute Hepatocellular Injury Afforded by Liver Fibrosis Is Independent of T Lymphocytes. *PLoS One* 2016; **11**: e0165360 [PMID: 27792745 DOI: 10.1371/journal.pone.0165360]

37 **Gao J**, Wei B, de Assuncao TM, Liu Z, Hu X, Ibrahim S, Cooper SA, Cao S, Shah VH, Kostallari E. Hepatic stellate cell autophagy inhibits extracellular vesicle release to attenuate liver fibrosis. *J Hepatol* 2020; **73**: 1144-1154 [PMID: 32389810 DOI: 10.1016/j.jhep.2020.04.044]

38 **Li XD**, Wu YP, Chen SH, Liang YC, Lin TT, Lin T, Wei Y, Xue XY, Zheng QS, Xu N. Fasudil inhibits actin polymerization and collagen synthesis and induces apoptosis in human urethral scar fibroblasts *via* the Rho/ROCK pathway. *Drug Des Devel Ther* 2018; **12**: 2707-2713 [PMID: 30214158 DOI: 10.2147/DDDT.S156095]

39 **Shi J**, Xiao P, Liu X, Chen Y, Xu Y, Fan J, Yin Y. Notch3 Modulates Cardiac Fibroblast Proliferation, Apoptosis, and Fibroblast to Myofibroblast Transition *via* Negative Regulation of the RhoA/ROCK/Hif1α Axis. *Front Physiol* 2020; **11**: 669 [PMID: 32695015 DOI: 10.3389/fphys.2020.00669]

40 **Galvão I**, Athayde RM, Perez DA, Reis AC, Rezende L, de Oliveira VLS, Rezende BM, Gonçalves WA, Sousa LP, Teixeira MM, Pinho V. ROCK Inhibition Drives Resolution of Acute Inflammation by Enhancing Neutrophil Apoptosis. *Cells* 2019; **8** [PMID: 31450835 DOI: 10.3390/cells8090964]

41 **Gao Y**, Yan Y, Fang Q, Zhang N, Kumar G, Zhang J, Song LJ, Yu J, Zhao L, Zhang HT, Ma CG. The Rho kinase inhibitor fasudil attenuates $A\beta_{1-42}$-induced apoptosis *via* the ASK1/JNK signal pathway in primary cultures of hippocampal neurons. *Metab Brain Dis* 2019; **34**: 1787-1801 [PMID: 31482248 DOI: 10.1007/s11011-019-00487-0]

42 **Wu Y**, Shu J, He C, Li M, Wang Y, Ou W, He Y. ROCK inhibitor Y27632 promotes proliferation and diminishes apoptosis of marmoset induced pluripotent stem cells by suppressing expression and activity of caspase 3. *Theriogenology* 2016; **85**: 302-314 [PMID: 26476594 DOI: 10.1016/j.theriogenology.2015.09.020]

43 **Wang Q**, Cheng F, Xu Y, Zhang J, Qi J, Liu X, Wang R. Thymol alleviates lipopolysaccharide-stimulated inflammatory response *via* downregulation of RhoA-mediated NF-κB signalling pathway in human peritoneal mesothelial cells. *Eur J Pharmacol* 2018; **833**: 210-220 [PMID: 29883671 DOI: 10.1016/j.ejphar.2018.06.003]

44 **Widjaja AA**, Singh BK, Adami E, Viswanathan S, Dong J, D'Agostino GA, Ng B, Lim WW, Tan J, Paleja BS, Tripathi M, Lim SY, Shekeran SG, Chothani SP, Rabes A, Sombetzki M, Bruinstroop E, Min LP, Sinha RA, Albani S, Yen PM, Schafer S, Cook SA. Inhibiting Interleukin 11 Signaling Reduces Hepatocyte Death and Liver Fibrosis, Inflammation, and Steatosis in Mouse Models of Nonalcoholic Steatohepatitis. *Gastroenterology* 2019; **157**: 777-792. e14 [PMID: 31078624 DOI: 10.1053/j.gastro.2019.05.002]

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ORIGINAL ARTICLE

Basic Study

Early genetic diagnosis of clarithromycin resistance in *Helicobacter pylori*

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Abstract

BACKGROUND

The drug resistance rate of clinical *Helicobacter pylori* (*H. pylori*) isolates has increased. However, the mechanism of drug resistance remains unclear. In this study, drug-resistant *H. pylori* strains were isolated from different areas and different populations of Chinese for genomic analysis.

AIM

To investigate drug-resistant genes in *H. pylori* and find the genes for the early diagnosis of clarithromycin resistance.

METHODS

Three drug-resistant *H. pylori* strains were isolated from patients with gastritis in Bama County, China. Minimal inhibitory concentrations of clarithromycin, metronidazole, and levofloxacin were determined and complete genome sequencing was performed with annotation. *Hp1181* and *hp1184* genes were found in these strains and then detected by reverse transcription polymerase chain reaction. The relationships between *hp1181* or *hp1184* and clarithromycin resistance were ascertained with gene mutant and drug-resistant strains. The homology of the strains with *hp26695* was assessed through complete genome detection and identification. Differences in genome sequences, gene quantity, and

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board at Youjiang Medical University for Nationalities.

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gene characteristics were detected amongst the three strains. Prediction and analysis of the function of drug-resistant genes indicated that the RNA expression of *hp1181* and *hp1184* increased in the three strains, which was the same in the artificially induced clarithromycin-resistant bacteria. After gene knockout, the drug sensitivity of the strains was assessed.

RESULTS

The strains showing a high degree of homology with *hp26695*, *hp1181*, and *hp1184* genes were found in these strains; the expression of the genes *hp1184* and *hp1181* was associated with clarithromycin resistance.

CONCLUSION

Hp1181 and *hp1184* mutations may be the earliest and most persistent response to clarithromycin resistance, and they may be the potential target genes for the diagnosis, prevention, and treatment of clarithromycin resistance.

Key Words: *Helicobacter pylori*; Clarithromycin-resistance; Diagnostic gene; Early genetic diagnosis; *Helicobacter pylori* strains

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Core Tip: The World Health Organization designated clarithromycin-resistant *Helicobacter pylori* (*H. pylori*) a high priority among bacteria for antibiotic research and development, but the clarithromycin resistance mechanism remains unclear. We isolated and cultured clinical *H. pylori* strains, determined their minimal inhibitory concentrations, completed genome sequencing of *hp1181* and *hp1184* genes, analyzed their mutations, and found that the expression of the genes *hp1184* and *hp1181* was associated with clarithromycin resistance, which suggested that they can be used as genes for early diagnosis. This research may prove useful in the diagnosis, prevention, and treatment of clarithromycin-resistant *H. pylori*.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is recognized as an important human pathogen that colonizes the gastric mucus, resulting in superficial gastritis, atrophic gastritis, and gastric cancer[1-3]. Present treatments for *H. pylori* infection include proton pump inhibitors, bismuth in combination with amoxicillin, metronidazole and clarithromycin [4,5]. The rate of drug resistance is increasing because of the wide use of antibiotics and high resistance rates to clarithromycin, metronidazole, and levofloxacin are associated with the failure of *H. pylori* eradication[6-8]. The World Health Organization designated clarithromycin-resistant *H. pylori* a high priority bacterium for antibiotic research and development[9].

At present, the mechanism of antibiotic resistance of *H. pylori* is not completely understood[10,11]. It is widely accepted that the resistance to these antimicrobials is related to mutations in *H. pylori* genes, and clarithromycin-resistant strains present three point mutations in the region of domain V of 23S ribosomal RNA (rRNA): A2142G, A2142C, and A2143G[12,13]. In addition to the mutations, the efflux pump cluster is also involved in the development of resistance to clarithromycin[14,15]. However, there may be gene mutation sites that are not yet known, and the mechanism of drug resistance warrants further study.

We isolated and cultured *H. pylori* from the population in Bama County, which is a township known for the longevity of its residents in Guangxi, and randomly selected three strains of multiple drug-resistant *H. pylori* with resistance to clarithromycin.

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Complete genome sequences were analyzed to study the genomic characteristics of the strains and to elucidate the underlying mechanism of drug resistance in *H. pylori*.

MATERIALS AND METHODS

Isolation and culture of H. pylori

This study had received a strict medical ethics review from Youjiang Medical University for Nationalities. Written informed consent was obtained from each patient. Gastric mucosa tissue samples were collected from the People's Hospital of Bama Yao Autonomous County in patients' gastric body and pylorus with gastritis or gastric ulcers. Isolation and culture of *H. pylori* were performed at the Research Center for the Prevention and Treatment of Drug Resistant Microbial Infection, Youjiang Medical University for Nationalities. Patients investigated had not taken any antibiotics for at least 4 wk before examination. The isolation and identification of *H. pylori* were performed as previously described[16,17]. The bacteria were cultured on Columbia agar plates containing 5% fresh defibrinated sheep blood. The microaerophilic conditions included 5% O_2, 10% CO_2, and 85% N_2 at 37 °C for 3 to 5 days. Suspicious colonies were confirmed by Gram staining, urease, oxidase, and catalase activity testing, and urease gene polymerase chain reaction (PCR).

Antibiotic susceptibility testing

The antibiotic resistance of *H. pylori* was measured by dilution methods with reference to the protocols of the Clinical and Laboratory Standards Institute (Wayne, PA, United States)[18]. Briefly, the density of *H. pylori* was adjusted to be 1 × 10^6 CFU/mL and incubated at 37 °C for 3 to 5 d under microaerophilic conditions. After incubation, the plates were visually examined and the minimal inhibitory concentration (MIC) was determined to be the lowest concentration that resulted in no turbidity. Metronidazole (Aladdin, d1707126), amoxicillin (Xiansheng pharmaceutical, Co., Ltd, China), levofloxacin (Shandong Lukang Pharmaceutical Group Saite Co., Ltd, China), and clarithromycin (Yangzi River Pharmaceutical Group Co., Ltd, China) were also used.

Complete genome sequencing and analysis

Drug-resistant strains were selected and sent to the Shenzhen Huada Gene Co., Ltd (China) for complete genome analysis. After the DNA samples were delivered, the quality of the samples was tested and then used to construct a BSlibrary. The purified genomic DNA samples including genomic DNA, bacterial artificial chromosomes, or long-length PCR products were sheared into smaller fragments by CovarisS/E210 or using a Bioruptor. The overhangs resulting from fragmentation were converted into blunt ends using T4 DNA polymerase, Klenow fragment, and T4 polynucleotide kinase. After adding an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, adapters were ligated to the ends of the DNA fragments. The desired fragments were purified though gel-electrophoresis, selectively enriched, and amplified by PCR. The index tag was introduced into the adapter at the PCR stage as appropriate and a library quality test was conducted. Finally, the qualified BSlibrary was used for sequencing. Genomic component and gene function analyses were performed, including gene prediction, tRNA, sRNA, and gene annotation, and prediction of open reading frames by GO.

Drug-resistant gene detection

Drug-resistant genes were predicted based on the results of the complete genome sequence analysis and selected for detection by reverse transcription PCR (RT-PCR). The reaction for cDNA synthesis was held at 25 °C for 10 min, 42 °C for 60 min, and then 99 °C for 5 min. The reaction consisted of 32 cycles with each cycle composed of 1 min at 95 °C, 4 min at 56 °C, and 7 min at 70 °C. After a final extension of 15 min at 72 °C, the RT-PCR products were visualized by electrophoresis on 1% agarose gel and 15% acrylamide gel with a 200-bp ladder size marker.

Knockout of mutant genes

Hp1181 and *hp1184* knockout mutants were constructed by insertion of the KAN resistance cassette. Double-knockout mutants were made by natural transformation of the KAN resistance cassette with pBSII KS (as presented by Bi HK, Laboratory of Nanjing Medical University, China) containing an internal fragment interrupted with a cat cassette from pAV35, with selection for both KAN- and CHL-resistant colonies.

Table 1 Drug resistance characteristics of three drug-resistant strains (minimal inhibitory concentration: μg/mL)

Strain	Metronidazole	Clarithromycin	Levofloxacin	Amoxicillin
Hpbs1	32	8	8	0.125
Hpbs2	16	8	0.125	0.125
Hpbs3	0.125	8	8	0.125

Table 2 Sequence information of three drug-resistant strains

Sample name	ID name	Sequence type	Sequence topology	Sequence number	Total length (bp)	GC content
Hpbs1	Chromosome1	Chromosome	Circular	1	1563701	38.90
	All	All	-	1	1563701	38.90
Hpbs2	Chromosome1	Chromosome	Circular	1	1534481	38.87
	All	All	-	1	1534481	38.87
Hpbs3	Chromosome1	Chromosome	Circular	1	1534930	38.90
	All	All	-	1	1534930	38.90

Sequence type: Chromosome or plasmid; Sequence topology: Circular or linear.

Table 3 Gene information of three drug-resistant strains

Sample name (#)	Genome size (#)	Total number (#)	Total length (bp)	Average length (#)	Length/genome length (%)	GC content (%)
Hpbs1	1563701	1571	1434202	912.92	91.72	39.49
Hpbs2	1534481	1792	1395399	778.68	90.94	39.44
Hpbs3	1534930	1732	1407495	812.64	91.70	39.49

Total number denotes the count of genes. Total length represents the total length of all genes. Average length refers to the average length of all genes. GC content is the content of G and C in a gene. Length/genome length is the proportion of gene length in the genome.

Table 4 Gene annotation statistics A

Sample name (#)	Total	P450 (#) (%)	VFDB (#) (%)	ARDB (#) (%)	CAZY (#)	SWISSPROT (#) (%)	NOG (#) (%)	COG (#) (%)	CARD (#) (%)	NR (#)
Hpbs1	1571	22 (1.4)	196 (12.47)	0 (0)	14 (0.89)	742 (47.23)	67 (4.26)	1084 (69)	14 (0.89)	1599 (99.23)
Hpbs2	1792	21 (1.17)	177 (9.87)	0 (0)	14 (0.78)	751 (41.9)	125 (6.97)	1111 (61.99)	13 (0.72)	1723 (96.14)
Hpbs3	1732	22 (1.27)	174 (10.04)	0 (0)	14 (0.75)	750 (43.3)	97 (5.6)	1113 (64.26)	15 (0.86)	1698 (98.03)

Insertion of the KAN and cat resistance cassette at the desired locations in the *H. pylori* putative efflux genes was validated by PCR.

Induction of drug resistance

The MIC of clarithromycin to hp26695 was detected. Drug resistance was induced by 1/4 MIC. The culture medium was changed every 2 d and MIC was detected every 4 d. The concentration of induced drug was changed with MIC.

Table 5 Gene annotation statistics B

Sample name (#)	DBCAN (#) (%)	T3SS (#) (%)	TREMBL (#) (%)	IPR (#)	PHI (#) (%)	KEGG (#) (%)	GO (#) (%)	KOG (#) (%)	Over all (#) (%)
Hpbs1	30 (1.9)	175 (11.13)	1557 (99.1)	1234 (78.54)	54 (3.43)	1026 (65.3)	957 (60.91)	142 (9.03)	1563 (99.49)
Hpbs2	29 (1.61)	197 (10.99)	1706 (95.2)	1372 (76.56)	52 (2.9)	1078 (60.15)	1056 (58.92)	144 (8.03)	1750 (97.65)
Hpbs3	30 (1.73)	209 (12.06)	1688 (97.45)	1340 (77.36)	51 (2.94)	1067 (61.6)	1030 (59.4)	139 (8.02)	1711 (98.78)

RESULTS

Bacterial resistance

Three drug-resistant strains were isolated and identified by Gram staining, urease, oxidase, catalase activity testing, and urease gene PCR. The drug resistance information of these strains is summarized in Table 1.

Bacterial sequence information

Based on the valid data from the previous sequencing platform, the CleanData could be assembled for each sample and the optimal assembly results were obtained after multiple adjustments. The assembly sequence was analyzed by correcting single base, circular judgment, and plasmid comparison. The results of the genome assembly statistics of each sample are displayed in Table 2. These three strains have been uploaded to the NCBI Biosample database: Hpbs1 (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10461767). Hpbs2 (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10663081), and Hpbs3 (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10663175).

Gene information

Gene prediction was applied to determine gene composition. The statistics are shown in Table 3.

Circular genome analysis

GC skew analysis was carried out using (G-C)/(G+C) calculations based on genomic sequences of strains. The results of gene distribution, ncRNA distribution, and gene annotation are demonstrated in Figure 1. Hpbs1 had 835 genes, 26 tRNAs, 6 rRNAs, and 2 sRNAs in the positive chain. It also had 736 genes, 10 tRNAs, 0 rRNAs, and 5 sRNAs in the negative chain and 157 repeats without positive or negative chain. There are 943 genes, 26 tRNAs, 6 rRNAs, 3 sRNAs, 849 genes, 10 tRNAs, 0 rRNA, 3 sRNAs, and 153 repeats in Hpbs2; there are 869 genes, 26 tRNAs, 6 rRNAs, 3 sRNAs, 863 genes, 10 tRNAs, 0 rRNA, 3 sRNAs, and 155 repeats in Hpbs3.

Gene annotation

Functional annotation was accomplished by analysis of protein sequences. We aligned genes with databases to obtain their corresponding annotations. To demonstrate the biological meaning, the highest quality alignment result was chosen as a gene annotation. Functional annotation was completed by blast resistance genes with different databases. In this project we have finished annotations using 17 databases, including P450, VFDB, ARDB, CAZY, SWISSPROT, NOG, COG, CARD, NR, DBCAN, T3SS, TREMBL, IPR, PHI, KEGG, GO, and KOG. The annotation results are shown in Tables 4 and 5.

Analysis of drug-resistant gene database

The drug resistance gene numbers of three strains were different in the CARD (Comprehensive Antibiotic Resistance Database), which are 14, 13, and 15 genes, respectively. However, after sorting, it was found that some genes were repetitive. The specific numbers and characteristics of genes are presented in the Tables 6 and 7. NP_207975.1 and NP_207972.1 were efflux pump genes of 26695 strain, *i.e.*, *hp1181* and *hp1184* genes. Their drug resistance was verified by RT-PCR, as illustrated in Figure 2. After knocking out the drug-resistant genes, drug sensitivity was significantly improved, as shown in Figure 3.

Table 6 Analysis of gene resistance in CARD

Gene ID	Subject ID	Align length	Mismatch	Gap	Gene start	Gene end	Subject start	Subject end	*E* value
GL000175	YP_208874.1	97	39	0	2	98	4	100	6.00E-40
GL000286	YP_006374661.1	398	88	2	1	397	29	421	0
GL000295	NP_312937.1	1389	658	21	8	1371	8	1339	0
GL000296	AAK44936.1	124	35	0	1	124	1	124	4.00E-63
GL000306	NP_207975.1	459	16	0	1	459	1	459	0
GL000309	NP_207972.1	443	10	0	1	443	1	443	0
GL000772	AIL15701	421	220	3	1	420	1	417	4.00E-126
GL000822	YP_002344422.1	853	293	6	3	818	2	851	0
GL000911	NP_415611.1	247	130	2	1	247	1	243	2.00E-66
GL000972	WP_005768149.1	810	390	18	3	773	12	809	0
GL001063	AJF83452.1	287	164	2	1	283	2	288	1.00E-71
GL001265	NP_415804.1	262	141	1	1	261	1	262	2.00E-80
GL001295	YP_001332362.1	222	123	4	1	221	1	216	7.00E-51
GL001455	AJF82049.1	254	141	2	4	255	7	260	2.00E-62

Identification of 23S rRNA gene mutations

As three strains were resistant to clarithromycin, so we analyzed and identified the sites of clarithromycin-resistant mutations. We found that three strains had mutations in A2142G, A2143G, G2144T, and some had mutations in other sites, as shown in Table 8.

Gene mutation induced in drug-resistant strains

After induction with clarithromycin, hp26695 drug resistance was enhanced on the 12th day, reached the highest level on day 16, and increased to 8 μg/mL on the 24th day. The expression of *hp1181* and *hp1184* was also increased with increasing clarithromycin resistance, especially *hp1184*, as shown in Figure 4. Only A2142G and A 2143G mutations were detected in 23S RNA, with no other mutation sites being found, as shown in Table 9. These data indicated that these two genes may be involved early in the regulation of clarithromycin resistance.

DISCUSSION

The treatment of *H. pylori* infection remains reliant on bismuth tetralogy at present. *H. pylori* is eradicated clinically using common antibiotics including clarithromycin, amoxicillin, metronidazole, tetracycline and levofloxacin. However, in recent years, the growing rate of antibiotic resistance has resulted in the failure of *H. pylori* eradication[19,20]. The most serious resistance has developed to drugs including metronidazole, clarithromycin, and levofloxacin star. The common mechanisms of bacterial resistance involve the production of inactivated enzymes, change in the target position of antibacterial drugs, change in the permeability of bacterial outer membrane, effects on the active outflow system, and formation of bacterial biofilm and cross resistance[21-23]. There are some differences in the mechanisms of drug resistance of each kind of bacteria; however, the same kind of bacteria still have different resistances to the same antibiotic in different areas[24]. The mechanism of drug resistance of *H. pylori* remains unclear and needs further study.

We selected drug-resistant strains using metronidazole, clarithromycin, and levofloxacin for genome sequencing analysis. We found that there were no significant differences in the number of drug-resistant genes in the CARD database. This may be because two kinds of antibiotic resistance can develop and the drug-resistant genes in *H. pylori* are mainly *hp1181* and *hp1184*. *Hp1181* encodes a putative NDA translocase that is related to the major facilitator superfamily and is an integral membrane protein; *hp1184* encodes another translocase that belongs to the MATE family, resulting in the aforementioned susceptibility. These can contribute to resistance *via* a multidrug-

Table 7 Characteristics of drug-resistant genes in CARD

Subject ID	ARO number	Definition of term
YP_208874.1	*Neisseria gonorrhoeae* FA 1090	rpsJ is a tetracycline resistance protein identified in *Neisseria gonorrhoeae*. Tetracycline resistance is conferred by binding to the ribosome as a 30S ribosomal protection protein[27]
YP_006374661.1	*Enterococcus faecium* DO	Sequence variants of *Enterococcus faecium* elongation factor Tu that can confer resistance to GE2270A[28]
NP_312937.1	*Escherichia coli* O157•H7 str. Sakai	Point mutation that occurs in *Escherichia coli* rpoB resulting in resistance to rifampicin[29]
AAK44936.1	*Mycobacterium tuberculosis* CDC1551	Ribosomal protein S12 stabilizes the highly conserved pseudoknot structure formed by 16S rRNA. Amino acid substitutions in RpsL affect the higher-order structure of 16S rRNA and confer streptomycin resistance by disrupting interactions between 16S rRNA and streptomycin[30-35]
NP_207975.1	*Helicobacter pylori* 26695	hp1184 is a translocase that belongs to the MATE efflux pump family. It is found in *H. pylori* and is involved in the active efflux of antibiotics[25,26]
NP_207972.1	*Helicobacter pylori* 26695	hp1181 is a translocase that is part of the MFS efflux pump family. It is found in *H. pylori* and plays a role in the active efflux of antibiotics[25]
AIL15701	*Escherichia coli* ATCC25922	murA or UDP-N-acetylglucosamine enolpyruvy1 transferase catalyzes the initial step in peptidoglycan biosynthesis and is inhibited by Fosfomycin. Over-expression of murA through mutations such as Asp369Asn and Leu370I1e confers fosfomycin resistance. Extensive evidence has shown the significance of C115 mutations in conferring fosfomycin resistance since this residue represents a primary binding site for the antibiotic across many species[36-39]
YP_002344422.1	*Campylobacter jejuni* subsp. *jejuni* NCTC 11168	*Campylobacter jejuni* is a major bacterial infectious agent associated with gastroenteritis. Quinolone resistance is reportedly conferred by a single C-257-T nucleotide substitution in the gyrA gene[40]
NP_415611.1	*Escherichia coli* str. K-12 substr. MG1655	Fab G is a 3-oxoacyl-acyl carrier protein reductase involved in lipid metabolism and fatty acid biosynthesis. The bacterial biocide Triclosan blocks the final reduction step in fatty acid elongation, inhibiting biosynthesis. Point mutations in fabG can confer resistance to Triclosan[41]
WP_005768149.1	*Bartonella bacilliformis* KC583	Point mutation in *Bartonella bacilliformis* results in amino coumarin resistance[42]
AJF83452.1	*Acinetobacter baumannii*	The LpxC gene is widely known to be involved in the biosynthesis of lipid A in Gram-negative bacteria and mutations to this gene may cause resistance to antimicrobial peptides that target the outer membrane[43,44]
NP_415804.1	*Escherichia coli* str. K-12 substr. MG1655	fabI is an enoyl-acy1 carrier reductase used in lipid metabolism and fatty acid biosynthesis. The bacterial biocide Triclosan blocks the final reduction step in fatty acid elongation, inhibiting biosynthesis. Point mutations in fabI can confer resistance to Triclosan and Isoniazid[41]
YP_001332362.1	*Staphylococcus aureus* subsp. *aureus* str. Newman	Ar1R is a response regulator that binds to the norA promoter to activate expression. Ar1R must first be phosphorylated by Ar1S[45]
AJF82049.1	*Acinetobacter baumannii*	The *LpxA* gene is widely known to be involved in the biosynthesis of lipid A in Gram-negative bacteria and mutations to this gene may cause resistance to antimicrobial peptides that target the outer membrane[43,44]

H. pylori: *Helicobacter pylori*.

resistant efflux protein, active-efflux of antibiotics, and other efflux pump genes, such as *HefA*. After knockout of these two genes, the MICs of the drugs were significantly decreased and the sensitivity was increased. It is noteworthy that in addition to these two genes, the *GE2270A* gene of *Enterococcus* and *MurA* gene of *Escherichia coli* also show a correlation. It is likely that the drug-resistant plasmids of other strains invade *H. pylori* through transformation or other mechanisms. Bacteria other than *H. pylori* in

Table 8 Mutations in the 23S rRNA genes of *Helicobacter pylori* strains

Nucleotide position	Ref	Mutation	Hpbs1	Hpbs2	Hpbs3
2143	A	G	+	+	+
2142	A	G	+	+	+
2144	G	T	+	+	+
2302	A	G	-	-	+
2182	T	C	-	+	-
2173	C	T	+	+	+
1513	G	A	-	+	+
2196	C	T	+	-	-
1280	A	G	+	-	-
1023	G	A	-	-	+

Table 9 23S rRNA mutations of *Helicobacter pylori* strains

Nucleotide position	Ref	Mutation	26695(S)	26695(R)
2142	A	G	-	+
2143	A	G	-	+

the gastric mucosa of patients can indirectly confirm this view. The main reason for this may be long-term acid resistant treatment, gastric erosion, or intestinal bacterial reflux. This will lead to drug resistance becoming more difficult to prevent and control. In addition, all three strains have clarithromycin resistance. The mechanism of resistance to clarithromycin is mainly reflected in the mutations A2142G, A2143G, and G2144T. In addition, it is common that there are several mutations in the same strain.

Hp1181 and *hp1184* are related to multidrug resistance and to clarithromycin resistance, which has been previously reported in the literature[25,26]. The RNA expression of *hp1181* and *hp1184* were increased with the emergence of clarithromycin resistance, with *hp1184* showing the fastest increase. Therefore, these genes are also involved in the regulation of drug resistance and may be one of the mechanisms of *H. pylori* resistance to clarithromycin. Compared with the clinical isolates, 23S RNA mutation sites of *H. pylori* were less frequent in artificially induced strains that had only A2142G and A2143G mutations. These may be attributed to the single factor of artificial induction that is not as complex as human stomach environment. More importantly, *hp1181* and *hp1184* mutations may be the earliest and most persistent response to clarithromycin resistance, and they may be the main target genes for the diagnosis, prevention, and treatment of clarithromycin resistance.

The genetic characteristics of multidrug-resistant strains in this area were preliminarily identified: The relationship between *hp1181* or *hp1184* and clarithromycin resistance was ascertained through genome sequencing analysis and gene function identification of drug-resistant *H. pylori* from Bama County, Guangxi Province. Our study further provided an improved experimental basis for the prevention and treatment of drug resistance of *H. pylori*.

CONCLUSION

Hp1181 and *hp1184* mutations may be the earliest and most persistent response to clarithromycin resistance, and they may be the main target genes for the diagnosis, prevention, and treatment of clarithromycin resistance.

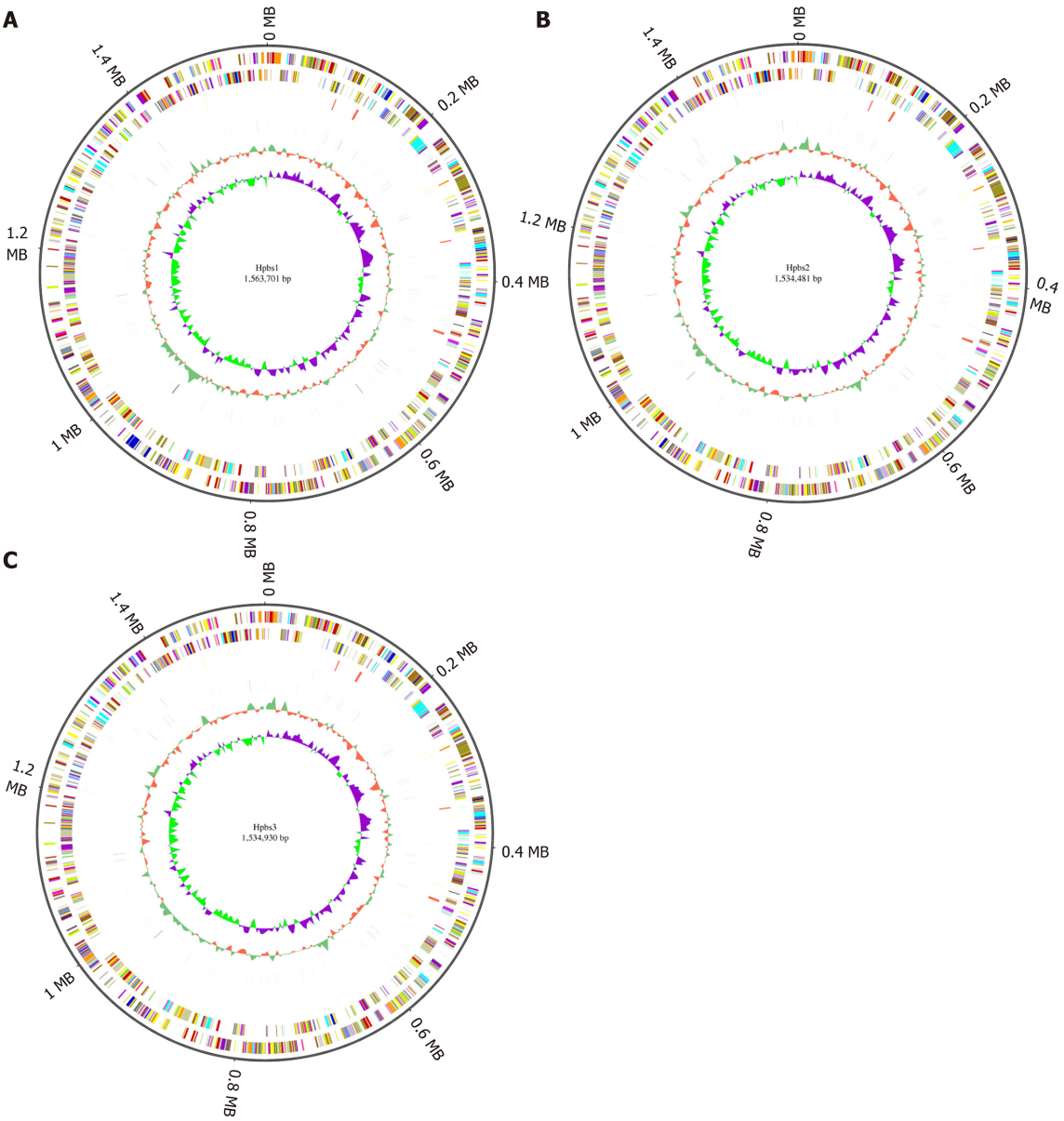


Figure 1 Circular genome analysis of three drug-resistant strains. A: Hpbs1; B: Hpbs2; C: Hpbs3.

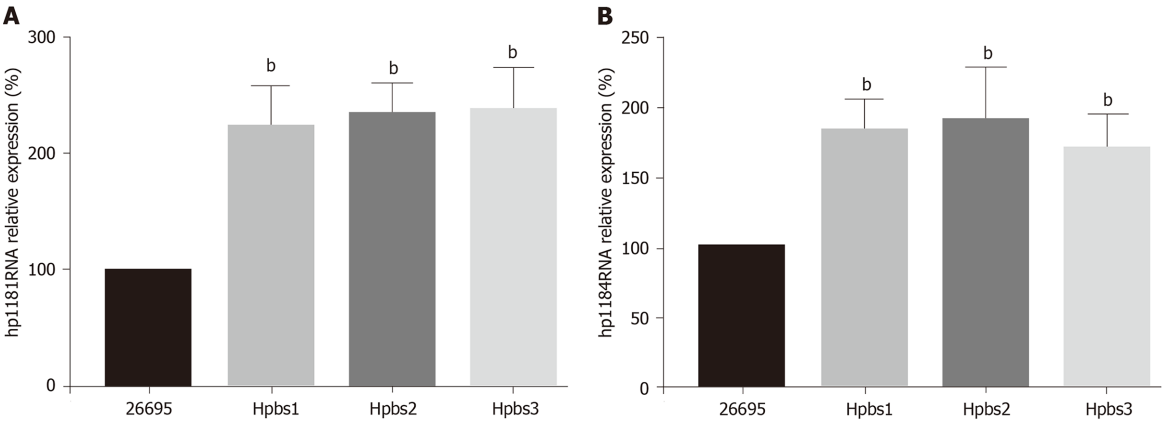


Figure 2 *Hp1181* and *hp1184* gene expression in drug-resistant strains. A: *Hp1181*; B: *Hp1184*. $^{b}P < 0.01$.

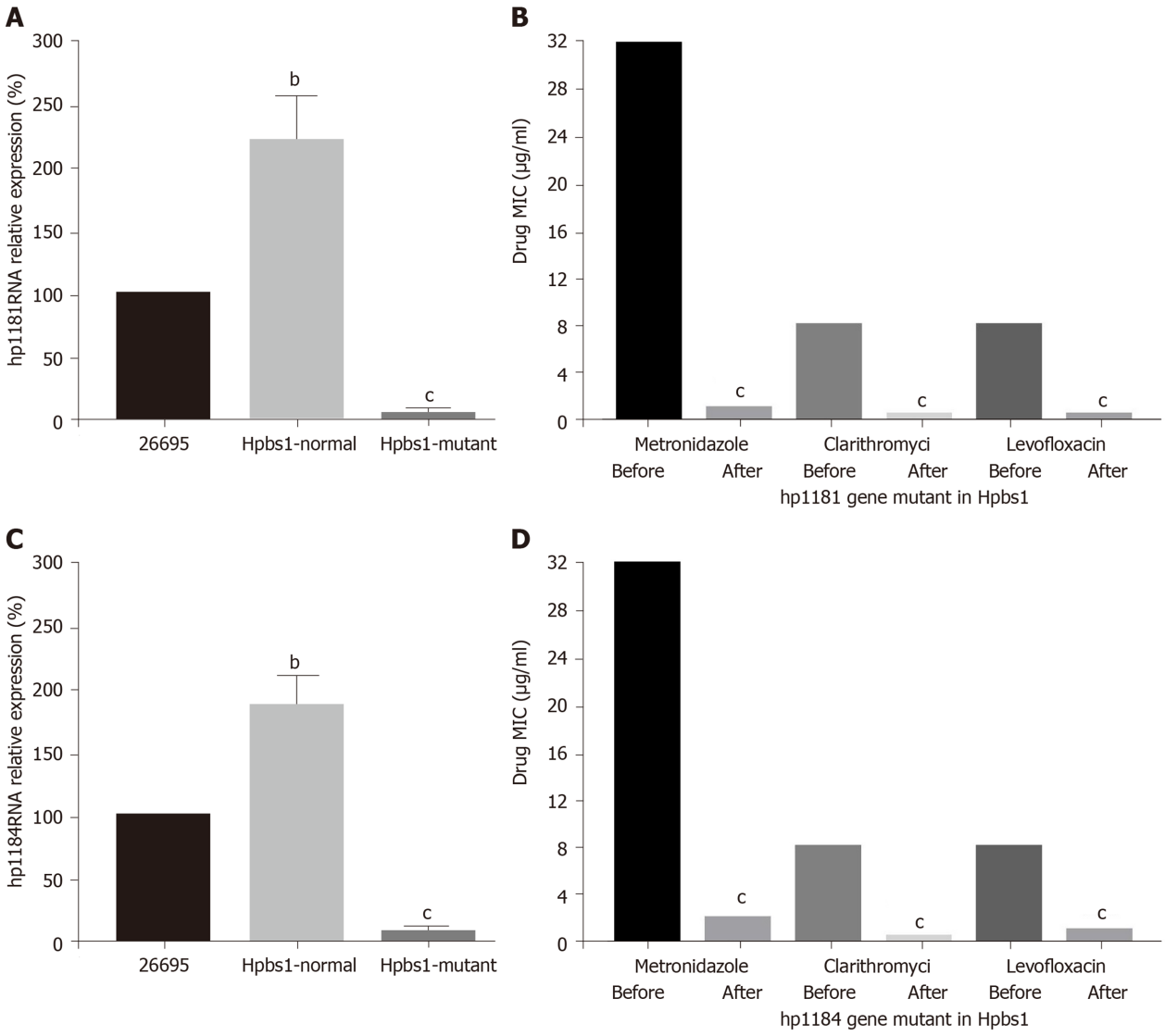


Figure 3 Drug sensitivity is improved after knockout of the drug-resistant genes. A: *Hp1181* knockout; B: Minimal inhibitory concentration (MIC) after *hp1181* knockout; C: *Hp1184* knockout; D: MIC after *hp1184* knockout. MIC: Minimal inhibitory concentration. $^{b}P < 0.01$; $^{c}P < 0.001$.

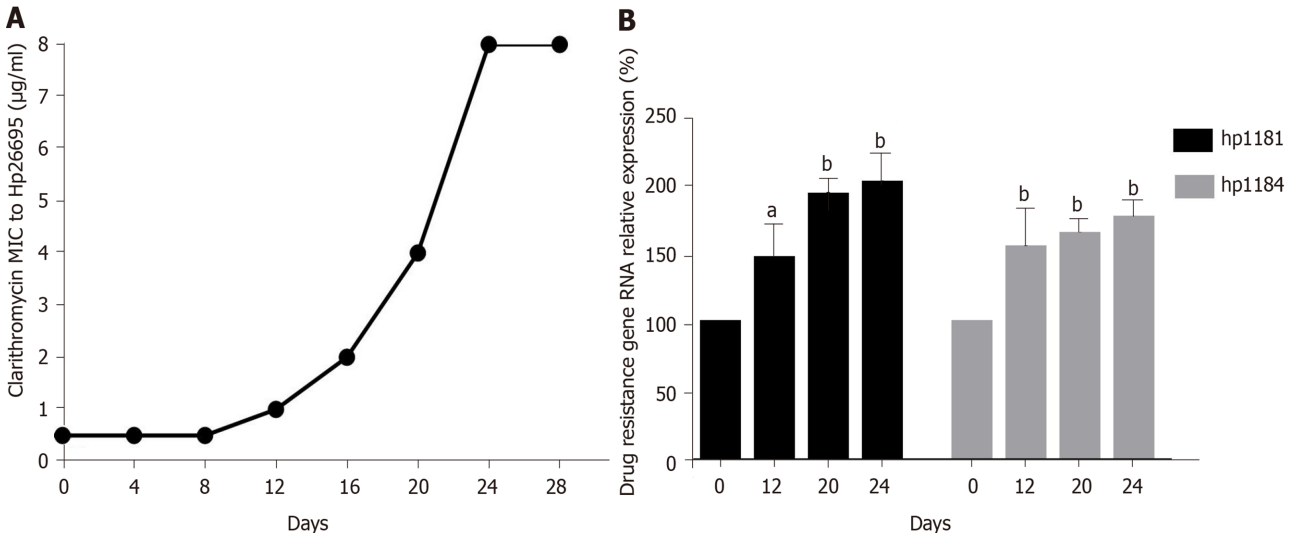


Figure 4 Induction of resistance to clarithromycin and expression of drug-resistant genes in *Helicobacter pylori*. A: Induction of clarithromycin resistance; B: Expression of drug-resistant genes. $^{a}P < 0.05$; $^{b}P < 0.01$.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) is recognized as an important human pathogen associated with superficial gastritis, atrophic gastritis, gastric cancer, *etc*., each of which has become a serious threat to human health and survival. The rate of drug resistance is increasing due to the wide use of antibiotics and high rates of resistance to clarithromycin, metronidazole, and levofloxacin are associated with the failure of *H. pylori* eradication. At present, the mechanism of antibiotic resistance of *H. pylori* is not completely understood. It is very difficult to prevent drug resistance and improve the rate of eradication of the target, thus warranting exploration of the mechanism of drug resistance to *H. pylori*, and provision of an experimental basis for the prevention and treatment of drug resistance.

Research motivation

Clarithromycin-resistant *H. pylori* urgently needs new antibiotics; however, antibiotic research and development are very difficult. If we can detect drug resistance by detecting drug-resistant genes in a timeous manner, this may help to alleviate the problem of clarithromycin resistance.

Research objectives

The objectives of this study were to investigate drug-resistant genes in *H. pylori*, and find a gene suited to early diagnosis of clarithromycin resistance, thereby rationalizing the rate of use of the drug.

Research methods

H. pylori strains were isolated and cultured, minimal inhibitory concentrations were measured, and complete genome sequence was determined. Prediction and analysis of the function of drug-resistant genes indicated that the RNA expression of *hp1181* and *hp1184* increased in the *H. pylori* strains, which was the same in the artificially induced clarithromycin-resistant bacteria. The relationships between *hp1181* or *hp1184* and clarithromycin resistance were confirmed with gene mutant and drug-resistant strains.

Research results

Hp1181 and *hp1184* genes were found in these *H. pylori* strains. Their expression was associated with clarithromycin resistance.

Research conclusions

Hp1181 and *hp1184* mutations may be the earliest and most persistent response to clarithromycin resistance, and they may be the main target genes for the diagnosis, prevention, and treatment of clarithromycin resistance.

Research perspectives

The relationship between *hp1181* or *hp1184* and clarithromycin resistance was demonstrated, providing an improved experimental basis for early diagnosis of clarithromycin resistance in *H. pylori*.

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REFERENCES

1 **Yoon K**, Kim N, Lee JW, Yoon H, Shin CM, Park YS, Lee DH. Annual eradication rate of bismuth-containing quadruple therapy as second-line treatment for Helicobacter pylori infection: A 15-year prospective study at a tertiary hospital in Korea. *Helicobacter* 2020; **25**: e12685 [PMID: 32141173 DOI: 10.1111/hel.12685]

2 **Fischbach W**, Malfertheiner P. Helicobacter Pylori Infection. *Dtsch Arztebl Int* 2018; **115**: 429-436 [PMID: 29999489 DOI: 10.3238/arztebl.2018.0429]

3 **Diaconu S**, Predescu A, Moldoveanu A, Pop CS, Fierbințeanu-Braticevici C. Helicobacter pylori infection: old and new. *J Med Life* 2017; **10**: 112-117 [PMID: 28616085]

4 **Lahner E**, Carabotti M, Annibale B. Treatment of *Helicobacter pylori* infection in atrophic gastritis. *World J Gastroenterol* 2018; **24**: 2373-2380 [PMID: 29904244 DOI: 10.3748/wjg.v24.i22.2373]

5 **Suzuki S**, Esaki M, Kusano C, Ikehara H, Gotoda T. Development of *Helicobacter pylori* treatment: How do we manage antimicrobial resistance? *World J Gastroenterol* 2019; **25**: 1907-1912 [PMID: 31086459 DOI: 10.3748/wjg.v25.i16.1907]

6 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]

7 **Savoldi A**, Carrara E, Graham DY, Conti M, Tacconelli E. Prevalence of Antibiotic Resistance in Helicobacter pylori: A Systematic Review and Meta-analysis in World Health Organization Regions. *Gastroenterology* 2018; **155**: 1372-1382. e17 [PMID: 29990487 DOI: 10.1053/j.gastro.2018.07.007]

8 **Liu DS**, Wang YH, Zeng ZR, Zhang ZY, Lu H, Xu JM, Du YQ, Li Y, Wang JB, Xu SP, Chen Y, Lan CH, Cheng H, Jiang MD, Zhang LX, Huo LJ, Chen SY, Zhang GX, Wu KC, Zhu X, Chen YX, Zhu Y, Shu X, Xie Y, Lu NH. Primary antibiotic resistance of Helicobacter pylori in Chinese patients: a multiregion prospective 7-year study. *Clin Microbiol Infect* 2018; **24**: 780.e5-780. e8 [PMID: 29138101 DOI: 10.1016/j.cmi.2017.11.010]

9 **Ge X**, Cai Y, Chen Z, Gao S, Geng X, Li Y, Jia J, Sun Y. Bifunctional Enzyme SpoT Is Involved in Biofilm Formation of Helicobacter pylori with Multidrug Resistance by Upregulating Efflux Pump Hp1174 (*gluP*). *Antimicrob Agents Chemother* 2018; **62** [PMID: 30181372 DOI: 10.1128/AAC.00957-18]

10 **Bińkowska A**, Biernat MM, Łaczmański Ł, Gościniak G. Molecular Patterns of Resistance Among *Helicobacter pylori* Strains in South-Western Poland. *Front Microbiol* 2018; **9**: 3154 [PMID: 30619218 DOI: 10.3389/fmicb.2018.03154]

11 **Zhang XY**, Shen WX, Chen CF, Sheng HH, Cheng H, Li J, Hu F, Lu DR, Gao HJ. Detection of the clarithromycin resistance of Helicobacter pylori in gastric mucosa by the amplification refractory mutation system combined with quantitative real-time PCR. *Cancer Med* 2019; **8**: 1633-1640 [PMID: 30864275 DOI: 10.1002/cam4.1986]

12 **Matta AJ**, Zambrano DC, Pazos AJ. Punctual mutations in *23S rRNA* gene of clarithromycin-resistant *Helicobacter pylori* in Colombian populations. *World J Gastroenterol* 2018; **24**: 1531-1539 [PMID: 29662291 DOI: 10.3748/wjg.v24.i14.1531]

13 **Alarcón-Millán J**, Fernández-Tilapa G, Cortés-Malagón EM, Castañón-Sánchez CA, De Sampedro-Reyes J, Cruz-Del Carmen I, Betancourt-Linares R, Román-Román A. Clarithromycin resistance and prevalence of Helicobacter pylori virulent genotypes in patients from Southern México with chronic gastritis. *Infect Genet Evol* 2016; **44**: 190-198 [PMID: 27355861 DOI: 10.1016/j.meegid.2016.06.044]

14 **Kouitcheu Mabeku LB**, Eyoum Bille B, Tepap Zemnou C, Tali Nguefack LD, Leundji H. Broad spectrum resistance in Helicobacter pylori isolated from gastric biopsies of patients with dyspepsia in Cameroon and efflux-mediated multiresistance detection in MDR isolates. *BMC Infect Dis* 2019; **19**: 880 [PMID: 31640588 DOI: 10.1186/s12879-019-4536-8]

15 **Attaran B**, Falsafi T, Ghorbanmehr N. Effect of biofilm formation by clinical isolates of *Helicobacter pylori* on the efflux-mediated resistance to commonly used antibiotics. *World J Gastroenterol* 2017; **23**: 1163-1170 [PMID: 28275296 DOI: 10.3748/wjg.v23.i7.1163]

16 **Su P**, Li Y, Li H, Zhang J, Lin L, Wang Q, Guo F, Ji Z, Mao J, Tang W, Shi Z, Shao W, Zhu X, Zhang X, Tong Y, Tu H, Jiang M, Wang Z, Jin F, Yang N. Antibiotic resistance of Helicobacter pylori isolated in the Southeast Coastal Region of China. *Helicobacter* 2013; **18**: 274-279 [PMID: 23418857 DOI: 10.1111/hel.12046]

17 **Ranjbar R**, Farsani FY, Dehkordi FS. Phenotypic analysis of antibiotic resistance and genotypic study of the *vacA*, *cagA*, *iceA*, *oipA* and *babA* genotypes of the *Helicobacter pylori* strains isolated from raw milk. *Antimicrob Resist Infect Control* 2018; **7**: 115 [PMID: 30288255 DOI: 10.1186/s13756-018-0409-y]

18 **Humphries RM**, Kircher S, Ferrell A, Krause KM, Malherbe R, Hsiung A, Burnham CA. The Continued Value of Disk Diffusion for Assessing Antimicrobial Susceptibility in Clinical Laboratories: Report from the Clinical and Laboratory Standards Institute Methods Development and Standardization Working Group. *J Clin Microbiol* 2018; **56** [PMID: 29743302 DOI: 10.1128/JCM.00437-18]

19 **Goderska K**, Agudo Pena S, Alarcon T. Helicobacter pylori treatment: antibiotics or probiotics. *Appl Microbiol Biotechnol* 2018; **102**: 1-7 [PMID: 29075827 DOI: 10.1007/s00253-017-8535-7]

20 **Thung I**, Aramin H, Vavinskaya V, Gupta S, Park JY, Crowe SE, Valasek MA. Review article: the global emergence of Helicobacter pylori antibiotic resistance. *Aliment Pharmacol Ther* 2016; **43**: 514-533 [PMID: 26694080 DOI: 10.1111/apt.13497]

21 **Munita JM**, Arias CA. Mechanisms of Antibiotic Resistance. *Microbiol Spectr* 2016; **4** [PMID: 27227291 DOI: 10.1128/microbiolspec.VMBF-0016-2015]

22 **Lee CR**, Lee JH, Park M, Park KS, Bae IK, Kim YB, Cha CJ, Jeong BC, Lee SH. Biology of *Acinetobacter baumannii*: Pathogenesis, Antibiotic Resistance Mechanisms, and Prospective Treatment Options. *Front Cell Infect Microbiol* 2017; **7**: 55 [PMID: 28348979 DOI:

10.3389/fcimb.2017.00055]
23 **Pang Z**, Raudonis R, Glick BR, Lin TJ, Cheng Z. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnol Adv* 2019; **37**: 177-192 [PMID: 30500353 DOI: 10.1016/j.biotechadv.2018.11.013]
24 **Chung The H**, Baker S. Out of Asia: the independent rise and global spread of fluoroquinolone-resistant Shigella. *Microb Genom* 2018; **4** [PMID: 29595412 DOI: 10.1099/mgen.0.000171]
25 **Falsafi T**, Ehsani A, Attaran B, Niknam V. Association of hp1181 and hp1184 Genes With the Active Efflux Phenotype in Multidrug-Resistant Isolates of Helicobacter pylori. *Jundishapur J Microbiol* 2016; **9**: e30726 [PMID: 27303615 DOI: 10.5812/jjm.30726]
26 **van Amsterdam K**, Bart A, van der Ende A. A Helicobacter pylori TolC efflux pump confers resistance to metronidazole. *Antimicrob Agents Chemother* 2005; **49**: 1477-1482 [PMID: 15793129 DOI: 10.1128/AAC.49.4.1477-1482.2005]
27 **Kubanov A**, Vorobyev D, Chestkov A, Leinsoo A, Shaskolskiy B, Dementieva E, Solomka V, Plakhova X, Gryadunov D, Deryabin D. Molecular epidemiology of drug-resistant Neisseria gonorrhoeae in Russia (Current Status, 2015). *BMC Infect Dis* 2016; **16**: 389 [PMID: 27506605 DOI: 10.1186/s12879-016-1688-7]
28 **Miele A**, Goldstein BP, Bandera M, Jarvis C, Resconi A, Williams RJ. Differential susceptibilities of enterococcal species to elfamycin antibiotics. *J Clin Microbiol* 1994; **32**: 2016-2018 [PMID: 7989561 DOI: 10.1128/JCM.32.8.2016-2018.1994]
29 **Jin DJ**, Gross CA. Mapping and sequencing of mutations in the Escherichia coli rpoB gene that lead to rifampicin resistance. *J Mol Biol* 1988; **202**: 45-58 [PMID: 3050121 DOI: 10.1016/0022-2836(88)90517-7]
30 **Ballif M**, Harino P, Ley S, Coscolla M, Niemann S, Carter R, Coulter C, Borrell S, Siba P, Phuanukoonnon S, Gagneux S, Beck HP. Drug resistance-conferring mutations in Mycobacterium tuberculosis from Madang, Papua New Guinea. *BMC Microbiol* 2012; **12**: 191 [PMID: 22943573 DOI: 10.1186/1471-2180-12-191]
31 **Finken M**, Kirschner P, Meier A, Wrede A, Böttger EC. Molecular basis of streptomycin resistance in Mycobacterium tuberculosis: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Mol Microbiol* 1993; **9**: 1239-1246 [PMID: 7934937 DOI: 10.1111/j.1365-2958.1993.tb01253.x]
32 **Okamoto S**, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, Suzuki Y, Ochi K. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Mol Microbiol* 2007; **63**: 1096-1106 [PMID: 17238915 DOI: 10.1111/j.1365-2958.2006.05585.x]
33 **Sreevatsan S**, Pan X, Stockbauer KE, Williams DL, Kreiswirth BN, Musser JM. Characterization of rpsL and rrs mutations in streptomycin-resistant Mycobacterium tuberculosis isolates from diverse geographic localities. *Antimicrob Agents Chemother* 1996; **40**: 1024-1026 [PMID: 8849220 DOI: 10.1128/AAC.40.4.1024]
34 **Nair J**, Rouse DA, Bai GH, Morris SL. The rpsL gene and streptomycin resistance in single and multiple drug-resistant strains of Mycobacterium tuberculosis. *Mol Microbiol* 1993; **10**: 521-527 [PMID: 7968530 DOI: 10.1111/j.1365-2958.1993.tb00924.x]
35 **Brzostek A**, Sajduda A, Sliwiński T, Augustynowicz-Kopeć E, Jaworski A, Zwolska Z, Dziadek J. Molecular characterisation of streptomycin-resistant Mycobacterium tuberculosis strains isolated in Poland. *Int J Tuberc Lung Dis* 2004; **8**: 1032-1035 [PMID: 15305490]
36 **Wanke C**, Amrhein N. Evidence that the reaction of the UDP-N-acetylglucosamine 1-carboxyvinyltransferase proceeds through the O-phosphothioketal of pyruvic acid bound to Cys115 of the enzyme. *Eur J Biochem* 1993; **218**: 861-870 [PMID: 8281938 DOI: 10.1111/j.1432-1033.1993.tb18442.x]
37 **Kim DH**, Lees WJ, Kempsell KE, Lane WS, Duncan K, Walsh CT. Characterization of a Cys115 to Asp substitution in the Escherichia coli cell wall biosynthetic enzyme UDP-GlcNAc enolpyruvyl transferase (MurA) that confers resistance to inactivation by the antibiotic fosfomycin. *Biochemistry* 1996; **35**: 4923-4928 [PMID: 8664284 DOI: 10.1021/bi952937w]
38 **Cheng G**, Hu Y, Lu N, Li J, Wang Z, Chen Q, Zhu B. Identification of a novel fosfomycin-resistant UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) from a soil metagenome. *Biotechnol Lett* 2013; **35**: 273-278 [PMID: 23143172 DOI: 10.1007/s10529-012-1074-5]
39 **Takahata S**, Ida T, Hiraishi T, Sakakibara S, Maebashi K, Terada S, Muratani T, Matsumoto T, Nakahama C, Tomono K. Molecular mechanisms of fosfomycin resistance in clinical isolates of Escherichia coli. *Int J Antimicrob Agents* 2010; **35**: 333-337 [PMID: 20071153 DOI: 10.1016/j.ijantimicag.2009.11.011]
40 **Hormeño L**, Palomo G, Ugarte-Ruiz M, Porrero MC, Borge C, Vadillo S, Píriz S, Domínguez L, Campos MJ, Quesada A. Identification of the main quinolone resistance determinant in Campylobacter jejuni and Campylobacter coli by MAMA-DEG PCR. *Diagn Microbiol Infect Dis* 2016; **84**: 236-239 [PMID: 26658311 DOI: 10.1016/j.diagmicrobio.2015.11.002]
41 **Khan R**, Kong HG, Jung YH, Choi J, Baek KY, Hwang EC, Lee SW. Triclosan Resistome from Metagenome Reveals Diverse Enoyl Acyl Carrier Protein Reductases and Selective Enrichment of Triclosan Resistance Genes. *Sci Rep* 2016; **6**: 32322 [PMID: 27577999 DOI: 10.1038/srep32322]
42 **Battisti JM**, Smitherman LS, Samuels DS, Minnick MF. Mutations in Bartonella bacilliformis gyrB confer resistance to coumermycin A1. *Antimicrob Agents Chemother* 1998; **42**: 2906-2913 [PMID: 9797224 DOI: 10.1128/AAC.42.11.2906]

43 **Moffatt JH**, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J, Boyce JD. Colistin resistance in Acinetobacter baumannii is mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother* 2010; **54**: 4971-4977 [PMID: 20855724 DOI: 10.1128/AAC.00834-10]

44 **Beceiro A**, Moreno A, Fernández N, Vallejo JA, Aranda J, Adler B, Harper M, Boyce JD, Bou G. Biological cost of different mechanisms of colistin resistance and their impact on virulence in Acinetobacter baumannii. *Antimicrob Agents Chemother* 2014; **58**: 518-526 [PMID: 24189257 DOI: 10.1128/AAC.01597-13]

45 **Fournier B**, Aras R, Hooper DC. Expression of the multidrug resistance transporter NorA from Staphylococcus aureus is modified by a two-component regulatory system. *J Bacteriol* 2000; **182**: 664-671 [PMID: 10633099 DOI: 10.1128/jb.182.3.664-671.2000]

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ORIGINAL ARTICLE

Case Control Study

Altered profiles of fecal bile acids correlate with gut microbiota and inflammatory responses in patients with ulcerative colitis

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Author contributions: Yang ZH designed and performed the study, analyzed the data, and drafted the manuscript; Liu F and Jia ZJ collected the samples and clinical data of the patients; Zhu XR and Suo FY took part in designing the study and analyzing the data; Yao SK designed the study, supervised the study performance, revised the manuscript, and obtained the funding.

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Abstract

BACKGROUND

Gut microbiota and its metabolites may be involved in the pathogenesis of inflammatory bowel disease. Several clinical studies have recently shown that patients with ulcerative colitis (UC) have altered profiles of fecal bile acids (BAs). It was observed that BA receptors Takeda G-protein-coupled receptor 5 (TGR5) and vitamin D receptor (VDR) participate in intestinal inflammatory responses by regulating NF-κB signaling. We hypothesized that altered profiles of fecal BAs might be correlated with gut microbiota and inflammatory responses in patients with UC.

AIM

To investigate the changes in fecal BAs and analyze the relationship of BAs with gut microbiota and inflammation in patients with UC.

METHODS

The present study used 16S rDNA sequencing technology to detect the differences in the intestinal flora between UC patients and healthy controls (HCs). Fecal BAs were measured by targeted metabolomics approaches. Mucosal TGR5 and VDR expression was analyzed using immunohistochemistry, and serum inflammatory cytokine levels were detected by ELISA.

RESULTS

Thirty-two UC patients and twenty-three HCs were enrolled in this study. It was found that the diversity of gut microbiota in UC patients was reduced compared with that in HCs. *Firmicutes*, *Clostridium IV*, *Butyricicoccus*, *Clostridium XlVa*, *Faecalibacterium*, and *Roseburia* were significantly decreased in patients with UC (P = 3.75E-05, P = 8.28E-07, P = 0.0002, P = 0.003, P = 0.0003, and P = 0.0004,

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respectively). *Proteobacteria*, *Escherichia*, *Enterococcus*, *Klebsiella*, and *Streptococcus* were significantly enriched in the UC group (P = 2.99E-09, P = 3.63E-05, P = 8.59E-05, P = 0.003, and P = 0.016, respectively). The concentrations of fecal secondary BAs, such as lithocholic acid, deoxycholic acid, glycodeoxycholic acid, glycolithocholic acid, and taurolithocholate, in UC patients were significantly lower than those in HCs (P = 8.1E-08, P = 1.2E-07, P = 3.5E-04, P = 1.9E-03, and P = 1.8E-02, respectively) and were positively correlated with *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium*, and *Clostridium XlVb* (P < 0.01). The concentrations of primary BAs, such as taurocholic acid, cholic acid, taurochenodeoxycholate, and glycochenodeoxycholate, in UC patients were significantly higher than those in HCs (P = 5.3E-03, P = 4E-02, P = 0.042, and P = 0.045, respectively) and were positively related to *Enterococcus*, *Klebsiella*, *Streptococcus*, *Lactobacillus*, and pro-inflammatory cytokines (P < 0.01). The expression of TGR5 was significantly elevated in UC patients (0.019 ± 0.013 *vs* 0.006 ± 0.003, P = 0.0003). VDR expression in colonic mucosal specimens was significantly decreased in UC patients (0.011 ± 0.007 *vs* 0.016 ± 0.004, P = 0.033).

CONCLUSION

Fecal BA profiles are closely related to the gut microbiota and serum inflammatory cytokines. Dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR.

Key Words: Ulcerative colitis; Gut microbiota; Bile acids; Takeda G-protein-coupled receptor 5; Vitamin D receptor

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Core Tip: This study comprehensively investigated the changes in gut microbiota and fecal bile acid profiles and analyzed the relationship of bile acids with gut microbiota and inflammation in patients with ulcerative colitis. It was demonstrated that fecal bile acid profiles are closely related to gut microbiota and serum inflammatory cytokines. Dysregulation of gut microbiota and altered constitution of fecal bile acids may participate in regulating inflammatory responses *via* the bile acid receptors Takeda G-protein-coupled receptor 5 and vitamin D receptor.

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INTRODUCTION

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD). It is characterized by continuous and diffuse inflammation starting in the rectum that can extend to proximal segments of the colon. The typical symptoms of UC include bloody diarrhea, abdominal pain, fecal urgency, and tenesmus. According to relevant epidemiological studies, the prevalence rates are high in Western developed countries, particularly in Europe (505 per 100000 in Norway) and North America (286 per 100000 in the United States)[1]. With the popularization of colonoscopy screening and changes in lifestyle and diet, the incidence and prevalence of UC have been increasing over time worldwide[2].

The natural history of UC includes periods of remission and flare-ups, and the goal of therapy is to induce and maintain clinical remission free of corticosteroids, thus minimizing the impact on quality of life[3]. Currently, treatments for UC include 5-aminosalicylates[4], corticosteroids[5], antitumor necrosis factor alpha drugs[6], antibiotics[7], probiotics[8], and immunosuppressants[9]. Studies indicate that a

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substantial proportion of patients who fail to respond to mesalamine for remission induction often rely on corticosteroids and/or immunomodulators to control the disease[10]. Corticosteroid resistance/refractoriness rates range from 8.9% to 25% in individuals with IBD[11,12]. Patients with long-standing IBD involving at least 1/3 of the colon are at an increased risk for colorectal cancer [13]. Colectomy is needed in up to 15% of patients with UC[14]. Therefore, it is necessary to explore the pathogenesis of UC, and effective therapies that can induce and maintain remission in UC without serious side effects are forthcoming.

The etiology and pathogenesis of IBD are still unclear. It is generally believed that various factors, such as the environment, genetics, immunity, and intestinal microbes, play a key role in the occurrence and development of IBD[15]. The human gut microbiota is a dynamic and diverse community of commensal bacteria, fungi, and viruses; among them, bacteria, of which there are over 1000 different species, constitute the majority[16,17]. Microorganisms regulate multiple aspects of host functions, including fermentation of dietary fibers[18], pathogen defense[19], metabolism, and immune maturation[20]. Multiple studies have indicated differences in the composition of the gut microbiota in UC, including reduction in diversity, decreased abundances of bacterial taxa within the *Firmicutes* and *Bacteroides*, and increases in the members of the *Proteobacteria phylum*, such as *Enterobacteriaceae*[21-23].

One of the primary modes by which the gut microbiota interacts with the host is by means of metabolites. Bile acid (BA) metabolites have recently drawn much attention in UC. BAs can be divided into two categories based on structure: Free BAs, including cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithochalic acid (LCA); and conjugated BAs, which are a combination of the abovementioned free BAs and glycine or taurine. BAs can also be divided into primary BAs and secondary BAs based on the source. The intestinal microbiota converts the primary BAs synthesized by host cells to secondary BAs. A few clinical studies have shown that there is a disorder of BA metabolism in patients with UC, which is characterized by enrichment of primary BAs and conjugated BAs and reduction of secondary BAs such as LCA and DCA[24-26]. Nevertheless, previous studies have only measured various fecal BAs but have not extensively investigated their relationships with the gut microbiota, its metabolites, and inflammatory cytokines in patients with UC.

BA receptors mediate the effects of BAs in the intestine, including nuclear receptors and membrane receptors. Nuclear receptors include farnesoid X receptor (FXR), pregnane X receptor, and vitamin D receptor (VDR). Membrane receptor refers to G protein-coupled bile acid receptor 1, also known as Takeda G protein-coupled receptor 5 (TGR5)[27,28]. The TGR5 and VDR signaling pathways play an important role in regulating inflammatory responses, cell proliferation, and apoptosis and controlling glycolipid and energy metabolism. Animal studies have shown that TGR5 and VDR participate in intestinal immune regulation and barrier function and reduce inflammatory responses[29]. However, the expression of the BA receptors VDR and TGR5 in colonic mucosal specimens from UC patients is still unclear.

Therefore, we hypothesized that dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR. This study focused on the changes in the gut microbiota, fecal BA profiles, and BA receptor expression in the colonic mucosa. Correlations between these parameters were also analyzed. The findings may provide new insights into the pathogenesis of UC and the development of effective therapeutic methods for UC.

MATERIALS AND METHODS

Recruitment of subjects and sample collection

Based on the sample size of other studies[26,30-32], a total of 32 UC patients and 23 age- and sex-matched healthy controls (HCs) were recruited in the study. All patients were treated at the gastroenterological department of China-Japan Friendship Hospital from April 2019 to January 2020. Ulcerative colitis usually presents with bloody diarrhea and is diagnosed by colonoscopy and histological findings, as well as the exclusion of infectious and noninfectious colitis. The Mayo score of disease activity of the enrolled patients was required to be 4-12 to ensure that the clinical symptoms were significant at the study entry.

The exclusion criteria for UC patients were as follows: (1) Below the age of 18 or above the age of 65 years; (2) Use of probiotics or prokinetics, antispasmodics, and analgesics within 2 wk, or antibiotics, corticosteroids, immunosuppressants, BA

sequestrants, and lipid-lowering agents within 3 mo; (3) Previous major abdominal surgery or organic diseases such as celiac disease; (4) Severe hypertension, diabetes, coronary heart disease, psychiatric disorders, or biliary or liver comorbidities; (5) Pregnancy, lactation, or planned pregnancy; and (6) Uncooperativeness. All subjects gave written informed consent before participation. The study protocol was approved by the Ethics Committee of the China-Japan Friendship Hospital (No. 2019-K16).

After enrollment, the clinical status of each subject was first assessed using validated questionnaires. Fasting blood specimens were collected from all subjects, and serum samples were obtained and stored at -80 °C until analysis. Stool samples were collected as soon as possible within 1 d of UC patients' visit to prevent initiation of medical treatment from changing the composition of the intestinal flora. The pharmacological agents aforementioned were not allowed throughout the study period. Each fecal sample was divided into two parts with sterile plastic tubes after defecation. Samples were frozen in liquid nitrogen immediately and stored at -80 °C. All subjects were required to maintain their daily dietary habits at least 1 wk before the collection of the stool samples and until all of the assessments were finished. The next day, subjects underwent colonoscopy after standard bowel preparation with polyethylene glycol electrolyte solution, and one mucosal pinch biopsy was taken from the colorectal lesion. The specimen was immediately fixed in 10% formalin for at least 72 h, embedded in paraffin, and sectioned (4 μm) for immunohistochemistry.

Clinical assessments

Clinical assessments were first conducted using standardized questionnaires. The severity indexes of UC were assessed using the previously validated Mayo score[33]. The scoring system determines the severity of UC based on the patient's bloody diarrhea, doctor's assessment, and colonoscopy. Montreal classification was used to assess the extent of UC[34]. The Bristol Stool Form Scale (BSFS), a 7-point scale, was used to measure stool form.

DNA extraction, 16S rDNA amplification, and Illumina sequencing

Total DNA extraction was performed according to the instructions of the E.Z.N.A. soil kit (Omega Biotek, Norcross, GA, United States). The DNA concentration and purity were determined using a Thermo NanoDrop2000, and the quality of DNA extraction was validated by 1% agarose gel electrophoresis; 341F (5′-CCTACGGGRSGCAGCAG-3′) and 806R (5′-GGACTACVVGGGTATCTAATC-3′) primers were used for PCR amplification of the V3-V4 variable region using the following amplification procedure: Predena-turation at 95 °C for 3 min, amplification for 27 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s), and final extension at 72 °C for 10 min (PCR instrument: ABI GeneAmp 9700). The 20 μL reaction mixture included 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mmol/L dNTPs, 0.8 μL of primer (5 μmol/L), 0.4 μL of FastPfu polymerase, and 10 ng of DNA template.

The PCR products were recovered using a 2% agarose gel, purified with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States), eluted with Tris-HCl, and detected by 2% agarose electrophoresis. Quantification was performed using QuantiFluor-ST (Promega, United States). Sequencing was performed using Illumina's MiSeq PE250 platform (Illumina, San Diego, United States).

Bile acid quantitation

BAs in feces were measured according to previously reported methods[35,36]. A Waters ACQUITY ultra-performance LC system coupled with a Waters XEVO TQ-S mass spectrometer with an ESI source controlled by MassLynx 4.1 software (Waters, Milford, MA) was used for all analyses. Chromatographic separations were performed with an ACQUITY BEH C18 column (1.7 μm, 100 mm × 2.1 mm internal dimensions; Waters, Milford, MA). UPLC-MS raw data obtained in negative mode were analyzed using TargetLynx Applications Manager version 4.1 (Waters Corp., Milford, MA) to obtain calibration equations and the concentration of each BA in the samples.

Immunohistochemistry

Paraffin sections were processed for immunohistochemistry. Following deparaffinization, antigen repair, endogenous peroxidase inhibition, and nonspecific antigen blocking, the sections were incubated with primary antibodies (rabbit monoclonal anti-TGR5 antibody, 1:100; rabbit monoclonal anti-VDR antibody, 1:100; Abcam, Cambridge, United Kingdom) overnight at 4 °C. Following thorough washing with

PBS, slides were incubated at room temperature for 1 h with horseradish peroxidase-conjugated anti-mouse rabbit secondary antibody (Zhongshan Gold Bridge, Beijing, China) and then visualized using diaminobenzidine. Finally, slides were counter-stained with hematoxylin and viewed under a light microscope.

For each section, five nonoverlapping fields at 400 × magnification were randomly selected and scanned under an OLYMPUS microscope. Images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, United States). The mean optical density of the mucosal staining area was used to measure the expression of TGR5 and VDR. All sections were inspected independently by two blinded observers, and the mean values of the readings were used for final analysis.

Statistical analysis

Statistical analyses were performed using SPSS software, version 24.0 (SPSS Inc, Chicago, IL, United States). The normality of the distribution of the variables was tested using the Shapiro-Wilk test. Normally distributed data are presented as the mean ± SD, and abnormally distributed data are expressed as the median [interquartile range (IQR)]. Comparisons between groups were performed using independent sample *t*-tests for normally distributed data with homogeneous variances or nonparametric Mann-Whitney *U* tests. The χ^2 test or Fisher's exact test was used to analyze qualitative data. Correlations between BA metabolites and other parameters were explored using Pearson's correlation analysis for normally distributed data or Spearman's correlation analysis for nonnormally distributed data or ranked data. *P* values were two-sided, and differences were considered significant at $P < 0.05$. Statistical charts were generated with GraphPad Prism 5.0 software (GraphPad Software Inc, La Jolla, CA, United States).

RESULTS

Characteristics of study subjects

The demographics and clinical characteristics of UC patients and HCs are presented in Table 1. Thirty-two UC patients (17 males and 15 females; median age 37.0 years, IQR: 32.00-49.75) and twenty-three HCs (13 males and 10 females; median age 32.0 years, IQR: 27.00-51.00) were enrolled in this study. There were no significant differences between the groups in age ($P = 0.570$), sex ($P = 0.803$), or body mass index ($P = 0.337$). In UC patients, the duration of disease ranged from 0.5 to 25 years (median 2 years). According to the Mayo scores (7.8 ± 1.9), 5 (15.6%) patients had mild UC, and 25 (78.1%) had moderate UC. The BSFS score [UC: 6.0 (6.0, 6.0) *vs* HC: 4.0 (4.0, 4.0)] was significantly higher in UC patients than in HCs.

Structural characteristics of gut microbiota in the UC and control groups

Among the 594 operational taxonomic units (OTUs) detected, a total of 317 OTUs were identified in the two groups, including 86 unique OTUs in the UC group and 191 unique OTUs in the control group (Figure 1A). The dilution curve analysis based on the Sobs index for community richness and the Shannon index for community diversity showed that the sequencing volume covered all the microorganisms in the samples and met the data analysis requirements (Figure 1B). The species accumulation curve based on whether the sample size is sufficient and the estimated species richness showed that the sequencing sample size was sufficient, which can reflect most of the microbial information in the sample (Figure 1C). Principal coordinate analysis was performed to assess the similarity of the bacterial communities, which clearly differentiated the intestinal flora of the UC group from the control group (Figures 1D). Chao community richness index or the Shannon and Simpson community alpha diversity indexes of the UC group were significantly lower than those of the control group, indicating that the diversity of flora was reduced (Figure 1E). The community compositions of the intestinal microbes in the UC group and control group were analyzed at the phylum and genus levels. At the phylum level, the dominant phyla found in both groups were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (the proportions in the two groups were 46.06%, 28.69%, 20.60%, and 3.61% *vs* 68.75%, 26.32%, 2.28%, and 1.66%, respectively), while at the genus level, *Bacteroides*, *Faecalibacterium*, *Escherichia*, *Prevotella*, and *Roseburia* were the top five genera (the proportions of which were 24.64%, 10.88%, 15.03%, 6.50, and 4.96% *vs* 21.05%, 24.59%, 0.63%, 7.12% and 8.32%, respectively) (Figure 1F).

Table 1 Demographics and clinical characteristics of the study subjects

Feature	UC patients	Controls	*P* value
n	32	23	NA
Age (yr)	37.0 (32.00, 49.75)	32.0 (27.00, 51.00)	0.570
Gender (male:female)	17:15	13:10	0.803
Body mass index (kg/m^2)	21.85 ± 3.64	22.75 ± 2.95	0.337
Duration of disease (yr)	2 (0.5, 8)	NA	NA
Mayo score	7.8 ± 1.9[b]	NA	< 0.001
BSFS score	6.0 (6.0, 6.0)[b]	4.0 (4.0, 4.0)	< 0.001
Inflammation location			
Rectum	9	NA	NA
Left colon	11	NA	NA
Whole colon	12	NA	NA
Disease activity		NA	NA
Mild activity	5	NA	NA
Moderate activity	25	NA	NA
Severe activity	2	NA	NA

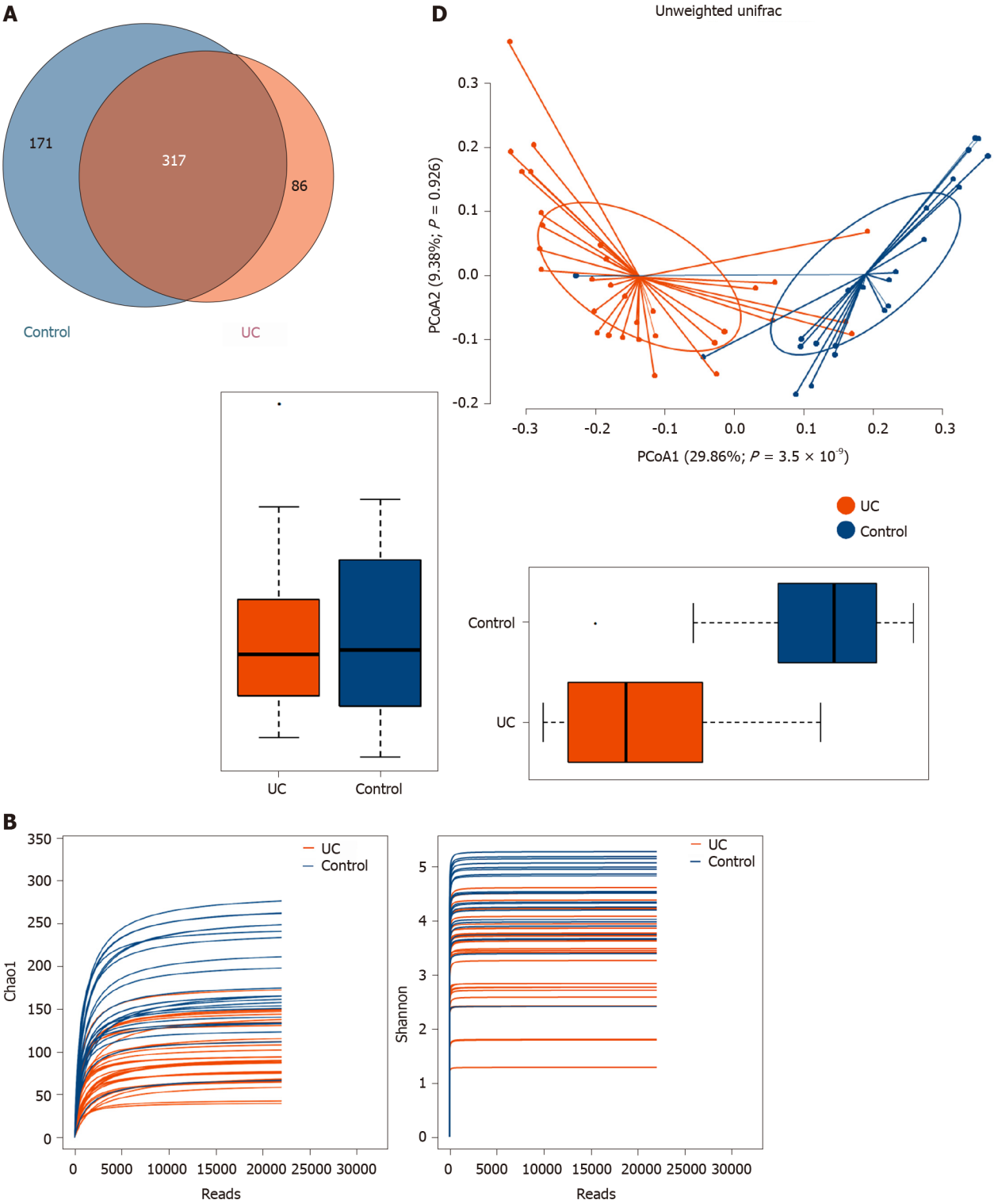
[b]$P < 0.01$ *vs* controls. The data are presented as the mean ± SD or median (interquartile range). UC: Ulcerative colitis; BSFS: Bristol stool form scale; NA: Not applicable.

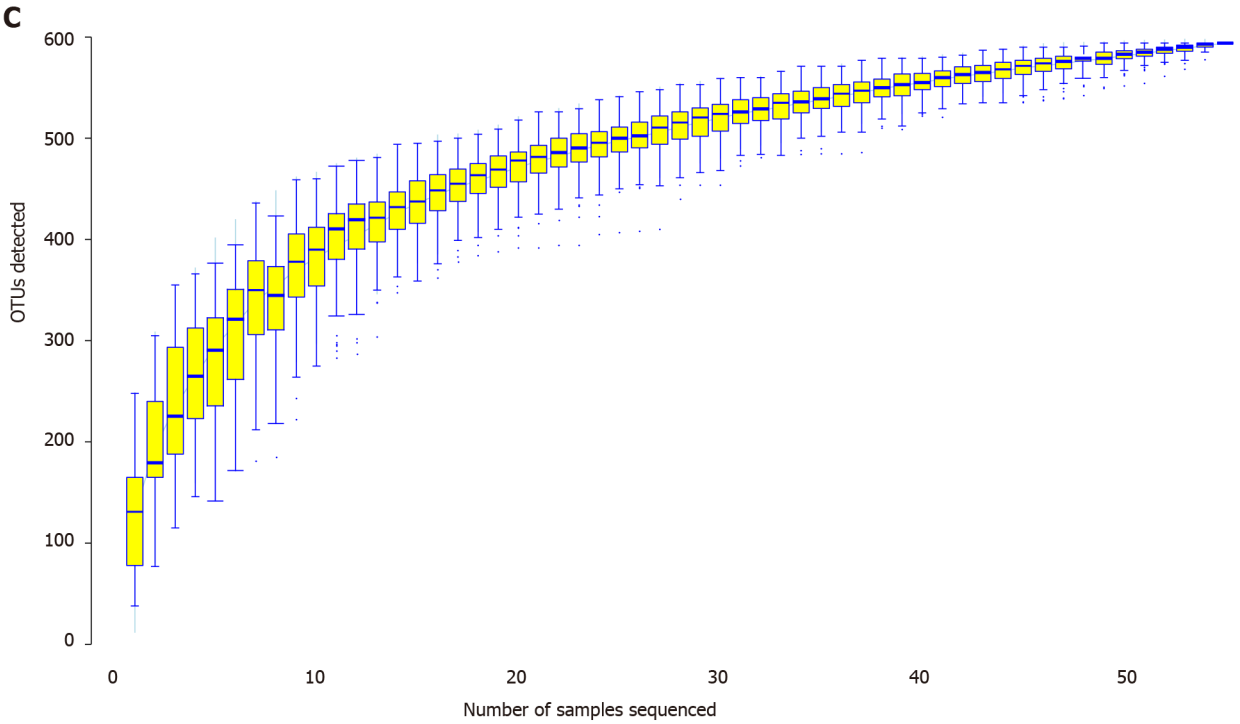
Screening of different key gut microbiota between the UC and control groups

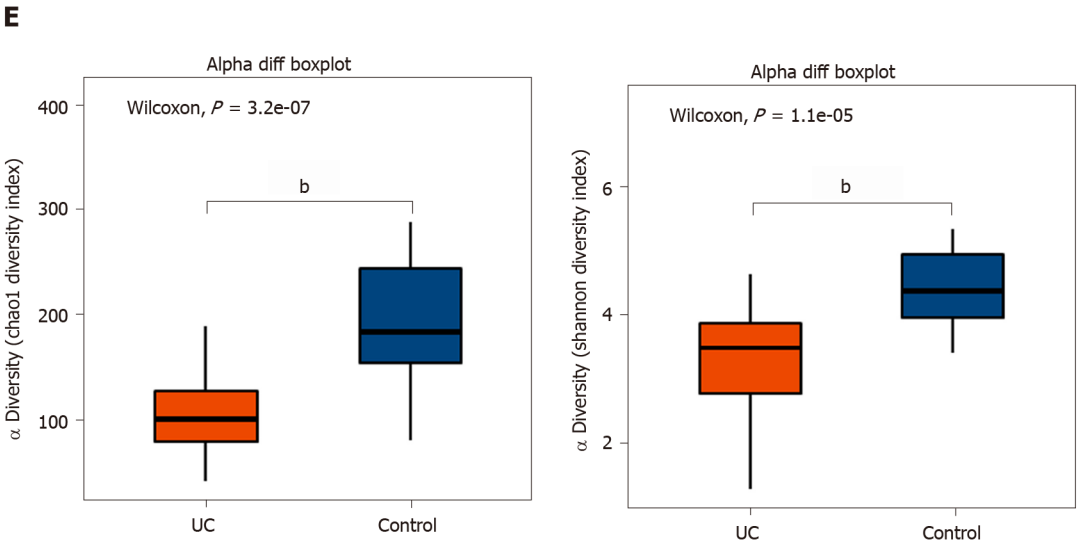
The Wilcoxon rank sum test of differential species between the two groups showed significant changes in the intestinal microbes between the UC and control groups (Figure 2A). At the phylum level, *Firmicutes* and *Proteobacteria* in the UC group were significantly different from those in the control group ($P = 3.75 \times 10^5$, $P = 2.99 \times 10^9$). At the genus level, the percentages of *Clostridium IV*, *Butyricicoccus*, *Clostridium XlVa*, *Faecalibacterium*, *Roseburia*, and *Coprococcus* in the UC group were significantly lower than those in the control group ($P = 8.28 \times 10^7$, $P = 0.0002$, $P = 0.003$, $P = 0.0003$, $P = 0.0004$, and $P = 7.38 \times 10^6$, respectively), and the percentages of *Escherichia*, *Enterococcus*, *Klebsiella*, and *Streptococcus* were significantly higher than those in the control group ($P = 3.63 \times 10^5$, $P = 8.59 \times 10^5$, $P = 0.003$, and $P = 0.016$, respectively). LEfSe analysis identified (threshold 2) the differential intestinal microbial communities between the two groups (Figure 2B). *Clostridia*, *Clostridiales*, *Firmicutes*, *Ruminococcaceae* and *Faecalibacterium* were significantly enriched in the control group. *Proteobacteria*, *Gammaproteobacteria*, *Enterobacteriaceae*, *Enterobacteriales*, and *Escherichia_Shigella* were significantly enriched in the UC group.

Analysis of difference in fecal BAs between the UC and control groups

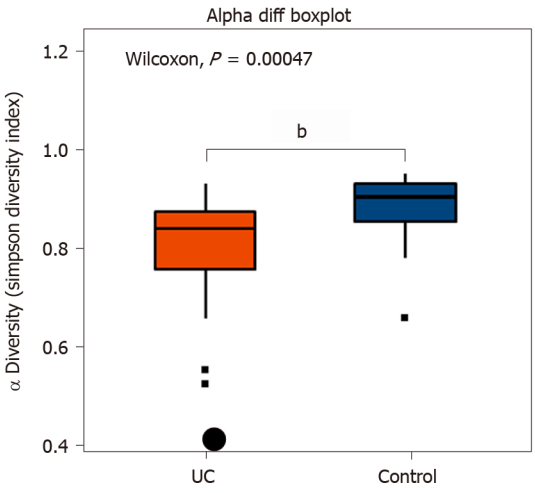
PCA was performed to evaluate the similarity of the fecal BAs of the two groups. Twenty-four BAs clearly distinguished the UC group from the control group (Figure 3). Fecal secondary BAs were significantly decreased in UC patients compared with healthy controls (Figure 3B). The concentrations of fecal secondary BAs such as LCA, DCA, 12_ketoLCA, glycol-deoxycholic acid (GDCA), glycol-lithocholic acid (GLCA), and tauro-lithocholic acid (TLCA) were significantly lower than those in healthy controls ($P = 8.1 \times 10^8$, $P = 1.2 \times 10^7$, $P = 6.3 \times 10^7$, $P = 3.5 \times 10^4$, $P = 1.9 \times 10^3$, and $P = 1.8 \times 10^2$, respectively) (Figure 3C-H). The concentrations of primary BAs such as taurocholic acid (TCA), CA, tauro-chenodeoxycholic acid (TCDCA), and glycol-chenodeoxycholic acid (GCDCA) were significantly higher than those in HCs ($P = 5.3 \times 10^3$, $P = 4 \times 10^2$, $P = 0.042$, and $P = 0.045$, respectively) (Figure 3I and M). The concentrations of CDCA and glycol-cholic acid (GCA) showed a tendency to increase in UC patients but failed to reach a significant level ($P = 0.138$ and $P = 0.074$, respectively) (Figure 3J and N).


A
171
317
86
Control
UC
D
Unweighted unifrac
PCoA2 (9.38%; P = 0.926)
PCoA1 (29.86%; P = 3.5 × 10-9)
UC
Control
Control
UC
UC
Control
B
Chao1
Reads
Shannon
Reads
UC
Control


C
OTUs detected
Number of samples sequenced
0
100
200
300
400
500
600
0
10
20
30
40
50


E
Alpha diff boxplot
Wilcoxon, P = 3.2e-07
α Diversity (chao1 diversity index)
b
UC
Control

Alpha diff boxplot
Wilcoxon, P = 1.1e-05
α Diversity (shannon diversity index)
b
UC
Control


Alpha diff boxplot
Wilcoxon, P = 0.00047
α Diversity (simpson diversity index)
b
UC
Control

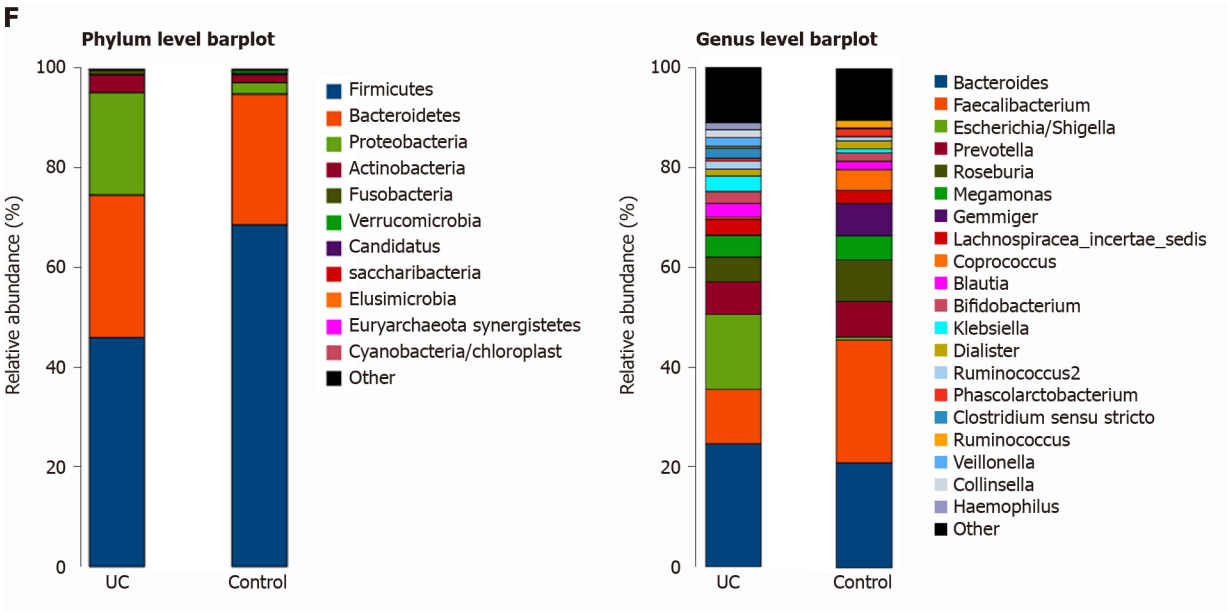


Figure 1 Structural characteristics of intestinal microbes in the ulcerative colitis and control groups. A: Among the 594 operational taxonomic units (OTUs) detected, a total of 317 OTUs were identified in the two groups, including 86 unique OTUs in the UC group and 191 unique OTUs in the control group. B: The dilution curve analysis based on the Chao1 index for community richness and the Shannon index for community diversity showed that the sequencing volume had covered all the microorganisms in the samples. C: The species accumulation curve shows that the sequencing sample size was sufficient, which reflected most of the microbial information in the sample. D: Principal coordinate analysis clearly differentiated the intestinal flora of the ulcerative colitis (UC) group from the control group. E: Chao community richness index or the Shannon and Simpson community α diversity indexes of the UC group were significantly lower than those of the control group. F: The community compositions of the intestinal microbes in the UC group and the control group were analyzed at the phylum and genus levels. UC: Ulcerative colitis; OTUs: Operational taxonomic units; PCoA: Principal coordinate analysis.

Correlations between fecal BAs and intestinal microbes in all subjects

Correlative assessments were made on the fecal BAs and intestinal microbes (Figure 4). The results showed that DCA, LCA, and 12_ketoLCA were negatively correlated with *Enterococcus*, *Klebsiella*, *Streptococcus*, and *Lactobacillus*. CA, CDCA, TCA, TCDCA, GCA, and GCDCA were positively related with *Enterococcus*, *Klebsiella*, *Streptococcus*, and *Lactobacillus*. *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium Ruminococcus*, *Clostridium XlVb*, *Coprococcus*, and *Alistipes* were negatively correlated with the concentrations of CA, CDCA, TCA, TCDCA, GCA, and GCDCA and positively correlated with the concentrations of DCA, LCA, 12_ketoLCA, GLCA, and GDCA.

Mucosal immunohistochemistry

Representative photomicrographs of the immunoreactivity of TGR5 and VDR in the mucosa of UC patients and HCs are shown in Figure 5A-D (× 400 magnification). The level of TGR5 in mucosal biopsies was significantly higher in UC patients than in HCs (0.019 ± 0.013 *vs* 0.006 ± 0.003, P=0.0003) (Figure 5E). VDR expression in colonic mucosal specimens decreased significantly in UC patients (0.011 ± 0.007 *vs* 0.016 ± 0.004, P = 0.033) (Figure 5F).

Serum inflammatory cytokine levels and correlations between fecal BAs and serum inflammatory cytokines

The levels of inflammatory cytokines in the serum of UC patients and HCs were quantified by ELISA. The levels of IL-1α, IL-1β, TNF-α, IL-2, and IL-6 were significantly higher in UC patients (P < 0.0001) (Figure 6A-E). TCA, GCA, and GCDCA were positively correlated with IL-1α (P < 0.05); TCA and TNF-α were positively correlated (P < 0.05); LCA, DCA, 12-KetoLCA, TLCA, GDCA, and 6-Keto-LCA were negatively correlated with the levels of IL-1α, IL-1β, TNF-α, and IL-6 (P < 0.01) (Figure 6F and G).

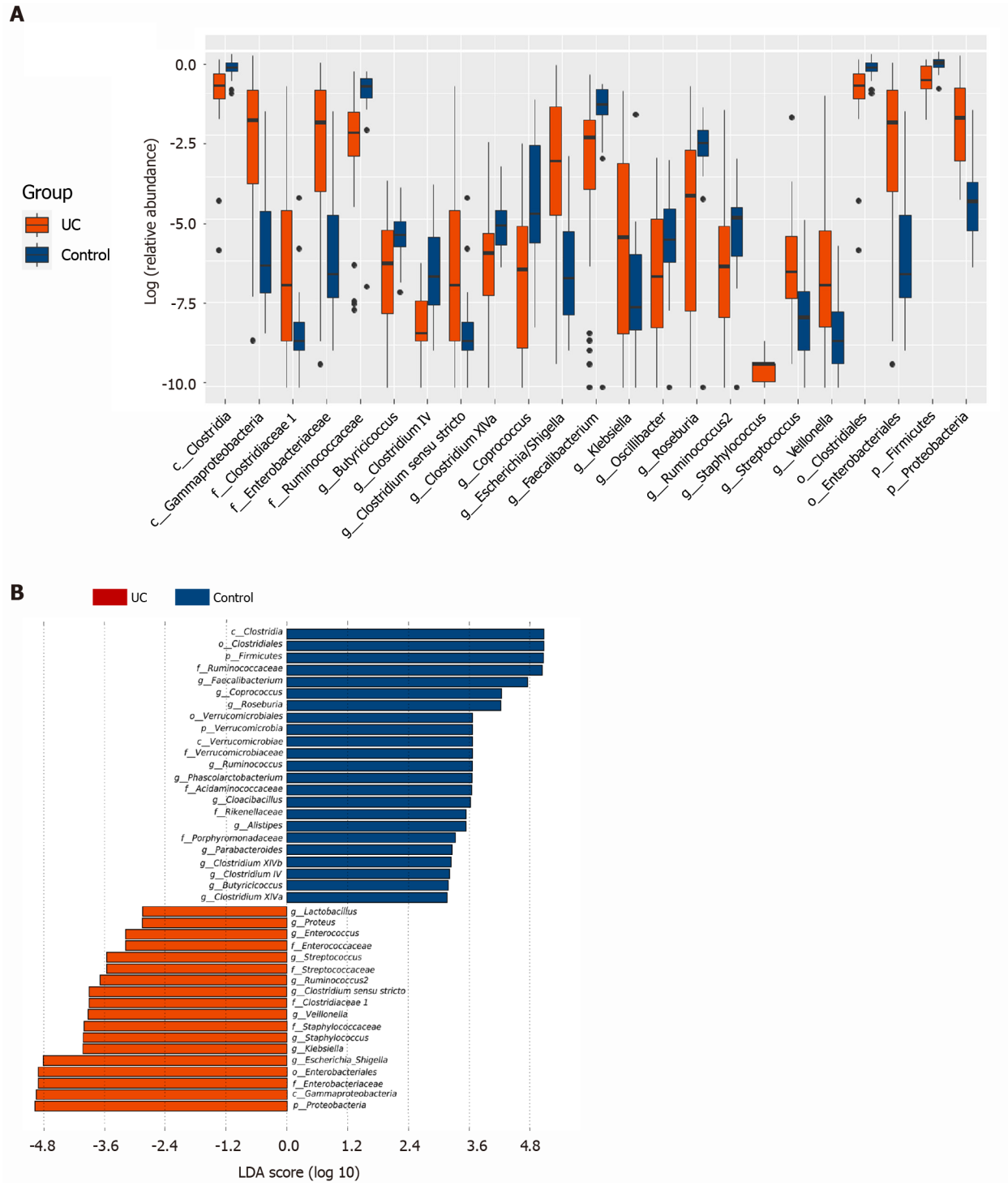
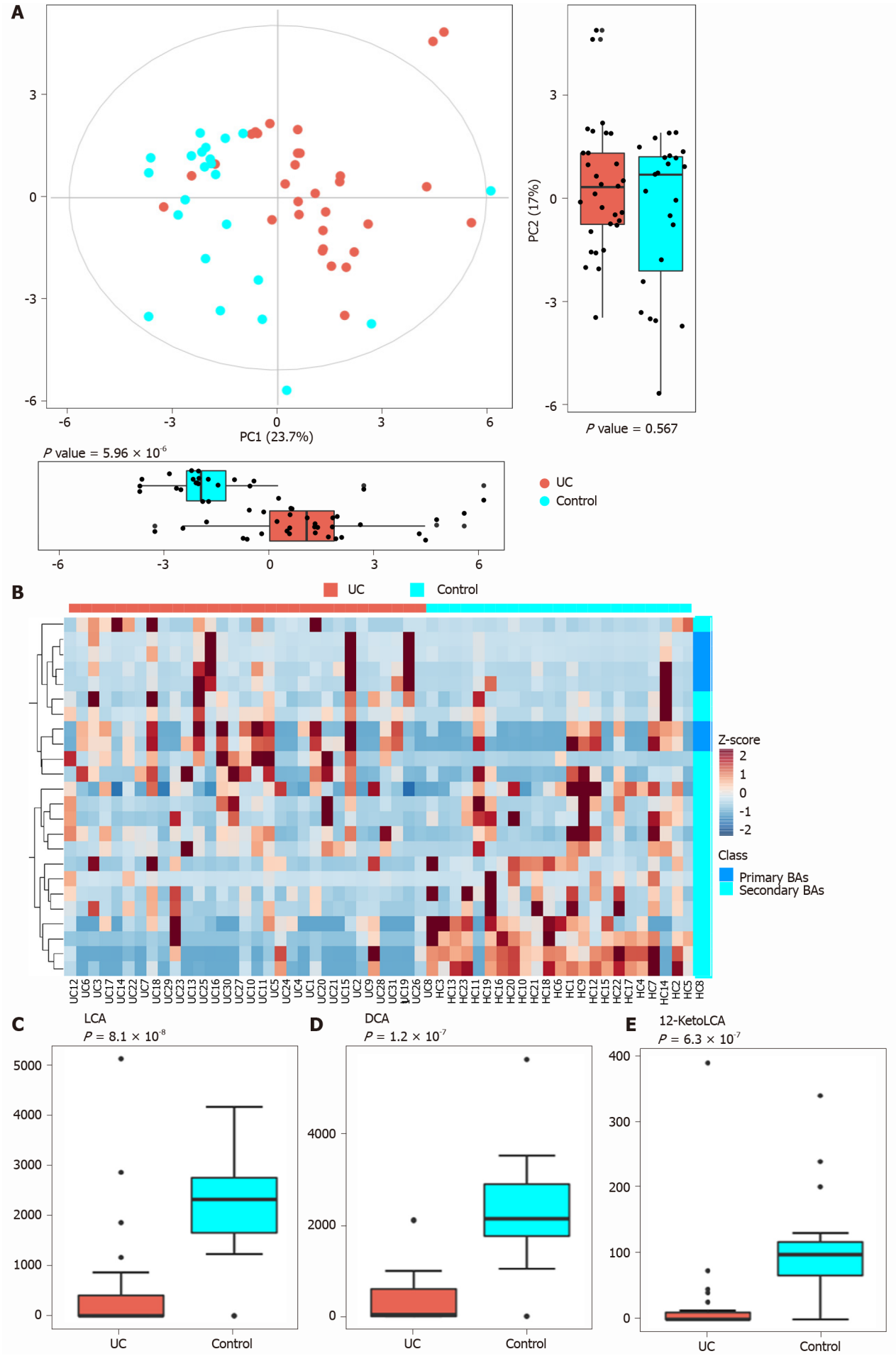


Figure 2 Screening of different key microorganisms between the ulcerative colitis and control groups. A: The Wilcoxon rank sum test of differential species between the two groups showed significant changes in the intestinal microbes between the ulcerative colitis and control groups; B: LEfSe analysis identified (threshold 2) the differential intestinal microbial communities between the two groups. UC: Ulcerative colitis; LDA: Linear discriminant analysis.

DISCUSSION

This study comprehensively investigated the changes in fecal BA profiles and analyzed the associations of BAs with the gut microbiota and inflammation in patients with UC. As expected, these data confirmed the differences in fecal BA compositions between UC patients and HCs, and the concentrations of some BAs were significantly correlated with the gut microbiota and serum inflammatory cytokines. Specifically, the concentrations of fecal secondary BAs such as LCA, DCA, GDCA, GLCA, and TLCA in UC patients were significantly lower than those in HCs and were positively correlated with *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium*, and


A
PC1 (23.7%)
PC2 (17%)
P value = 0.567
P value = 5.96 × 10-6
UC
Control
B
UC
Control
Z-score
2
1
0
-1
-2
Class
Primary BAs
Secondary BAs
C
LCA
P = 8.1 × 10-8
D
DCA
P = 1.2 × 10-7
E
12-KetoLCA
P = 6.3 × 10-7

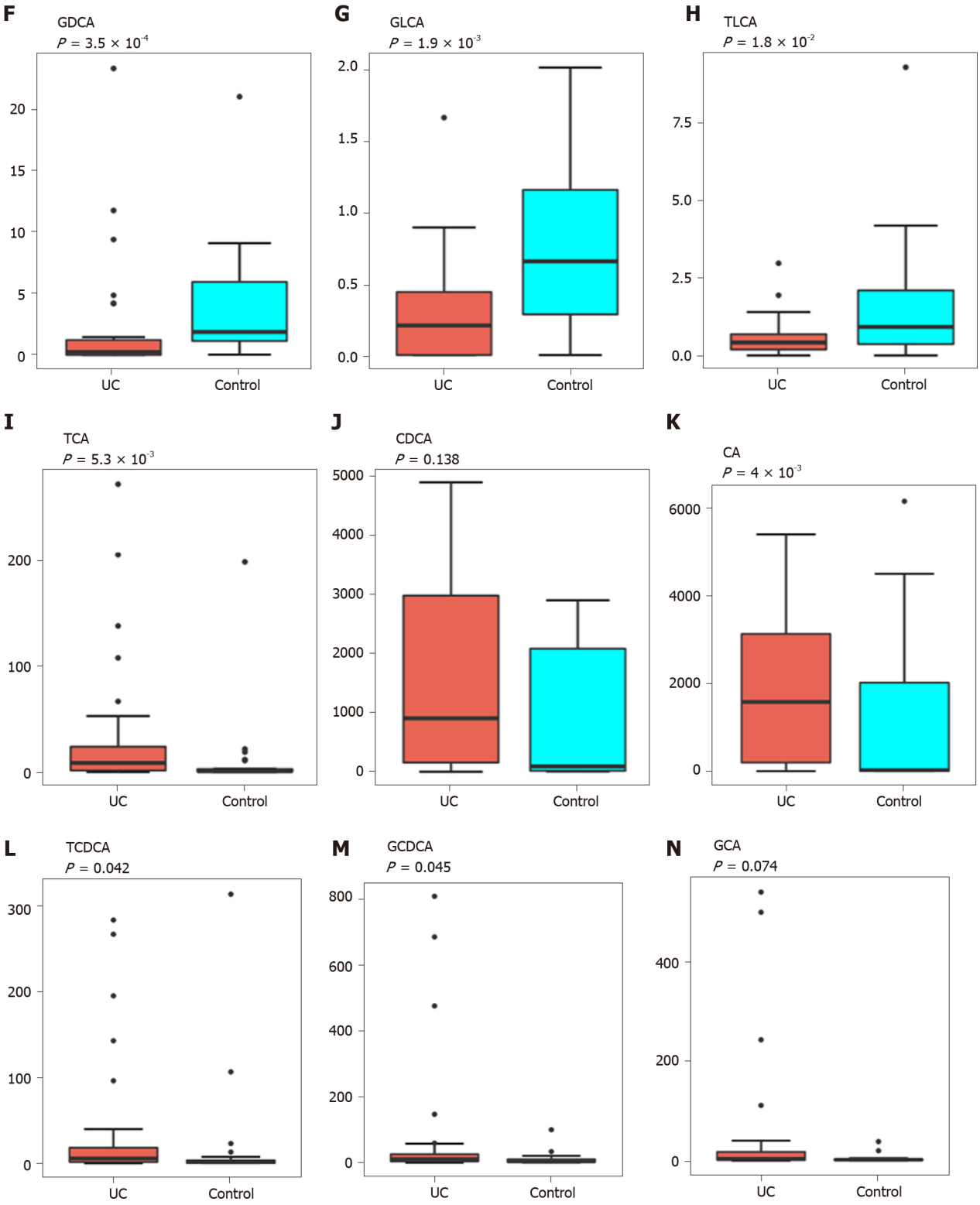


Figure 3 Analysis of difference in fecal bile acids between the ulcerative colitis and control groups. A: Principal component analysis was performed to evaluate the similarity of the fecal BAs of the two groups. Twenty-four BAs clearly distinguished the ulcerative colitis (UC) group from the control group; B: Heatmap showing the individual BA concentrations in the samples (log-transformed). Shades of red and blue represent high and low BA concentrations, respectively (see color scale); C-H: Fecal secondary BAs in UC patients, such as lithocholic acid, deoxycholic acid, glyco-deoxycholic acid, glyco-lithocholic acid, and tauro-lithocholate, were significantly lower than those in healthy controls; I and K-M: The primary BAs such as tauro-cholic acid, cholic acid, tauro-chenodeoxycholic acid, and glyco-chenodeoxycholic acid were significantly higher than those in healthy controls; J and N: The concentrations of chenodeoxycholic acid and glyco-cholic acid showed a tendency to increase in UC patients but the increases were not significant. UC: Ulcerative colitis; PCA: Principal component analysis; BAs: Bile acids; LCA: Lithocholic acid; DCA: Deoxycholic acid; GDCA: Glyco-deoxycholic acid; GLCA: Glyco-lithocholic acid; TLCA: Tauro-lithocholate; TCA: Tauro-cholic acid; CA: Cholic acid; TCDCA: Tauro-chenodeoxycholic acid; GCDCA: Glyco-chenodeoxycholic acid; CDCA: Chenodeoxycholic acid; GCA: Glyco-cholic acid.

Clostridium XlVb. The concentrations of primary BAs such as TCA, CA, TCDCA, and

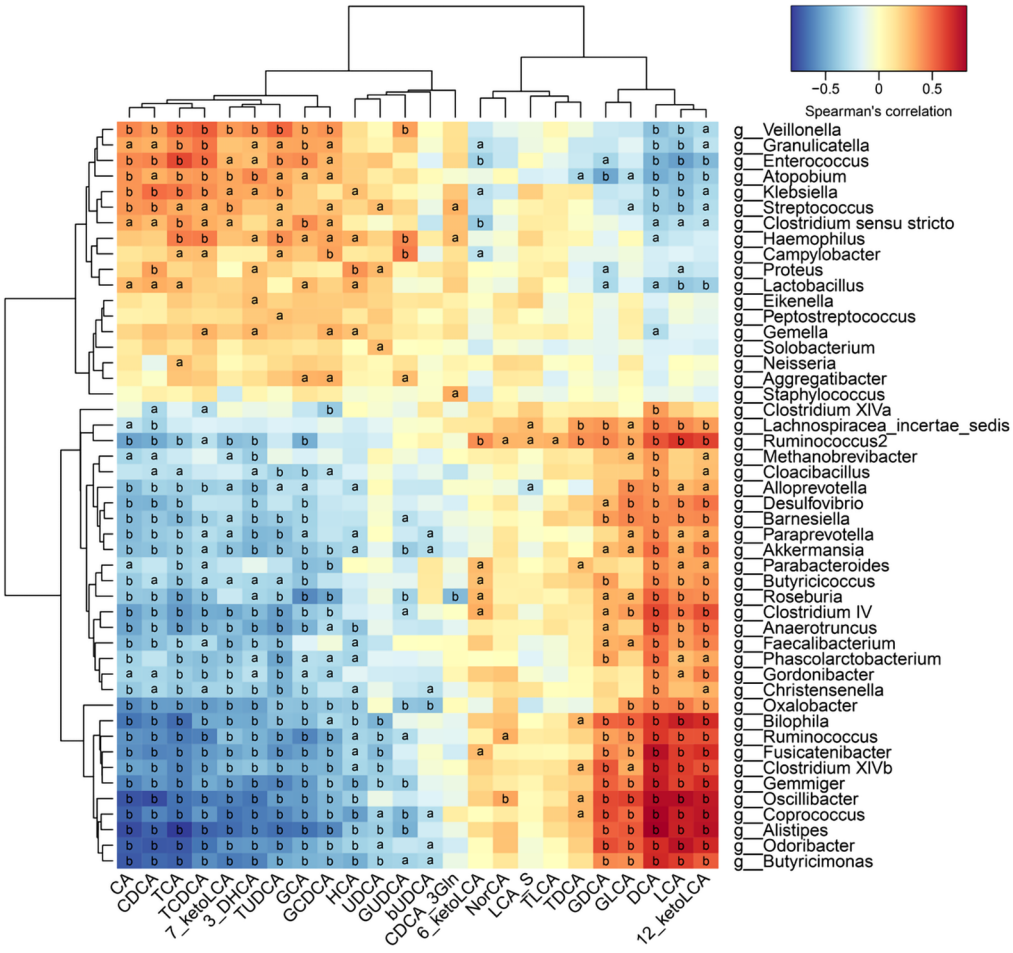


Figure 4 Correlations between fecal bile acids and intestinal microbes. A heatmap of correlative assessments was made on the fecal bile acid metabolites and intestinal microbes ($^{a}P < 0.05$, $^{b}P < 0.01$). LCA: Lithocholic acid; DCA: Deoxycholic acid; GDCA: Glyco-deoxycholic acid; GLCA: Glyco-lithocholic acid; TLCA: Tauro-lithocholate; TDCA: Tauro-deoxycholic acid; UDCA: Ursodeoxycholic acid; TUDCA: Tauro-ursodeoxycholic acid; GUDCA: Glyco-ursodeoxycholic acid; TCA: Tauro-cholic acid; CA: Cholic acid; TCDCA: Tauro-chenodeoxycholic acid; GCDCA: Glyco-chenodeoxycholic acid; CDCA: Chenodeoxycholic acid; GCA: Glyco-cholic acid.

GCDCA in UC patients were significantly higher than those in HCs and positively correlated with *Enterococcus, Klebsiella, Streptococcus, Lactobacillus*, and proinflammatory cytokines. The mucosal expression of the BA membrane receptor TGR5 was significantly elevated in UC patients. Additionally, BA nuclear receptor VDR expression in colonic mucosal specimens was significantly decreased in UC patients. Based on these findings, we concluded that dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR.

For demographics, 32 UC patients (17 males and 15 females; median age 37.0 years, IQR: 32.00-49.75) were enrolled in this study. Similar to previous studies, no sex predominance existed in UC, and the peak age of disease onset was between ages 30 years and 40 years[37]. In order to prevent initiation of medical treatment from changing the composition of the intestinal flora of UC patients, we collected stool samples as soon as possible within 1 d of UC patients' visit to ensure that the clinical symptoms were significant at the study entry. Except for five patients who took mesalazine for a short period of time, the remaining active (relapse) patients included in our study did not receive any treatment before collecting stool and serum samples. Although the time of active disease is not specified, the Mayo score of disease activity of the enrolled patients was required to be 4-12.

Gut microbes were demonstrated to be an essential factor in intestinal inflammation in UC. It has been consistently shown that there is a decrease in biodiversity and species richness in UC[38]. Changes in the composition of the gut microbiota led to metabolite alterations that are likely to have a role in UC pathogenesis[39]. The intestinal microbiota converts ingested food or host products into metabolites that target either the intestinal microbial population or host cells. Hence, the presence of metabolites depends on microbial metabolic activity[40,41]. It is estimated that more

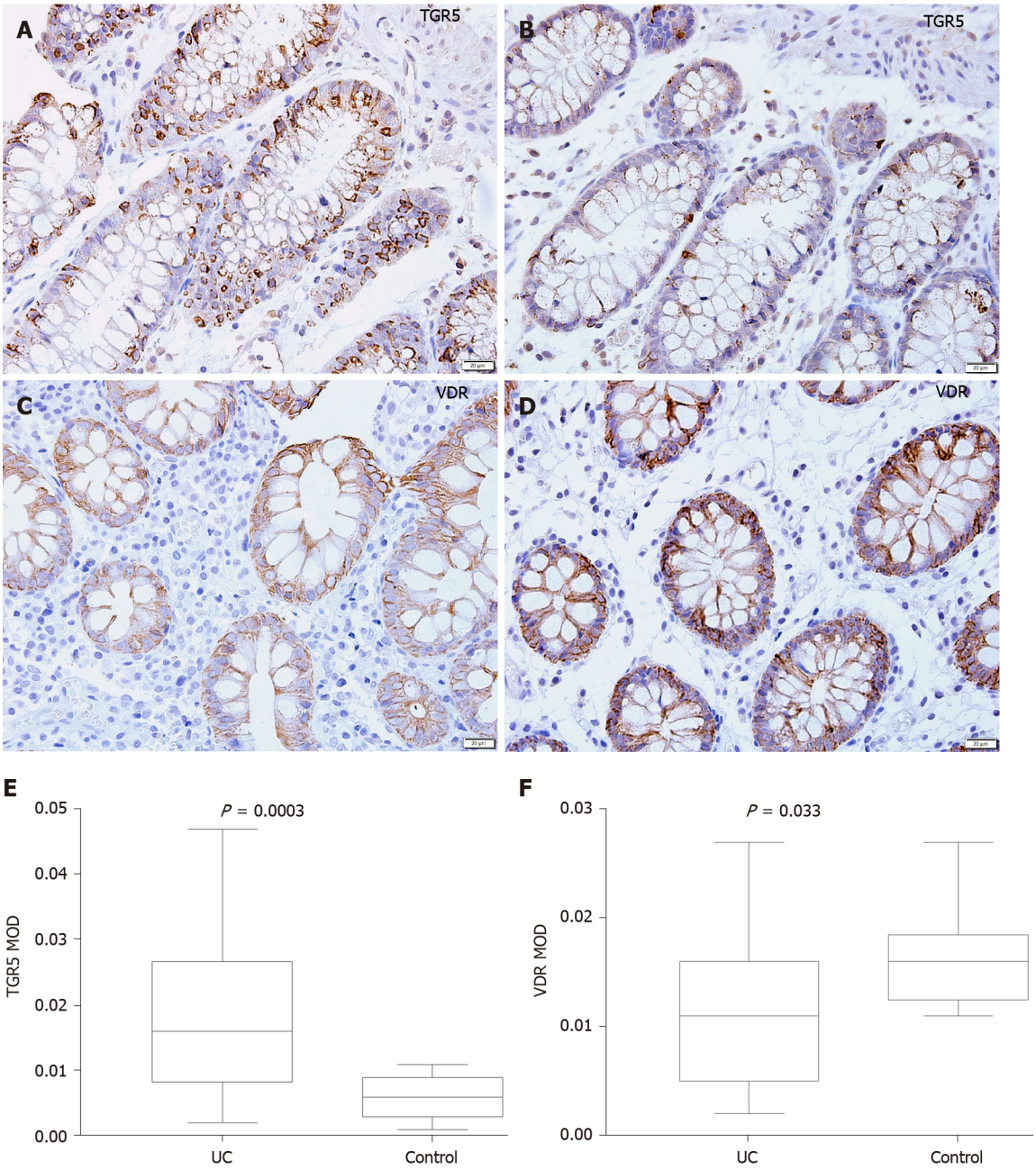


Figure 5 Mucosal immunohistochemistry in patients with ulcerative colitis and healthy controls. A and B: Takeda G protein-coupled receptor 5 (TGR5) immunoreactivity was mainly scattered in the epithelium in ulcerative colitis (UC) patients (scale bar = 20 μm); C and D: Vitamin D receptor (VDR) immunoreactivity was distributed in the epithelium and lamina propria in UC patients and healthy controls (Scale bar = 20 μm); E: The mean optical density of TGR5 in the colonic mucosa in UC patients was significantly higher than that in healthy controls (P = 0.0003); F: The mean optical density of VDR in the colonic mucosa decreased significantly in patients (P = 0.033). UC: Ulcerative colitis; TGR5: Takeda G protein-coupled receptor 5; VDR: Vitamin D receptor; MOD: Mean optical density.

than 50% of metabolites found in fecal matter and urine are derived from or modified by the intestinal microbiota[42]. It is particularly noteworthy that the intestinal flora has an important influence on the composition of BA metabolites. The BAs in feces are mainly secondary BAs but also contain a small amount of primary BAs, trace conjugated BAs, and ursodeoxycholic acid (UDCA)[43]. The conversion of conjugated BAs to free BAs depends on the bile salt hydrolase that exists in the intestinal flora [44], which has been identified in *Bacteroides fragilis, Clostridium, Lactobacillus,* and *Bifidobacterium*. Thus, we infer that the imbalance of the intestinal flora affects the deconjugation of BAs, leading to an increase in the concentration of conjugated BAs. In agreement with these studies, we found that the concentrations of conjugated BAs such as TCA, TCDCA, and GCDCA in UC patients were significantly higher than those in HCs and negatively related to *Clostridium IV*, *Faecalibacterium*, *Ruminococcus*, and *Clostridium XlVb*.

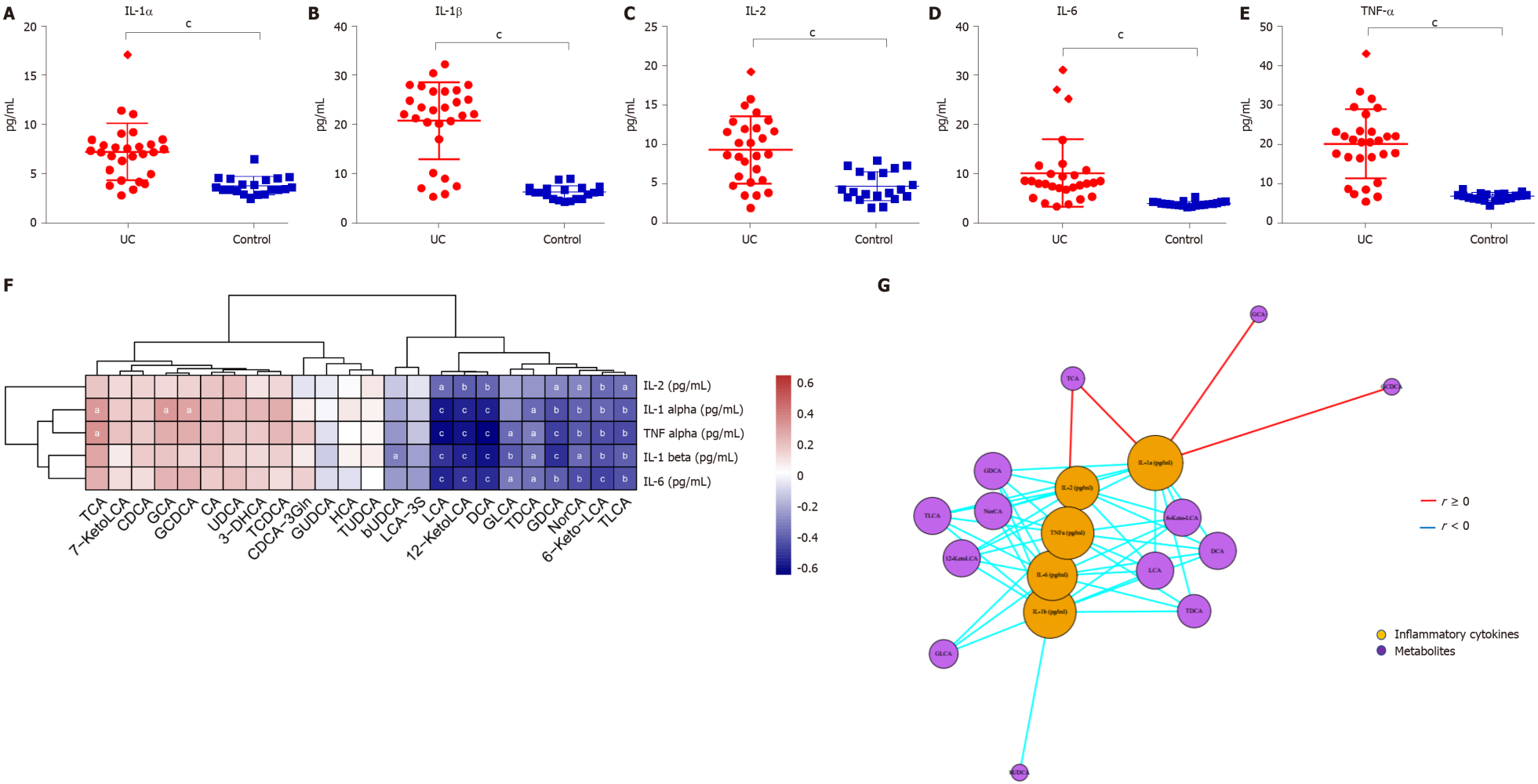


Figure 6 Serum inflammatory cytokine levels and correlations between fecal bile acids and serum inflammatory cytokines in all subjects. A-E: The levels of IL-1α, IL-1β, TNF-α, IL-2, and IL-6 were significantly higher in UC patients ($P < 0.0001$); F: A heatmap of correlative assessments was made of the fecal bile acids and serum inflammatory cytokines ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$); G: A network diagram of correlative assessments was made on the fecal bile acid metabolites and serum inflammatory cytokines (purple nodes represent bile acid metabolites and orange nodes represent inflammatory cytokines; red lines represent positive correlations and blue lines represent negative correlations). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. LCA: Lithocholic acid; DCA: Deoxycholic acid; GDCA: Glyco-deoxycholic acid; GLCA: Glyco-lithocholic acid; TLCA: Tauro-lithocholate; TDCA: Tauro-deoxycholic acid; UDCA: Ursodeoxycholic acid; TUDCA: Tauro-ursodeoxycholic acid; GUDCA: Glyco-ursodeoxycholic acid; TCA: Tauro-cholic acid; CA: Cholic acid; TCDCA: Tauro-chenodeoxycholic acid; GCDCA: Glyco-chenodeoxycholic acid; CDCA: Chenodeoxycholic acid; GCA: Glyco-cholic acid.

A sequence of enzymatic reactions in the liver converts cholesterol to primary BAs in humans. The intestinal microbiota converts primary BAs to secondary BAs by various reactions, including deconjugation, dehydroxylation, esterification, and desulfatation[29,45-47]. Dehydroxylation only occurs after deconjugation and is catalyzed by the Firmicutes phylum, including *Clostridium* and *Eubacterium*. Desulfatation driven by BA sulfatase is catalyzed by *Clostridium*, *Peptococcus*, *Fusobacterium*, and *Pseudomonas*[47,48]. Therefore, dysregulation of gut microbiota impairs the deconjugation, dehydroxylation, and desulfatation of BAs. As a result, patients with UC have increased secondary BAs and decreased primary BAs. In accordance with these studies, we also found that the concentrations of fecal secondary BAs such as LCA, DCA, GDCA, GLCA, and TLCA in UC patients were significantly lower than those in HCs and were positively correlated with *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium*, and *Clostridium XlVb*.

Moreover, we demonstrated that altered constitution of fecal BAs may participate in regulating inflammatory responses *via* BA receptors. TGR5 is a BA reactive receptor expressed in various cell types and is widely distributed throughout the gastrointestinal tract[49]. Different types of BAs have different agonistic effects on TGR5: LCA > DCA > CDCA > UDCA > CA[50]. TGR5 also plays a role in inflammation, energy balance, and insulin signaling[51]. Studies have shown that TGR5 negatively regulates liver inflammation in mice by antagonizing NF-κB signaling. Compared with WT mice, mice with *TGR5* gene deficiency have significantly higher levels of serum inflammation markers after induction with LPS, but treating WT mice with TGR5 agonists can reduce inflammatory responses[52]. Nevertheless, TGR5 is increased in experimental colitis, and the mRNA expression of *TGR5* is upregulated in patients with Crohn's disease[53,54]. The anti-inflammatory properties of TGR5 indicate that TGR5 activation may be beneficial to IBD, which might be a compensatory mechanism to counterbalance the vicious cycle of inflammation in IBD.

In addition, this study found that BA metabolites can regulate the immune response in a VDR-dependent fashion. The nuclear receptor VDR is highly expressed in the small intestine and colon and is an essential regulator of intestinal cell proliferation, barrier function, and immunity[55,56]. Evidence strongly supports a protective effect of VDR in UC, and the underlying mechanism may be that VDR can ameliorate intestinal inflammation by downregulating NF-κB signaling and activating autophagy [57,58]. In experimental models of colitis, *Vdr* whole-body knockout mice are known to develop severe colitis[59]. The secondary BA LCA, as a VDR ligand that is produced by *Clostridium* bacteria in the gut lumen, controls Th1 immune responses and suppresses the production of the Th1 cytokines IFNγ and TNFα by activating VDR [60]. *Vdr* knockout mice have lower *Clostridium* in the gut, illustrating the influence of crosstalk between the microbiome and VDR signaling in immunity[61]. Consistent with previous studies[62], the current study showed that the low expression of VDR in the intestine of patients with UC may be related to the imbalance of the flora and the decrease of secondary BAs such as DCA and LCA.

There were several limitations in this study. First, due to limited time and conditions, the sample size was relatively small in this study, and subgroup analysis of the microbiota composition and BA profiles with different disease activities and stages of UC patients has not been performed yet. Previous studies have shown that there are differences in the intestinal flora of UC patients during active and remission periods [63,64]. Longitudinal analyses revealed reduced temporal microbiota stability in UC, particularly in patients with changes in disease activity[65,66]. As the number of subjects increases, we will collect stool samples from a large population of patients with UC at different time points during periods of active and remission disease and rank the contribution of variables to microbiota composition and BA profiles. Second, our conclusions are based on observational research, and such cross-sectional studies do not provide information about the timing of dysbiosis relative to disease onset and, therefore, should be interpreted with caution particularly with regards to cause–effect relationships[39]. We will later conduct intervention studies and animal experiments to verify their relationship. Third, considering that the short-term modification of a diet can rapidly disturb the gut microbiota[67], all subjects were required to maintain their daily dietary habits before the collection of the stool samples. However, dietary constituents have been shown to affect the inflammatory status, in great part mediated through the modulation of the microbiota[68,69], so it is better to supply a standardized diet for subjects. The standardized diet minimizes diet-induced deviations in the gut microbiota and BA metabolites, but masks the gut microbiota under usual dietary habits. Therefore, the measures of gut microbiota and BAs before and after a standardized diet combined with a detailed assessment of the usual dietary habits of patients are necessary for a future study. Finally, since the nuclear FXR is mostly

distributed in hepatocytes, the small intestine, and macrophages[50], this study did not detect the expression of FXR in the colonic mucosa.

CONCLUSION

In conclusion, this study provides new evidence that fecal BAs are closely related to the gut microbiota and serum inflammatory cytokines. Dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR. This study provides a preliminary exploration for possible involvement of the gut microbiota and BA metabolites in the inflammatory responses of UC in humans.

ARTICLE HIGHLIGHTS

Research background

The gut microbiota and its metabolites are involved in the pathogenesis of inflammatory bowel disease. Bile acid (BA) metabolites have recently drawn much attention in ulcerative colitis (UC). Animal studies have shown that secondary BAs participate in intestinal inflammatory responses *via* the BA receptors Takeda G-protein-coupled receptor 5 (TGR5) and vitamin D receptor (VDR). However, there are few studies about the quantitative analysis of fecal BAs and intestinal TGR5 and VDR expression in patients with UC. The relationship between BAs and inflammatory cytokines has not been investigated in UC patients. It was hypothesized that BA metabolites may play a role in the pathogenesis of UC.

Research motivation

The main topics of this study included clinical assessments, screening different key gut microbiota and BAs, examining BA receptor expression in UC patients and healthy controls (HCs), performing correlation analyses between these parameters, and clarifying whether there were similar mechanisms to those of animal studies in UC patients. The findings suggested a mechanism by which the gut microbiota and BAs may participate in the pathophysiology of UC and may provide new insights into the management of UC.

Research objectives

The aims of this study were to compare differences in the gut microbiota, fecal BAs, and BA receptor expression in the intestinal mucosa between UC patients and HVs and to analyze the relationship of BAs with the gut microbiota and inflammatory cytokines.

Research methods

The present study used 16S rDNA sequencing technology to detect the differences in the intestinal flora between UC patients and HCs. Fecal BAs were measured by targeted metabolomics approaches. Mucosal TGR5 and VDR expression was analyzed using immunohistochemistry, and serum inflammatory cytokine levels were detected by ELISA.

Research results

It was found that the diversity of gut microbes in UC patients was reduced compared with that in HCs. The concentrations of fecal secondary BAs such as lithochalic acid, deoxycholic acid, glycodeoxycholic acid, glycolithocholic acid, and taurolithocholic acid in UC patients were significantly lower than those in HCs and were positively correlated with *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium*, and *Clostridium XlVb*. The concentrations of primary BAs such as taurocholic acid, cholic acid, taurochenodeoxycholic acid, and glycochenodeoxycholic acid in UC patients were significantly higher than those in HCs and positively correlated with *Enterococcus*, *Klebsiella*, *Streptococcus*, *Lactobacillus* and proinflammatory cytokines. The mucosal expression of TGR5 was significantly elevated in UC patients. VDR expression in colonic mucosal specimens was significantly decreased in UC patients.

Research conclusions

Fecal BAs are closely related to the gut microbiota and serum inflammatory cytokines. Dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR. These findings not only contribute to the understanding of the role of the gut microbiota and metabolites in UC pathogenesis but also offer a valuable reference for future research and more effective therapies.

Research perspectives

This preliminary study investigated the changes in the fecal BA metabolite profile and analyzed the relationship between metabolites, the gut microbiota, and inflammation in patients with UC. In the future, we will focus on the following aspects. First, due to limited time and conditions, the sample size was relatively small, which may impact the reliability of the conclusion. Second, we cannot draw causal inferences in this cross-sectional study. Therefore, conclusions need to be further verified by well-designed large-sample clinical studies and basic studies. Third, diet was not standardized during the study period. It is necessary to standardize diet in future studies to avoid the influence of diet on the intestinal flora and metabolites.

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REFERENCES

1 **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 2017; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]

2 **Kaplan GG**, Ng SC. Understanding and Preventing the Global Increase of Inflammatory Bowel Disease. *Gastroenterology* 2017; **152**: 313-321. e2 [PMID: 27793607 DOI: 10.1053/j.gastro.2016.10.020]

3 **D'Haens GR**, Sartor RB, Silverberg MS, Petersson J, Rutgeerts P. Future directions in inflammatory bowel disease management. *J Crohns Colitis* 2014; **8**: 726-734 [PMID: 24742736 DOI: 10.1016/j.crohns.2014.02.025]

4 **Nikfar S**, Rahimi R, Rezaie A, Abdollahi M. A meta-analysis of the efficacy of sulfasalazine in comparison with 5-aminosalicylates in the induction of improvement and maintenance of remission in patients with ulcerative colitis. *Dig Dis Sci* 2009; **54**: 1157-1170 [PMID: 18770034 DOI: 10.1007/s10620-008-0481-x]

5 **De Cassan C**, Fiorino G, Danese S. Second-generation corticosteroids for the treatment of Crohn's disease and ulcerative colitis: more effective and less side effects? *Dig Dis* 2012; **30**: 368-375 [PMID: 22796798 DOI: 10.1159/000338128]

6 **Cohen BL**, Sachar DB. Update on anti-tumor necrosis factor agents and other new drugs for inflammatory bowel disease. *BMJ* 2017; **357**: j2505 [PMID: 28630047 DOI: 10.1136/bmj.j2505]

7 **Nitzan O**, Elias M, Peretz A, Saliba W. Role of antibiotics for treatment of inflammatory bowel disease. *World J Gastroenterol* 2016; **22**: 1078-1087 [PMID: 26811648 DOI: 10.3748/wjg.v22.i3.1078]

8 **Derwa Y**, Gracie DJ, Hamlin PJ, Ford AC. Systematic review with meta-analysis: the efficacy of probiotics in inflammatory bowel disease. *Aliment Pharmacol Ther* 2017; **46**: 389-400 [PMID: 28653751 DOI: 10.1111/apt.14203]

9 **Zabana Y**, Rodríguez L, Lobatón T, Gordillo J, Montserrat A, Mena R, Beltrán B, Dotti M, Benitez O, Guardiola J, Domènech E, Garcia-Planella E, Calvet X, Piqueras M, Aceituno M, Fernández-Bañares F, Esteve M. Relevant Infections in Inflammatory Bowel Disease, and Their Relationship With Immunosuppressive Therapy and Their Effects on Disease Mortality. *J Crohns Colitis* 2019; **13**: 828-837 [PMID: 30668662 DOI: 10.1093/ecco-jcc/jjz013]

10 **Baumgart DC**, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* 2007; **369**: 1641-1657 [PMID: 17499606 DOI: 10.1016/S0140-6736(07)60751-X]

11 **Ho GT**, Chiam P, Drummond H, Loane J, Arnott ID, Satsangi J. The efficacy of corticosteroid therapy in inflammatory bowel disease: analysis of a 5-year UK inception cohort. *Aliment Pharmacol Ther* 2006; **24**: 319-330 [PMID: 16842459 DOI: 10.1111/j.1365-2036.2006.02974.x]

12 **Li J**, Wang F, Zhang HJ, Sheng JQ, Yan WF, Ma MX, Fan RY, Gu F, Li CF, Chen DF, Zheng P, Gu YP, Cao Q, Yang H, Qian JM, Hu PJ, Xia B. Corticosteroid therapy in ulcerative colitis: Clinical response and predictors. *World J Gastroenterol* 2015; **21**: 3005-3015 [PMID: 25780299 DOI:

10.3748/wjg.v21.i10.3005]
13 **Clarke WT**, Feuerstein JD. Colorectal cancer surveillance in inflammatory bowel disease: Practice guidelines and recent developments. *World J Gastroenterol* 2019; **25**: 4148-4157 [PMID: 31435169 DOI: 10.3748/wjg.v25.i30.4148]
14 **Magro F**, Rodrigues A, Vieira AI, Portela F, Cremers I, Cotter J, Correia L, Duarte MA, Tavares ML, Lago P, Ministro P, Peixe P, Lopes S, Garcia EB. Review of the disease course among adult ulcerative colitis population-based longitudinal cohorts. *Inflamm Bowel Dis* 2012; **18**: 573-583 [PMID: 21793126 DOI: 10.1002/ibd.21815]
15 **Glassner KL**, Abraham BP, Quigley EMM. The microbiome and inflammatory bowel disease. *J Allergy Clin Immunol* 2020; **145**: 16-27 [PMID: 31910984 DOI: 10.1016/j.jaci.2019.11.003]
16 **Qin J**, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Jian M, Zhou Y, Li Y, Zhang X, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J; MetaHIT Consortium, Bork P, Ehrlich SD. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010; **464**: 59-65 [PMID: 20203603 DOI: 10.1038/nature08821]
17 **Ley RE**, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 2006; **124**: 837-848 [PMID: 16497592 DOI: 10.1016/j.cell.2006.02.017]
18 **Wong JM**, de Souza R, Kendall CW, Emam A, Jenkins DJ. Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol* 2006; **40**: 235-243 [PMID: 16633129 DOI: 10.1097/00004836-200603000-00015]
19 **Buffie CG**, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, No D, Liu H, Kinnebrew M, Viale A, Littmann E, van den Brink MR, Jenq RR, Taur Y, Sander C, Cross JR, Toussaint NC, Xavier JB, Pamer EG. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. *Nature* 2015; **517**: 205-208 [PMID: 25337874 DOI: 10.1038/nature13828]
20 **Fulde M**, Hornef MW. Maturation of the enteric mucosal innate immune system during the postnatal period. *Immunol Rev* 2014; **260**: 21-34 [PMID: 24942679 DOI: 10.1111/imr.12190]
21 **Ananthakrishnan AN**. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 205-217 [PMID: 25732745 DOI: 10.1038/nrgastro.2015.34]
22 **Knox NC**, Forbes JD, Peterson CL, Van Domselaar G, Bernstein CN. The Gut Microbiome in Inflammatory Bowel Disease: Lessons Learned From Other Immune-Mediated Inflammatory Diseases. *Am J Gastroenterol* 2019; **114**: 1051-1070 [PMID: 31232832 DOI: 10.14309/ajg.0000000000000305]
23 **Sartor RB**, Wu GD. Roles for Intestinal Bacteria, Viruses, and Fungi in Pathogenesis of Inflammatory Bowel Diseases and Therapeutic Approaches. *Gastroenterology* 2017; **152**: 327-339. e4 [PMID: 27769810 DOI: 10.1053/j.gastro.2016.10.012]
24 **Lloyd-Price J**, Arze C, Ananthakrishnan AN, Schirmer M, Avila-Pacheco J, Poon TW, Andrews E, Ajami NJ, Bonham KS, Brislawn CJ, Casero D, Courtney H, Gonzalez A, Graeber TG, Hall AB, Lake K, Landers CJ, Mallick H, Plichta DR, Prasad M, Rahnavard G, Sauk J, Shungin D, Vázquez-Baeza Y, White RA 3rd; IBDMDB Investigators, Braun J, Denson LA, Jansson JK, Knight R, Kugathasan S, McGovern DPB, Petrosino JF, Stappenbeck TS, Winter HS, Clish CB, Franzosa EA, Vlamakis H, Xavier RJ, Huttenhower C. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 2019; **569**: 655-662 [PMID: 31142855 DOI: 10.1038/s41586-019-1237-9]
25 **Franzosa EA**, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, Vatanen T, Hall AB, Mallick H, McIver LJ, Sauk JS, Wilson RG, Stevens BW, Scott JM, Pierce K, Deik AA, Bullock K, Imhann F, Porter JA, Zhernakova A, Fu J, Weersma RK, Wijmenga C, Clish CB, Vlamakis H, Huttenhower C, Xavier RJ. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat Microbiol* 2019; **4**: 293-305 [PMID: 30531976 DOI: 10.1038/s41564-018-0306-4]
26 **Duboc H**, Rajca S, Rainteau D, Benarous D, Maubert MA, Quervain E, Thomas G, Barbu V, Humbert L, Despras G, Bridonneau C, Dumetz F, Grill JP, Masliah J, Beaugerie L, Cosnes J, Chazouillères O, Poupon R, Wolf C, Mallet JM, Langella P, Trugnan G, Sokol H, Seksik P. Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases. *Gut* 2013; **62**: 531-539 [PMID: 22993202 DOI: 10.1136/gutjnl-2012-302578]
27 **Martinot E**, Sèdes L, Baptissart M, Lobaccaro JM, Caira F, Beaudoin C, Volle DH. Bile acids and their receptors. *Mol Aspects Med* 2017; **56**: 2-9 [PMID: 28153453 DOI: 10.1016/j.mam.2017.01.006]
28 **Lieu T**, Jayaweera G, Bunnett NW. GPBA: a GPCR for bile acids and an emerging therapeutic target for disorders of digestion and sensation. *Br J Pharmacol* 2014; **171**: 1156-1166 [PMID: 24111923 DOI: 10.1111/bph.12426]
29 **Wahlström A**, Sayin SI, Marschall HU, Bäckhed F. Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. *Cell Metab* 2016; **24**: 41-50 [PMID: 27320064 DOI: 10.1016/j.cmet.2016.05.005]
30 **Kolho KL**, Pessia A, Jaakkola T, de Vos WM, Velagapudi V. Faecal and Serum Metabolomics in Paediatric Inflammatory Bowel Disease. *J Crohns Colitis* 2017; **11**: 321-334 [PMID: 27609529 DOI: 10.1093/ecco-jcc/jjw158]
31 **Bjerrum JT**, Wang Y, Hao F, Coskun M, Ludwig C, Günther U, Nielsen OH. Metabonomics of human fecal extracts characterize ulcerative colitis, Crohn's disease and healthy individuals. *Metabolomics* 2015; **11**: 122-133 [PMID: 25598765 DOI: 10.1007/s11306-014-0677-3]
32 **Zhang YL**, Cai LT, Qi JY, Lin YZ, Dai YC, Jiao N, Chen YL, Zheng L, Wang BB, Zhu LX, Tang

ZP, Zhu RX. Gut microbiota contributes to the distinction between two traditional Chinese medicine syndromes of ulcerative colitis. *World J Gastroenterol* 2019; **25**: 3242-3255 [PMID: 31333315 DOI: 10.3748/wjg.v25.i25.3242]

33 **Dignass A**, Eliakim R, Magro F, Maaser C, Chowers Y, Geboes K, Mantzaris G, Reinisch W, Colombel JF, Vermeire S, Travis S, Lindsay JO, Van Assche G. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. *J Crohns Colitis* 2012; **6**: 965-990 [PMID: 23040452 DOI: 10.1016/j.crohns.2012.09.003]

34 **Satsangi J**, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 2006; **55**: 749-753 [PMID: 16698746 DOI: 10.1136/gut.2005.082909]

35 **Xie G**, Wang X, Jiang R, Zhao A, Yan J, Zheng X, Huang F, Liu X, Panee J, Rajani C, Yao C, Yu H, Jia W, Sun B, Liu P. Dysregulated bile acid signaling contributes to the neurological impairment in murine models of acute and chronic liver failure. *EBioMedicine* 2018; **37**: 294-306 [PMID: 30344125 DOI: 10.1016/j.ebiom.2018.10.030]

36 **Xie G**, Zhong W, Li H, Li Q, Qiu Y, Zheng X, Chen H, Zhao X, Zhang S, Zhou Z, Zeisel SH, Jia W. Alteration of bile acid metabolism in the rat induced by chronic ethanol consumption. *FASEB J* 2013; **27**: 3583-3593 [PMID: 23709616 DOI: 10.1096/fj.13-231860]

37 **Cosnes J**, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1785-1794 [PMID: 21530745 DOI: 10.1053/j.gastro.2011.01.055]

38 **Zuo T**, Ng SC. The Gut Microbiota in the Pathogenesis and Therapeutics of Inflammatory Bowel Disease. *Front Microbiol* 2018; **9**: 2247 [PMID: 30319571 DOI: 10.3389/fmicb.2018.02247]

39 **Ni J**, Wu GD, Albenberg L, Tomov VT. Gut microbiota and IBD: causation or correlation? *Nat Rev Gastroenterol Hepatol* 2017; **14**: 573-584 [PMID: 28743984 DOI: 10.1038/nrgastro.2017.88]

40 **Krishnan S**, Ding Y, Saedi N, Choi M, Sridharan GV, Sherr DH, Yarmush ML, Alaniz RC, Jayaraman A, Lee K. Gut Microbiota-Derived Tryptophan Metabolites Modulate Inflammatory Response in Hepatocytes and Macrophages. *Cell Rep* 2018; **23**: 1099-1111 [PMID: 29694888 DOI: 10.1016/j.celrep.2018.03.109]

41 **Levy M**, Thaiss CA, Elinav E. Metabolites: messengers between the microbiota and the immune system. *Genes Dev* 2016; **30**: 1589-1597 [PMID: 27474437 DOI: 10.1101/gad.284091.116]

42 **Zheng X**, Xie G, Zhao A, Zhao L, Yao C, Chiu NH, Zhou Z, Bao Y, Jia W, Nicholson JK. The footprints of gut microbial-mammalian co-metabolism. *J Proteome Res* 2011; **10**: 5512-5522 [PMID: 21970572 DOI: 10.1021/pr2007945]

43 **Bajor A**, Gillberg PG, Abrahamsson H. Bile acids: short and long term effects in the intestine. *Scand J Gastroenterol* 2010; **45**: 645-664 [PMID: 20334475 DOI: 10.3109/00365521003702734]

44 **Long SL**, Gahan CGM, Joyce SA. Interactions between gut bacteria and bile in health and disease. *Mol Aspects Med* 2017; **56**: 54-65 [PMID: 28602676 DOI: 10.1016/j.mam.2017.06.002]

45 **Gérard P**. Metabolism of cholesterol and bile acids by the gut microbiota. *Pathogens* 2013; **3**: 14-24 [PMID: 25437605 DOI: 10.3390/pathogens3010014]

46 **Chen ML**, Takeda K, Sundrud MS. Emerging roles of bile acids in mucosal immunity and inflammation. *Mucosal Immunol* 2019; **12**: 851-861 [PMID: 30952999 DOI: 10.1038/s41385-019-0162-4]

47 **Sittipo P**, Shim JW, Lee YK. Microbial Metabolites Determine Host Health and the Status of Some Diseases. *Int J Mol Sci* 2019; **20** [PMID: 31653062 DOI: 10.3390/ijms20215296]

48 **Lavelle A**, Sokol H. Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat Rev Gastroenterol Hepatol* 2020; **17**: 223-237 [PMID: 32076145 DOI: 10.1038/s41575-019-0258-z]

49 **Kawamata Y**, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M. A G protein-coupled receptor responsive to bile acids. *J Biol Chem* 2003; **278**: 9435-9440 [PMID: 12524422 DOI: 10.1074/jbc.M209706200]

50 **Fiorucci S**, Distrutti E. The Pharmacology of Bile Acids and Their Receptors. *Handb Exp Pharmacol* 2019; **256**: 3-18 [PMID: 31201555 DOI: 10.1007/164_2019_238]

51 **Duboc H**, Taché Y, Hofmann AF. The bile acid TGR5 membrane receptor: from basic research to clinical application. *Dig Liver Dis* 2014; **46**: 302-312 [PMID: 24411485 DOI: 10.1016/j.dld.2013.10.021]

52 **Wang YD**, Chen WD, Yu D, Forman BM, Huang W. The G-protein-coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor κ light-chain enhancer of activated B cells (NF-κB) in mice. *Hepatology* 2011; **54**: 1421-1432 [PMID: 21735468 DOI: 10.1002/hep.24525]

53 **Cipriani S**, Mencarelli A, Chini MG, Distrutti E, Renga B, Bifulco G, Baldelli F, Donini A, Fiorucci S. The bile acid receptor GPBAR-1 (TGR5) modulates integrity of intestinal barrier and immune response to experimental colitis. *PLoS One* 2011; **6**: e25637 [PMID: 22046243 DOI: 10.1371/journal.pone.0025637]

54 **Yoneno K**, Hisamatsu T, Shimamura K, Kamada N, Ichikawa R, Kitazume MT, Mori M, Uo M, Namikawa Y, Matsuoka K, Sato T, Koganei K, Sugita A, Kanai T, Hibi T. TGR5 signalling inhibits the production of pro-inflammatory cytokines by *in vitro* differentiated inflammatory and intestinal macrophages in Crohn's disease. *Immunology* 2013; **139**: 19-29 [PMID: 23566200 DOI: 10.1111/imm.12045]

55 **Wu S**, Liao AP, Xia Y, Li YC, Li JD, Sartor RB, Sun J. Vitamin D receptor negatively regulates

bacterial-stimulated NF-kappaB activity in intestine. *Am J Pathol* 2010; **177**: 686-697 [PMID: 20566739 DOI: 10.2353/ajpath.2010.090998]

56 **Barbáchano A**, Fernández-Barral A, Ferrer-Mayorga G, Costales-Carrera A, Larriba MJ, Muñoz A. The endocrine vitamin D system in the gut. *Mol Cell Endocrinol* 2017; **453**: 79-87 [PMID: 27913273 DOI: 10.1016/j.mce.2016.11.028]

57 **Wu S**, Zhang YG, Lu R, Xia Y, Zhou D, Petrof EO, Claud EC, Chen D, Chang EB, Carmeliet G, Sun J. Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. *Gut* 2015; **64**: 1082-1094 [PMID: 25080448 DOI: 10.1136/gutjnl-2014-307436]

58 **Chen Y**, Zhang J, Ge X, Du J, Deb DK, Li YC. Vitamin D receptor inhibits nuclear factor κB activation by interacting with IκB kinase β protein. *J Biol Chem* 2013; **288**: 19450-19458 [PMID: 23671281 DOI: 10.1074/jbc.M113.467670]

59 **Kong J**, Zhang Z, Musch MW, Ning G, Sun J, Hart J, Bissonnette M, Li YC. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G208-G216 [PMID: 17962355 DOI: 10.1152/ajpgi.00398.2007]

60 **Pols TWH**, Puchner T, Korkmaz HI, Vos M, Soeters MR, de Vries CJM. Lithocholic acid controls adaptive immune responses by inhibition of Th1 activation through the Vitamin D receptor. *PLoS One* 2017; **12**: e0176715 [PMID: 28493883 DOI: 10.1371/journal.pone.0176715]

61 **Jin D**, Wu S, Zhang YG, Lu R, Xia Y, Dong H, Sun J. Lack of Vitamin D Receptor Causes Dysbiosis and Changes the Functions of the Murine Intestinal Microbiome. *Clin Ther* 2015; **37**: 996-1009. e7 [PMID: 26046242 DOI: 10.1016/j.clinthera.2015.04.004]

62 **Liu W**, Chen Y, Golan MA, Annunziata ML, Du J, Dougherty U, Kong J, Musch M, Huang Y, Pekow J, Zheng C, Bissonnette M, Hanauer SB, Li YC. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. *J Clin Invest* 2013; **123**: 3983-3996 [PMID: 23945234 DOI: 10.1172/JCI65842]

63 **Prosberg M**, Bendtsen F, Vind I, Petersen AM, Gluud LL. The association between the gut microbiota and the inflammatory bowel disease activity: a systematic review and meta-analysis. *Scand J Gastroenterol* 2016; **51**: 1407-1415 [PMID: 27687331 DOI: 10.1080/00365521.2016.1216587]

64 **Schirmer M**, Denson L, Vlamakis H, Franzosa EA, Thomas S, Gotman NM, Rufo P, Baker SS, Sauer C, Markowitz J, Pfefferkorn M, Oliva-Hemker M, Rosh J, Otley A, Boyle B, Mack D, Baldassano R, Keljo D, LeLeiko N, Heyman M, Griffiths A, Patel AS, Noe J, Kugathasan S, Walters T, Huttenhower C, Hyams J, Xavier RJ. Compositional and Temporal Changes in the Gut Microbiome of Pediatric Ulcerative Colitis Patients Are Linked to Disease Course. *Cell Host Microbe* 2018; **24**: 600-610. e4 [PMID: 30308161 DOI: 10.1016/j.chom.2018.09.009]

65 **Clooney AG**, Eckenberger J, Laserna-Mendieta E, Sexton KA, Bernstein MT, Vagianos K, Sargent M, Ryan FJ, Moran C, Sheehan D, Sleator RD, Targownik LE, Bernstein CN, Shanahan F, Claesson MJ. Ranking microbiome variance in inflammatory bowel disease: a large longitudinal intercontinental study. *Gut* 2021; **70**: 499-510 [PMID: 32536605 DOI: 10.1136/gutjnl-2020-321106]

66 **Halfvarson J**, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, D'Amato M, Bonfiglio F, McDonald D, Gonzalez A, McClure EE, Dunklebarger MF, Knight R, Jansson JK. Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol* 2017; **2**: 17004 [PMID: 28191884 DOI: 10.1038/nmicrobiol.2017.4]

67 **David LA**, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014; **505**: 559-563 [PMID: 24336217 DOI: 10.1038/nature12820]

68 **Rapozo DC**, Bernardazzi C, de Souza HS. Diet and microbiota in inflammatory bowel disease: The gut in disharmony. *World J Gastroenterol* 2017; **23**: 2124-2140 [PMID: 28405140 DOI: 10.3748/wjg.v23.i12.2124]

69 **Kostovcikova K**, Coufal S, Galanova N, Fajstova A, Hudcovic T, Kostovcik M, Prochazkova P, Jiraskova Zakostelska Z, Cermakova M, Sediva B, Kuzma M, Tlaskalova-Hogenova H, Kverka M. Diet Rich in Animal Protein Promotes Pro-inflammatory Macrophage Response and Exacerbates Colitis in Mice. *Front Immunol* 2019; **10**: 919 [PMID: 31105710 DOI: 10.3389/fimmu.2019.00919]

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ORIGINAL ARTICLE

Retrospective Study

High rate of complete histopathological response in hepatocellular carcinoma patients after combined transarterial chemoembolization and stereotactic body radiation therapy

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Abstract

BACKGROUND

Liver transplantation (LT) presents a curative treatment option in patients with early stage hepatocellular carcinoma (HCC) who are not eligible for resection or ablation therapy. Due to a risk of up 30% for waitlist drop-out upon tumor progression, bridging therapies are used to halt tumor growth. Transarterial chemoembolization (TACE) and less commonly stereotactic body radiation therapy (SBRT) or a combination of TACE and SBRT, are used as bridging therapies in LT. However, it remains unclear if one of those treatment options is superior. The analysis of explant livers after transplantation provides the unique opportunity to investigate treatment response by histopathology.

AIM

To analyze histopathological response to a combination of TACE and SBRT in HCC in comparison to TACE or SBRT alone.

METHODS

In this multicenter retrospective study, 27 patients who received liver transplantation for HCC were analyzed. Patients received either TACE or SBRT alone, or a combination of TACE and SBRT as bridging therapy to liver transplantation. Liver explants of all patients who received at least one TACE and/or SBRT were analyzed for the presence of residual vital tumor tissue by histopathology to assess differences in treatment response to bridging therapies. Statistical analysis was performed using Fisher-Freeman-Halton exact test, Kruskal-Wallis and Mann-Whitney-*U* tests.

RESULTS

Fourteen patients received TACE only, four patients SBRT only, and nine patients a combination therapy of TACE and SBRT. There were no significant differences between groups regarding age, sex, etiology of underlying liver disease or number and size of tumor lesions. Strikingly, analysis of liver explants revealed that almost all patients in the TACE and SBRT combination group (8/9, 89%) showed no residual vital tumor tissue by histopathology, whereas TACE or SBRT alone resulted in significantly lower rates of complete histopathological response (0/14, 0% and 1/4, 25%, respectively, P value < 0.001).

CONCLUSION

Our data suggests that a combination of TACE and SBRT increases the rate of complete histopathological response compared to TACE or SBRT alone in bridging to liver transplantation.

Key Words: Hepatocellular carcinoma; Transarterial chemoembolization; Stereotactic body radiation therapy; Bridging therapy; Liver transplantation

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Core Tip: In patients with early-stage hepatocellular carcinoma (HCC) who are not eligible for resection or ablation, liver transplantation presents a curative treatment option. To halt tumor growth during waiting time, bridging therapies such as transar-

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terial chemoembolization (TACE), ablation, and stereotactic body radiation therapy (SBRT) are used prior to liver transplantation. In a multicenter retrospective trial with 27 HCC patients who received either TACE or SBRT alone, or a combination of TACE and SBRT, explant histopathology was analyzed to assess treatment response. Strikingly, almost all patients in the combination group exhibited no residual vital tumor by histopathology, whereas TACE or SBRT alone resulted in significantly lower rates of complete histopathological response.

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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks among the leading causes of cancer-associated deaths worldwide. In very early [1 tumor < 2 cm, Barcelona clinic liver cancer (BCLC) 0, United Network for Organ Sharing (UNOS) T1] and early (1 tumor 2-5 cm or 2-3 tumors ≤ 3 cm, BCLC A, UNOS T2) stage HCC, surgical resection or local ablation is the treatment of choice. However, accompanying cirrhosis and tumor location often preclude these curative treatment approaches. Additionally, recurrence rates after resection of early HCC (BCLC A) are high with up to 70% after 2 years[1]. In contrast, liver transplantation (LT) is a curative treatment option not only for the tumor but also for the underlying precancerous condition (*i.e.* liver cirrhosis, chronic HBV infection, non-alcoholic fatty liver disease) with excellent 5-year survival (65%-78%) and low recurrence rates (11%-18%) if Milan criteria (MC, 1 tumor ≤ 5 cm or 3 tumors ≤ 3 cm, without vascular invasion) are fulfilled[2,3]. Acceptable outcomes after LT are even achieved in patients outside MC, though 5-year survival rates are noticeably lower (46%-60%), depending on size and number of tumor lesions, alpha-fetoprotein (AFP), and treatment response[2,4,5]. Therefore, MC are widely accepted to identify HCC patients who will benefit from LT.

Currently about 30%-35% of patients on the waiting list for LT in Europe suffer from HCC[3]. In the US, the percentage is lower, but has been steadily rising over the recent years[6]. Due to organ shortage, waiting periods are long with a high risk for tumor progression and therefore drop-out from the waiting list. Without any bridging therapy, tumor progression beyond MC has been reported in up to 30% of cases[7]. To avoid tumor progression, locoregional therapies are used as bridging to LT and several countries have now implemented response to locoregional therapies into their transplant allocation systems[8,9].

The most commonly used locoregional bridging therapies are transarterial chemoembolization (TACE) and thermal ablation, such as radio frequency ablation (RFA) and microwave ablation (MWA). These therapies are also recommended in current guidelines for the treatment of BCLC A or B stage HCC[3,9]. However, in patients with tumors not suitable for these standard treatment modalities, individual treatment approached such as 90Y radioembolization or stereotactic body radiation therapy (SBRT) have been used to control tumor growth[10-12].

RFA or MWA are established in the treatment of early and very early stage HCC (BCLC A and 0, respectively) as a curative approach or as bridging with excellent long term outcomes after LT[13-16]. However, thermal ablation is not technically feasible in all patients. Tumor location can preclude safe and successful treatment, for example in subcapsular HCC close to the diaphragm or in lesions close to the liver hilum[17]. If these lesions are not amenable for resection, TACE and less commonly alternative treatment options such as SBRT or radioembolization are used to achieve tumor control in a pre-transplant setting[12].

TACE, which also presents the standard of care in patients with intermediate stage HCC, is a widely used bridging therapy that can efficiently halt tumor growth in the pre-transplant setting[2,3,9,18-21]. Even in patients initially outside MC, who achieve

down-staging to fulfill MC after TACE, overall survival rates are comparable to patients that were never outside MC [5,22]. On the other hand, insufficient response to TACE is a predictor of post-transplant HCC recurrence[20]. With longer waiting times due to organ shortage, the risk of tumor progression after treatment with TACE remains a substantial concern.

SBRT is a local ablative treatment option for patients not suitable for resection or thermal ablation. In particular, SBRT can also be applied to tumors close to large blood vessels or wherever tumor location precludes RFA or MWA[17]. Even with excellent local control of tumor lesions and a good safety profile, current guidelines do not regard SBRT as primary treatment option due to a lack of large randomized trials[23-26]. SBRT is therefore mainly performed as an individualized treatment approach in selected cases. In a pre-transplant setting, complete histopathological response after SBRT in 3 of 11 tumor lesions (27%) in a small cohort of 10 patients has been reported [27]. In a larger retrospective analysis of 30 patients treated with SBRT prior to transplantation, drop-out rate (16.7%) and 5-year survival (61%) were not different from patients treated with TACE or RFA[10].

A combination of TACE and SBRT is an alternative local treatment option with therapeutic benefits and a good safety profile, though data from large randomized controlled trials is still missing[28,29]. A recent retrospective analysis of SBRT and TACE (n = 49) compared to TACE alone (n = 98) showed significantly better disease control, progression free survival, and 3-year overall survival for the TACE and SBRT combination group[30]. In a larger study of 199 patients with tumors ≤ 5 cm, combination therapy lead to improved local control rates, but did not have any effect on overall survival[31]. To date, TACE and SBRT combination therapy has been mainly used as a palliative treatment approach and only rarely as bridging to LT. Therefore, data on histopathologic response is limited with only one study reporting on two tumors which showed near complete tumor response after treatment[12]. Three ongoing prospective trials are currently recruiting patients to evaluate TACE and SBRT combination therapy in comparison to TACE (NCT01918683; NCT02513199; NCT03895359).

Given the promising results achieved with TACE and SBRT combination therapy, we aimed to analyze treatment response prior to liver transplantation in patients within MC who could not be treated with resection or ablation and were treated with TACE and SBRT.

MATERIALS AND METHODS

Patients

This multi-center retrospective trial was conducted to specify treatment options that may improve prognosis in patients with HCC and possible LT. Three German transplant centers, University Hospital rechts der Isar, Munich, University Hospital of Munich, and Hannover Medical School participated in the study. Protocols for patient analysis were reviewed and approved by the local ethics committee of each participating center. Decisions for tumor treatment were discussed in a multidisciplinary tumor board. Patients received treatment as standard of care and data were collected retrospectively.

For this study, medical records of all patients with liver cirrhosis and HCC within MC who underwent LT between 2007 and 2019 were retrospectively reviewed. From Hannover Medical School, only patients who received TACE and SBRT or SBRT alone prior to LT were screened. Patients who received at least one TACE with or without SBRT (TACE only: 8 patients at University Hospital rechts der Isar, Munich; 6 patients at University Hospital of Munich; TACE + SBRT: 4 patients at University Hospital rechts der Isar, Munich; 5 patients at University Hospital of Munich), or at least one SBRT alone (2 patients at University Hospital of Munich; 2 patients at Hannover Medical School), were included in our study. Patients who received additional tumor therapies as bridging such as resection of individual lesions, RFA, or MWA were not included into our study since these therapies are established as a curative treatment option. Additionally, data showing excellent response by radiology and histopathology to these therapies is already available[12,32].

Observation period started with initial diagnosis through December 2019. To compare the different dose and fractionation regimens used for SBRT, the biological equivalent dose (BED) of the surrounding isodose was calculated according to the formula BED = nd (1+ d/$alphabeta$) (with n: Number of fractions, d: Single dose and $alpha/beta$ set to 10).

Number and size of HCCs were documented by magnetic resonance imaging or computed tomography scan at the time of diagnosis. Number of treatment cycles, time of treatment, and radiation dose were assessed when applicable. Additionally, age, sex, cause of liver cirrhosis (alcohol, chronic viral hepatitis, other) and serum AFP levels were analyzed. After transplantation, the presence of vital tumor tissue in explant livers was analyzed. Specifically, size and number of any remaining tumor nodules were determined macroscopically and by histopathology in order to identify differences in tumor response. The absence of vital tumor tissue was considered as complete response.

Statistical analysis

The study was designed as a retrospective multicenter longitudinal survey. All data were analyzed using Microsoft Excel (version 16) and SPSS (version 25). Statistics were performed using Fisher-Freeman-Halton test. Due to the limited sample size, no multivariate analysis was performed. Kruskal-Wallis tests as well as Mann-Whitney-*U* tests were used for comparisons of variables between groups, when appropriate. All statistical tests were performed two-sided using a significance level of $\alpha = 5\%$.

RESULTS

The study cohort comprised 27 subjects with HCC of whom 14 received TACE only, four SBRT only, and nine a combination of TACE and SBRT. Within the study cohort, 20 (74%) patients were male, 7 (26%) female. Mean patient age was 60 (SD ± 6) years ranging from 48 to 71 years. All patients suffered from cirrhosis, mostly due to alcohol (11/27; 40%) or hepatitis C (10/27; 37%).

Most patients had a single tumor lesion (20/27; 74%). Of the seven patients with two lesions, one patient received a combination of TACE and SBRT and one patient SBRT only, the others were in the TACE only group. At the time of diagnosis, mean tumor size was 29.3 mm (SD ± 9.5 mm). Median AFP was 8.0 ng/mL, with 1st quartile 5.0 ng/mL and 3rd quartile 58.0 ng/mL (range 1.2 to 2515 ng/mL).

Treatment plans were tailored to each individual patient and varied in number of TACE cycles (median = 2, range 1 to 5) and SBRT radiation dose (range 18.9 to 54 Gy, in 3 to 9 fractions, prescribed to the surrounding isodose) (Table 1). The most common schemes were 3 × 12.5 Gy prescribed to the 65% -isodose and 3 × 15 Gy prescribed to the 60%-isodose delivered every other day. There were no statistically significant differences in age, gender, origin of cirrhosis, tumor size or number of tumor lesions between groups (Table 1, Figure 1). LT was performed after a median interval of 114 d (range 1 to 786 d) from SBRT treatment (Supplementary Figure 1).

Analysis of explant livers by histopathology showed different treatment responses. In 9/27 patients (33%), no vital tumor was detected microscopically, which was considered as complete response (Figure 2A-D, Supplementary Figure 2). Strikingly, for the majority of patients in the TACE and SBRT combination therapy group a complete response was observed (8/9, 89%), compared to none in the TACE only group (0/14, 0%) and only one in the SBRT only (1/4, 25%) group ($P < 0.001$) (Table 2, Figure 2E). When tumor size at the time of initial diagnosis was compared to tumor size in liver explants, treatment with TACE alone led to a stabilization or a decrease in tumor size in the majority of patients, but could not stop tumor growth in all cases. In the combination group, the only sample with vital tumor showed disease stabilization (increase in size < 20%) with most lesions being completely necrotic by histopathology as described above. In the SBRT group, one completely necrotic tumor was observed, but no conclusions on treatment response could be made due to the small sample size (Figure 3).

Of note, the only patient with vital tumor in the TACE and SBRT group had by far the highest AFP level (2515 ng/mL, Figure 4) and the shortest time interval between SBRT and LT (29 d). The only patient with a complete response in the SBRT only group had the smallest tumor in this group (12 mm, BCLC 0), the longest time between SBRT and LT (256 d) and was transplanted due to deterioration of liver function. While there was a weak correlation between tumor size and treatment response in the overall patient cohort, the difference was not statistically significant (Supplementary Figure 3).

On follow-up, two patients suffered from extrahepatic recurrence after LT, of whom one was in the TACE only group (with vital tumor tissue by explant histology) and one patient was in the TACE and SBRT group (no vital tumor detected in explanted liver).

Table 1 Patients characteristics of all patients and separated into treatment groups, *n* (%)

Characteristics	Total number of patients (n^1 = 27)	TACE only (*n* = 14)	Combination of TACE and SBRT (*n* = 9)	SBRT only (*n* = 4)	*P* value
Male/female	20 (74)/7 (26)	12 (86)/2 (14)	5 (56)/4 (44)	3 (75)/1 (25)	
age < 60/≥ 60 yr	13 (48)/14 (52)	6 (43)/8 (57)	5 (56)/4 (44)	2 (50)/2 (50)	
mean age yr ± SD	60 ± 6	59.5 ± 8	61 ± 4	60 ± 2	0.963
Genesis of cirrhosis					
1 alcohol	11 (41)	6 (44)	3 (33)	2 (50)	
2 viral[2]	12 (44)	8 (57)	3 (33)	1 (25)	
3 others[3]	4 (15)	0 (0)	3 (33)	1 (25)	
Numbers of TACE treatment cycle[4]					
1	12 (44)	5 (36)	7 (78)	NA	
2	6 (22)	4 (29)	2 (22)		
3 or more	5 (19)	5 (36)	0 (0)		
Mean radiation dose in Gy		NA	40.00 ± 3.75	36.80 ± 17.56	0.586

[1]*n* = 27 is the number of patients included into our study.
[2]Ten patients suffered from hepatitis C virus (HCV), two from hepatitis B virus.
[3]One patient with combination of alcohol and HCV, three patients with autoimmune hepatitis.
[4]No statistical testing due to different therapeutic approaches. Dichotomous variables are presented in number and percentage, continuous variables in mean ± SD. TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy.

Table 2 Tumor response by treatment (including tumor characteristics), *n* (%)

	Total number of patients (n^1 = 27)	TACE only (*n* = 14)	Combination of TACE and SBRT (*n* = 9)	SBRT only (*n* = 4)	*P* value
Complete response	9 (33.3)	0 (0)	8 (88.89)	1 (25)	< 0.001
Number of tumor lesions					0.517
1	20 (74)	9 (64)	8 (89)	3 (75)	
2	7 (26)	5 (36)	1 (11)	1 (25)	
Mean tumor size[2]	29.3 ± 9.46	29.50 ± 7.63	27.67 ± 9.54	26.67 ± 14.50	0.389
BCLC[4]					
0	1 (4)	0 (0)	0 (0)	1 (25)	
A	26 (96)	14 (100)	9 (100)	3 (75)	
Median AFP[3,5]	8.0, 5.0/58.0	8.05, 5.2/84.2	8.0, 5.0/17.7	9.85, 8.0/11.85	

[1]*n* = 27 is the maximum number of patients included into our study.
[2]mean size of largest tumor in mm ± SD at time of diagnosis.
[3]Median AFP in ng/ml with 1st/3rd quartile at time of diagnosis.
[4]No statistical testing due to small sample size.
[5]No statistical testing due to high variation and SD. Patients characteristics of all patients and separated into treatment groups. Dichotomous variables are presented in number and percentage, continuous variables in mean ± SD. AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy; BCLC: Barcelona clinic liver cancer.

DISCUSSION

In this study, patients with HCC who received a combination therapy of TACE and SBRT before LT had a significantly higher rate of complete histopathological response than patients who received TACE or SBRT alone.

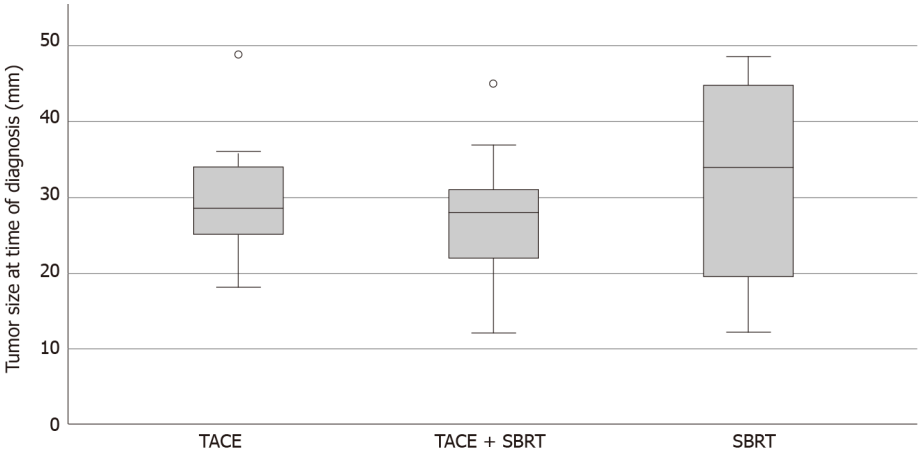


Figure 1 Box plot showing tumor size in each treatment group. Median is represented by bars, 25%-75% percentiles by boxes and outliers by markers. There were no statistically significant differences between groups. TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy.

Current bridging strategies to LT aim to stabilize the disease but are not sufficient to delay tumor growth in all patients[7]. Thermal ablation and TACE are the most commonly used bridging therapies before LT. However, RFA or MWA are not technically feasible in all patients mostly due to tumor location, and a complete pathologic response to TACE alone is found in less than 35% of patients receiving LT for HCC[21,33]. Disease progression poses a risk in these patients – especially in countries with long waiting times such as Germany, with an average of two to three patients per center removed due to tumor progression each year, revoking any curative treatment option. Based on the outcomes of previous studies indicating improved response after TACE and SBRT combination *vs* TACE alone in HCC[34,35], we used TACE and SBRT combination therapy as an individualized treatment approach in patients at risk for tumor progression beyond MC to achieve long term disease stabilization.

While a better outcome of the combination of TACE and SBRT was expected[28,30], the rate of complete tumor response by histopathology was surprisingly high in our patient cohort. In almost all patients who received combined TACE and SBRT, no residual vital tumor was detected in explant livers (TACE and SBRT 89% *vs* TACE alone 0%; $P < 0.001$). The only patient in the TACE and SBRT group with vital tumor tissue by histopathology had a very high AFP (2515 ng/mL) and was transplanted less than one month after SBRT treatment. On the other hand, one patient with complete response in the SBRT-only group had a lesion < 2 cm, an interval of more than 6 mo between SBRT and LT, and was transplanted for deterioration of liver function.

In the small group of patients treated with SBRT alone (four patients in which chemoembolization was not feasible for anatomical reasons or where treatment decision was made at an external hospital), only one of four patients had no vital tumor by histomorphology. Importantly, we had no indications for differences regarding SBRT schemes between groups in our study cohort (Table 1, Supplementary Figure 4 and 5). However, from a sample size this small and above all a very short time interval between SBRT and LT in three out of four patients in the SBRT only group (Supplementary Figure 1) it cannot be ruled out that SBRT alone might be equally efficient to TACE and SBRT combination therapy. However, recently published data from a cohort of 14 patients showing complete response by histopathology in 23.1% of tumor nodules in liver explants is in line with our data[36] – indicating that complete tumor necrosis is not commonly achieved after SBRT alone.

Importantly, none of our patients showed any higher grade treatment-related toxicities, which is in line with previous analyses[37,38]. While treatment side effects were not prospectively evaluated, there were no reports of deterioration of liver function in the TACE and SBRT group, or other higher-grade side effects observed at our centers. However, our study comprises a relatively small group of patients and almost all patients of our cohort had well-preserved liver function. Therefore, outcomes might have been different in patients with impaired liver function[39,40]. Clearly, long-term hepatic toxicity, which is mostly negligible in a pre-transplant setting, might be limiting in palliative treatment strategies[38].

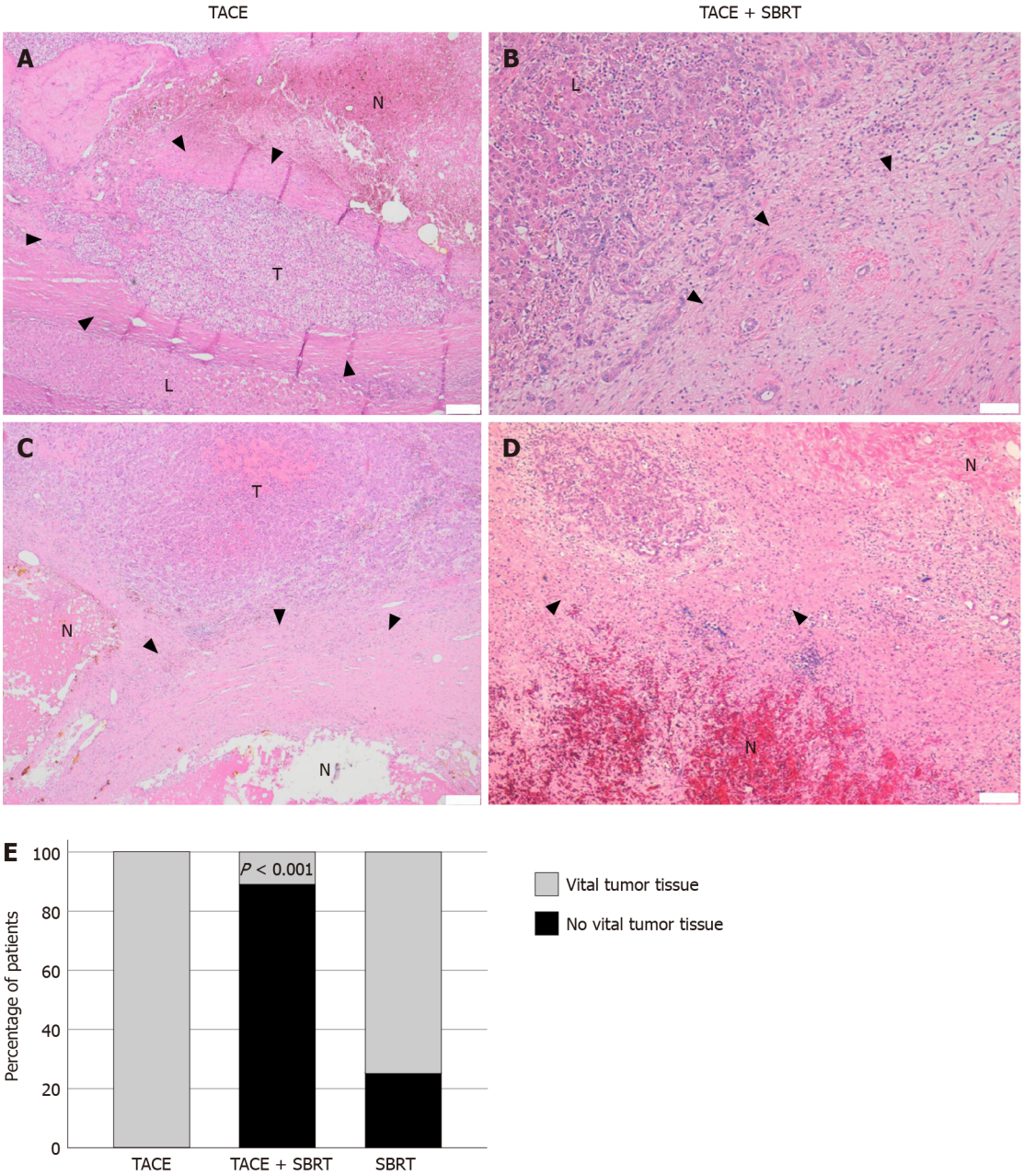


Figure 2 Tumor response by histopathology for each treatment group. A-D: Representative histopathology (Hematoxylin and eosin stain) of tumor lesions in explant livers after transarterial chemoembolization (TACE) (A, C; scale bar 200 μm) or TACE + stereotactic body radiation therapy (SBRT) (B, D; scale bar 100 μm). Samples show necrosis with granulation tissue and organization by connective tissue at the border area (arrowheads) to normal liver. Residual tumor tissue was observed in TACE only samples, while no vital tumor cells could be detected in most patients in the TACE + SBRT group (B, D); E: Bar graph displaying the proportion of vital tumor tissue in each treatment group. Combination therapy with TACE and SBRT leads to a statistically significantly lower number of residual tumor tissue in explant livers ($P < 0.001$). TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy; N: Necrosis; L: Normal liver; T: Tumor tissue.

Together, data from our study strongly indicate that TACE and SBRT combination therapy might lead to higher rates of complete histopathological tumor response than TACE alone. Nevertheless, this study has some limitations. A sample bias due to the multicenter, retrospective design, large duration of study recruitment and little opportunity to adjust for possible confounders due to small sample size cannot be ruled out. Furthermore, most of the patients with two tumor lesions were in the TACE group, which might have biased the results towards a lower percentage of complete response in this cohort. Additionally, the limited number of patients in this study does not allow to draw any conclusions on tumor recurrence or even overall survival. Our cohort accounts for less than 25% of all patients that were transplanted with HCC in Munich transplant centers as most HCC patients received additional bridging therapies such as thermal ablation or even resection whenever feasible. Therefore, whether these histopathological findings will translate into a survival benefit remains to be investigated prospectively in a larger patient cohort. For example, one patient

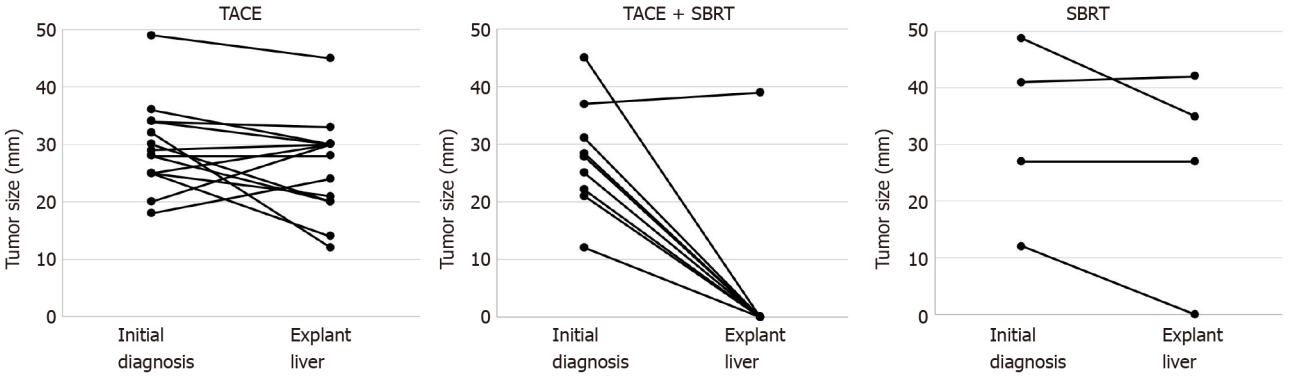


Figure 3 Tumor size at time of diagnosis and in explant histology for each treatment group. Tumor size at initial diagnosis was determined by radiology. When more than one tumor was present, the size of the largest tumor was graphed. In cases where no vital tumor tissue was detected, a size of 0 mm was graphed. TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy.

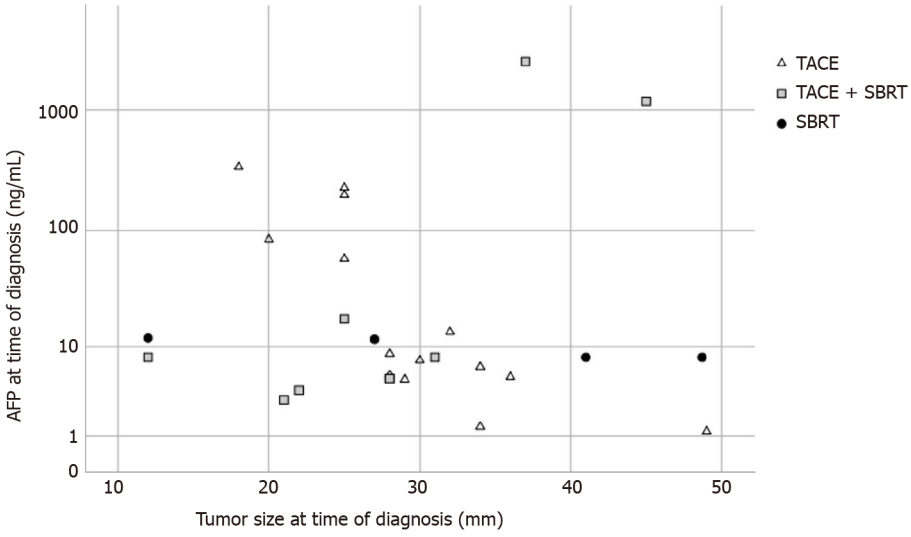


Figure 4 Correlation of alpha-fetoprotein and tumor size for each group. Scatter chart showing the correlation of alpha-fetoprotein in ng / mL and tumor size in mm for each group. AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy.

who received TACE and SBRT combination therapy and showed a complete response in the explant liver developed metachronous metastatic disease less than 6 mo after liver transplantation. In this patient, metastases first occurred in the skull that was not routinely screened by standard tumor staging procedures while the patient was on the waiting list. If bone metastases to the skull were already present before completing SBRT bridging therapy before LT cannot be determined retrospectively but is certainly a possibility. The development of extrahepatic metastases therefore remains an eminent risk even with excellent local tumor control, yet it occurs very rarely at this stage. On the other hand, a patient with high AFP (2515 ng/mL) indicating limited prognosis was successfully transplanted after TACE and SBRT combination therapy. Despite only partial response with 20% of vital tumor tissue by histopathology, he shows no signs of tumor recurrence more than 4 years after LT.

More recently, down-staging to MC has been implemented in organ allocation criteria in several countries. In our cohort, a complete response with decrease of tumor size and loss of arterial hyperperfusion was routinely observed in the combination cohort (Figure 5).

Even though our study did not include any patients beyond MC, TACE and SBRT combination therapy might be efficient for down-staging patients to MC to reach requirements for LT. As a sample bias cannot be excluded due to the limited number of patients, retrospective design, and long recruitment time, further studies in a larger patient cohort are needed to confirm high treatment response to TACE and SBRT combination therapy and to clarify if these findings translate into a decreased number of waitlist removals due to tumor progression or into reduced rates of tumor

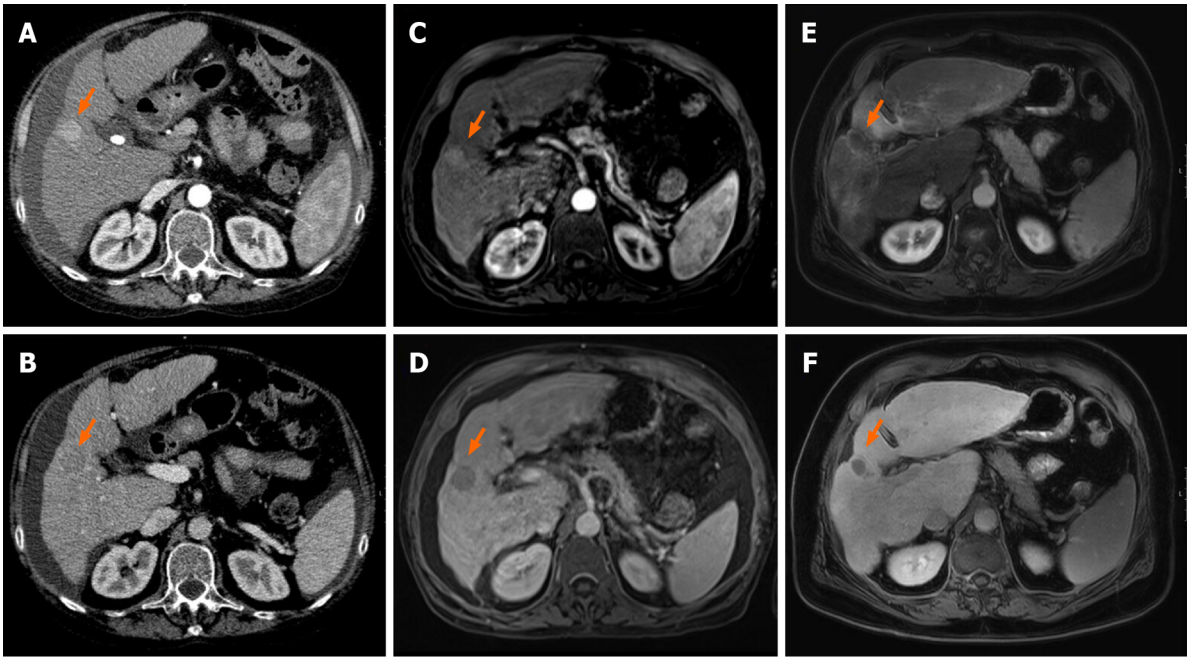


Figure 5 Imaging from before and after combination therapy in one patient in the transarterial chemoembolization + stereotactic body radiation therapy cohort. A, B: Contrast enhanced computed tomography (CT); C-F: Magnetic resonance imaging (MRI). Contrast enhanced CT and MRI, arterial phase (top row) and portal venous phase (bottom row) cross sectional imaging from before (A–D) and after (E, F) treatment. At baseline CT and MRI, a well-defined nodular lesion with typical contrast agent dynamics is noted in the right liver lobe (Arrows). After treatment, typical radiation induced peri-lesional hyperenhancement and no hepatocellular carcinoma-specific contrast agent uptake is noted.

recurrence after liver transplantation.

CONCLUSION

In summary, data from our study shows that patients not eligible for ablation or resection who received TACE and SBRT combination therapy were significantly more likely to have complete histopathological tumor response in explanted livers compared to patients treated with TACE or SBRT only. Whether TACE and SBRT combination therapy results in decreased number of waiting list removals and/or a reduced rate of tumor recurrence after LT needs to be evaluated prospectively in a larger patient cohort. Additionally, future studies will need to show if patients within MC who are not eligible for LT because of old age or relevant co-morbidities could benefit from TACE and SBRT combination therapy if curative resection or ablation is not possible due to tumor location.

ARTICLE HIGHLIGHTS

Research background

In patients with hepatocellular carcinoma (HCC) who are not eligible for resection or ablation therapy, liver transplantation presents a curative treatment option. Due to organ shortage there are long waiting times with the risk of tumor progression. Therefore, efficient bridging therapies are needed.

Research motivation

This study evaluated the treatment response to a combination therapy of transarterial chemoembolization (TACE) and stereotactic body radiation therapy (SBRT) as bridging to liver transplantation.

Research objectives

This study aimed to establish a pathologic response in explant livers after TACE and SBRT.

Research methods

Retrospective multicenter analysis of 27 patients that underwent liver transplantation and received either TACE or SBRT alone or a combination therapy of TACE and SBRT as bridging to liver transplantation.

Research results

About 89% of the patients in the TACE and SBRT combination group had no residual tumor tissue by histopathology, whereas 0% in the TACE only and 25% in the SBRT only group had a complete histopathological response.

Research conclusions

A combination of TACE and SBRT shows superior pathologic response in comparison to TACE or SBRT alone for bridging to liver transplantation in patients with HCC.

Research perspectives

If complete histopathological response in the TACE and SBRT combination group translates into a better progression free and overall survival needs to be evaluated in larger studies.

REFERENCES

1 **Chan AWH**, Zhong J, Berhane S, Toyoda H, Cucchetti A, Shi K, Tada T, Chong CCN, Xiang BD, Li LQ, Lai PBS, Mazzaferro V, García-Fiñana M, Kudo M, Kumada T, Roayaie S, Johnson PJ. Development of pre and post-operative models to predict early recurrence of hepatocellular carcinoma after surgical resection. *J Hepatol* 2018; **69**: 1284-1293 [PMID: 30236834 DOI: 10.1016/j.jhep.2018.08.027]

2 **Mazzaferro V**, Bhoori S, Sposito C, Bongini M, Langer M, Miceli R, Mariani L. Milan criteria in liver transplantation for hepatocellular carcinoma: an evidence-based analysis of 15 years of experience. *Liver Transpl* 2011; **17** Suppl 2: S44-S57 [PMID: 21695773 DOI: 10.1002/lt.22365]

3 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

4 **Yao FY**. Liver transplantation for hepatocellular carcinoma: beyond the Milan criteria. *Am J Transplant* 2008; **8**: 1982-1989 [PMID: 18727702 DOI: 10.1111/j.1600-6143.2008.02351.x]

5 **Clavien PA**, Lesurtel M, Bossuyt PM, Gores GJ, Langer B, Perrier A; OLT for HCC Consensus Group. Recommendations for liver transplantation for hepatocellular carcinoma: an international consensus conference report. *Lancet Oncol* 2012; **13**: e11-e22 [PMID: 22047762 DOI: 10.1016/S1470-2045(11)70175-9]

6 **Kwong A**, Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, Noreen SM, Foutz J, Miller E, Snyder JJ, Israni AK, Kasiske BL. OPTN/SRTR 2018 Annual Data Report: Liver. *Am J Transplant* 2020; **20** Suppl s1: 193-299 [PMID: 31898413 DOI: 10.1111/ajt.15674]

7 **Bhoori S**, Sposito C, Germini A, Coppa J, Mazzaferro V. The challenges of liver transplantation for hepatocellular carcinoma on cirrhosis. *Transpl Int* 2010; **23**: 712-722 [PMID: 20492616 DOI: 10.1111/j.1432-2277.2010.01111.x]

8 **Cillo U**, Burra P, Mazzaferro V, Belli L, Pinna AD, Spada M, Nanni Costa A, Toniutto P; I-BELT (Italian Board of Experts in the Field of Liver Transplantation). A Multistep, Consensus-Based Approach to Organ Allocation in Liver Transplantation: Toward a "Blended Principle Model". *Am J Transplant* 2015; **15**: 2552-2561 [PMID: 26274338 DOI: 10.1111/ajt.13408]

9 **Marrero JA**, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; **68**: 723-750 [PMID: 29624699 DOI: 10.1002/hep.29913]

10 **Sapisochin G**, Barry A, Doherty M, Fischer S, Goldaracena N, Rosales R, Russo M, Beecroft R, Ghanekar A, Bhat M, Brierley J, Greig PD, Knox JJ, Dawson LA, Grant DR. Stereotactic body radiotherapy vs. TACE or RFA as a bridge to transplant in patients with hepatocellular carcinoma. An intention-to-treat analysis. *J Hepatol* 2017; **67**: 92-99 [PMID: 28257902 DOI: 10.1016/j.jhep.2017.02.022]

11 **Bauschke A**, Altendorf-Hofmann A, Ardelt M, Kissler H, Tautenhahn HM, Settmacher U. Impact of successful local ablative bridging therapy prior to liver transplantation on long-term survival in patients with hepatocellular carcinoma in cirrhosis. *J Cancer Res Clin Oncol* 2020; **146**: 1819-1827 [PMID: 32356179 DOI: 10.1007/s00432-020-03215-9]

12 **Rubinstein MM**, Kaubisch A, Kinkhabwala M, Reinus J, Liu Q, Chuy JW. Bridging therapy effectiveness in the treatment of hepatocellular carcinoma prior to orthotopic liver transplantation. *J Gastrointest Oncol* 2017; **8**: 1051-1055 [PMID: 29299366 DOI: 10.21037/jgo.2017.08.11]

13 **Cho YK**, Kim JK, Kim WT, Chung JW. Hepatic resection versus radiofrequency ablation for very early stage hepatocellular carcinoma: a Markov model analysis. *Hepatology* 2010; **51**: 1284-1290

[PMID: 20099299 DOI: 10.1002/hep.23466]
14 **Lu DS**, Yu NC, Raman SS, Limanond P, Lassman C, Murray K, Tong MJ, Amado RG, Busuttil RW. Radiofrequency ablation of hepatocellular carcinoma: treatment success as defined by histologic examination of the explanted liver. *Radiology* 2005; **234**: 954-960 [PMID: 15681691 DOI: 10.1148/radiol.2343040153]
15 **Lee MW**, Raman SS, Asvadi NH, Siripongsakun S, Hicks RM, Chen J, Worakitsitisatorn A, McWilliams J, Tong MJ, Finn RS, Agopian VG, Busuttil RW, Lu DSK. Radiofrequency ablation of hepatocellular carcinoma as bridge therapy to liver transplantation: A 10-year intention-to-treat analysis. *Hepatology* 2017; **65**: 1979-1990 [PMID: 28170115 DOI: 10.1002/hep.29098]
16 **Wang X**, Hu Y, Ren M, Lu X, Lu G, He S. Efficacy and Safety of Radiofrequency Ablation Combined with Transcatheter Arterial Chemoembolization for Hepatocellular Carcinomas Compared with Radiofrequency Ablation Alone: A Time-to-Event Meta-Analysis. *Korean J Radiol* 2016; **17**: 93-102 [PMID: 26798221 DOI: 10.3348/kjr.2016.17.1.93]
17 **Wells SA**, Hinshaw JL, Lubner MG, Ziemlewicz TJ, Brace CL, Lee FT Jr. Liver Ablation: Best Practice. *Radiol Clin North Am* 2015; **53**: 933-971 [PMID: 26321447 DOI: 10.1016/j.rcl.2015.05.012]
18 **De Giorgio M**, Vezzoli S, Cohen E, Armellini E, Lucà MG, Verga G, Pinelli D, Nani R, Valsecchi MG, Antolini L, Colledan M, Fagiuoli S, Strazzabosco M. Prediction of progression-free survival in patients presenting with hepatocellular carcinoma within the Milan criteria. *Liver Transpl* 2010; **16**: 503-512 [PMID: 20373461 DOI: 10.1002/lt.22039]
19 **Kollmann D**, Selzner N, Selzner M. Bridging to liver transplantation in HCC patients. *Langenbecks Arch Surg* 2017; **402**: 863-871 [PMID: 28755240 DOI: 10.1007/s00423-017-1609-2]
20 **Tsochatzis E**, Garcovich M, Marelli L, Papastergiou V, Fatourou E, Rodriguez-Peralvarez ML, Germani G, Davies N, Yu D, Luong TV, Dhillon AP, Thorburn D, Patch D, O'Beirne J, Meyer T, Burroughs AK. Transarterial embolization as neo-adjuvant therapy pretransplantation in patients with hepatocellular carcinoma. *Liver Int* 2013; **33**: 944-949 [PMID: 23530918 DOI: 10.1111/liv.12144]
21 **Graziadei IW**, Sandmueller H, Waldenberger P, Koenigsrainer A, Nachbaur K, Jaschke W, Margreiter R, Vogel W. Chemoembolization followed by liver transplantation for hepatocellular carcinoma impedes tumor progression while on the waiting list and leads to excellent outcome. *Liver Transpl* 2003; **9**: 557-563 [PMID: 12783395 DOI: 10.1053/jlts.2003.50106]
22 **Chapman WC**, Majella Doyle MB, Stuart JE, Vachharajani N, Crippin JS, Anderson CD, Lowell JA, Shenoy S, Darcy MD, Brown DB. Outcomes of neoadjuvant transarterial chemoembolization to downstage hepatocellular carcinoma before liver transplantation. *Ann Surg* 2008; **248**: 617-625 [PMID: 18936575 DOI: 10.1097/SLA.0b013e31818a07d4]
23 **Liu E**, Stenmark MH, Schipper MJ, Balter JM, Kessler ML, Caoili EM, Lee OE, Ben-Josef E, Lawrence TS, Feng M. Stereotactic body radiation therapy for primary and metastatic liver tumors. *Transl Oncol* 2013; **6**: 442-446 [PMID: 23908687 DOI: 10.1593/tlo.12448]
24 **Méndez Romero A**, Wunderink W, Hussain SM, De Pooter JA, Heijmen BJ, Nowak PC, Nuyttens JJ, Brandwijk RP, Verhoef C, Ijzermans JN, Levendag PC. Stereotactic body radiation therapy for primary and metastatic liver tumors: A single institution phase i-ii study. *Acta Oncol* 2006; **45**: 831-837 [PMID: 16982547 DOI: 10.1080/02841860600897934]
25 **Lasley FD**, Mannina EM, Johnson CS, Perkins SM, Althouse S, Maluccio M, Kwo P, Cárdenes H. Treatment variables related to liver toxicity in patients with hepatocellular carcinoma, Child-Pugh class A and B enrolled in a phase 1-2 trial of stereotactic body radiation therapy. *Pract Radiat Oncol* 2015; **5**: e443-e449 [PMID: 25899219 DOI: 10.1016/j.prro.2015.02.007]
26 **Nabavizadeh N**, Mitin T, Dawson LA, Hong TS, Thomas CR Jr. Stereotactic body radiotherapy for patients with hepatocellular carcinoma and intermediate grade cirrhosis. *Lancet Oncol* 2017; **18**: e192 [PMID: 28368254 DOI: 10.1016/S1470-2045(17)30162-6]
27 **O'Connor JK**, Trotter J, Davis GL, Dempster J, Klintmalm GB, Goldstein RM. Long-term outcomes of stereotactic body radiation therapy in the treatment of hepatocellular cancer as a bridge to transplantation. *Liver Transpl* 2012; **18**: 949-954 [PMID: 22467602 DOI: 10.1002/lt.23439]
28 **Huo YR**, Eslick GD. Transcatheter Arterial Chemoembolization Plus Radiotherapy Compared With Chemoembolization Alone for Hepatocellular Carcinoma: A Systematic Review and Meta-analysis. *JAMA Oncol* 2015; **1**: 756-765 [PMID: 26182200 DOI: 10.1001/jamaoncol.2015.2189]
29 **Takeda A**, Sanuki N, Tsurugai Y, Iwabuchi S, Matsunaga K, Ebinuma H, Imajo K, Aoki Y, Saito H, Kunieda E. Phase 2 study of stereotactic body radiotherapy and optional transarterial chemoembolization for solitary hepatocellular carcinoma not amenable to resection and radiofrequency ablation. *Cancer* 2016; **122**: 2041-2049 [PMID: 27062278 DOI: 10.1002/cncr.30008]
30 **Wong TC**, Chiang CL, Lee AS, Lee VH, Yeung CS, Ho CH, Cheung TT, Ng KK, Chok SH, Chan AC, Dai WC, Wong FC, Luk MY, Leung TW, Lo CM. Better survival after stereotactic body radiation therapy following transarterial chemoembolization in nonresectable hepatocellular carcinoma: A propensity score matched analysis. *Surg Oncol* 2019; **28**: 228-235 [PMID: 30851906 DOI: 10.1016/j.suronc.2019.01.006]
31 **Jun BG**, Kim SG, Kim YD, Cheon GJ, Han KH, Yoo JJ, Kim YS, Jeong SW, Jang JY, Lee SH, Park S, Kim HS. Combined therapy of transarterial chemoembolization and stereotactic body radiation therapy *vs* transarterial chemoembolization for ≤5cm hepatocellular carcinoma: Propensity score matching analysis. *PLoS One* 2018; **13**: e0206381 [PMID: 30379885 DOI: 10.1371/journal.pone.0206381]
32 **Bale R**, Schullian P, Eberle G, Putzer D, Zoller H, Schneeberger S, Manzl C, Moser P, Oberhuber G. Stereotactic Radiofrequency Ablation of Hepatocellular Carcinoma: a Histopathological Study in

Explanted Livers. *Hepatology* 2019; **70**: 840-850 [PMID: 30520063 DOI: 10.1002/hep.30406]

33 **Zhang W**, Xu AH, Wang W, Wu YH, Sun QL, Shu C. Radiological appearance of hepatocellular carcinoma predicts the response to trans-arterial chemoembolization in patients undergoing liver transplantation. *BMC Cancer* 2019; **19**: 1041 [PMID: 31690274 DOI: 10.1186/s12885-019-6265-1]

34 **Honda Y**, Kimura T, Aikata H, Kobayashi T, Fukuhara T, Masaki K, Nakahara T, Naeshiro N, Ono A, Miyaki D, Nagaoki Y, Kawaoka T, Takaki S, Hiramatsu A, Ishikawa M, Kakizawa H, Kenjo M, Takahashi S, Awai K, Nagata Y, Chayama K. Stereotactic body radiation therapy combined with transcatheter arterial chemoembolization for small hepatocellular carcinoma. *J Gastroenterol Hepatol* 2013; **28**: 530-536 [PMID: 23216217 DOI: 10.1111/jgh.12087]

35 **Jacob R**, Turley F, Redden DT, Saddekni S, Aal AK, Keene K, Yang E, Zarzour J, Bolus D, Smith JK, Gray S, White J, Eckhoff DE, DuBay DA. Adjuvant stereotactic body radiotherapy following transarterial chemoembolization in patients with non-resectable hepatocellular carcinoma tumours of ≥ 3 cm. *HPB (Oxford)* 2015; **17**: 140-149 [PMID: 25186290 DOI: 10.1111/hpb.12331]

36 **Wang YF**, Dai YH, Lin CS, Chang HC, Shen PC, Yang JF, Hsiang CW, Lo CH, Huang WY. Clinical outcome and pathologic correlation of stereotactic body radiation therapy as a bridge to transplantation for advanced hepatocellular carcinoma: a case series. *Radiat Oncol* 2021; **16**: 15 [PMID: 33446231 DOI: 10.1186/s13014-020-01739-5]

37 **Honda Y**, Kimura T, Aikata H, Nakahara T, Naeshiro N, Tanaka M, Miyaki D, Nagaoki Y, Kawaoka T, Takaki S, Hiramatsu A, Waki K, Ishikawa M, Kakizawa H, Kenjo M, Awai K, Nagata Y, Chayama K. Pilot study of stereotactic body radiation therapy combined with transcatheter arterial chemoembolization for small hepatocellular carcinoma. *Hepatogastroenterology* 2014; **61**: 31-36 [PMID: 24895789]

38 **Jang WI**, Bae SH, Kim MS, Han CJ, Park SC, Kim SB, Cho EH, Choi CW, Kim KS, Hwang S, Kim JH, Chang AR, Park Y, Kim ES, Kim WC, Jo S, Park HJ. A phase 2 multicenter study of stereotactic body radiotherapy for hepatocellular carcinoma: Safety and efficacy. *Cancer* 2020; **126**: 363-372 [PMID: 31747476 DOI: 10.1002/cncr.32502]

39 **Gerum S**, Heinz C, Belka C, Walter F, Paprottka P, De Toni EN, Roeder F. Stereotactic body radiation therapy (SBRT) in patients with hepatocellular carcinoma and oligometastatic liver disease. *Radiat Oncol* 2018; **13**: 100 [PMID: 29843752 DOI: 10.1186/s13014-018-1048-4]

40 **Culleton S**, Jiang H, Haddad CR, Kim J, Brierley J, Brade A, Ringash J, Dawson LA. Outcomes following definitive stereotactic body radiotherapy for patients with Child-Pugh B or C hepatocellular carcinoma. *Radiother Oncol* 2014; **111**: 412-417 [PMID: 24906626 DOI: 10.1016/j.radonc.2014.05.002]

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ORIGINAL ARTICLE

Retrospective Study

Stem cell injection for complex anal fistula in Crohn's disease: A single-center experience

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Author contributions: Schwandner O performed the research and wrote the paper.

Institutional review board statement: According to the Ethics Committee of the Bavarian State Chamber of Physicians retrospective reviews of anonymized patient data do not require ethical approval, No. 2021-1022.

Informed consent statement: Patients were not required to give informed consent to the study because the retrospective analysis used anonymized clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The author had honoraria from Takeda and Medtronic for invited presentations during advisory board meetings and satellite symposia. Neither funding nor financial support was administered for the current study.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article that was

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Abstract

BACKGROUND

Despite tremendous progress in medical therapy and optimization of surgical strategies, considerable failure rates after surgery for complex anal fistula in Crohn's disease have been reported. Therefore, stem cell therapy for the treatment of complex perianal fistula can be an innovative option with potential long-term healing.

AIM

To evaluate the results of local administration of allogenic, adipose-derived mesenchymal stem cells (darvadstrocel) for complex anal Crohn's fistula.

METHODS

All patients with complex anal fistulas associated with Crohn's disease who were amenable for definite fistula closure within a defined observation period were potential candidates for stem cell injection (darvadstrocel) if at least one conventional or surgical attempt to close the fistula had failed. Darvadstrocel was only indicated in patients without active Crohn's disease and without presence of anorectal abscess. Local injection of darvadstrocel was performed as a standardized procedure under general anesthesia including single-shot antibiotic prophylaxis, removal of seton drainage, fistula curettage, closure of the internal openings and local stem cell injection. Data collection focusing on healing rates, occurrence of abscess and follow-up was performed on a regular basis of quality control and patient care. Data were retrospectively analyzed.

RESULTS

Between July 2018 and January 2021, 12 patients (6 females, 6 males) with a mean age of 42.5 (range: 26-61) years underwent stem cell therapy. All patients had a minimum of one complex fistula, including patients with two complex fistulas in 58.3% (7/12). Two of the 12 patients had horse-shoe fistula and 3 had one complex fistula. According to Parks classification, the majority of fistulas were trans-

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sphincteric (76%) or suprasphincteric (14%). All patients underwent removal of seton, fistula curettage, transanal closure of internal opening by suture (11/12) or mucosal flap (1/12) and stem cell injection. At a mean follow-up of 14.3 (range: 3-30) mo, a healing rate was documented in 66.7% (8/12); mean duration to achieve healing was 12 (range: 6-30) wk. Within follow-up, 4 patients required reoperation due to perianal abscess (33.3%). Focusing on patients with a minimum follow-up of 12 mo (6/12) or 24 mo (4/12), long-term healing rates were 66.7% (4/6) and 50.0% (2/4), respectively.

CONCLUSION

Data of this single-center experience are promising but limited due to the small number of patients and the retrospective analysis.

Key Words: Complex anal fistula; Crohn's disease; Stem cell therapy; Mesenchymal stem cells; Darvadstrocel; Treatment; Surgery; Outcomes

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Core Tip: This is a retrospective single-center study on the application of allogenic, adipose-derived mesenchymal stem cells (darvadstrocel) for complex perianal fistula associated with Crohn's disease, providing structured inclusion and exclusion criteria for 12 patients. The current data demonstrate that the management by local stem cell therapy is safe and effective. However, based on the small patient sample size and the retrospective analysis of routine clinical data, a general conclusion remains limited. Therefore, further prospective controlled studies are mandatory to assess the definite role of adipose-derived mesenchymal stem cells for complex anal fistula in Crohn's disease.

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INTRODUCTION

Both medical and surgical options for complex perianal fistulas associated with Crohn's disease remain difficult and challenging[1-5]. Despite recent progress in medical treatment including biologicals and actual trends in sphincter-preserving surgical techniques, considerable recurrence rates after definite surgery for Crohn's anal fistula have been documented[2,3,5]. As an interdisciplinary treatment regimen is the prerequisite for disease control and potential long-term remission of perianal fistulizing Crohn's disease, therapeutic goals include symptom improvement (*e.g.*, reduction of secretion, absence of pain), prevention of recurrent perianal abscess requiring further surgery, preservation of continence, improvement of quality of life and, finally, healing of the fistula.

Focusing on surgical procedures for complex anal fistulas, conventional procedures such as advancement flap repair have shown considerable failure rates for Crohn's fistulas, whereas innovative surgical options such as ligation of the intersphincteric fistula tract (LIFT), biomaterials (*e.g.*, plug) or video-assisted anal fistula treatment seem to have a 50% healing rate after 12 mo without significant impairment of continence[6]. Recently, several reports and randomized studies have demonstrated that stem cell therapy for Crohn's complex anal fistula has raised the healing rates after 12 mo[2,7,8].

Impressed by the encouraging healing rates reported from the ADMIRE trial[7,8], it was the aim of this retrospective single-center study to evaluate the current results of stem cell therapy for complex anal fistulas in Crohn's disease with special focus on indication, patient selection and long-term outcome.

MATERIALS AND METHODS

Clinical setting

Stem cell injection was performed by administration of human, allogenic, expanded adipose-derived mesenchymal stem cells (darvadstrocel) for complex anal fistulas associated with Crohn's disease in adult patients. For the current study, Alofisel® (5 million cells/mL) was used (Takeda GmbH, Konstanz, Germany). Upon authorization of the European Medicines Agency (initial authorization on December 14, 2017), Alofisel® is indicated in patients with non-active or mildly active Crohn's disease when fistulas show an inadequate response (failure either due to persistence or recurrence) to at least one conventional, biological or surgical therapy. Darvadstrocel was only administered if the fistulas had conditioning by curettage or seton drainage. All potential candidates for stem cell injection had fully informed consent of the innovative method and underwent a selection process by multidisciplinary evaluation and discussion (gastroenterology and proctology); finally, the center was trained by an educational and hands-on workshop for the use of darvadstrocel in complex fistulas in Crohn's disease to ensure that only specialist physicians perform the procedure. Moreover, intrainstitutional education was conducted for darvadstrocel administration (*e.g.*, pharmacy, surgical team).

Study population

All patients with complex anorectal fistulas associated with Crohn's disease who were amenable for definite fistula closure were potential candidates for darvadstrocel administration if at least one conventional or surgical attempt to close the fistula had failed. All patients suffered from complex fistula according to American Gastroenterological Association and Parks classification[9,10]. All patients had a minimum of one complex fistula, including patients with two complex fistulas in 58.3% (7/12). According to Parks classification, 76% of the fistulas were transsphincteric and 14% were suprasphincteric. Darvadstrocel was only indicated in patients without active Crohn's disease, ruled out by ileocolonoscopy, and without the presence of anorectal abscess assessed by clinical examination and/or pelvic magnetic resonance imaging (MRI). Additionally, all patients had interdisciplinary discussion prior to surgical treatment. Specific inclusion and exclusion criteria are outlined in Table 1.

Study design and outcome evaluation

To evaluate the outcome of patients who underwent local stem cell injection by the application of darvadstrocel, a retrospective analysis of existing routine clinical data was performed. In detail, regular clinical data of patient care and quality control (including patients' characteristics, type of therapy, outcome evaluation during routine clinical follow-up) were retrospectively analyzed in an anonymized fashion. Patients underwent darvadstrocel administration not for primary study purpose but for quality assurance and patient care. To assess outcome, criteria of healing included that both internal and external openings were closed, no abscess or fluid collection was present and if the patient was free of symptoms (*e.g.*, pain, secretion). Healing was assessed by clinical examination and proctoscopy. Strict criteria and definitions of persistence and recurrence are outlined in Table 2.

Special consideration for coronavirus disease 2019 pandemic

Due to the coronavirus disease pandemic in the beginning of 2020, the coronavirus disease 2019 (COVID-19) outbreak had direct implications for patients with perianal fistulizing Crohn's disease regarding their schedule for definite surgery. As a result of the primary outbreak in March 2020, no patients had elective surgery for Crohn's associated complex anal fistulas at our institution between March 2020 and July 2020 according to governmental restrictions (German Ministry of Health). Moreover, according to a second restriction period for elective procedures at our institution starting in November 2020, potential procedures were postponed to 2021. Finally, a relevant number of patients with fistulizing perianal Crohn's disease under immunosuppression and/or maintenance therapy (*e.g.*, biologicals) postponed their surgery to 2021.

Preoperative assessment

All patients amenable for stem cell injection were seen in the proctological office 2-4 wk prior to darvadstrocel treatment. Informed consent was given, including for efficacy from the ADMIRE study, innovation of the current technique, and monitoring of side effects or adverse events, surgical technique and follow-up observation. Procto-

Table 1 Inclusion and exclusion criteria for administration of darvadstrocel

Inclusion	Exclusion
Complex anal fistulas associated with Crohn's disease (including horse-shoe fistula)	Simple fistulas associated with Crohn's disease, rectovaginal fistula
At least one conventional, biological or surgical therapy prior to stem cell injection	No medical, biological or surgical therapy prior to stem cell injection
No active Crohn's disease confirmed by ileocolonoscopy	Active Crohn's disease confirmed by ileocolonoscopy
No perianal abscess or sepsis confirmed by clinical exam, endoanal ultrasound and/or pelvic MRI	Perianal abscess or significant inflammation in the pelvis or anorectal region confirmed by MRI
Interdisciplinary recommendation	No recommendation by multidisciplinary team
Patient compliant for medical consultation, follow-up examination and interdisciplinary monitoring of disease	Non-compliance of patient
Pretreatment of fistulas by seton drainage ("conditioning")	Fistulas without seton drainage or conditioning by curettage
Maximum of two fistulas with two internal and two external openings (each)	More than two complex fistulas
No anorectal stenosis	Presence of anorectal stenosis
Fully informed consent possible	No informed consent possible

MRI: Magnetic resonance imaging.

Table 2 Definitions of outcome

Parameter	Definition	Supplementary definition
Healing	Complete healing of internal and external opening; no abscess, no symptoms	Assessment by clinical examination including proctoscopy
Persistence	No complete healing with persistence of fistula (external opening) and/or symptoms	Defined postoperative observation period
Recurrence	Recurrence of fistula after period with complete healing and interval without symptoms	With or without occurrence of abscess

logical examination included clinical examination and proctorectoscopy to rule out abscess or proctitis in the immediate preoperative phase. No antibiotic treatment was administered; setons were controlled to be correct in place. No routine curettage of fistulas drained by setons was performed in the preoperative period, and no specific change for medical treatment was performed.

Surgical technique

All surgical procedures were performed under general anesthesia. The patient was placed in lithotomy position. A single-shot antibiotic prophylaxis was routinely administered (cefuroxime/metronidazole). Initially, a careful examination under anesthesia was performed to ensure that there was no presence of abscess or proctitis; moreover, assessment of fistula length, anatomy and topography according to Parks classification[10] was performed. After removal of setons, a vigorous curettage of the fistula tracts was performed by using a metallic curette. Additionally, injection of a sodium chloride solution (0.9%) was administered after curettage. No excision of the fistula tracts were conducted; however, the external openings were sparingly excised. Afterwards, the internal openings were closed by either direct suturing (absorbable suture, Vicryl 3/0; Ethicon EndoSurgery, Norderstedt, Germany) or by mucosal flap (absorbable suture, Vicryl 3/0) if necessary.

After preparing and resuspension of the stem cells and gentle aspiration by using a syringe and injection needles (22-G), darvastrocel was injected according to the manufacturer`s recommendations: Two vials were injected around the internal openings (transanal approach), and the other two vials were injected along the fistula tracts creating small deposits of the cell suspension along the fistulas from external openings (perianal approach). After injection, a soft massage along the fistula region was performed; finally, a sterile bandage was placed around the anal region.

Postoperatively, no specific restrictions related to feeding and mobility were present; after wound control on the first postoperative day, the patient was discharged from hospital. No specific wound management was proposed (only clear water twice a day). Maintenance therapy was given as planned prior to surgery. Neither intravenous nor oral antibiotic therapy was given in the postoperative period.

Follow-up

Regular follow-up examination was performed 2, 4 and 6 wk after surgery in the proctological office, to obtain clinical data related to quality assurance and patient care. Moreover, follow-up examination was advised at 6, 12 and 24 mo after stem cell injection. Additionally, gastroenterological monitoring was advised to provide regular monitoring of Crohn's disease. As clinical follow-up was primarily indicated for routine quality control and was not indicated for study purpose, regular follow-up did not include postoperative MRI as routine examination.

RESULTS

Study population and patient characteristics

Between July 2018 and January 2021, a total of 12 patients (6 females, 6 males) with a mean age of 42.5 (range: 26-61) years underwent stem cell therapy for complex anal fistula associated with Crohn's disease. All patients had medical therapy (infliximab, adalimumab, vedolizumab or azathioprine), and complex fistulas did not respond to either medical or surgical treatment prior to stem cell therapy. Mean duration of Crohn's disease was 19.3 years (range: 7-36 years), and median duration of perianal fistulizing Crohn's disease was 8.7 years (range: 1-16 years). All fistulas had at least one conventional surgical approach to close the fistula (except seton drainage) prior to stem cell therapy; mean number of surgical attempts to close the fistula was 2.0 (range: 1-4). Details on study population and patients' characteristics are detailed in Table 3.

Fistula characterization

All patients underwent fistula conditioning by seton drainage for a mean duration of 31.5 (range: 6-72) wk, and 4 patients had undergone fecal diversion due to severe perianal sepsis. All patients had a minimum of one complex fistula, including patients with two complex fistulas in 58.3% (7/12). Two of 12 patients had horse-shoe fistula, and 3 of 12 had one complex fistula. In total, 21 fistula tracts were documented in 12 patients. According to Parks classification, the majority of fistulas were trans-sphincteric (76%) or suprasphincteric (14%).

Surgery

All patients underwent removal of seton, fistula curettage, transanal closure of internal opening by suture (11/12) or mucosal flap (1/12) and stem cell injection. No intraoperative complications or side effects were noted. Postoperative morbidity included 1 patient with fewer (without signs of perianal sepsis) on the second postoperative day. No serious adverse events were documented.

Outcome

Details on efficacy evaluation and outcome are outlined in Table 4. At a mean follow-up of 14.3 mo (range: 3-30 mo), a healing rate was documented in 66.7% (8/12); mean duration to achieve healing was 12 wk (range: 6-30 wk). Four patients had either fistula persistence (n = 2, 16.7%) or fistula recurrence (n = 2; 16.7%). Within follow-up, 4 patients developed perianal abscess (33.3%) and required reoperation. From 4 patients with ileostomy, one stoma reversal was performed 2 years after fistula healing.

Focusing on patients with a minimum follow-up of 12 mo (6/12) or 24 mo (4/12), long-term healing rates were 66.7% (4/6) and 50.0% (2/4), respectively.

DISCUSSION

Despite significant progress in medical treatment, including biologicals and cell-based therapies, development of sphincter-saving surgical techniques and interdisciplinary co-working, failure rates after definite surgery for perianal fistulizing Crohn's disease are still high[3,11-13]. Moreover, in patients with severe and refractory perianal

Table 3 Patient population

	1	2	3	4	5	6	7	8	9	10	11	12
Age	26 yr	31 yr	52 yr	49 yr	39 yr	35 yr	61 yr	26 yr	54 yr	44 yr	45 yr	48 yr
Sex	Male	Female	Female	Female	Male	Female	Male	Female	Male	Male	Male	Female
CPF Parks classification (number of fistulas)	Transsphincteric (2)	Transsphincteric (2)	Suprasphincteric (1)	Transsphincteric (2)	Transsphincteric (1) Intersphincteric (1)	Transsphincteric (2)	Transsphincteric (2)	Transsphincteric (1)	Suprasphincteric (1) Transsphincteric (1)	Transsphincteric horseshoe-fistula (2)	Transsphincteric horseshoe-fistula (2)	Suprasphincteric (1)
Number of internal/external openings	2/2	2/2	1/1	2/2	2/2	1/2	2/2	1/1	2/2	1/2	1/2	1/1
Length of fistula tract	3 cm each	3 cm each	4 cm	2 cm; 5 cm	10 cm; 1.5 cm	12 cm; 1.5 cm	3 cm; 7 cm	3 cm	6.5 cm; 2.5 cm	2.5 cm; 2.5 cm	3.0 cm; 3.0 cm	3.0 cm
Previous medical treatment	Azathioprin	Azathioprin Infliximab	Azathioprin	Azathioprin	Azathioprin	Azathioprin	Azathioprin Infliximab	Azathioprin	Adalimumab	Azathioprin Adalimumab	Infliximab	Azathiopron Antibiotics
Actual medical treatment	Adalimumab	Ustekinumab	Azathioprin	None	Ustekinumab	Vedolizumab	Ustekinumab	Infliximab	Infliximab	Infliximab	Adalimumab	None
Previous surgical treatment for abscess and fistula	Multiple abscess excisions, previous fistula surgery (1)	Multiple abscess excisions, previous fistula surgery (1)	Multiple abscess excisions,previous fistula surgery (3)	Multiple abscess excisions, previous fistula surgery (2)	Multiple abscess excisions, previous fistula surgery (3)	Multiple abscess excisions, previous fistula surgery (2)	Multiple abscess and fistula surgeries (11)	Multiple abscess and fistula surgery (4)	Multiple abscess and fistula surgery (4)	Multiple abscess and fistula surgery (4)	Multiple abscess and fistula surgery (3)	Multiple abscess and fistula surgery (9)
Previous treatment (stoma)	No	Yes	No	Yes	No	No	No	No	No	Yes	Yes	No
Seton drainage prior to Darvadstrocel (duration)	Yes (6 wk)	Yes (4 mo)	Yes (10 mo)	Yes (18 mo)	Yes (8 wk)	Yes (12 wk)	Yes (9 mo)	Yes (60 wk)	Yes (36 wk)	Yes (48 wk)	Yes (24 wk)	Yes (12 wk)
Method of closure of internal opening	Suture (1), mucosal flap (1)	Suture (2)	Suture (1)	Suture (2)	Suture (2)	Suture (1)	Suture (2)	Suture (1)	Suture (2)	Suture (1)	Suture (1)	Suture (1)
Intraoperative adverse events	No	No	No	No	No	No	No	No	No	No	No	No
Postoperative adverse events	No	No	No	No	No	No	No	No	No	No	Fewer (day 2)	No

CD: Crohn's disease; CPF: Crohn's perianal fistula.

Table 4 Efficacy evaluation and outcomes

	1	2	3	4	5	6	7	8	9	10	11	12
Length of follow-up (mo)	27	30	8	24	26	16	12	6	8	6	6	3
Efficacy evaluation (clinical)	Complete healing	Complete healing	Complete healing	Recurrence	Persistence	Complete healing	Complete healing	Complete healing	Persistence	Complete healing	Recurrence	Complete healing
Efficacy evaluation (MRI)	Not performed	Complete healing	Not performed	Recurrence	Persistence	Not performed	Not performed	Not performed	Not performed	Persistence	Recurrence	Not performed
Time to fistula closure	6 wk	12 wk	9 wk	---	---	12 wk	30 wk	9 wk	--	9 wk	---	10 wk
Incidence of abscess during follow-up	Yes	No	No	No	Yes	No	No	No	Yes	No	Yes	No
Further surgery during follow-up	Yes; Abscess excision (twice)	No	No	Seton drainage	Yes; Abscess excision	No	No	No	Yes; Abscess excision	No	Yes; Abscess excision	No
Maintenance therapy	Adalimumab	Ustekinumab	Azathioprin	Infliximab	Ustekinumab	Vedolizumab	Ustekinumab	Ceased	Infliximab	Infliximab	Infliximab	None
Stoma reversal (if present)	No stoma	Stoma reversal	No stoma	No	No stoma	No stoma	No stoma	No stoma	No stoma	No	No	No stoma

MRI: Magnetic resonance imaging.

disease, the decision to perform fecal diversion or even proctectomy has a tremendous impact on quality of life, particularly in younger patients. Impressed by the preliminary results of limited single-center studies and the encouraging data of the ADMIRE study[7,8], this was a retrospective single-center study analyzing routine clinical data on the application of allogenic, adipose-derived mesenchymal stem cells (darvadstrocel) for complex perianal fistula associated with Crohn's disease, providing structured inclusion and exclusion criteria in 12 patients.

In general, the optimal surgical treatment of complex anal fistulas in patients with Crohn's disease remains challenging. As the majority of patients need surgery for perianal fistulizing Crohn's disease and a high proportion of patients need further surgery due to abscess and recurrent fistula, the integrity of the anal sphincter is essential for preservation of continence[3]. Therefore, the risk of deterioration of continence status increases with the number of surgical procedures and the invasiveness of procedures performed. For complex anal fistulas according to

American Gastroenterological Association and Parks classification[9,10], transrectal flap procedures (advancement or mucosal flap repair) and the LIFT procedure can be considered as effective surgical options in stable disease and absence of proctitis and/or anorectal stenosis with acceptable healing rates[11-18]; however, fecal incontinence and soiling is reported in approximately 10% of patients after endorectal flap procedures[6,11,18]. Focusing on the LIFT procedure, the risk of incontinence is low, but about half of the patients need additional surgery due to recurrence[14-16]. The main "disadvantage" or limitation of both procedures are high complex fistulas with suprasphincteric course or multi-tract fistulas with two internal and external openings ("multiple branching tracts"). In these patients, long-term seton drainage is the favored option; alternatively, proctectomy can be considered. Based on this "surgical dilemma", the novel therapeutic approach of local stem cell therapy seems to be an alternative for highly selected patients with multi-tract, complex fistulas.

In the meantime, a variety of limited studies demonstrated that local injection of mesenchymal stem cells can induce long-term fistula healing without the risk of incontinence and without serious adverse advents related to the mesenchymal stem cells themselves[19-22]. Following the results of the multicenter, phase III randomized control trial (ADMIRE), the application of allogenic, adipose-derived, mesenchymal stem cells combined with transanal closure of the internal opening and fistula curettage, a 50% healing rate of stem cell therapy compared with a 34% success in the placebo group was documented after 24 wk, and healing rates were sustained to 52 wk [7,8].

Impressed by these results and personally searching for the "best option" in patients with multi-branching or two-tract complex fistula, it was the aim of the current single-center experience to evaluate the outcome of local injection of darvadstrocel in a highly selected group of patients. Following the current single-center experience with stem cell therapy, strict inclusion and exclusion criteria were defined. In accordance with the inclusion criteria of the ADMIRE study[7,8], only patients with complex fistulas associated with Crohn's disease–in the majority two complex fistula, suprasphincteric fistula and horse-shoe-fistula, without active luminal Crohn's disease (stable disease under medical treatment) and no evidence of perianal sepsis were included. In contrast, simple fistulas or rectovaginal fistulas were excluded for stem cell therapy. Moreover, in the current study, patients with single-tract intersphincteric or transsphincteric fistulas as well as relatively short single-tract fistulas (fistula length less than 3 cm) were not candidates for stem cell therapy; in these patients, advancement flap repair, mucosal flap repair or LIFT procedure was the preferred option. Within the observation period, a total of 28 patients underwent flap or LIFT procedure for complex fistula associated with Crohn's disease (data not shown).

All patients had drainage of fistulas by seton for a minimum of 6 wk. In contrast to the ADMIRE study[7,8], 4 patients who underwent fecal diversion due to perianal sepsis related to complex fistulas years before admission to our center were included in the current study after interdisciplinary discussion, as the only alternative surgical treatment would have been a proctectomy in these patients, and patients refused this option. Finally, all 12 patients had at least one attempt to surgically close the fistula with no success; thus, all fistulas treated with stem cell therapy were recurrent complex fistula.

Focusing on surgical technique and administration of darvadstrocel, no difficulties or intraoperative morbidity were documented. One patient had fewer on the second postoperative day without any signs of perianal sepsis. No serious adverse events in the immediate postoperative course were noted.

After a mean follow-up of 14.3 mo (range: 3-30 mo), healing rate was 66.7% (8 of 12 patients). Healing was based on strict criteria and was assessed by clinical examination and proctoscopy in the follow-up period. In terms of postoperative MRI (5/12 patients), 1 female patient with clinical evidence of fistula healing also had radiological healing as documented by MRI, whereas 3 patients had radiological recurrence (2) or persistence (1). One patient with clinical healing had radiologic persistence of fistula without signs of contrast enhancement. Focusing on patients with a minimum follow-up of 12 mo (6/12) or 24 mo (4/12), long-term healing rates were 66.7% (4/6) and 50.0% (2/4), respectively.

The occurrence of perianal abscess during follow-up was relatively frequent in the current collective. Four patients (33.3%) developed perianal abscess in the postoperative course, and surgery (abscess excision) was required (1 patient had abscess formation twice). Interestingly, the occurrence of perianal abscess was late (3, 4, 7, 9 and 22 mo after darvadstrocel administration), and abscess localization was near to one former external opening. The incidence of abscess was related to recurrence in 1 patient (supralevatoric abscess with proctosigmoiditis), occurred with exacerbation of

systemic disease (subcutaneous abscess with active inflammation of ileocolic region and proctitis), and was associated with fistula persistence in 2 patients. As a consequence of the relatively high incidence of perianal abscess following stem cell therapy in the current series, a more wide or radical excision of the external opening should be recommended in future procedures to prevent perianal abscess. Moreover, patients with the occurrence of abscess could not be defined "in remission"; however, there was no change in medical treatment. Therefore, the differentiation of abscess as a local problem following fistula surgery or as a problem of systemic disease with direct implications for medical treatment should be more in focus between gastroenterologists and surgeons.

Analyzing the further course of patients with long-term healing in terms of medical therapy, 1 patient had ceased maintenance therapy after recommendation of the gastroenterologist. Focusing on patients who had fecal diversion in the past, one female patient underwent stoma reversal 2 years after stem cell injection. In the other 3 patients, follow-up is still too short, as stoma reversal should be advised not prior to a minimum period of 6 mo after definite fistula surgery (1 patient), and the other two patients had recurrence.

Specifically addressing the observation period related to the COVID-19 pandemic, stem cell therapy was not performed within March 2020 and July 2020 and within a second period starting in November 2020 due to general restrictions concerning elective operations in Germany. Based on governmental restrictions and in-hospital limitations (*e.g.*, reduced capacity in the surgical theater) as well as logistic reasons (*e.g.*, transportation of stem cells under specific conditions) patients with stable disease and without signs of active perianal disease were postponed. This was in accordance with other European and United States experiences related to the management of patients with inflammatory bowel disease[23,24].

The current results clearly demonstrate that an innovative sphincter-sparing surgical approach including fistula curettage, transanal closure of internal openings and local injection of darvadstrocel leads to promising long-term healing rates in patients with no "effective" alternative, such as flap repair or LIFT procedure. However, fundamental limitations of this single-center experience are the small number of patients, the retrospective design, and the absence of a control group. Therefore, the definite role of local application of mesenchymal stem cells has to be discussed within multidisciplinary round tables or consensus conferences to have clear position statements[25-28]. Actually, we are still in the episode of a "learning curve" in terms of patient selection, ideal surgical technique, interdisciplinary treatment discussion, role of maintenance therapy and evaluation of outcomes, among others. Therefore, generally accepted indications and pathways of stem cell therapy for complex fistulas in Crohn's disease cannot be derived; however, the current results should be a plea for further standardization and interdisciplinary consensus.

CONCLUSION

These single-center data demonstrate that local injection of adipose-derived mesenchymal stem cells (darvadstrocel) is safe and effective in patients suffering from perianal fistulizing Crohn's disease. Providing long-term healing in 66.7% of patients, stem cell therapy seems to be an innovative and promising surgical therapy for complex anal fistula associated with Crohn's disease. However, this single-center experience is limited due to the small number of patients and the retrospective assessment of routine clinical data related to quality control. As a technical consequence of a high incidence of perianal abscess during follow-up, a wide excision of the external opening should be recommended for future procedures. Finally, further interdisciplinary efforts including controlled studies are necessary to evaluate the definite role of stem cell therapy for complex anal fistula in Crohn's disease.

ARTICLE HIGHLIGHTS

Research background

Despite significant progress in medical therapy and surgical options, definite surgery for complex anal fistula in Crohn's disease remains challenging.

Research motivation

At present, failure rates after surgery for complex anal fistula associated with Crohn's disease are still high, and surgical options in patients with recurrent and/or multi-tract fistula are limited.

Research objectives

The primary objective was to assess whether local stem cell injection is associated with acceptable healing rates in a routine clinical setting.

Research methods

Providing strict inclusion and exclusion criteria, 12 patients with complex anal fistulas associated with Crohn's disease underwent local application of allogenic, adipose-derived mesenchymal stem cells (darvadstrocel). Darvadstrocel was only indicated in patients without active Crohn's disease confirmed by ileocolonoscopy and without presence of anorectal abscess. Study design was retrospective and routine clinical data were analyzed.

Research results

Twelve patients (6 females, 6 males) with complex anal fistula associated with Crohn's disease underwent fistula curettage, transanal closure of internal openings and local darvadstrocel administration. Fifty-eight percent of patients had two complex fistulas, and seventy-six percent of the fistulas were transsphincteric. After a mean follow-up of 14.3 mo, a healing rate of 66.7% (8/12) was documented. Perianal abscess occurred in 33.3% of patients during follow-up.

Research conclusions

This single-center experience demonstrates that local stem cell injection for complex perianal fistulizing disease is safe and provides acceptable healing rates. However, conclusions are limited due to the small number of patients and the retrospective study design.

Research perspectives

Based on the current results, local stem cell injection could be a new "puzzle piece" for effective treatment of complex anal fistulas associated with Crohn's disease.

REFERENCES

1 **Kelley KA**, Kaur T, Tsikitis VL. Perianal Crohn's disease: challenges and solutions. *Clin Exp Gastroenterol* 2017; **10**: 39-46 [PMID: 28223835 DOI: 10.2147/CEG.S108513]

2 **Panes J**, Reinisch W, Rupniewska E, Khan S, Forns J, Khalid JM, Bojic D, Patel H. Burden and outcomes for complex perianal fistulas in Crohn's disease: Systematic review. *World J Gastroenterol* 2018; **24**: 4821-4834 [PMID: 30479468 DOI: 10.3748/wjg.v24.i42.4821]

3 **Lightner AL**. Perianal Crohn's Disease. *Dis Colon Rectum* 2020; **63**: 1023-1026 [PMID: 32692067 DOI: 10.1097/DCR.0000000000001748]

4 **Scharl M**, Rogler G. Pathophysiology of fistula formation in Crohn's disease. *World J Gastrointest Pathophysiol* 2014; **5**: 205-212 [PMID: 25133023 DOI: 10.4291/wjgp.v5.i3.205]

5 **de Groof EJ**, Sahami S, Lucas C, Ponsioen CY, Bemelman WA, Buskens CJ. Treatment of perianal fistula in Crohn's disease: a systematic review and meta-analysis comparing seton drainage and anti-tumour necrosis factor treatment. *Colorectal Dis* 2016; **18**: 667-675 [PMID: 26921847 DOI: 10.1111/codi.13311]

6 **Schwandner O**. [Quality indicators in the treatment of anal fistulas]. *Chirurg* 2019; **90**: 270-278 [PMID: 30683947 DOI: 10.1007/s00104-019-0794-7]

7 **Panés J**, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi L, Grimaud JC, de la Portilla F, Goldin E, Richard MP, Leselbaum A, Danese S; ADMIRE CD Study Group Collaborators. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet* 2016; **388**: 1281-1290 [PMID: 27477896 DOI: 10.1016/S0140-6736(16)31203-X]

8 **Panés J**, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi L, Grimaud JC, de la Portilla F, Goldin E, Richard MP, Diez MC, Tagarro I, Leselbaum A, Danese S; ADMIRE CD Study Group Collaborators. Long-term Efficacy and Safety of Stem Cell Therapy (Cx601) for Complex Perianal Fistulas in Patients With Crohn's Disease. *Gastroenterology* 2018; **154**: 1334-1342. e4 [PMID: 29277560 DOI: 10.1053/j.gastro.2017.12.020]

9 **Sandborn WJ**, Fazio VW, Feagan BG, Hanauer SB; American Gastroenterological Association Clinical Practice Committee. AGA technical review on perianal Crohn's disease. *Gastroenterology* 2003; **125**: 1508-1530 [PMID: 14598268 DOI: 10.1016/j.gastro.2003.08.025]

10 **Parks AG**, Gordon PH, Hardcastle JD. A classification of fistula-in-ano. *Br J Surg* 1976; **63**: 1-12 [PMID: 1267867 DOI: 10.1002/bjs.1800630102]

11 **Emile SH**, Elfeki H, Shalaby M, Sakr A. A Systematic review and meta-analysis of the efficacy and safety of video-assisted anal fistula treatment (VAAFT). *Surg Endosc* 2018; **32**: 2084-2093 [PMID: 29052068 DOI: 10.1007/s00464-017-5905-2]

12 **Guizzetti L**, Zou G, Khanna R, Dulai PS, Sandborn WJ, Jairath V, Feagan BG. Development of Clinical Prediction Models for Surgery and Complications in Crohn's Disease. *J Crohns Colitis* 2018; **12**: 167-177 [PMID: 29028958 DOI: 10.1093/ecco-jcc/jjx130]

13 **Sugrue J**, Mantilla N, Abcarian A, Kochar K, Marecik S, Chaudhry V, Mellgren A, Nordenstam J. Sphincter-Sparing Anal Fistula Repair: Are We Getting Better? *Dis Colon Rectum* 2017; **60**: 1071-1077 [PMID: 28891851 DOI: 10.1097/DCR.0000000000000885]

14 **Malakorn S**, Sammour T, Khomvilai S, Chowchankit I, Gunarasa S, Kanjanasilp P, Thiptanakij C, Rojanasakul A. Ligation of Intersphincteric Fistula Tract for Fistula in Ano: Lessons Learned From a Decade of Experience. *Dis Colon Rectum* 2017; **60**: 1065-1070 [PMID: 28891850 DOI: 10.1097/DCR.0000000000000880]

15 **Rojanasakul A**. LIFT procedure: a simplified technique for fistula-in-ano. *Tech Coloproctol* 2009; **13**: 237-240 [PMID: 19636496 DOI: 10.1007/s10151-009-0522-2]

16 **Sirany AM**, Nygaard RM, Morken JJ. The ligation of the intersphincteric fistula tract procedure for anal fistula: a mixed bag of results. *Dis Colon Rectum* 2015; **58**: 604-612 [PMID: 25944433 DOI: 10.1097/DCR.0000000000000374]

17 **Schwandner O**. Video-assisted anal fistula treatment (VAAFT) combined with advancement flap repair in Crohn's disease. *Tech Coloproctol* 2013; **17**: 221-225 [PMID: 23179892 DOI: 10.1007/s10151-012-0921-7]

18 **Soltani A**, Kaiser AM. Endorectal advancement flap for cryptoglandular or Crohn's fistula-in-ano. *Dis Colon Rectum* 2010; **53**: 486-495 [PMID: 20305451 DOI: 10.1007/DCR.0b013e3181ce8b01]

19 **Zabot GP**, Cassol O, Saad-Hossne R, Bemelman W. Modern surgical strategies for perianal Crohn's disease. *World J Gastroenterol* 2020; **26**: 6572-6581 [PMID: 33268947 DOI: 10.3748/wjg.v26.i42.6572]

20 **Garcia-Olmo D**, Guadalajara H, Rubio-Perez I, Herreros MD, de-la-Quintana P, Garcia-Arranz M. Recurrent anal fistulae: limited surgery supported by stem cells. *World J Gastroenterol* 2015; **21**: 3330-3336 [PMID: 25805941 DOI: 10.3748/wjg.v21.i11.3330]

21 **Dailey FE**, Turse EP, Naseer M, Bragg JD, Tahan V. Review of stem cells as promising therapy for perianal disease in inflammatory bowel disease. *World J Transplant* 2018; **8**: 97-101 [PMID: 30148075 DOI: 10.5500/wjt.v8.i4.97]

22 **Turse EP**, Dailey FE, Naseer M, Partyka EK, Tahan V. One more chance of fistula healing in inflammatory bowel disease: Stem cell therapy. *World J Clin Cases* 2018; **6**: 493-500 [PMID: 30397605 DOI: 10.12998/wjcc.v6.i12.493]

23 **Remzi FH**, Panis Y, Spinelli A, Kotze PG, Mantzaris G, Söderholm JD, d'Hoore A, Bemelman WA, Yamamoto T, Pemberton JH, Tiret E, Øresland T, Fleshner P. International Organization for the Study of IBD Recommendations for Surgery in Patients With IBD During the Coronavirus Disease 2019 Pandemic. *Dis Colon Rectum* 2020; **63**: 870-873 [PMID: 32355056 DOI: 10.1097/DCR.0000000000001718]

24 **Carlson GL**. Prioritizing Access to Surgical Care During the Coronavirus Pandemic. *Dis Colon Rectum* 2020; **63**: 879-880 [PMID: 32251144 DOI: 10.1097/DCR.0000000000001698]

25 **Horaist C**, de Parades V, Abramowitz L, Benfredj P, Bonnaud G, Bouchard D, Fathallah N, Sénéjoux A, Siproudhis L, Staumont G, Viguier M, Marteau P. Elaboration and validation of Crohn's disease anoperineal lesions consensual definitions. *World J Gastroenterol* 2017; **23**: 5371-5378 [PMID: 28839437 DOI: 10.3748/wjg.v23.i29.5371]

26 **Pellino G**, Keller DS, Sampietro GM, Annese V, Carvello M, Celentano V, Coco C, Colombo F, Cracco N, Di Candido F, Franceschi M, Laureti S, Mattioli G, Pio L, Sciaudone G, Sica G, Villanacci V, Zinicola R, Leone S, Danese S, Spinelli A, Delaini G, Selvaggi F; the Italian Society of Colorectal Surgery (SICCR). Inflammatory bowel disease (IBD) position statement of the Italian Society of Colorectal Surgery (SICCR): general principles of IBD management. *Tech Coloproctol* 2020; **24**: 105-126 [PMID: 31983044 DOI: 10.1007/s10151-019-02145-0]

27 **Lightner AL**, Vogel JD, Carmichael JC, Keller DS, Shah SA, Mahadevan U, Kane SV, Paquette IM, Steele SR, Feingold DL. The American Society of Colon and Rectal Surgeons Clinical Practice Guidelines for the Surgical Management of Crohn's Disease. *Dis Colon Rectum* 2020; **63**: 1028-1052 [PMID: 32692069 DOI: 10.1097/DCR.0000000000001716]

28 **Adamina M**, Bonovas S, Raine T, Spinelli A, Warusavitarne J, Armuzzi A, Bachmann O, Bager P, Biancone L, Bokemeyer B, Bossuyt P, Burisch J, Collins P, Doherty G, El-Hussuna A, Ellul P, Fiorino G, Frei-Lanter C, Furfaro F, Gingert C, Gionchetti P, Gisbert JP, Gomollon F, González Lorenzo M, Gordon H, Hlavaty T, Juillerat P, Katsanos K, Kopylov U, Krustins E, Kucharzik T, Lytras T, Maaser C, Magro F, Marshall JK, Myrelid P, Pellino G, Rosa I, Sabino J, Savarino E, Stassen L, Torres J, Uzzan M, Vavricka S, Verstockt B, Zmora O. ECCO Guidelines on Therapeutics in Crohn's Disease: Surgical Treatment. *J Crohns Colitis* 2020; **14**: 155-168 [PMID: 31742338 DOI: 10.1093/ecco-jcc/jjz187]

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ORIGINAL ARTICLE

Observational Study

Laparoscopic lateral lymph node dissection in two fascial spaces for locally advanced lower rectal cancer

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Abstract

BACKGROUND

The procedure for lateral lymph node (LLN) dissection (LLND) is complicated and can result in complications. We developed a technique for laparoscopic LLND based on two fascial spaces to simplify the procedure.

AIM

To clarify the anatomical basis of laparoscopic LLND in two fascial spaces and to evaluate its efficacy and safety in treating locally advanced low rectal cancer (LALRC).

METHODS

Cadaveric dissection was performed on 24 pelvises, and the fascial composition related to LLND was observed and described. Three dimensional-laparoscopic total mesorectal excision with LLND was performed in 20 patients with LALRC, and their clinical data were analyzed.

RESULTS

The cadaver study showed that the fascia propria of the rectum, urogenital fascia, vesicohypogastric fascia and parietal fascia lie side by side in a medial-lateral direction constituting the dissection plane for curative rectal cancer surgery, and the last three fasciae formed two spaces (Latzko's pararectal space and paravesical space) which were the surgical area for LLND. Laparoscopic LLND in two fascial spaces was performed successfully in all 20 patients. The median operating time, blood loss and postoperative hospitalization were 178 (152-243) min, 55 (25-150) mL and 10 (7-20) d, respectively. The median number of harvested LLNs was 8.6

written consent prior to study enrollment.

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Data sharing statement: No additional data are available.

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(6-12), and pathologically positive LLN metastasis was confirmed in 7 (35.0%) cases. Postoperative complications included lower limb pain in 1 case and lymph leakage in 1 case.

CONCLUSION
Our preliminary surgical experience suggests that laparoscopic LLND based on fascial spaces is a feasible, effective and safe procedure for treating LALRC.

Key Words: Locally advanced low rectal cancer; Lateral lymph node dissection; Fascial anatomy; Visceral fascia; Vesicohypogastric fascia; Cardinal ligament

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Core Tip: The procedure for lateral lymph node dissection (LLND) is complicated, with a high incidence of complications. We developed a technique of laparoscopic LLND based on two fascial spaces to simplify the procedure. By cadaveric dissection, we found that urogenital fascia, vesicohypogastric fascia and parietal fascia lie side by side and formed two spaces (Latzko's pararectal space and paravesical space) which were the surgical area for LLND. 3D-Laparoscopic LLND in two fascial spaces was performed successfully in 20 patients with locally advanced low rectal cancer, and the results showed that it was a feasible, effective and safe procedure.

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INTRODUCTION

For rectal cancers below the peritoneal reflection, the incidence of lateral lymph node (LLN) metastasis is reported to be 15%-20%[1-3]. However, routine LLN dissection (LLND) for locally advanced low rectal cancer (LALRC) is still a controversial issue. In addition to the controversy on the indication for LLND, another argument against LLND is that the procedure is complicated, and consequently results in a high incidence of complications[4]. Earlier studies showed that LLND was associated with 30%-70% of urinary disorders and 80%-100% of sexual dysfunction at the initial stage [5]. Despite the introduction of pelvic autonomic nerve preservation, a Japanese clinical trial showed that urinary dysfunction and postoperative complications occurred in 59% and 5% of patients, respectively[6]. In this study, we do not discuss the indications for LLND but focus on how to improve the accuracy and safety of the procedure for laparoscopic LLND based on the fascial space approach.

Total mesorectal excision (TME) is the gold standard surgery for rectal cancer and has stimulated a tremendous upsurge of interest in "fascia anatomy" in colorectal surgery[7-10]. The theory of "fascia anatomy" has changed the traditional view of surgical anatomy focusing on organs and blood vessels, and holds that surgical dissection should be performed along a pre-existing embryological plane (space) formed by fasciae. However, the detailed anatomy of fasciae and spaces related to LLND is not clear, and therefore standard surgical procedures have not been established. In the last few years, there have been numerous anatomical research studies on pelvic fascia[11,12]. Based on the new understanding of fascial composition in the pelvis, we have developed a laparoscopic LLND in two fascial spaces formed by three layers of fasciae to treat LALRC. We describe the anatomical basis, surgical procedure and summarize our initial clinical results.

P-Editor: Ma YJ

MATERIALS AND METHODS

Cadavers

Twenty-three formalin-preserved and 1 fresh cadavers (12 males and 12 females) were dissected at the Anatomy Department of Shanghai Jiaotong University School of Medicine. Eighteen specimens (9 males and 9 females) were separated into two hemi-pelvises through the mid-sagittal plane. Detailed dissections were performed macroscopically with the assistance of binocular loupes (Heine, Germany, HR 2.5 mm × 340 mm). Cadavers with malformations, abdominal or pelvic adhesions, or a history of abdominal or pelvic surgery were excluded.

Patients

From July 2018 to October 2020, a total of 20 patients with LALRC underwent 3D-laparoscopic TME with LLND at Yangpu Hospital affiliated to Tongji University School of Medicine. There were 16 males and 4 females, with a median age of 58.2 years (range: 48-68 years). All patients were pathologically diagnosed by endoscopic biopsy and evaluated by computed tomography (CT) and magnetic resonance imaging (MRI) before surgery. All tumors were located below the peritoneal reflection, and the median distance between the inferior margin of the tumor and anal verge was 4.7 cm (range: 2.5-6.8 cm). The preoperative T stage was T3 in 13 patients and T4 in 7 patients. Preoperative MRI showed uni-LLN enlargement (the largest short diameter > 7 mm) in all cases.

Methods

This was a descriptive study, and was approved by the local Ethics Committee (LL-2020-KXJS-004) and performed in accordance with the Declaration of Helsinki[13].

Cadaver dissection: The details of the dissection procedure were described in our previous studies[11,12]. The main observation points were as follows: (1) The distribution of visceral fascia in the pelvic cavity and its association with the urogenital and vesicohypogastric fasciae; (2) The association of the hypogastric nerve and ureter with the urogenital fascia; (3) The association of the urogenital and vesicohypogastric fasciae with the cardinal ligament; (4) The association of Denonvilliers' fascia with the pelvic plexus; (5) The association of internal iliac blood vessels and their branches with the vesicohypogastric fascia; and (6) The distribution and variation of the branches of the internal iliac artery.

Surgical technique: TME: All operations were performed by the same experienced surgical team using the Karl Storz Image1 S™ 3D system. The patient was placed in a modified lithotomy position. Laparoscopic TME was performed using a five-trocar method through the traditional medial-to-lateral approach, and a detailed description of this surgical procedure was given previously[14].

LLND: (1) Separation of the urogenital fascia to expose the medial boundary of Latzko's pararectal space (Figure 1). The peritoneum was incised lateral to the ureter, and the dissection continued to the vas deferens (male) or the round ligament of the uterus (female). The ureter was pulled medially to expose the urogenital fascia containing the hypogastric nerve. There is an embryonic plane between the urogenital fascia and internal iliac lymph nodes. Dissection was continued along the surface of the urogenital fascia, and was stopped at the level of the pelvic plexus to avoid injuring the neurovascular bundle; (2) Separation of the obturator fascia (parietal fascia) to expose the lateral boundary of the paravesical space (Figure 2). The external iliac lymph nodes were removed along the external iliac artery, and the dissection was continued along the medial surface of the iliopsoas muscle deep into the obturator fascia. An avascular plane exists between the obturator lymph nodes and the obturator fascia. The obturator nerve and vessels were identified at the obturator. A further dissection along the obturator fascia was performed to expose the parietal fascia covering the levator ani muscle (superior fascia of the pelvic diaphragm), and then the dissection plane was connected to the TME surgical plane; (3) Separation of the vesicohypogastric fascia to expose the medial boundary of the paravesical space (Figure 3). The umbilical artery was identified, and the dissection was continued along the lateral border of the umbilical artery to expose the vesicohypogastric fascia. The vesicohypogastric fascia covers the lateral side of the branches of the internal iliac artery. An avascular plane also exists between the obturator lymph nodes and the vesicohypogastric fascia. The superior border of the distal part of the vesicohypogastric fascia fuses with the obturator fascia where the obturator was easily identified, and the obturator nerve and vessels were exposed. The inferior border of the

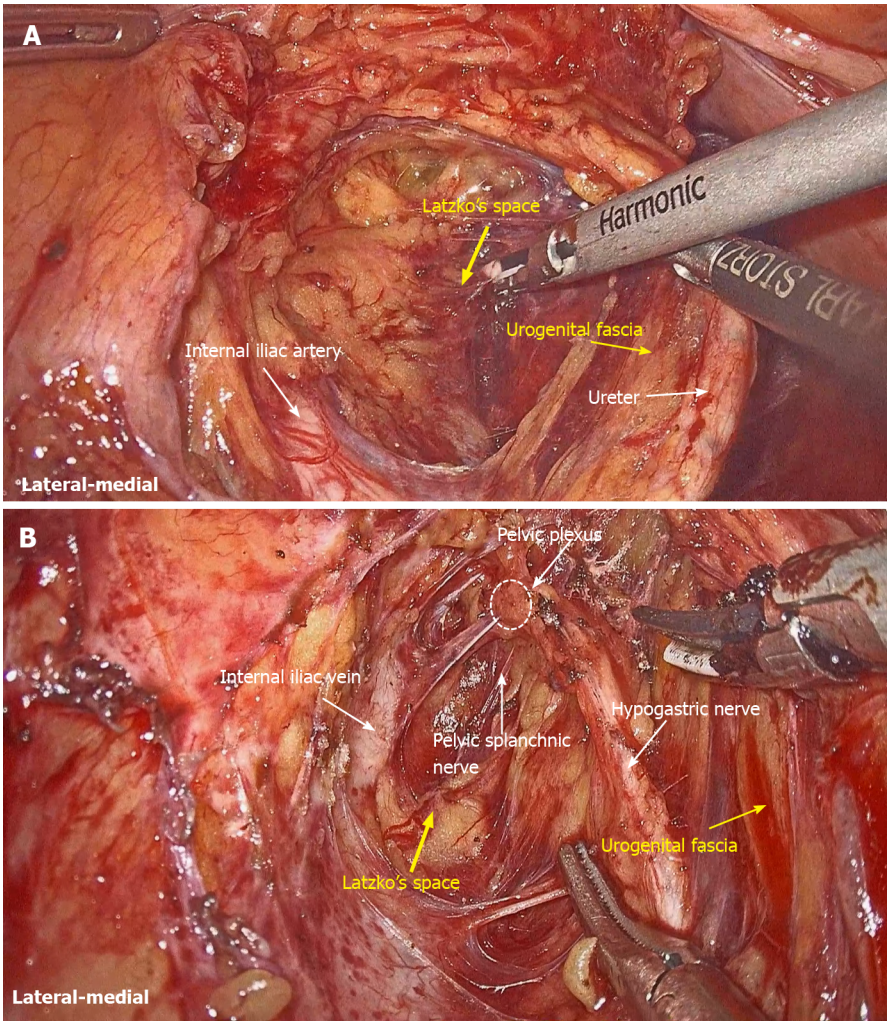


Figure 1 Urogenital fascia is separated to expose the medial boundary of the Latzko's pararectal space, and the dissection continues to the level of the pelvic plexus. A: Medial boundary of the Latzko's pararectal space; B: Dissection continues to the level of the pelvic plexus.

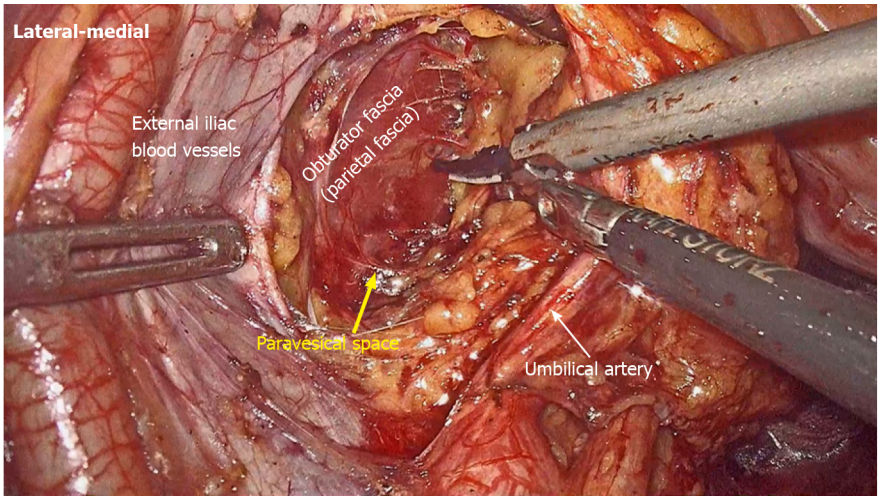


Figure 2 Obturator fascia is separated to expose the lateral boundary of the paravesical space.

vesicohypogastric fascia reaches the superior fascia of the pelvic diaphragm; (4) Dissection of the obturator lymph nodes in the paravesical space between the vesicohypogastric and parietal fasciae (Figure 4). Dissection of the paravesical space included three steps. At the lateral side, the obturator nerve was skeletonized, and the distal obturator vessels were cut and ligated at the obturator canal. At the medial side,

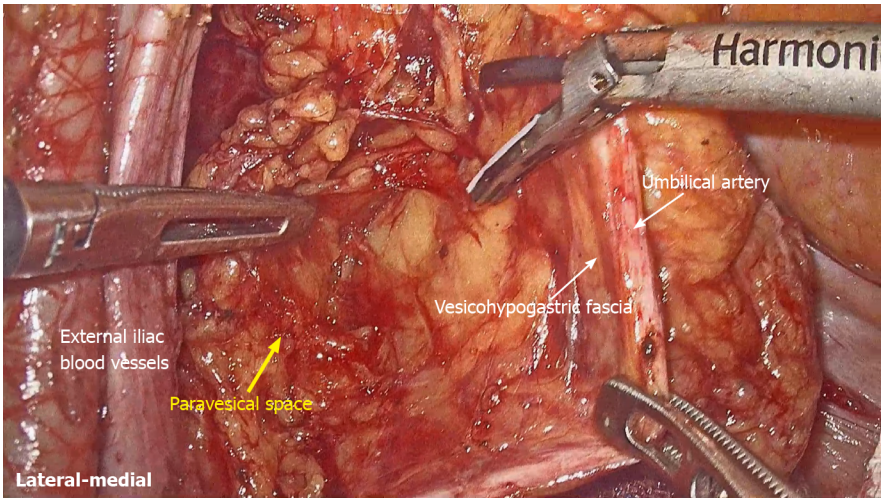


Figure 3 Vesicohypogastric fascia is separated to expose the medial boundary of the paravesical space.

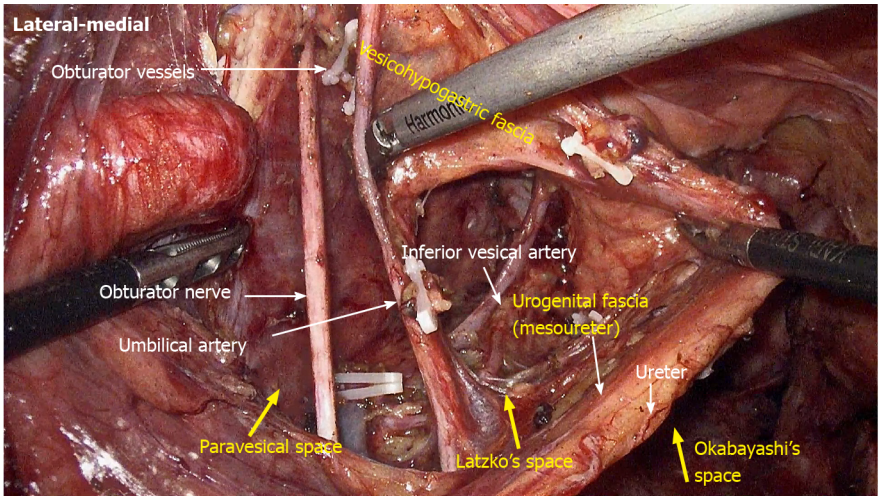


Figure 4 Obturator lymph nodes are dissected in the paravesical space.

the dissection began with removal of the common iliac lymph nodes, followed by dissection along the bifurcation of the internal and external blood vessels and the proximal vesicohypogastric fascia. The proximal obturator vessels were ligated and cut at their origins. At the dorsal side, after exposing the lumbosacral trunk, the dissection started from the bottom of the obturator fossa, and then extended ventrally, to expose Alcock's canal (pudendal canal) and the superior fascia of the pelvic diaphragm. The dissection also allowed the paravesical space to connect to the surgical plane of TME; and (5) Dissection of the internal iliac lymph nodes in Latzko's pararectal space between the urogenital and vesicohypogastric fasciae (Figure 5). The medial side of the vesicohypogastric fascia was dissected to expose the lateral boundary of Latzko's pararectal space. After identifying the trunks of the internal iliac vessels, the dissection was performed along the lateral surface of the vesicohypogastric fascia, in order to sever the branches of the internal iliac vessels at their origins. It should be noted that there was no need to identify each branch. Dissection around the umbilical artery, superior vesical artery, internal pudendal artery, and inferior vesical artery (male) or uterine artery (female) was required for internal iliac lymph nodes cleaning.

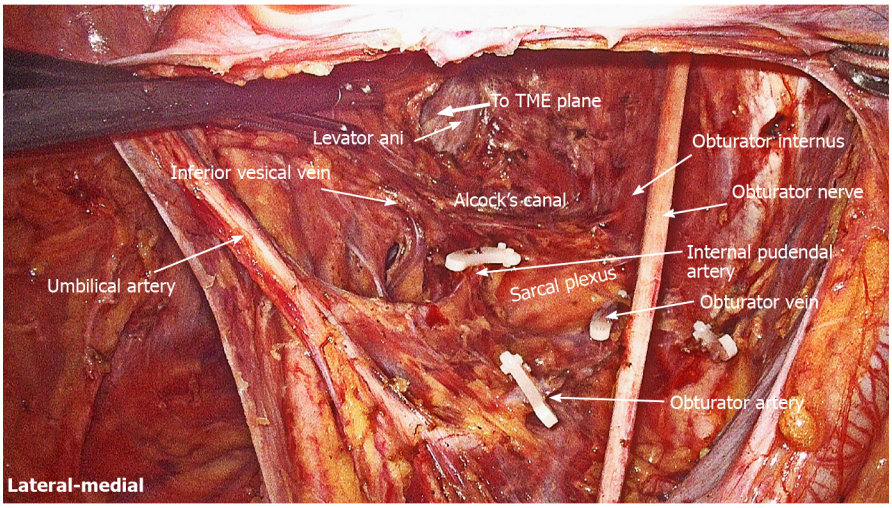


Figure 5 Internal iliac lymph nodes are dissected in the Latzko's pararectal space.

RESULTS

Anatomical observations

Fasciae related to LLND: (1) Fascia propria of the rectum (Figure 6). The fascia propria of the rectum is a thin fascia layer surrounding the mesorectum. It extends downward and shows tight contact with the visceral fascia below the level of Waldeyer's fascia; (2) Urogenital fascia (Figure 7). The urogenital fascia extends posterolateral to the rectum, providing a hammock-like support to the rectum. It can be recognized as the dense connective tissue which contains the hypogastric nerve and ureter. The urogenital fascia connects with Denonvilliers' fascia anterior to the rectum. The urogenital fascia is actually the visceral fascia described by Heald. The mesoureter is defined as a sheet of connective tissue surrounding the ureter. Actually, it is part of the urogenital fascia suspended below the ureter (Figure 4); and (3) Vesicohypogastric fascia (Figure 8). The vesicohypogastric fascia is a triangle-shaped structure, and its boundaries are formed by the umbilical artery, the tendinous arch of the pelvic fascia and the lateral surface of the bladder. The urogenital and vesicohypogastric fasciae blend with each other in the tendinous arch of the pelvic fascia, and present in a V-shape relationship.

Spaces related to LLND: (1) Pararectal space (Figure 9). The pararectal space is a potential space between the rectum and internal iliac vessels, and is divided into Latzko's and Okabayashi's pararectal spaces by the mesoureter. In the view of "fascia anatomy", Latzko's pararectal space is the space between the urogenital fascia and vesicohypogastric fascia, and Okabayashi's pararectal space is the space between the fascia propria of the rectum and the urogenital fascia; and (2) Paravesical space (Figure 9). The paravesical space is a potential space between the umbilical artery and the external iliac vein. From the perspective of "fascia anatomy", the paravesical space is the space between the vesicohypogastric fascia and parietal fascia.

Vascular anatomy related to LLND: As shown in Figure 10, the anatomical relationship between the branches of the internal iliac blood vessel is complicated, but there are still some anatomical characteristics to discriminate them. The first branch of the internal iliac artery is the umbilical artery. The superior vesical artery always arises from a common trunk with the umbilical artery. The vessels crossing above and below the ureter are the uterine artery and deep uterine vein, respectively. The vesical veins drain into the superficial and deep uterine veins or the internal iliac vein and are distributed along the bladder and uterus/vagina in a sagittal plane. The inferior gluteal artery and internal pudendal artery are two major branches arising from the internal iliac artery and enter into the lesser sciatic foramen. However, the inferior gluteal artery passes between the S2 and S3 nerves, and then enters into the lesser sciatic foramen behind the S3 nerves; while the internal pudendal artery goes directly into the lesser sciatic foramen. As the course of the internal pudendal artery in the pelvic cavity is longer, it is regarded as the terminal branch of the internal iliac artery.

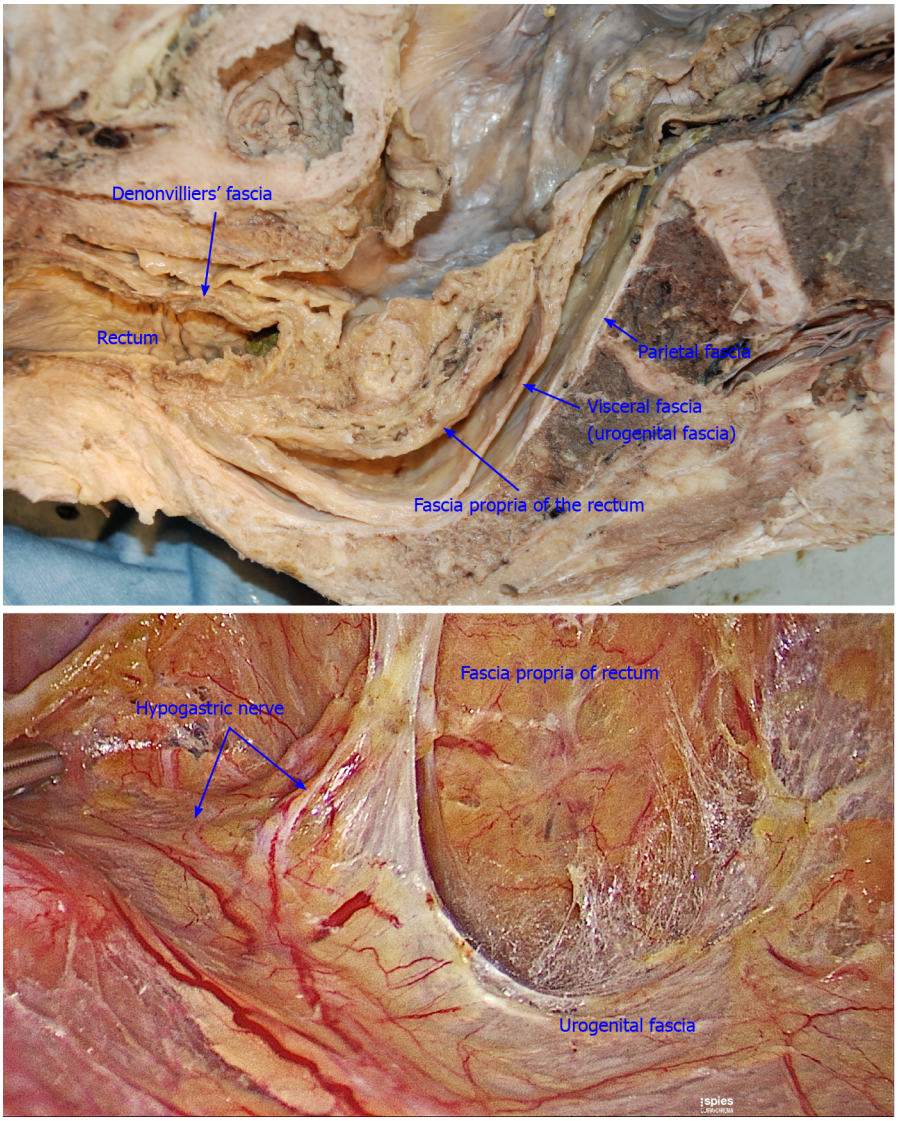


Figure 6 Fascia propria is the innermost fascia around the rectum, and appears as a thin and transparent fascia.

Nerve anatomy related to LLND: As shown in Figure 10, the hypogastric nerve is found within the urogenital fascia, which passes from the posterior to the anterior-lateral aspect of the rectum and fuses with Denonvilliers' fascia in a fan shape. The pelvic plexus is located exactly external to the junction of the urogenital and Denonvilliers' fascia. The pelvic plexus is made up of the hypogastric nerve and the pelvic splanchnic nerve, and give off branches to the bladder and the uterus. All these nerves are located in the same connective tissue plane and presents as a T-shape "pelvic nerve plate".

LLND based on fascial anatomy

3D-Laparoscopic LLND was performed successfully in all 20 patients without conversion to laparotomy. Of these patients, 12 underwent low anterior resection, 7 cases underwent intersphincteric resection, and 1 case underwent abdominoperineal resection. The median operating time for LLND was 178 (range: 152-243) min, with a median blood loss of 55 (range: 25-150) mL. The median length of postoperative hospitalization was 10 (range: 7-20) d.

All patients received uni-LLND, and the postoperative pathology showed that LLN metastasis was present in 7 (35.0%) cases. Of these, 6 cases had both mesenteric and internal iliac lymph node metastases, and 1 case had only internal iliac lymph node metastasis. The median number of harvested LLNs was 8.6 (range: 6-12).

Postoperative complications included lymph leakage in 1 case and lower limb pain in 1 case (grade I based on Clavien-Dindo classification). No other postoperative complication was observed. The former was cured after fasting for 4 d, and the latter

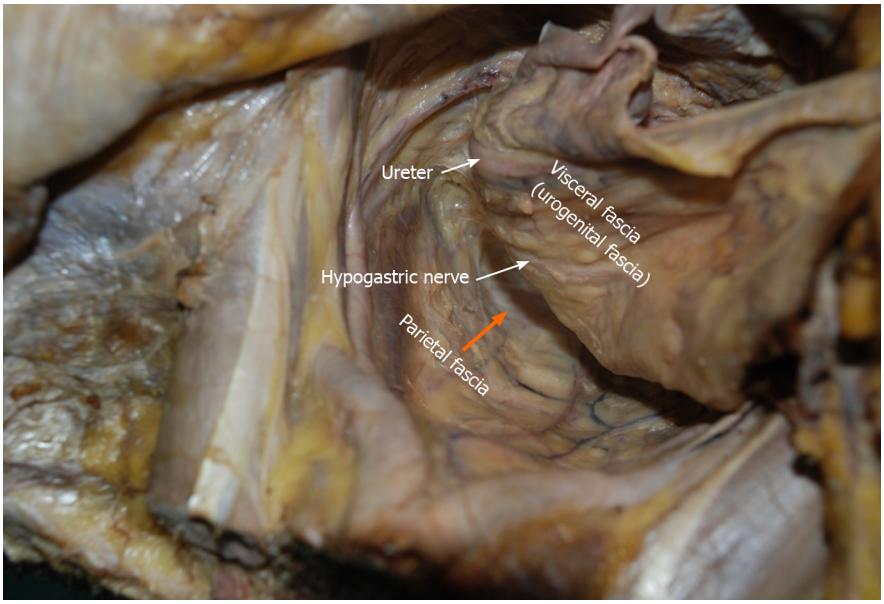


Figure 7 Urogenital fascia extends posterolaterally to the rectum, with the hypogastric nerve and ureter running in it. The "Holy plane" for total mesorectal excision is between the urogenital fascia (visceral fascia) and the parietal fascia (orange arrowhead).

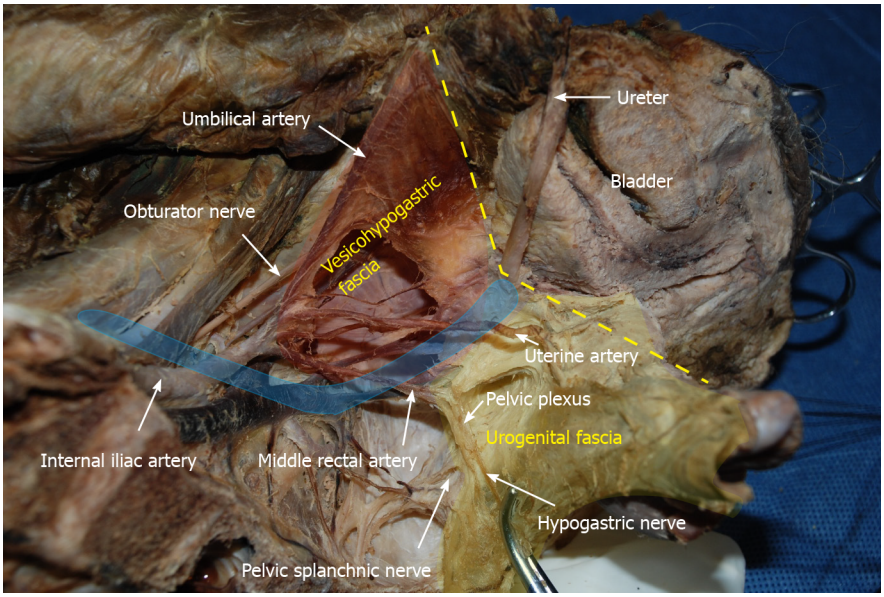


Figure 8 Vesicohypogastric fascia and urogenital fascia. Vesicohypogastric fascia is a triangular fascia composed of umbilical artery, tendinous arch of pelvic fascia and bladder (red shadow). The urogenital fascia (yellow shadow) and the vesicohypogastric fascia blend with each other and appear as a V-shape (yellow dotted line). The ureter is pulled up and the actual position is shown in blue shadow.

resolved spontaneously 1 mo after hospital discharge.

DISCUSSION

With the application of TME in clinical practice, the concept of performing surgery along the embryological space formed by fasciae has been established in colorectal surgery[15]. However, there is still a lack of data to illustrate fascial composition related to LLND; therefore, it is impossible to establish standard LLND procedures based on fascial anatomy. This is the first study to outline the terminology of fasciae and spaces used in colorectal surgery and gynecology to clarify the surgical plane of LLND. We have put forward the concept of two-space dissection for LLND, and located important blood vessels and nerves using landmark fasciae in each space. By

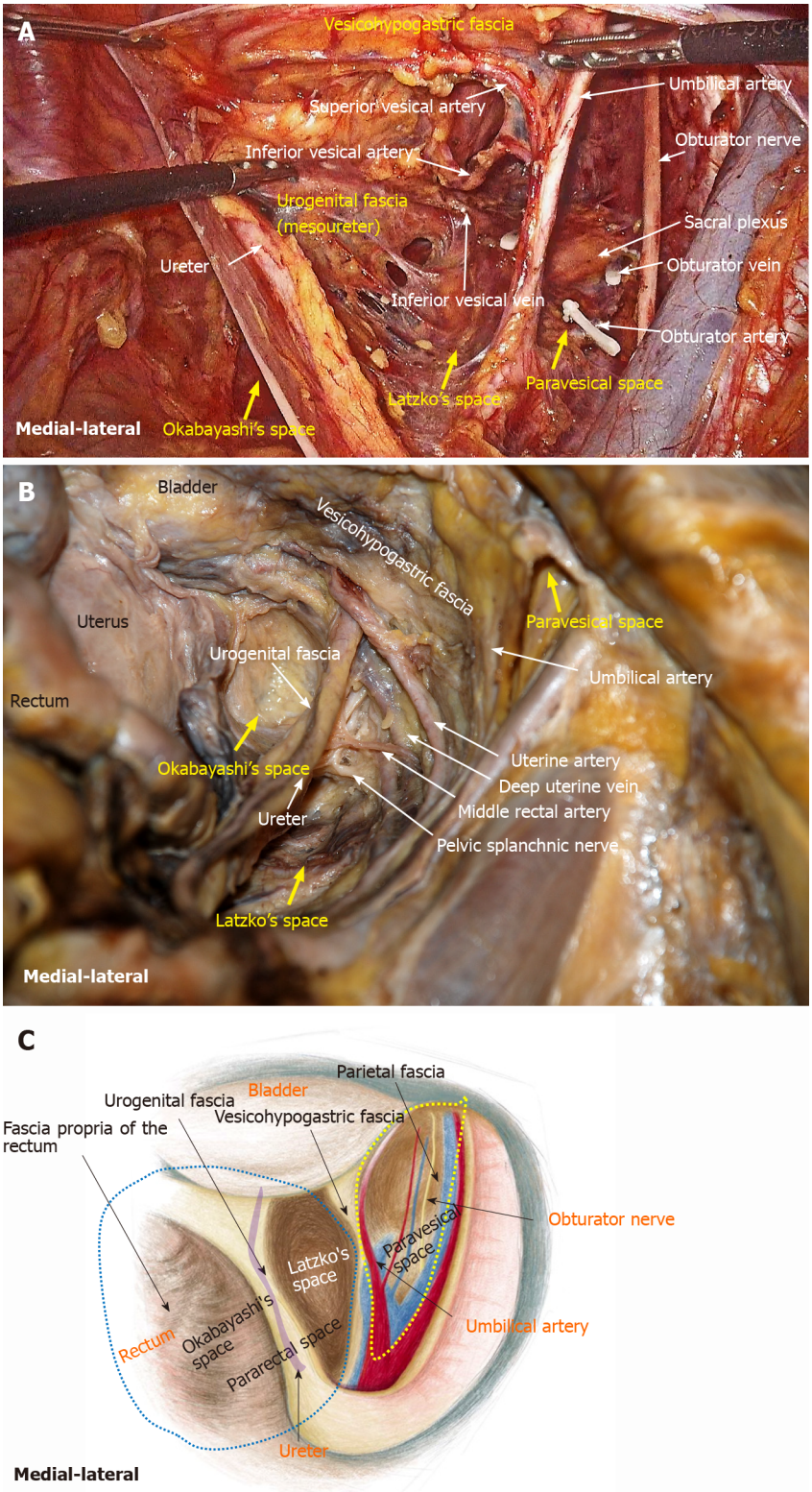


Figure 9 Fascial spaces related to rectal cancer surgery are showed in photograph of operation, photograph of cadaver dissection and diagram. A: Photograph of operation; B: Photograph of cadaver dissection; C: Photograph of diagram. The pararectal space (blue dotted line) can be divided into Okabayashi's space and Latzko's space. Fascia propria of the rectum, urogenital fascia, vesicohypogastric fascia and parietal fascia form Okabayashi's, Latzko's space and paravesical space (yellow dotted line). The last two spaces are the surgical area for lateral lymph node dissection.

applying these anatomical findings in practice, the initial clinical results showed that the fascial space approach can significantly reduce intraoperative blood loss and maintain a clear surgical field. Moreover, this approach can easily expose and protect the ureter, the hypogastric nerve, pelvic plexus, lumbosacral trunk and sacral plexus, which significantly decreased the incidence of postoperative urogenital dysfunction.

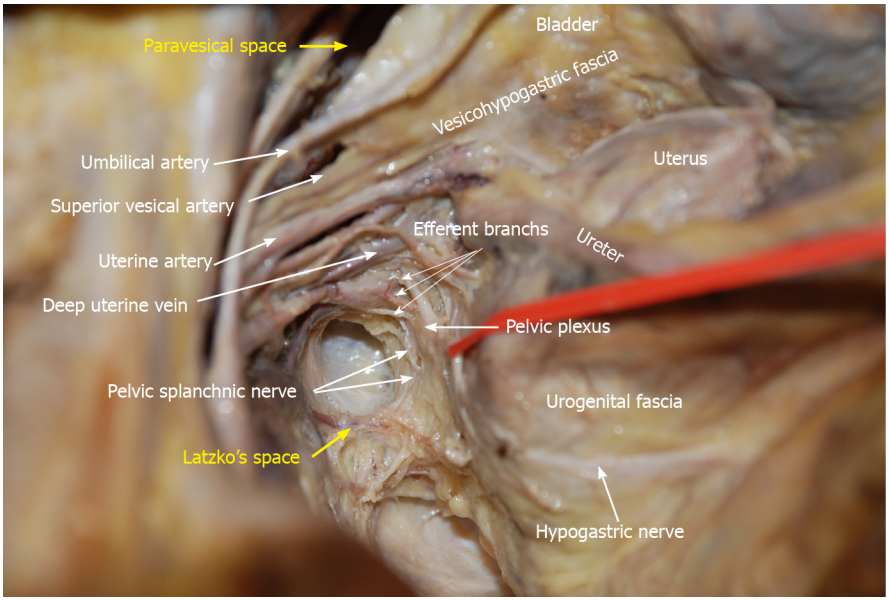


Figure 10 Anatomy of the branches of the internal iliac blood vessel and nerves related to lateral lymph node dissection. The hypogastric nerve, pelvic splanchnic nerves and pelvic plexus are located in the same connective tissue plane and presents as a T-shape "pelvic nerve plate".

There are many diverse descriptions on the fascia composition related to rectal cancer surgery[15]. As described in the literature, the fascia propria of the rectum is part of the visceral fascia, and the visceral fascia together with Denonvilliers' fascia constitute the morphology of the "mesorectum"[16]. This has become the knowledge on classical pelvic anatomy and has been widely accepted by colorectal surgeons. However, we found that the fascia propria of the rectum and visceral fascia are two independent layers of fascia. The fascia propria of the rectum presents as a barely visible translucent layer which is the innermost layer of fascia surrounding the rectum and perirectal fat (Figure 6). The visceral fascia was the densest portion of pelvic fascia, which stretched "like a hammock" posterolateral to the rectum and fused anteriorly with Denonvilliers' fascia (Figure 7). In fact, "visceral fascia" is a general term used to describe the fascia ensheathing organs[17], and therefore, it is inappropriately used to describe specific fascia. Actually, the visceral fascia described by Heald *et al*[7] in TME is just the urogenital fascia. The urogenital fascia originates from the fusion of the anterior renal fascia and posterior renal fascia below the inferior pole of the kidney. Muntean *et al*[18] described the hypogastric nerve and the ureter embedded in this fascia, hence the name urogenital fascia. However, Kinugasa demonstrated a protective fascia overlying the hypogastric nerves and proposed that "prehypogastric nerve fascia" seemed more appropriate[19]. In some patients with an enlarged hypogastric nerve, the hypogastric nerve was identified posterior to the urogenital fascia in this study (Figure 1B). The urogenital fascia carries the ureter and the hypogastric nerve to the bladder and continues with the vesicohypogastric fascia, and the two fasciae blend with each other anterior-laterally in the tendinous arch of the pelvic fascia (Figure 8). The obturator fascia is one of three parts of the parietal fascia [20]. In summary, the fascia propria of the rectum, urogenital fascia, vesicohypogastric fascia and parietal fascia lie side by side in a medial-lateral direction, and constitute the dissection plane for curative rectal cancer surgery (Figure 9).

In gynecological anatomy, the paravesical space is a potential space between the umbilical artery and the external iliac vein[21]. Given that the umbilical artery is the landmark of the vesicohypogastric fascia and the obturator fascia (parietal fascia) follow the same plane as the external iliac vein, the paravesical space can be considered to be located between the vesicohypogastric fascia and the parietal fascia (Figure 9). Gynecological anatomy also defines the pararectal space as a loose connective tissue space between the rectum and the internal iliac vessels, and is further divided into the medial Okabayashi's space and lateral Latzko's space by the ureter [21]. According to our findings, the fascia propria of the rectum is the innermost layer covering the rectum, the mesoureter is a part of the urogenital fascia, and the umbilical artery constituting the superior border of the vesicohypogastric fascia is the first branch of the internal iliac artery. Therefore, in the view of fascia anatomy, Okabayashi's space can be described as the space between the fascia propria of the

rectum and the urogenital fascia, and Latzko's space lies between the urogenital fascia and the vesicohypogastric fascia (Figure 9). Okabayashi's space corresponds to the resection area of TME, while Latzko's pararectal space and the paravesical space constitute the surgical field for LLND. Moreover, the vesicohypogastric fascia divides the LLNs into obturator lymph nodes and internal iliac lymph nodes.

The advantage of the fascial space approach lies in its ability to accurately locate important anatomical structures, for example, the hypogastric nerve is located in Latzko's space, while the lumbosacral trunk and sacral plexus are located in the paravesical space (Figures 1B and 5). Dissection of Latzko's space mainly involves the hypogastric nerve and inferior vesical artery. As the hypogastric nerve runs in the urogenital fascia, the urogenital fascia is recommended to be separated first and pulled medially to protect the hypogastric nerve. Separation of the urogenital fascia is always stopped at the level of the pelvic plexus to avoid damage to the neurovascular bundle. In fact, the hypogastric nerve and the pelvic plexus are easily exposed and protected using the fascial anatomy approach. Therefore, we consider that possible nerve damage depends more on the surgical procedures for TME rather than those for LLND. In TME, if we follow the traditional view to dissect in the "Holy plane" between the visceral fascia (urogenital fascia) and parietal fascia, the hypogastric nerve will undoubtedly be impaired (Figure 7). According to our viewpoint, the appropriate plane for TME is between the fascia propria of the rectum and the visceral fascia (urogenital fascia) (Figure 6), which is consistent with the opinions of Kinugasa *et al* [19]. We also showed that the mesoureter is a part of the urogenital fascia (Figure 9B); thus, care should be taken to preserve the integrity of the mesoureter to avoid damage to the hypogastric nerve, and the traditional procedure such as isolating the ureter and picking it up by a rubber band should be abandoned.

The inferior vesical artery is a common site for internal iliac lymph node metastasis [22]. It is the last branch of the internal iliac artery, and located just above the infrapiriformis foramen. Therefore, the inferior vesical artery can be considered the distal end of the vascular dissection in LLND. It should also be noted that the branches of the internal iliac artery are varied in their number, origin, location and course. For instance, our previous study showed that the middle rectal artery was only observed in 29.2% (7/24) specimens, and its diameter was mostly less than 1.5 mm[12]. As a result, we suggest that it is unnecessary to expose each branch of the internal iliac vessels in LLND. The blood vessels requiring dissection include the umbilical artery, superior vesical artery, internal pudendal artery, and inferior vesical artery (male) or uterine artery (female). Many classic anatomical works believe that the inferior vesical artery does not exist in females. In "Gray's anatomy", the inferior vesical artery is described as being replaced by the ovarian artery or uterine artery[23]. However, a cadaver study by de Treigny *et al*[24] indicated that the inferior vesical artery does exist in females, and originates mostly from the common trunk with the umbilical or uterine arteries. Whichever is the case, it is clear that it is difficult to identify the inferior vesical artery in females during LLND.

Dissection of the paravesical space can be completed from three directions. The lateral dissection begins at the external iliac lymph nodes and extends downward to the obturator fascia (parietal fascia). The medial dissection is performed along the vesicohypogastric fascia. The vesical veins change direction sagittally and are distributed in the distal part of the vesicohypogastric fascia. Therefore, special attention should be paid to avoid damage to the vesical veins when the distal end of the obturator vessels (at the obturator) is isolated and ligated. The proximal dissection begins at the bifurcation of the internal and external iliac vessels, followed by exposing the lumbosacral trunk. After dissecting the bottom of the obturator fossa, Alcock's canal and the superior fascia of the pelvic diaphragm are exposed, forming a connection between the paravesical space and the TME surgical plane. Although the sacral plexus is formed by the lumbosacral trunk and other rami of S1-S4, they have different anatomical locations. The sacral plexus is located between the obturator vein and internal pudendal artery (Figure 11). The sacral plexus is usually covered by the parietal fascia, which should be preserved to avoid postoperative limb pain. This might be the reason why one patient in this study developed limb pain after surgery. In addition, the lymphatic chain connects with the inguinal lymph nodes and the obturator nodes at the distal external iliac vein; therefore, the distal end should be ligated to avoid lymph leakage. In this study, one patient suffered postoperative lymph leakage, possibly because the lymphatic vessels were not securely ligated when dissecting the superior inguinal lymph node (stated by Fujii *et al*[25]) located in the angle between the external iliac vein and pubis bone.

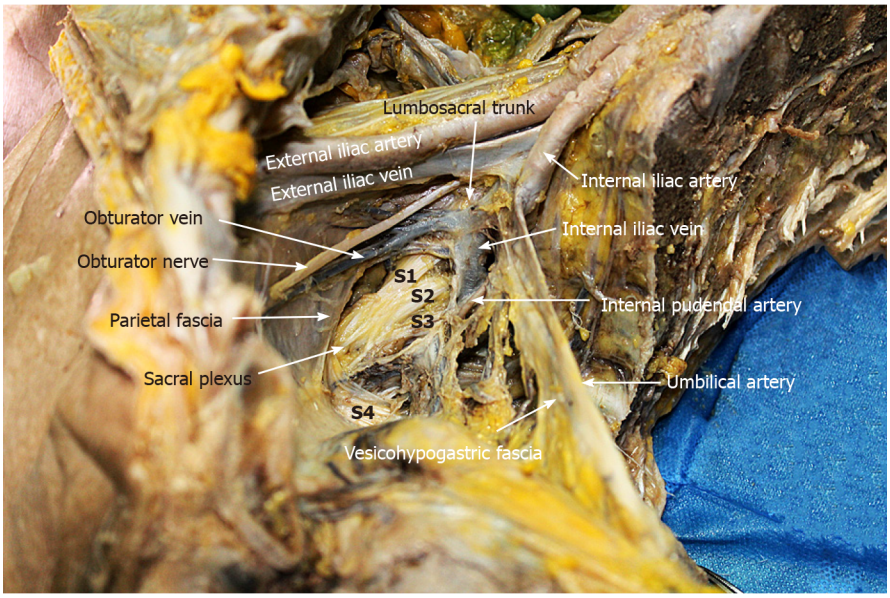


Figure 11 Lumbosacral trunk is located at the bifurcation of internal and external iliac vessels, while sacral plexus is located between obturator vein and internal pudendal artery. Sacral plexus was covered by parietal fascia.

CONCLUSION

In conclusion, laparoscopic LLND in two fascial spaces has several significant advantages, including easy identification of the surgical plane and clear location of nerves and blood vessels. Our preliminary surgical experience has shown that it is a feasible surgical approach for treating LALRC, which not only improves surgical safety but also ensures radical resection of the tumor. However, further studies are warranted to validate these results and optimize the surgical procedure.

ARTICLE HIGHLIGHTS

Research background

Lateral lymph node (LLN) metastasis is a major cause for local recurrence in rectal cancer. In Japan, LLN dissection (LLND) is the standard treatment for locally advanced low rectal cancer (LALRC). However, the procedure is complicated with significant morbidity. In recent years, with the rise of "fascia anatomy", more and more surgeons began to explore LLND based on the fascial space approach.

Research motivation-

LLND is a challenging procedure due to its technical difficulty and higher incidence of complications. The development of "fascia anatomy" provides a new sight for improving the accuracy and safety of laparoscopic LLND. However, the detailed anatomy is not clear and a standard surgical procedure has not yet been established.

Research objectives

We developed a technique of laparoscopic LLND in two fascial spaces formed by three layers of fasciae. This study aimed to describe the surgical procedure on an anatomical basis and to summarizes our preliminary surgical experiences in the treatment of LALRC.

Research methods

Detailed pelvic dissections were performed in 24 cadavers, and the fasciae and spaces related to LLND were observed and described. 20 patients with LALRC received 3D-laparoscopic total mesorectal excision with LLND at our hospital from July 2018 to October 2020, and their surgical videos and clinical data were analyzed.

Research results

The urogenital fascia lies posterolateral to the rectum, and the hypogastric nerve and ureter are observed to be enveloped in it; vesicohypogastric fascia shows a triangle shape formed by the umbilical artery, the tendinous arch of the pelvic fascia and the lateral border of the bladder. In all 24 cadavers, urogenital fascia, vesicohypogastric fascia and obturator fascia (parietal fascia) were located lateral to the rectum in a medial-to-lateral direction and form the Okabayashi's pararectal space and paravesical space, respectively, which were the surgical area for LLND. Laparoscopic LLND was performed successfully in all 20 LALRC patients with a median postoperative hospitalization of 10 (7-20) d. The median operating time was 178 (152-243) min, with a median blood loss of 55 (25-150) mL. The median number of harvested LLNs was 8.6 (6-12), and 7 patients (35.0 %) had LLN metastasis. Postoperative complications included lymph leakage and lower limb pain in 1 case, respectively.

Research conclusions

This study indicated that urogenital fascia, vesicohypogastric fascia and parietal fascia lie side by side in the pelvis and formed two spaces (Latzko's pararectal space and paravesical space), which were the surgical area for LLND. Performing LLND in two fascial spaces is a feasible surgical approach, which improves surgical safety while ensuring radical tumor resection.

Research perspectives

The present study preliminarily explored the clinical significance of laparoscopic LLND in two fascial spaces for treating LALRC. However, large studies with long-term follow-up and more detailed clinical data are needed to confirm these findings.

REFERENCES

1 **Akiyoshi T**, Watanabe T, Miyata S, Kotake K, Muto T, Sugihara K; Japanese Society for Cancer of the Colon and Rectum. Results of a Japanese nationwide multi-institutional study on lateral pelvic lymph node metastasis in low rectal cancer: is it regional or distant disease? *Ann Surg* 2012; **255**: 1129-1134 [PMID: 22549752 DOI: 10.1097/SLA.0b013e3182565d9d]

2 **Ueno H**, Mochizuki H, Hashiguchi Y, Ishiguro M, Miyoshi M, Kajiwara Y, Sato T, Shimazaki H, Hase K. Potential prognostic benefit of lateral pelvic node dissection for rectal cancer located below the peritoneal reflection. *Ann Surg* 2007; **245**: 80-87 [PMID: 17197969 DOI: 10.1097/01.sla.0000225359.72553.8c]

3 **Takahashi T**, Ueno M, Azekura K, Ohta H. Lateral node dissection and total mesorectal excision for rectal cancer. *Dis Colon Rectum* 2000; **43**: S59-S68 [PMID: 11052480 DOI: 10.1007/BF02237228]

4 **Nakamura T**, Watanabe M. Lateral lymph node dissection for lower rectal cancer. *World J Surg* 2013; **37**: 1808-1813 [PMID: 23703637 DOI: 10.1007/s00268-013-2072-z]

5 **Hojo K**, Koyama Y. The effectiveness of wide anatomical resection and radical lymphadenectomy for patients with rectal cancer. *Jpn J Surg* 1982; **12**: 111-116 [PMID: 7109356 DOI: 10.1007/BF02469377]

6 **Ito M**, Kobayashi A, Fujita S, Mizusawa J, Kanemitsu Y, Kinugasa Y, Komori K, Ohue M, Ota M, Akazai Y, Shiozawa M, Yamaguchi T, Akasu T, Moriya Y; Colorectal Cancer Study Group of Japan Clinical Oncology Group. Urinary dysfunction after rectal cancer surgery: Results from a randomized trial comparing mesorectal excision with and without lateral lymph node dissection for clinical stage II or III lower rectal cancer (Japan Clinical Oncology Group Study, JCOG0212). *Eur J Surg Oncol* 2018; **44**: 463-468 [PMID: 29428473 DOI: 10.1016/j.ejso.2018.01.015]

7 **Heald RJ**. The 'Holy Plane' of rectal surgery. *J R Soc Med* 1988; **81**: 503-508 [PMID: 3184105]

8 **Bissett IP**, Hill GL. Extrafascial excision of the rectum for cancer: a technique for the avoidance of the complications of rectal mobilization. *Semin Surg Oncol* 2000; **18**: 207-215 [PMID: 10757886 DOI: 10.1002/(sici)1098-2388(200004/05)18:3<207::aid-ssu4>3.0.co;2-d]

9 **Shen J**, Dong X, Liu Z, Wang G, Yang J, Zhou F, Lu M, Ma X, Li Y, Tang C, Luo X, Zhao Q, Zhang J. Modularized laparoscopic regional en bloc mesogastrium excision (rEME) based on membrane anatomy for distal gastric cancer. *Surg Endosc* 2018; **32**: 4698-4705 [PMID: 30054740 DOI: 10.1007/s00464-018-6375-x]

10 **Coffey JC**, Dillon M, Sehgal R, Dockery P, Quondamatteo F, Walsh D, Walsh L. Mesenteric-Based Surgery Exploits Gastrointestinal, Peritoneal, Mesenteric and Fascial Continuity from Duodenojejunal Flexure to the Anorectal Junction--A Review. *Dig Surg* 2015; **32**: 291-300 [PMID: 26138509 DOI: 10.1159/000431365]

11 **Lin M**, Chen W, Huang L, Ni J, Ding W, Yin L. The anatomic basis of total mesorectal excision. *Am J Surg* 2011; **201**: 537-543 [PMID: 20385366 DOI: 10.1016/j.amjsurg.2009.12.010]

12 **Lin M**, Chen W, Huang L, Ni J, Yin L. The anatomy of lateral ligament of the rectum and its role in total mesorectal excision. *World J Surg* 2010; **34**: 594-598 [PMID: 20052469 DOI: 10.1007/s00268-009-0371-1]

13 **General Assembly of the World Medical Association**. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *J Am Coll Dent* 2014; **81**: 14-18 [PMID: 25951678]

14 **Han Y**, He YG, Zhang HB, Lv KZ, Zhang YJ, Lin MB, Yin L. Total laparoscopic sigmoid and rectal surgery in combination with transanal endoscopic microsurgery: a preliminary evaluation in China. *Surg Endosc* 2013; **27**: 518-524 [PMID: 22806529 DOI: 10.1007/s00464-012-2471-5]

15 **Mike M**, Kano N. Laparoscopic surgery for colon cancer: a review of the fascial composition of the abdominal cavity. *Surg Today* 2015; **45**: 129-139 [PMID: 24515451 DOI: 10.1007/s00595-014-0857-9]

16 **Chapuis P**, Bokey L, Fahrer M, Sinclair G, Bogduk N. Mobilization of the rectum: anatomic concepts and the bookshelf revisited. *Dis Colon Rectum* 2002; **45**: 1-8; discussion 8 [PMID: 11786754 DOI: 10.1007/s10350-004-6104-5]

17 **Stecco C**, Sfriso MM, Porzionato A, Rambaldo A, Albertin G, Macchi V, De Caro R. Microscopic anatomy of the visceral fasciae. *J Anat* 2017; **231**: 121-128 [PMID: 28466969 DOI: 10.1111/joa.12617]

18 **Muntean V**. The surgical anatomy of the fasciae and the fascial spaces related to the rectum. *Surg Radiol Anat* 1999; **21**: 319-324 [PMID: 10635095 DOI: 10.1007/BF01631332]

19 **Kinugasa Y**, Murakami G, Suzuki D, Sugihara K. Histological identification of fascial structures posterolateral to the rectum. *Br J Surg* 2007; **94**: 620-626 [PMID: 17330242 DOI: 10.1002/bjs.5540]

20 **Ercoli A**, Delmas V, Fanfani F, Gadonneix P, Ceccaroni M, Fagotti A, Mancuso S, Scambia G. Terminologia Anatomica versus unofficial descriptions and nomenclature of the fasciae and ligaments of the female pelvis: a dissection-based comparative study. *Am J Obstet Gynecol* 2005; **193**: 1565-1573 [PMID: 16202758 DOI: 10.1016/j.ajog.2005.05.007]

21 **Ceccaroni M**, Clarizia R, Roviglione G, Ruffo G. Neuro-anatomy of the posterior parametrium and surgical considerations for a nerve-sparing approach in radical pelvic surgery. *Surg Endosc* 2013; **27**: 4386-4394 [PMID: 23783554 DOI: 10.1007/s00464-013-3043-z]

22 **Konishi T**. Laparoscopic Lateral Pelvic Lymph Node Dissection. In: Kim J, Garcia-Aguilar J. Minimally Invasive Surgical Techniques for Cancers of the Gastrointestinal Tract 2020. Springer, Cham; 2020

23 **Standring S**. Gray's Anatomy. 41th ed. Elsevier Churchill Livingstone: London; 2016

24 **de Treigny OM**, Roumiguie M, Deudon R, de Bonnecaze G, Carfagna L, Chaynes P, Rimailho J, Chantalat E. Anatomical study of the inferior vesical artery: is it specific to the male sex? *Surg Radiol Anat* 2017; **39**: 961-965 [PMID: 28229186 DOI: 10.1007/s00276-017-1828-9]

25 **Fujii S**, Sekiyama K. Precise Neurovascular Anatomy for Radical Hysterectomy 2002. Springer Nature Singapore Pte Ltd: Singapore

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SYSTEMATIC REVIEWS

Disorders of the brain-gut interaction and eating disorders

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Author contributions: Stanculete MF, Chiarioni G, Popa SL, Dumitrascu DI searched the literature, analyzed the results and made substantial contribution to the writing of the manuscript; Dumitrascu DL gave the idea of this review, suggested the methodology, analyzed the results, and revised the manuscript; all authors approved the final version of the manuscript.

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Abstract

BACKGROUND

Eating disorders (ED) involve both the nervous system and the gastrointestinal tract. A similar double involvement is also found in disorders of the brain-gut interaction (DGBI) and symptoms are sometimes similar.

AIM

To find out where there is an association and a cause-effect relationship, we looked for the comorbidity of DGBI and ED.

METHODS

A systematic review was undertaken. A literature search was performed. Inclusion criteria for the articles retained for analysis were: Observational cohort population-based or hospital-based and case-control studies, examining the relationship between DGBI and ED. Exclusion criteria were: Studies written in other languages than English, abstracts, conference presentations, letters to the Editor and editorials. Selected papers by two independent investigators were critically evaluated and included in this review.

RESULTS

We found 29 articles analyzing the relation between DGBI and ED comprising 13 articles on gastroparesis, 5 articles on functional dyspepsia, 7 articles about

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Italy

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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S-Editor: Fan JR
L-Editor: A
P-Editor: Ma YJ

functional constipation and 4 articles on irritable bowel syndrome.

CONCLUSION
There is no evidence for a cause-effect relationship between DGBI and ED. Their common symptomatology requires correct identification and a tailored therapy of each disorder.

Key Words: Eating disorders; Dyspepsia; Constipation; Irritable bowel syndrome, Anorexia; Gastroparesis

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Core Tip: Functional gastrointestinal disorders actually defined disorders of the brain-gut interaction (DGBI) by the new Rome IV criteria share similar symptoms with eating disorders (ED). Etiology is ill defined for both disorders thou a negative impact on quality of life and a high socio-economic burden is reported. We looked for the comorbidity of DGBI and ED, in order to find out where there is an association and a cause-effect relationship.

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INTRODUCTION

Eating disorders (ED) are psychiatric disorders defined by abnormal eating habits that negatively affect a person's physical or mental health and frequently begin in late childhood or early adulthood[1]. The pathogenesis of ED is still unclear, although it has been demonstrated that both genetic and environmental factors are involved. The most frequent ED include binge ED (eating a large amount of food in a short time), anorexia nervosa (eating an extremely small quantity of food due to a fear of gaining weight in contrast with low body weight in reality), bulimia nervosa (characterized by binge eating followed by purging, an attempt to get rid of the food consumed by vomiting or taking laxatives), pica (eating non-food items like hair, paper, sharp objects, metal objects, soil or glass), night ED (delayed circadian pattern of food intake) and avoidant/restrictive food intake disorder (eating only within an extremely narrow repertoire of foods)[1,2].

Epidemiological studies show that in the developed world, binge eating disorder affects about 1.6% of women and 0.8% of men, anorexia nervosa about 0.4%, and bulimia about 1.3% of young women[3,4]. At some point in their lifetime, more than 4% of women have anorexia, 2% have bulimia, and 2% have a binge eating disorder. In less developed countries, the rates of ED are lower[3,4]. Females are nine times more frequent than males to suffer from ED. ED do not include obesity. Notwithstanding visible progress in the current therapeutic methods[4], ED result in more than 7000 deaths a year, covering the highest mortality rate in all mental illnesses.

Disorders of the brain-gut interaction (DGBI) previously known as functional gastrointestinal disorders, involve visceral hypersensitivity and motility disturbances of different parts of the gastrointestinal tract[5]. DBGI are defined by several variable combinations of chronic or recurrent gastrointestinal symptoms that do not have an identified underlying pathophysiology. In the absence of any biological marker or endoscopic modifications, the identification and classification of DGBIs is based on symptoms[6,7]. Patients suffering from DGBIs typically present with various symptoms as early satiety, postprandial fullness, bloating, nausea, emesis, and epigastric pain[6-8]. Gastrointestinal motility disorders are the result of the dysfunction of the extrinsic nervous system, enteric nervous system, interstitial cells of Cajal (or intestinal pacemakers), or smooth muscle[6-8]. The type of disorder in transit (delay or acceleration) and the region of the gastrointestinal tract affected are the main criteria that are used for the classification. The neural control of the gastrointestinal

tract is a subject that is still generating interest and debates[9]. The close connections between the central nervous system and the enteric system are realized by ascending and descending fibers[10]. The ascending tract sends information from the digestive tube to the spine, hypothalamus, diencephalon and the cerebral cortex[9,10]. The descending tracts are responsible for the motility of the muscular layers of the digestive tract as well as glandular secretion[9]. A large part of the descending neurons has its origin in the prefrontal cerebral cortex[10]. This area is responsible for emotions and personality. Therefore, gastrointestinal motility is influenced by psychological factors[11-14].

Therefore, we looked for the comorbidity of DGBI and ED, in order to find out where there is an association and where there is a cause-effect relationship.

MATERIALS AND METHODS

A thorough literature search was undertaken. PubMed, Cochrane Library, EMBASE, and WILEY databases were screened for relevant publications regarding DGBI in ED. The search terms included: ("Eating disorders" OR "disorders of gut-brain interaction" OR "functional gastrointestinal disorders" OR "neurogastroenterological disorders" OR "gastrointestinal motility disorders") AND ("anorexia nervosa" OR "bulimia nervosa" OR "binge eating disorders") AND ("gastroparesis" OR "functional dyspepsia" OR "irritable bowel syndrome" OR "functional bowel disorders"). Inclusion criteria of original articles in this systematic review were as follows: Observational cohort population-based or hospital-based and case-control studies, examining the relationship between gastrointestinal disorders and ED. Exclusion criteria were: Studies written in languages other than English, abstracts, conference presentations, letters to the editor and editorials (Figure 1). The articles included in this review refer only to DGBI present in ED. Searches were carried out by two independent investigators.

RESULTS

We found 29 articles analyzing the relation between DGBI and ED comprising 13 articles regarding gastroparesis (Table 1), 5 articles on functional dyspepsia (FD) (Table 2), 7 articles regarding functional constipation (FC) (Table 3) and 4 articles on irritable bowel syndrome (IBS).

Gastroparesis

Gastroparesis is a neuromuscular disorder of the upper gastrointestinal tract characterized by delayed gastric emptying in the absence of mechanical obstruction of the stomach[15,16]. The clinical outcome of the delayed gastric emptying is a combination of symptoms, including early satiety, postprandial fullness, nausea, vomiting, belching, and bloating. The complicated overlap between upper gastrointestinal symptoms leads to a difficult diagnosis between gastroparesis and other DGBIs, such as FD, gastroparesis like syndrome and other organic gastrointestinal disorders[17]. A precise diagnosis requires measurement of gastric emptying using gastric scintigraphy, which is considered a gold standard diagnosis technique or using breath testing[15-17].

The etiology of gastroparesis is multifactorial: Diabetes mellitus, infectious, connective tissue diseases, prior gastric surgery, visceral ischemia, myopathic diseases, neurological diseases (most frequent Parkinson disease), coma or artificial ventilation and medications (especially opiate narcotic analgesics and anticholinergic agents)[17]. Nevertheless, the etiology cannot be established in more than 50% cases, representing idiopathic gastroparesis. The pathogenesis of gastroparesis is also complex, and abnormalities in fundic tone, gastroduodenal dyscoordination, a weak antral pump, gastric dysrhythmias, abnormal duodenal feedback justify the reason why gastroparesis is considered a part of a broader spectrum of gastric neuromuscular dysfunction that includes impaired gastric accommodation[15-17]. Epidemiological evidence is still lacking. The number of gastroparesis patients in the United States is estimated to be more than 4 million[17]. Gastroparesis is associated with ED. A study performed by Szmukler *et al*[18] investigated 20 patients presenting ED using gastric scintigraphy [18]. From a total of 20 patients, 8 had anorexia nervosa, 10 had both anorexia nervosa and bulimia nervosa, and 2 with bulimia nervosa alone. In this group, gastric half-

Table 1 Studies investigating the association between gastroparesis and eating disorders

Ref.	Aims	Study population	Assessment instruments	Results and conclusions
Szmukler *et al*[18], 1990	To determine the natural history of delayed gastric emptying of solid foods in AN	20 consecutive female inpatients. 8 restrictive AN. 10 AN and BN. 2BN. Mean age: 22.8 ± 5.2 yr. Duration of illness: 49.0 ± 37.4 mo	Scintigraphy; HET; BMI	HET > 110 min. HET significant negative correlation with BMI; delayed gastric emptying in AN improves quite rapidly as feeding recommences
Hutson and Wald[19], 1990	To measure: Gastric emptying of a mixed liquid and solid meal in patients with AN, BN, and HC; the relationship of body weight and gastrointestinal symptoms to gastric emptying	11 BN. 10 AN. A sex-matched HC	A dual radioisotope technique	Gastric emptying of solids in patients with BN was similar to that in HC (gastric T1/2 131 ± 15 min *vs* 119 ± 7 min; mean ± SEM). AN patients had overall delayed emptying (182 ± 31 min; $P < 0.05$); gastric emptying of liquids was similar in the BN and HC (gastric T1/2 48 ± 5 min and 49 ± 4 min, respectively), AN tended to have prolonged gastric emptying (65 ± 11 min, P = NS). There was no correlation between body weight, gastrointestinal symptoms, and gastric emptying
Benini *et al* [20], 2004	To compare dyspeptic symptoms and gastric emptying times. To examine the relationship between dyspeptic symptoms, gastric motility, behavioral and psychological features of eating disorders and general psychopathology. To study the effect of simple reefeding and of long-term rehabilitation on gastric symptoms and on parameters of psychopathological distress	23 AN. 12 binge/purging subtype. Mean age 19.9 ± 0.7 yr; mean BMI 13.2 ± 0.6, 11 restricting subtype; mean age 25.4 ± 1.1 yr; mean BMI 15.5 ± 0.7. 24 HC age and sex matched	Ultrasonographic gastric-emptying test, psychopathological questionnaires (SCL-90, EDI, EDE-Q). The bowel symptom questionnaires. VAS for hunger and epigastric fullness	Gastric symptom scores: Markedly higher in AN than in HC; improved significantly with treatment; no correlation between entry values of gastric emptying symptoms and questionnaire score was found; long-term rehabilitation improves gastrointestinal symptoms, gastric emptying and psychopathological distress in an independent manner, but not short-term refeeding
Inui *et al* [21], 1995	Analyzing gastrointestinal motility abnormalities in ED patients	26 female patients. 9 AN (mean age 22.5 ± 2.0 yr). 10 AN and BN (mean age 22.2 ± 1.6 yr). 7 BN (mean age 19.2 ± 1.2 yr). 9 HC	Gastric emptying: Radionuclide technique SDS; CAS	ED patients had delayed gastric emptying after ingestion of a solid meal. The patients has high depression and anxiety scores
Dubois *et al* [22], 1979	Measure of gastric emptying and gastric output concurrently in a group of patients with AN before and after weight gain	15 female AN age 14-32 yr; weight 34 ± 1 kg; 11 HC (8 male and 3 female) age 20-31 years old weight 68 ± 3 kg	Dye dilution technique; Barium meal x-ray examination	Fractional gastric emptying rate was significantly less in AN patients than in controls during basal conditions and following a water load, but not during maximal doses of pentagastrin. Emptying is inversely correlated with body weight in healthy controls. Gastric emptying is abnormally low in AN patients, even after weight gain
Kamal *et al* [23], 1991	To determine whether small bowel transit time or colonic transit time is delayed in AN and BN. To determine whether delays in gastrointestinal transit are correlated with symptoms of constipation or bloating	10 AN (9 female, 1 male). 18 BN (15 female, 3 male). 10 female HC	Whole-gut transit was tested by the radiopaque marker technique, mouth-to-cecum transit time was assessed by the lactulose breath test	Whole-gut transit time was significantly delayed in both AN (66.6 ± 29.6 h) and BN (70.2 ± 32.4 h) compared with HC (38.0 ± 19.6 h). Mouth-to-cecum transit time longer in AN (109.0 ± 33.5 min) and BN (106.2 ± 24.5 min) than in HC (84.0 ± 27.7 min), but these differences were not statistically significant
Robinson *et al*[24], 1988	Determinants of delayed gastric emptying in AN and BN patients	22 AN patients (21 female and 1 male). 10 BN female. 10 HC (8 female and 2 male)	Gamma camera technetium ^{99}m-sulphur colloid	Only gastric emptying rates of the solid meal and glucose solution were significantly delayed. The gastric disturbance was confined to patients with AN patients selecting their own diet. Patients receiving adequate nutrition on the ward had normal gastric emptying and weight gain in this group had no significant effect on emptying. Slow emptying was observed in patients who maintained a low weight solely by food restriction as well as in patients whose AN was complicated by episodes of bulimia. Gastric emptying in BN was normal
Bluemel *et al*[26], 2017	Relationship of postprandial gastrointestinal motor and sensory function with body weight	24 AN [BMI 14.4 (11.9–16.0) kg/m^2]. 16 OB [34.9 (29.6-41.5) kg/m^2]. 20 HC [21.9 (18.9-24.9) kg/m^2]	MRI and 13C-lactose-ureide breath test	Gastric half-emptying time (t_{50}) was slower in AN than HC ($P = 0.016$) and OB ($P = 0.007$). A negative association between t_{50} and BMI was observed between BMI 12 and 25 kg/m^2 ($P = 0.0$). Antral contractions and oro-cecal transit were not different. Self-reported postprandial fullness was greater in AN than in HC or OB ($P < 0.001$). After weight rehabilitation, t_{50} in AN tended to become shorter ($P = 0.09$) and postprandial fullness was less marked ($P < 0.01$)

Holt *et al* [27], 1981	Gastric emptying of the solid and liquid components of a physiological test meal	10 AN female patients, age 17-32 yr, mean weight 42 kg. 12 HC (6 females, 6 males, age 32-65 yr; mean weight 67 kg	Scintiscanning method	Significantly slower gastric emptying was found for both the liquid and the solid components of the meal in AN patients compared with HC. Emptying during the early phase (0-40 mm after meal ingestion) was not significantly differently in the two groups
Abell *et al* [28], 1987	Gastrointestinal and neurohormonal function measuring gastric electrical activity, antral phasic pressure activity, gastric emptying of solids and liquids, and hormonal and autonomic function in AN patients	8 AN (2 male and 6 female), age: 16-31 yr. 8 HC (2 male and 6 female) age19-34 yr	Gastric electrogastrography and manometry (fasting and postprandially), radioscintigraphic gastric emptying test, cold pressor test	AN patients: Increased episodes of gastric dysrhythmia (mean percentage of dysrhythmic time: 9.75 patients *vs* 0.48 controls during fasting, $P < 0.02$; 7.21 patients *vs* 0.18 controls postcibally, $P < 0.001$); impaired antral contractility (mean motility index, 12.8 patients *vs* 14.2 controls, $P < 0.002$); delayed emptying of solids; decreased postcibal blood levels of norepinephrine and neurotensin; impaired autonomic function
Rigaud *et al* [29], 1988	Effects of renutrition on gastric emptying in AN patients	14 AN inpatients (13 female and 1 male); duration of illness: 9 mo-40 yr; mediane 5.9 yr); age 18-61 yr	Double-isotope technique (^{111}In) DTPA and ^{99m}Tc-ovalbumin	Gastric emptying can be improved by a renutrition program in AN
Waldholtz *et al*[30], 1990	To determine the type and frequency of gastrointestinal symptoms. To follow symptoms during refeeding prospectively. To develop guidelines for gastrointestinal testing and intervention in hospitalized AN patients	16 AN consecutive patients in their early 20 s, chronically ill (4.5 ± 1.2 yr); 71.6% ± 2.9% of matched population weight, 12 HC	AN patients rated on 12 gastrointestinal symptoms before and after nutritional rehabilitation. GISS (24 questions); blood tests physical examination	Belching did not improve during treatment. No patients required endoscopy, x-ray evaluation, or antipeptic regimens. Although severe gastrointestinal symptoms are common in AN, they improve significantly with refeeding
Murray *et al* [33], 2020	To identify the frequency of FED symptoms and evaluate the relations between FED symptoms, gastrointestinal symptoms, and gastric retention	288 patients (ages 17-78 yr; 77.5% female). Age 42.7 ± 16.3 yr; BMI 26.3 ± 6.5 (kg/m^2). AN 5 (2.0%) Other Specified FED 23 (9.4%) Unspecified FED-Restrictive 24 (8.3%)	GES, NIAS, EDDS, PAGI-SYM, GCSI	FED symptoms: Were common (55%), particularly ARFID symptoms (23%-40%); Were associated with greater GI symptom severity, but not gastric retention

GP: Gastroparesis; GI: Gastrointestinal; AN: Anorexia nervosa; BN: Bulimia nervosa; ED: Eating disorders; HC: Healthy controls; HET: Initial gastric half-emptying time; BMI: Body mass index; SCL-90: Symptom Check List-90; EDI: Eating disorders inventory; EDE-Q: Eating disorders examination-questionnaire; VAS: Visual analogue scale; SDS: Self-rating depression scale; CAS: Cattell anxiety scale; OB: Obesity; MRI: Magnetic resonance imaging; DTPA: Diethylenetriaminepentacetic acid; GCSI: Gastroparesis cardinal symptom index; PAGI-SYM: Patient assessment of upper GI symptoms; GCSI: Gastroparesis cardinal symptom inventory; EDDS: Eating disorder diagnostic scale; FED: Feeding or eating disorder; NIAS: Nine item avoidant/restrictive food intake disorder survey; GES: Gastric emptying scintigraphy; ARFID: Avoidant/restrictive food intake disorder.

emptying time (HET) showed a significant negative correlation with body mass and no correlation with age, gender, duration of illness or use of psychiatric medication. In the second part of the study, 12 patients with delayed gastric emptying were retested after one month, and HET had improved in 9 of 12 ($P = 0.0005$)[18]. Further, the normalization of the majority of scintigraphy parameters occurred while the body mass index was still subnormal (less than 20.3) and with amenorrhea still present. The conclusion of the study was that delayed gastric emptying is present in ED and improves quite rapidly after restarting normal feeding. Therefore, the gastrointestinal motility disorder is secondary to insufficient food intake and is not the primary disorder.

A study performed by Hutson *et al*[19] analyzed gastric emptying of a mixed liquid and solid meal in 11 patients with bulimia nervosa and was compared with ten patients with anorexia nervosa and a sex-matched control population[19]. The authors of the study decided to use a dual radioisotope technique in order to measure gastric

Table 2 Studies analyzing the association between functional dyspepsia and eating disorders

Ref.	Aims	Study population	Assessment instruments	Results and conclusions
Santonicola *et al*[37], 2012	Prevalence of FD	20 AN, 6 BN, 10 EDNOS, 9 CT, 32 OB, 22 HC	Rome III criteria (18 questions diagnosis of FD and its subgroups PDS and EPS)	90% AN, 83.3% BN, 90% EDNOS, 55.6% OB and 18.2% CT met PDS criteria. Emesis was present in 100% BN patients, 20% EDNOS, 15% AN, 22% of CT subjects, 5.6% HC. Postprandial fullness intensity-frequency score was significantly higher in AN, BN, EDNOS. Nausea and epigastric pressure were increased in BN and EDNOS
Porcelli *et al* [38], 1998	Presence of lifetime ED in patients referred for FGID	127 consecutive patients (42 FD, 28 IBS 20 FAP, 37 with FD and IBS; male and 83 females; 163 control subjects gallstone disease	GSRS; HADS (HADS-A and HADS-D)	Past ED were significantly more prevalent in FGID (15.7%) than in gallstone disease patients (3.1%) (chi-square = 14.6, $P < 0.001$). FGID patients with past ED were significantly younger, more educated, more psychologically distressed, more dyspeptic, and more were women than FGID patients without past ED
Cremonini *et al*[39], 2009	Severity of BE episodes would be associated with upper and lower GI symptoms	4096 subjects (population-based survey of community residents found through the medical record linkage system) > 18 yr	Questionnaire measuring GI symptoms, frequency of BE episodes and physical activity level	BE disorder: Was present in 6.1% subjects, was independently associated with upper. GI symptoms: Acid regurgitation heartburn, dysphagia, bloating and upper abdominal pain, was associated with lower GI symptoms: diarrhea, urgency, constipation and feeling of anal blockage. The associations independent of the level of obesity
Jáuregui *et al* [40], 2011	QoL in FD patients psychopathological features that underlie the FD	245 people (mean age 28.36 ± 11.26 yr; 189 female and 56 male) 78 patients with ED (70 female and 8 male, mean age 22.88 ± 8.28 yr), 90 university students with associated FD (76 female and 14 male, mean age 22.49 ± 4.27 yr); 77 psychiatric patients (non-ED) (43 female and 34 male, mean age 40.78 ± 9.40 yr)	NDI-SF, BDI, STAI, TSF-Q, VAS	Satiation and bloating were significantly higher in ED patients. Correlations between dyspepsia and TSF were initially positive and significant in all cases, but significance was only maintained in the group of ED patients. Predictors of quality of life in ED patients: dyspepsia, depressive symptomatology, TSF-conceptual, TSF-interpretative and total TSF
Santonicola *et al*[41], 2019	Relationship among anhedonia, BED and upper gastrointestinal symptoms in 2 group of morbidly OB with and without SG	81 OB without SG, 45 OB with SG, 55 HC	BDI, STAI, SHAPS, ROME IV criteria for FD and its subtypes	OB without SG showed a higher prevalence of PDS, mood disorders and anxiety when positive for BE behavior compared to those negative for BE behavior, no differences were found in SHAPS score. OB with SG showed a higher prevalence of PDS compared to OB without SG. BED and depression are less frequent in the OB with SG, while state and trait anxiety are significantly higher. The more an OB with SG is anhedonic, less surgical success was achieved

FD: Functional dyspepsia; ED: Eating disorders; HC: Healthy controls; AN: Anorexia nervosa; BN: Bulimia nervosa; EDNOS: Eating disorders not otherwise specified; CT: Constitutional thinners; OB: Obesity; PDS: Postprandial distress syndrome; EPS: Epigastric pain syndrome; FGID: Functional gastrointestinal disorders; IBS: Irritable bowel syndrome; FAP: Functional abdominal pain; GSRS: Gastrointestinal symptoms rating scale; HADS: Hospital Anxiety and Depression Scale; BE: Binge eating; GI: Gastrointestinal; QoL: Quality of life; NDI-SF: Nepean Dyspepsia Index-Short Form; BDI: Beck Depression Inventory; STAI: State-Trait Anxiety Inventory; TSF-Q: Thought Shape Fusion-Questionnaire; VAS: Visual Analogue Scales; SG: Sleeve gastrectomy; SHAPS: Snaith-Hamilton pleasure scale.

emptying. Similar to previous studies, the relationship between body mass index and gastrointestinal symptoms were also examined. Authors reported at gastric emptying of solids in patients with bulimia nervosa was similar to that in controls (gastric T1/2 131 ± 15 min *vs* 119 ± min; mean ± SEM) and anorexia nervosa patients had overall significantly delayed emptying (182 ± 31 min[19]. Gastric emptying of liquids was similar in bulimic patients and healthy controls[19]. The study did not find any correlation between body mass index, gastrointestinal symptoms, and gastric emptying, indicating that gastrointestinal symptoms are unreliable criteria of gastric emptying in patients with ED[19]. Although gastric scintigraphy is the gold standard technique for measuring gastric emptying, not all studies use it. A study performed by

Table 3 Studies analyzing the association between functional constipation and eating disorders

Ref.	Aims	Study population	Assessment instruments	Results and conclusions
Chun *et al*[47], 1997	Colorectal function measuring colonic transit and anorectal function in AN with constipation during treatment with a refeeding program	Prospective study 13 AN females; 20 age-matched, female HC	Radiopaque marker technique; anorectal manometry	Colonic transit is normal/returns to normal in the majority of AN patients once they are consuming a balanced weight gain or weight maintenance diet for at least 3 wk
Sileri *et al*[48], 2014	Prevalence and type of defecatory disorders in AN patients	85 patients (83 female and 2 male); mean age 28 ± 13 yr; BMI 16 ± 2 kg/m^2; 57 HC, BMI 22 ± 3 kg/m^2	WCS, OD score, FISI	All results influenced by the severity of the disease (BMI; duration). The percentage of defecatory disorders rises from 75 to 100% when BMI is < 18 kg/m^2 and from 60% to 75% when the duration of illness is ≥ 5 yr (P < 0.001 and P = 0.021)
Chiarioni *et al*[49], 2000	Anorectal and colonic function in AN patients complaining of chronic constipation	12 AN female (age 19-29 yr) chronic constipation. 12 female HC	Anorectal manometry; radiopaque technique; test of rectal sensation	AN patients: anorectal motor abnormalities (slow colonic tranzit time, pelvic floor dysfunction)
Boyd *et al*[50], 2005	Prevalence and type of FGIDs in AN, BN and EDNOS patients; relationships between psychological features, eating-disordered attitudes and behaviours, demographic characteristics and the type and number of FGIDs	101 consecutive female AN (n = 45, 44%), EDNOS (n = 34, 34%), BN (n = 22, 22%). Mean age 21 yr	Rome II modular questionnaire GI, ENS, BDI, STAI, BSI somatization subscale, EEE-C, version 4, EDI-2, EAT	52% IBS (constipation-predominant 22%, diarrhoea-predominant 6%, alternating 24%), FH (51%), FAB (31%), FC (24%), FDys (23%), FAno (22%). 52% of patients exhibited 3 or more coexistent FGID diagnoses. Psychological variables (somatization, neuroticism, state and trait anxiety), age and binge eating were significant predictors of specific, and > 3 coexistent FGIDs
Murray *et al*[51], 2020	Frequency of and relation between EDs and constipation in patients with chronic constipation referred for anorectal manometry	279 patients with chronic constipation (79.2% female). Average age (SD) 46.6 ± 17.2 yr	EAT, PAC-SYM, HADS, VSI, ARM, colonic transit testing (24 radiopaque markers)	19% had clinically significant ED pathology. ED pathology might contribute to constipation *via* gastrointestinal-specific anxiety
Dykes *et al*[52], 2001	Past and current psychological factors associated with slow and normal transit constipation.	28 consecutive constipated female patients, mean age 38.2 yr (SD 10.8 yr)	SCID, SF-36, EAT	1/5 current affective disorder, 2/3 previous affective disorder, 1/3 distorted attitudes to food
Waldholtz *et al*[30], 1990	Type and frequency of GI symptoms. To follow symptoms during refeeding prospectively. Guidelines for gastrointestinal testing and intervention in hospitalized AN patients	16 consecutive AN patients chronically ill (4.5 ± 1.2 yr); 71.6% ± 2.9% of matched population weight, 12 HC	AN patients rated on 12 gastrointestinal symptoms before and after nutritional rehabilitation GISS (24 questions); blood tests physical examination	Belching did not improve during treatment; no patients required endoscopy, x-ray evaluation, or antipeptic regimens; although severe gastrointestinal symptoms are common in AN, they improve significantly with refeeding

AN: Anorexia nervosa; BN: Bulimia nervosa; EDNOS: Eating disorders not otherwise specified; HC: Healthy controls; BMI: Body mass index; WCS: Wexner constipation score; OD score: Obstructed defecation score; FIOSI: Fecal incontinence severity index; FGIDs: Functional gastrointestinal disorders; ENS: Eysenck neuroticism scale; BDI: Beck Depression Inventory; STAI: The State-Trait Anxiety Inventory; BSI: Brief Symptom Inventory; EEE-C: Eating and Exercise Examination/computerized; EDI-2: Eating Disorder Inventory-2; EAT: Eating attitudes test; ED: Eating disorders; IBS: Irritable bowel syndrome; FH: Functional heartburn; FAB: Functional abdominal pain disorder; FC: Functional constipation; Fano: Functional anorectal pain disorder; PAC-SYM: Patient Assessment of Constipation Symptom Questionnaire; VSI: Visceral Sensitivity Index; ARM: High-Definition Anorectal Manometry; SCID: Structured Clinical Interview for DSM; SF-36: Short Form (36) Health Survey.

Benini *et al*[20] analyzed 23 anorexic patients using an ultrasonographic gastric-emptying test and psychopathological questionnaires, before and after 4 and 22 wk rehabilitation[20]. The result was that gastric symptom scores were markedly higher in patients than in controls and improved significantly with treatment. Further, no correlation between entry values of gastric emptying symptoms and questionnaire score was found[20]. The study summarized that long-term rehabilitation improves gastrointestinal symptoms, gastric emptying, and psychopathological distress, and short-term does not.

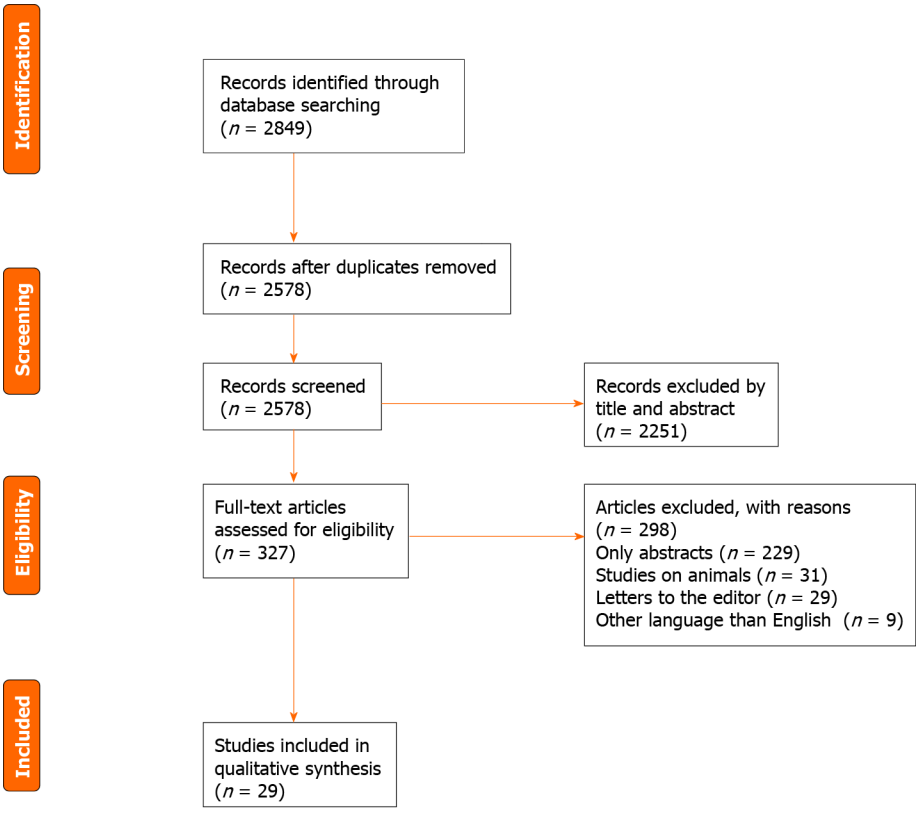


Figure 1 PRISMA flow diagram for study selection.

A study performed by Inui *et al*[21] analyzed gastrointestinal motility abnormalities in 26 female patients who met the DSM-III-R criteria for ED[21]. Gastric emptying was measured using a radionuclide technique, and all patients were additionally evaluated using a self-rating depression scale and the Cattell anxiety scale. Nine patients were diagnosed with anorexia nervosa, 10 with anorexia nervosa and bulimia nervosa, and 7 with bulimia nervosa. In addition, the time expressed in minutes at which half the meal was emptied from the stomach was measured. Patients with anorexia nervosa, anorexia nervosa with bulimia nervosa, and bulimia nervosa all had delayed gastric emptying as compared to nine normal healthy controls and had delayed gastric emptying after ingestion of a solid meal, regardless of DSM-III-R classification[21]. The authors concluded that impaired gastric motility might be caused not only by food restriction or emesis but also by other independent factors from the patient nutritional state[21].

The intricate effect of depression and anxiety shows that gastroparesis pathogenesis is more complex, and psychotherapy may have an essential role in the nutritional rehabilitation of patients with ED. Anorexia nervosa and bulimia nervosa were the most frequent ED associated with gastroparesis, a fact demonstrated by numerous studies over the last 6 decades[22-30]. Limited evidence regarding the association of gastroparesis and pica, night eating disorder and avoidant/restrictive food intake disorder was found[22-30].

FD

FD is one of the most frequent DGBIs and is defined using the Rome IV criteria as any combination of the following symptoms: Postprandial fullness, early satiety, epigastric pain, and epigastric burning that are severe enough to interfere with the usual activities and occur at least three days *per* week over the past three months with an onset of at least six months before presentation[31].

FD includes three syndromes: (1) Postprandial distress syndrome; (2) Epigastric pain syndrome; and (3) Overlapping postprandial distress syndrome and epigastric pain syndrome[31-33]. The pathophogensis of FD is multifactorial and incompletely understood. Dysfunctional gastrointestinal motility (antral hypomotility, delayed or rapid gastric emptying, impaired gastric accommodation), psychological stress, visceral hypersensitivity, psychiatric disorders (depressive disorder, anxiety disorder),

gastric or duodenal hypersensitivity to specific types of food and gastric distension, have been incriminated as pathogenesis mechanisms[34-44]. Smokers, women, patients with Helicobacter pylori infection, ED patients, and nonsteroidal anti-inflammatory drug users have an increased risk of developing FD[34-44].

The dyspeptic symptoms may be encountered in ED. Thus, a study performed by Santonicola *et al*[37] analyzed the prevalence of FD in ED patients, constitutional thinner subjects with no pathology, obese patients, and healthy volunteers[37]. The patients were recruited from a clinic specialized in treating ED, and the study groups included 20 anorexia nervosa patients, six bulimia nervosa patients, ten unspecified eating disorder patients, nine constitutional thinner subjects, 32 obese patients and 22 healthy controls[37]. The presence of epigastric pain syndrome and postprandial distress syndrome were diagnosed according to Rome III criteria. The intensity and frequency score of early satiety, epigastric fullness, epigastric pain, epigastric burning, epigastric pressure, belching, nausea, and vomiting were measured by a standardized questionnaire[37]. The result was that 90% of anorexia nervosa patients, 83.3% of bulimia nervosa patients, 90% of unspecified eating disorder patients, 55.6% of constitutionally thin subjects and 18.2% of the healthy volunteers met the postprandial distress syndrome criteria (χ^2, $P < 0.001$) and only one bulimia nervosa patient met the epigastric pain syndrome criteria[37]. Emesis was present in 100% of bulimia nervosa patients, in 20% of ED not otherwise specified patients, in 15% of anorexia nervosa patients, in 22% of constitutional thinner subjects, and, in 5.6% healthy volunteers (χ^2, $P < 0.001$)[37]. The pathologic eating behavior causes dysfunctional gastrointestinal sensitivity and motility, and after a variable period of time, the resulting DGBIs can persist independently of the eating disorder that originally caused the motility dysfunction[38].

A study performed by Cremonini *et al*[39] analyzed the gastrointestinal symptoms associated with binge ED[39]. A population-based survey of community residents through a mailed questionnaire was performed, and a total of 4096 subjects were included in the study. Binge eating disorder was present in 6.1% of subjects and was associated with the following gastrointestinal symptoms: Acid regurgitation, heartburn, dysphagia, bloating and epigastric pain, diarrhea, constipation and feeling the sensation of anal blockage[39]. The study demonstrated that both upper and lower gastrointestinal symptoms appear in binge ED.

Although the number of studies analyzing ED and FD is small, dyspeptic symptoms are more common in anorexia nervosa and bulimia nervosa patients.

A limitation of most studies regarding FD in ED is that a clear delimitation between meal-related gastrointestinal symptoms is lacking, and future studies analyzing the theoretical and clinical implications might help in developing a more efficient diagnosis and therapeutic scheme[40,41].

FC

FC is defined according to the Rome IV criteria as a change in bowel habit, or defecatory behavior that results in acute or chronic symptoms or diseases that would be resolved with the relief of constipation and a patient must have experienced at least two of the symptoms over the preceding three months[45,46]. FC is present also in ED. Thus, a study by Chun *et al*[47] analyzed the prevalence of FC in anorexic patients by measuring colonic transit and anorectal function[47]. The first study group consisted of 13 anorexic females, and the second study group consisted of 20 healthy female control subjects. Colonic transit was measured using a radiopaque marker technique, and anal sphincter function, rectal sensation, expulsion dynamics, and rectal compliance were measured with anorectal manometry[47]. The result showed that colonic transit returns to normal in the majority of patients after they start to finish a specialized refeeding program[47].

A study performed by Sileri *et al*[48] analyzed the prevalence of defecatory disorders in anorexic patients[48]. The Wexner constipation score (WCS), Altomare's obstructed defecation score (ODS), and the fecal incontinence severity index were used to evaluate constipation and incontinence of 83 female anorexic patients and 57 healthy volunteers. The result showed that constipation (defined as WCS ≥ 5) was present in 83% of anorexic patients and in 12% of healthy controls ($P = 0.001$), while obstructed defecation syndrome (defined as ODS ≥ 10) was present in 84% of anorexic patients and in 12% of healthy controls ($P < 0.001$)[48].

A study performed by Chiarioni *et al*[49] evaluated the prevalence and pathogenetic mechanisms of FC in 12 female anorexic patients and 12 healthy female controls[49]. Pelvic floor dysfunction was analyzed using an anorectal manometry, and colonic transit time was measured by a radiopaque marker technique.

A subgroup of 8 patients was retested after a specialized refeeding program[49]. The results showed that 66.7% of anorexic patients had slow colonic transit times, and 41.7% had pelvic floor dysfunction. The specialized refeeding program normalized the colonic transit time in the subgroup of 8 patients, but pelvic floor dysfunction did not normalize in these patients[49]. The study demonstrated that anorectal motility dysfunctions and delayed colonic transit are frequent in anorexic patients, a result also shown by other studies[30,50-52].

Although, the relation between FC and ET was analyzed in a limited number of studies, a significant association between the disorders was found.

IBS

IBS is one of the most frequent DGBIs characterized by abdominal pain and altered bowel habit in the absence of a specific organic pathology[53,54]. Epidemiologic studies show that the prevalence of irritable bowel syndrome is 10%-20% and the incidence of 1%-2% *per* year[53,54]. Perkins *et al*[55] performed a study about the prevalence of IBS in a large sample of ED patients analyzing the timing of onset of the ED symptomatology and assessing if they are predictors of IBS. The result of the study was that 64% of ED patients met the Manning criteria for IBS and 87% had developed their ED before the onset of IBS symptoms, with a mean period of time of 10 years between the onset of ED and IBS demonstrating that EDs increase the risk of IBS[55].

A study performed by Dejong *et al*[56] examined the prevalence of IBS in patients with bulimia nervosa and showed a high prevalence of IBS in the bulimia nervosa group (68.8%) [b]. IBS diagnosis was assessed using the Manning criteria and even if the study demonstrated a high incidence of IBS in patients with BN, the relationship between those disorders remain unclear[56]. A study performed by Sullivan *et al*[57] on 48 IBS patients, 32 ED patients, 31 inflammatory bowel disease (IBD) patients and 28 healthy controls analyzed the relationship between IBS, IBD and ED. The results showed that the eating Attitudes Test score for the IBS group was higher than IBD and control group (P = 0.05), demonstrating the correlation between IBS and ED[57].

The relation between clinical characteristics of ED and IBS was investigated in a study performed by Tang *et al*[58] on 60 IBS patients. The result was that the severity of IBS symptomatology was correlated with Perfectionism and Ineffectiveness and severe episodes of emesis were associated with self-reported binge-purge behaviors measured by the Bulimia subscale of the EDI[58]. Bulimia nervosa was more common in IBS patients, but no data about the effect of psychiatric therapy on IBS symptoms was found.

DISCUSSION

In this review we looked for current evidence of the association of neurogastroenterological disorders with ED. We identified 29 studies, focusing on four gastrointestinal disorders associated with ED (gastroparesis, FD, FC and irritable bowel syndrome). Despite progress in the field of neurogastroenterology, we were not able yet to identify a causative relation between neurogastroenterological disorders and ED.

One of the strengths of this study is that it highlights the overlap between ED and neurogastroenterological disorders, a fact of paramount importance for the management of those patients who often are affected with both type of disorders, but, just as often, are treated for only one disorder, depending on whether they visit the psychiatrist or the gastroenterologist.

No relation between gastroesophageal reflux disease and ED was found[11-14]. A limited number of studies investigated the rumination syndrome but we were not able to find supportive data about the association between rumination and ED.

The small number of patients included in the majority of studies represent the main limitation and future studies under the support of international collaboration might help in developing a more efficient therapy. Another limit of most studies regarding FD is that a clear delimitation between meal-related gastrointestinal symptoms is lacking, and future studies using an animal model might help in developing precise diagnosis tools[40-44]. Anorexia nervosa and bulimia nervosa were the most frequent ED associated with FD[37-39]. Most studies about FC showed that anorectal motility dysfunctions and delayed colonic transit are frequent in anorexic patients and anorexia nervosa was the most frequent eating disorder associated with FC[47-53]. IBS was associated with anorexia nervosa and bulimia nervosa[55-58].

Anorexia nervosa and bulimia nervosa were the most frequent ED associated with gastroparesis[18-21]. A major limit in studies concerning gastroparesis was the fact

that not all studies use gastric scintigraphy for diagnosis, although it is the gold standard technique for measuring gastric emptying. Thereby, a lack of a clear distinction between gastroparesis and FD was present[20,21,59].

Clear evidence for a cause effect relationship between ED and DGBI, still does not exist. More powerful studies are required. In respect to therapy of DGBI in ED, the absence of randomized controlled trials (RCTs) is the main reason why no guidelines for gastrointestinal symptomatology in ED exist. These limitations can be overcome by projecting larger RCTs with significant samples of ED and DGBI patients.

CONCLUSION

There is no evidence for a cause-effect relationship between DGBI and ED. Their common symptomatology required correct identification and a tailored therapy of each disorder.

ARTICLE HIGHLIGHTS

Research background

Eating disorders (ED) involve both the nervous system and the gastrointestinal tract. A similar double involvement is also found in disorders of the brain-gut interaction (DGBI) and symptoms are sometimes similar.

Research motivation

We aimed to understand the management of patients who often are affected with both DGBI and ED, but, just as often, are treated for only one disorder, depending on whether they visit the psychiatrist or the gastroenterologist.

Research objectives

This systematic review aimed to evaluate the comorbidity of DGBI and ED, in order to find out where there is an association and a cause-effect relationship.

Research methods

A thorough literature search was undertaken. PubMed, Cochrane Library, EMBASE, and WILEY databases were screened for relevant publications regarding DGBI in ED.

Research results

We found 29 articles analyzing the relation between DGBI and ED comprising 13 articles on gastroparesis, 85 articles on functional dyspepsia, 7 articles about functional constipation and 4 articles on irritable bowel syndrome.

Research conclusions

There is no evidence for a cause-effect relationship between DGBI and ED. Their common symptomatology requires correct identification and a tailored therapy of each disorder.

Research perspectives

The absence of randomized controlled trials (RCTs) is the main reason why no guidelines for gastrointestinal symptomatology in ED exist. These limitations can be overcome by projecting larger RCTs with significant samples of ED and DGBI patients.

REFERENCES

1 **Hay P**. Current approach to eating disorders: a clinical update. *Intern Med J* 2020; **50**: 24-29 [PMID: 31943622 DOI: 10.1111/imj.14691]

2 **Rantala MJ**, Luoto S, Krama T, Krams I. Eating Disorders: An Evolutionary Psychoneuroimmunological Approach. *Front Psychol* 2019; **10**: 2200 [PMID: 31749720 DOI: 10.3389/fpsyg.2019.02200]

3 **Smink FR**, van Hoeken D, Hoek HW. Epidemiology, course, and outcome of eating disorders. *Curr Opin Psychiatry* 2013; **26**: 543-548 [PMID: 24060914 DOI: 10.1097/YCO.0b013e328365a24f]

4 **Smink FR**, van Hoeken D, Hoek HW. Epidemiology of eating disorders: incidence, prevalence and

mortality rates. *Curr Psychiatry Rep* 2012; **14**: 406-414 [PMID: 22644309 DOI: 10.1007/s11920-012-0282-y]
5 **Drossman DA**. Functional Gastrointestinal Disorders: History, Pathophysiology, Clinical Features and Rome IV. *Gastroenterology* 2016 [PMID: 27144617 DOI: 10.1053/j.gastro.2016.02.032]
6 **Abraham S**, Kellow JE. Do the digestive tract symptoms in eating disorder patients represent functional gastrointestinal disorders? *BMC Gastroenterol* 2013; **13**: 38 [PMID: 23448363 DOI: 10.1186/1471-230X-13-38]
7 **Corazziari E**. Definition and epidemiology of functional gastrointestinal disorders. *Best Pract Res Clin Gastroenterol* 2004; **18**: 613-631 [PMID: 15324703 DOI: 10.1016/j.bpg.2004.04.012]
8 **Schmulson M**. How to use Rome IV criteria in the evaluation of esophageal disorders. *Curr Opin Gastroenterol* 2018; **34**: 258-265 [PMID: 29846258 DOI: 10.1097/MOG.0000000000000443]
9 **Levinthal DJ**, Strick PL. Multiple areas of the cerebral cortex influence the stomach. *Proc Natl Acad Sci USA* 2020; **117**: 13078-13083 [PMID: 32434910 DOI: 10.1073/pnas.2002737117]
10 **Browning KN**, Travagli RA. Central nervous system control of gastrointestinal motility and secretion and modulation of gastrointestinal functions. *Compr Physiol* 2014; **4**: 1339-1368 [PMID: 25428846 DOI: 10.1002/cphy.c130055]
11 **Person H**, Keefer L. Psychological comorbidity in gastrointestinal diseases: Update on the brain-gut-microbiome axis. *Prog Neuropsychopharmacol Biol Psychiatry* 2021; **107**: 110209 [PMID: 33326819 DOI: 10.1016/j.pnpbp.2020.110209]
12 **Allen AP**, Dinan TG, Clarke G, Cryan JF. A psychology of the human brain-gut-microbiome axis. *Soc Personal Psychol Compass* 2017; **11**: e12309 [PMID: 28804508 DOI: 10.1111/spc3.12309]
13 **Midenfjord I**, Borg A, Törnblom H, Simrén M. Cumulative Effect of Psychological Alterations on Gastrointestinal Symptom Severity in Irritable Bowel Syndrome. *Am J Gastroenterol* 2020 [PMID: 33156010 DOI: 10.14309/ajg.0000000000001038]
14 **Lee SY**, Ryu HS, Choi SC, Jang SH. A Study of Psychological Factors Associated with Functional Gastrointestinal Disorders and Use of Health Care. *Clin Psychopharmacol Neurosci* 2020; **18**: 580-586 [PMID: 33124590 DOI: 10.9758/cpn.2020.18.4.580]
15 **Kim BJ**, Kuo B. Gastroparesis and Functional Dyspepsia: A Blurring Distinction of Pathophysiology and Treatment. *J Neurogastroenterol Motil* 2019; **25**: 27-35 [PMID: 30509017 DOI: 10.5056/jnm18162]
16 **Stein B**, Everhart KK, Lacy BE. Gastroparesis: A Review of Current Diagnosis and Treatment Options. *J Clin Gastroenterol* 2015; **49**: 550-558 [PMID: 25874755 DOI: 10.1097/MCG.0000000000000320]
17 **Camilleri M**, Chedid V, Ford AC, Haruma K, Horowitz M, Jones KL, Low PA, Park SY, Parkman HP, Stanghellini V. Gastroparesis. *Nat Rev Dis Primers* 2018; **4**: 41 [PMID: 30385743 DOI: 10.1038/s41572-018-0038-z]
18 **Szmukler GI**, Young GP, Lichtenstein M, Andrews JT. A serial study of gastric emptying in anorexia nervosa and bulimia. *Aust N Z J Med* 1990; **20**: 220-225 [PMID: 2372271 DOI: 10.1111/j.1445-5994.1990.tb01023.x]
19 **Hutson WR**, Wald A. Gastric emptying in patients with bulimia nervosa and anorexia nervosa. *Am J Gastroenterol* 1990; **85**: 41-46 [PMID: 2296962]
20 **Benini L**, Todesco T, Dalle Grave R, Deiorio F, Salandini L, Vantini I. Gastric emptying in patients with restricting and binge/purging subtypes of anorexia nervosa. *Am J Gastroenterol* 2004; **99**: 1448-1454 [PMID: 15307858 DOI: 10.1111/j.1572-0241.2004.30246.x]
21 **Inui A**, Okano H, Miyamoto M, Aoyama N, Uemoto M, Baba S, Kasuga M. Delayed gastric emptying in bulimic patients. *Lancet* 1995; **346**: 1240 [PMID: 7475706 DOI: 10.1016/s0140-6736(95)92954-1]
22 **Dubois A**, Gross HA, Ebert MH, Castell DO. Altered gastric emptying and secretion in primary anorexia nervosa. *Gastroenterology* 1979; **77**: 319-323 [PMID: 376392]
23 **Kamal N**, Chami T, Andersen A, Rosell FA, Schuster MM, Whitehead WE. Delayed gastrointestinal transit times in anorexia nervosa and bulimia nervosa. *Gastroenterology* 1991; **101**: 1320-1324 [PMID: 1936803 DOI: 10.1016/0016-5085(91)90083-w]
24 **Robinson PH**, Clarke M, Barrett J. Determinants of delayed gastric emptying in anorexia nervosa and bulimia nervosa. *Gut* 1988; **29**: 458-464 [PMID: 3371714 DOI: 10.1136/gut.29.4.458]
25 **Stacher G**. Gut function in anorexia nervosa and bulimia nervosa. *Scand J Gastroenterol* 2003; **38**: 573-587 [PMID: 12825864 DOI: 10.1080/00365520310002120]
26 **Bluemel S**, Menne D, Milos G, Goetze O, Fried M, Schwizer W, Fox M, Steingoetter A. Relationship of body weight with gastrointestinal motor and sensory function: studies in anorexia nervosa and obesity. *BMC Gastroenterol* 2017; **17**: 4 [PMID: 28056812 DOI: 10.1186/s12876-016-0560-y]
27 **Holt S**, Ford MJ, Grant S, Heading RC. Abnormal gastric emptying in primary anorexia nervosa. *Br J Psychiatry* 1981; **139**: 550-552 [PMID: 7332861 DOI: 10.1192/bjp.139.6.550]
28 **Abell TL**, Malagelada JR, Lucas AR, Brown ML, Camilleri M, Go VL, Azpiroz F, Callaway CW, Kao PC, Zinsmeister AR. Gastric electromechanical and neurohormonal function in anorexia nervosa. *Gastroenterology* 1987; **93**: 958-965 [PMID: 3653645 DOI: 10.1016/0016-5085(87)90557-9]
29 **Rigaud D**, Bedig G, Merrouche M, Vulpillat M, Bonfils S, Apfelbaum M. Delayed gastric emptying in anorexia nervosa is improved by completion of a renutrition program. *Dig Dis Sci* 1988; **33**: 919-925 [PMID: 3391083 DOI: 10.1007/BF01535985]
30 **Waldholtz BD**, Andersen AE. Gastrointestinal symptoms in anorexia nervosa. A prospective study. *Gastroenterology* 1990; **98**: 1415-1419 [PMID: 2338185 DOI: 10.1016/0016-5085(90)91070-m]

31 **Grundy D**, Al-Chaer ED, Aziz Q, Collins SM, Ke M, Taché Y, Wood JD. Fundamentals of neurogastroenterology: basic science. *Gastroenterology* 2006; **130**: 1391-1411 [PMID: 16678554 DOI: 10.1053/j.gastro.2005.11.060]

32 **Talley NJ**. Functional dyspepsia: new insights into pathogenesis and therapy. *Korean J Intern Med* 2016; **31**: 444-456 [PMID: 27048251 DOI: 10.3904/kjim.2016.091]

33 **Burton Murray H**, Jehangir A, Silvernale CJ, Kuo B, Parkman HP. Avoidant/restrictive food intake disorder symptoms are frequent in patients presenting for symptoms of gastroparesis. *Neurogastroenterol Motil* 2020; **32**: e13931 [PMID: 32677153 DOI: 10.1111/nmo.13931]

34 **Popa SL**, Chiarioni G, David L, Dumitrascu DL. The Efficacy of Hypnotherapy in the Treatment of Functional Dyspepsia. *Am J Ther* 2019; **26**: e704-e713 [PMID: 31425161 DOI: 10.1097/MJT.0000000000001033]

35 **Talley NJ**, Hunt RH. What role does Helicobacter pylori play in dyspepsia and nonulcer dyspepsia? *Gastroenterology* 1997; **113**: S67-S77 [PMID: 9394764 DOI: 10.1016/s0016-5085(97)80016-9]

36 **Karamanolis G**, Caenepeel P, Arts J, Tack J. Association of the predominant symptom with clinical characteristics and pathophysiological mechanisms in functional dyspepsia. *Gastroenterology* 2006; **130**: 296-303 [PMID: 16472585 DOI: 10.1053/j.gastro.2005.10.019]

37 **Santonicola A**, Siniscalchi M, Capone P, Gallotta S, Ciacci C, Iovino P. Prevalence of functional dyspepsia and its subgroups in patients with eating disorders. *World J Gastroenterol* 2012; **18**: 4379-4385 [PMID: 22969202 DOI: 10.3748/wjg.v18.i32.4379]

38 **Porcelli P**, Leandro G, De Carne M. Functional gastrointestinal disorders and eating disorders. Relevance of the association in clinical management. *Scand J Gastroenterol* 1998; **33**: 577-582 [PMID: 9669626 DOI: 10.1080/00365529850171819]

39 **Cremonini F**, Camilleri M, Clark MM, Beebe TJ, Locke GR, Zinsmeister AR, Herrick LM, Talley NJ. Associations among binge eating behavior patterns and gastrointestinal symptoms: a population-based study. *Int J Obes (Lond)* 2009; **33**: 342-353 [PMID: 19139750 DOI: 10.1038/ijo.2008.272]

40 **Jáuregui Lobera I**, Santed MA, Bolaños Ríos P. Impact of functional dyspepsia on quality of life in eating disorder patients: the role of thought-shape fusion. *Nutr Hosp* 2011; **26**: 1363-1371 [PMID: 22411384 DOI: 10.1590/S0212-16112011000600025]

41 **Santonicola A**, Gagliardi M, Guarino MPL, Siniscalchi M, Ciacci C, Iovino P. Eating Disorders and Gastrointestinal Diseases. *Nutrients* 2019; **11** [PMID: 31842421 DOI: 10.3390/nu11123038]

42 **Zipfel S**, Sammet I, Rapps N, Herzog W, Herpertz S, Martens U. Gastrointestinal disturbances in eating disorders: clinical and neurobiological aspects. *Auton Neurosci* 2006; **129**: 99-106 [PMID: 16962383 DOI: 10.1016/j.autneu.2006.07.023]

43 **Robinson PH**. The gastrointestinal tract in eating disorders. *Eur Eat Disord Rev* 2000; **8**: 88-97

44 **Robinson PH**. Gastric function in eating disorders. *Ann N Y Acad Sci* 1989; **575**: 456-64; discussion 464 [PMID: 2699203 DOI: 10.1111/j.1749-6632.1989.tb53266.x]

45 **Drossman DA**, Hasler WL. Rome IV-Functional GI Disorders: Disorders of Gut-Brain Interaction. *Gastroenterology* 2016; **150**: 1257-1261 [PMID: 27147121 DOI: 10.1053/j.gastro.2016.03.035]

46 **Simren M**, Palsson OS, Whitehead WE. Update on Rome IV Criteria for Colorectal Disorders: Implications for Clinical Practice. *Curr Gastroenterol Rep* 2017; **19**: 15 [PMID: 28374308 DOI: 10.1007/s11894-017-0554-0]

47 **Chun AB**, Sokol MS, Kaye WH, Hutson WR, Wald A. Colonic and anorectal function in constipated patients with anorexia nervosa. *Am J Gastroenterol* 1997; **92**: 1879-1883 [PMID: 9382057]

48 **Sileri P**, Franceschilli L, De Lorenzo A, Mezzani B, Todisco P, Giorgi F, Gaspari AL, Jacoangeli F. Defecatory disorders in anorexia nervosa: a clinical study. *Tech Coloproctol* 2014; **18**: 439-444 [PMID: 24030784 DOI: 10.1007/s10151-013-1068-x]

49 **Chiarioni G**, Bassotti G, Monsignori A, Menegotti M, Salandini L, Di Matteo G, Vantini I, Whitehead WE. Anorectal dysfunction in constipated women with anorexia nervosa. *Mayo Clin Proc* 2000; **75**: 1015-1019 [PMID: 11040849 DOI: 10.4065/75.10.1015]

50 **Boyd C**, Abraham S, Kellow J. Psychological features are important predictors of functional gastrointestinal disorders in patients with eating disorders. *Scand J Gastroenterol* 2005; **40**: 929-935 [PMID: 16170899 DOI: 10.1080/00365520510015836]

51 **Murray HB**, Flanagan R, Banashefski B, Silvernale CJ, Kuo B, Staller K. Frequency of Eating Disorder Pathology Among Patients With Chronic Constipation and Contribution of Gastrointestinal-Specific Anxiety. *Clin Gastroenterol Hepatol* 2020; **18**: 2471-2478 [PMID: 31923640 DOI: 10.1016/j.cgh.2019.12.030]

52 **Dykes S**, Smilgin-Humphreys S, Bass C. Chronic idiopathic constipation: a psychological enquiry. *Eur J Gastroenterol Hepatol* 2001; **13**: 39-44 [PMID: 11204807 DOI: 10.1097/00042737-200101000-00007]

53 **Saha L**. Irritable bowel syndrome: pathogenesis, diagnosis, treatment, and evidence-based medicine. *World J Gastroenterol* 2014; **20**: 6759-6773 [PMID: 24944467 DOI: 10.3748/wjg.v20.i22.6759]

54 **Popa SL**, Leucuta DC, Dumitrascu DL. Pressure management as an occupational stress risk factor in irritable bowel syndrome: A cross-sectional study. *Medicine (Baltimore)* 2018; **97**: e13562 [PMID: 30544474 DOI: 10.1097/MD.0000000000013562]

55 **Perkins SJ**, Keville S, Schmidt U, Chalder T. Eating disorders and irritable bowel syndrome: is there a link? *J Psychosom Res* 2005; **59**: 57-64 [PMID: 16185999 DOI: 10.1016/j.jpsychores.2004.04.375]

56 **Dejong H**, Perkins S, Grover M, Schmidt U. The prevalence of irritable bowel syndrome in outpatients with bulimia nervosa. *Int J Eat Disord* 2011; **44**: 661-664 [PMID: 21997430 DOI: 10.1002/eat.20901]

57 **Sullivan G**, Blewett AE, Jenkins PL, Allison MC. Eating attitudes and the irritable bowel syndrome. *Gen Hosp Psychiatry* 1997; **19**: 62-64 [PMID: 9034814 DOI: 10.1016/s0163-8343(96)00106-5]

58 **Tang TN**, Toner BB, Stuckless N, Dion KL, Kaplan AS, Ali A. Features of eating disorders in patients with irritable bowel syndrome. *J Psychosom Res* 1998; **45**: 171-178 [PMID: 9753389 DOI: 10.1016/s0022-3999(97)00300-0]

59 **Usai-Satta P**, Bellini M, Morelli O, Geri F, Lai M, Bassotti G. Gastroparesis: New insights into an old disease. *World J Gastroenterol* 2020; **26**: 2333-2348 [PMID: 32476797 DOI: 10.3748/wjg.v26.i19.2333]

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SYSTEMATIC REVIEWS

Weight loss interventions in living donor liver transplantation as a tool in expanding the donor pool: A systematic review and meta-analysis

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Author contributions: Trakroo S designed the study, did the data acquisition, helped analyzed and interpreted data, drafted and made critical revisions to manuscript content and made final revisions before publication; Bhardwaj N helped analyze the data, drafted the manuscript, made critical revisions to the manuscript and helped make final revisions before publication; Garg R substantially contributed to data analysis and interpretation, made critical revisions to the manuscript and helped in the final approval of article before publication; Modaresi Esfeh J conceptualized the study, designed the study, helped with drafting of the article, made critical revisions to the content of manuscript, helped with data analysis and did the final approval of the version of the article before publication.

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Abstract

BACKGROUND

With increasing rates of liver transplantation and a stagnant donor pool, the annual wait list removals have remained high. Living donor liver transplantation (LDLT) is an established modality in expanding the donor pool and is the primary method of liver donation in large parts of the world. Marginal living donors, including those with hepatic steatosis, have been used to expand the donor pool. However, due to negative effects of steatosis on graft and recipient outcomes, current practice excludes overweight or obese donors with more than 10% macro vesicular steatosis. This has limited a potentially important source to help expand the donor pool. Weight loss is known to improve or resolve steatosis and rapid weight loss with short-term interventions have been used to convert marginal donors to low-risk donors in a small series of studies. There is, however, a lack of a consensus driven standardized approach to such interventions.

AIM

To assess the available data on using weight loss interventions in potential living liver donors with steatotic livers and investigated the feasibility, efficacy, and safety of using such donors on the donor, graft and recipient outcomes. The principal objective was to assess if using such treated donor livers, could help expand the donor pool.

METHODS

We performed a comprehensive literature review and meta-analysis on studies

The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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examining the role of short-term weight loss interventions in potential living liver donors with hepatic steatosis with the aim of increasing liver donation rates and improving donor, graft, and recipient outcomes.

RESULTS
A total of 6 studies with 102 potential donors were included. Most subjects were males (71). All studies showed a significant reduction in body mass index post-intervention with a mean difference of -2.08 (-3.06, 1.10, I^2 = 78%). A significant reduction or resolution of hepatic steatosis was seen in 93 of the 102 (91.2%). Comparison of pre- and post-intervention liver biopsies showed a significant reduction in steatosis with a mean difference of -21.22 (-27.02, -15.43, I^2 = 56%). The liver donation rates post-intervention was 88.5 (74.5, 95.3, I^2 = 42%). All donors who did not undergo LDLT had either recipient reasons or had fibrosis/steato-hepatitis on post intervention biopsies. Post-operative biliary complications in the intervention group were not significantly different compared to controls with an odds ratio of 0.96 [(0.14, 6.69), I^2 = 0]. The overall post-operative donor, graft, and recipient outcomes in treated donors were not significantly different compared to donors with no steatosis.

CONCLUSION
Use of appropriate short term weight loss interventions in living liver donors is an effective tool in turning marginal donors to low-risk donors and therefore in expanding the donor pool. It is feasible and safe, with comparable donor, graft, and recipient outcomes, to non-obese donors. Larger future prospective studies are needed.

Key Words: Living donor liver transplant; Living liver donors; Liver steatosis; Weight loss interventions; Donor outcomes; Recipient outcomes

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Core Tip: Living donor liver transplantation is an established modality in expanding the donor pool but is limited by donor safety concerns and recipient and graft outcomes due to high prevalence of hepatic steatosis in obese or overweight donors. Weight loss is known to improve or resolve steatosis and help convert marginal donors to low-risk donors in a small series of studies. Our meta-analysis demonstrates that short term weight loss intervention, is feasible and safe in significantly reducing hepatic steatosis in living liver donors undergoing donor evaluation and has the potential to safely expand the donor pool.

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INTRODUCTION

With the steady increase in liver transplantation (LT) over the last 2 decades, and the donor pool remaining largely stagnant, the shortage of organs for transplantation has become even more pressing. This has led to an increase in median time on the wait list for transplantation, especially in patients with a model for end-stage liver disease (MELD) greater than 15[1]. Consequently, as per United Network for Organ Sharing (UNOS) data, every year more than 1200 patients are being removed from the liver transplant wait list[1].

Living donor LT (LDLT) has the potential of increasing the donor pool and lowering the wait list mortality. LDLT offers recipients the advantage of a high-quality graft and the possibility of avoiding delisting, deconditioning over time, or death due to a change in clinical status. In addition, LDLT has the benefit of scheduling the

transplantation as an elective surgery[2]. In parts of the world including South Korea, Japan, India, and Taiwan, LDLT is the primary modality of offering organs to patients in need for LT[3].

Although the number of LDLTs has steadily increased in the United States in the last few years, the total number of LDLTs has lagged in comparison to high volume centers in Asia. The number of deceased donor liver transplants in the United States in 2019 was 8372. The number of LDLTs during the same year was 524, a mere 6% of total liver transplants performed[4].

Most patients listed for liver transplant struggle to find a suitable living donor[5,6]. One way to address the shortage of donors is to use marginal living donors, including those with hepatic steatosis. The negative effects of such steatotic grafts on liver donation and transplantation are well known, including higher incidence of severe ischemic damage resulting in primary dysfunction or primary non function of graft, biliary strictures, and decreased one-year graft survival[7-9]. In a study by Gabrielli *et al*[10], recipients who received non-heart beating liver grafts with macrovesicular steatosis had significantly lower 3-year overall survival.

Compounding the problem of organ shortage is the dramatically rising rate of obesity around the world[11]. In the United States in 2012, 69% of the population was overweight [body mass index (BMI) > 25] and 35% was obese (BMI > 30)[12]. Obesity is a strong risk factor for hepatic steatosis and steatosis is seen on liver biopsy in 76% of potential living liver donors with a BMI of more than 28[13].

The aim of our study was to summarize the current evidence on the role of short-term interventions for weight loss, such as diet and medications, in obese or overweight potential living liver donors with steatotic livers. The objectives were to assess the effectiveness of these interventions in reducing donor BMI and liver steatosis, turning marginal donors to low-risk donors, and examining the impact of steatosis reduction or resolution on short-term donor and recipient morbidity, mortality, and graft outcomes.

MATERIALS AND METHODS

We used PubMed as our primary electronic search database. Keywords used for search criteria included LDLT, living liver donors, diet therapy, fatty liver, steatosis, and short-term weight loss interventions. Studies were analyzed using the Population, Intervention, Comparison, and Outcomes methodology and all studies that met our eligibility criteria were included (Table 1).

Studies that investigated weight loss strategies for potential living liver donors were reviewed. Our eligibility criteria included adult (age > 18 years), overweight or obese potential living liver donors with biopsy proven and or radiologically assessed hepatic steatosis who underwent weight loss interventions and who had post intervention assessment of liver steatosis, with liver biopsy with or without radiologic modalities and post intervention assessment of weight loss. Studies should have also analyzed donor, recipient, and graft outcomes, including perioperative complications as graded by Clavien-Dindo classification[14], and donor and recipient morbidity and mortality. Six studies that met our criteria were finally included in our study.

Variables that were examined in each study included exclusion criteria, treatment modality, diagnostic modalities to assess for pre- and post-intervention hepatic steatosis (such as liver biopsies, computed tomography, or magnetic resonance imaging), pre- and post-intervention BMIs, total bilirubin, liver transaminases [aspartate transaminase, alanine amiotransferase (ALT)]. In addition, liver donation rates, donor and recipient perioperative complications (graded according to Clavien's scale), and donor, graft and recipient outcomes were examined with each study.

Statistical analysis

We used meta-analysis techniques to calculate the pooled estimates following the methods suggested by DerSimonian and Laird using the random-effects model. Mean difference and odds ratio were calculated using random-effects model for continuous and binary variables, respectively. When the incidence of an outcome was zero in a study, a continuity correction of 0.5 was added to the number of incident cases before statistical analysis. Heterogeneity was assessed between study-specific estimates by using Cochran Q statistical test for heterogeneity, and the I^2 statistics. I^2 values of < 30%, 30%-60%, 61%-75%, and > 75% were suggestive of low, moderate, substantial, and considerable heterogeneity, respectively. A P value of ≥ 0.05 was used 'a-priori' to define statistical significance. The analysis was done using RStudio and RevMan

Table 1 Overview of interventions used for body mass index and steatosis reduction, and donor, recipient and graft outcomes following liver transplantation

Study number	Ref.	*n*	Type of intervention	Treatment duration	BMI reduction	Steatosis reduction	Liver donation	Donor, graft, recipient outcomes
1	Fujii *et al*[15], 2020	8	< 1600 Kcal/d + exercise 20 min x 3/wk ± statins	Median of 58 d	Yes (P = 0.0009)	Yes (P = 0.0006)	8	No significant difference from controls
2	Doyle *et al* [16], 2016	16	Optifast VLCD: 1000 kcal/ d	Median of 7.3 wk	Yes (P < 0.001)	Yes (P < 0.001)	14 (1 inadequate volume, 1 fibrosis)	No significant difference from controls
3	Choudhary *et al*[17], 2015	16	1200 kcal/d + 200 to 400 kcal/d exercise ± statins	Mean 28 ± 10 d	Yes (P = 0.006)	Yes (P = 0.008)	14 (2 had NASH/fibrosis)	No reported complication in perioperative period
4	Oshita *et al* [18], 2012	42	800 to 1400 kcal/d diet + 100 to 400 kcal/d exercise	Median 2.9 mo	Yes (P < 0.0001)	Yes, to < 20 %	41 (1 had stage 2 fibrosis)	No different from control group
5	Nakamuta *et al* [19], 2005	11	1000 kcal/d diet + exercise (600 kcal/d) + Bezafibrate	Mean 37.8 ± 4.6 d	Yes(P = 0.0033)	Yes(P= 0.0028)	7 (2 recipient deaths, 1 inadequate GRWR)	No different from control group
6	Hwang *et al* [20], 2004	9	Diet (25-30 calories x ideal body weight) + exercise	Median of 3 mo	Yes (P = 0.0001)	Yes (P = 0.006)	9	No different from control group

BMI: Body mass index; GRWR: Graft weight/recipient weight ratios; NASH: Nonalcoholic steatohepatitis; VLCD: Very low calorie diet.

software.

RESULTS

Six studies were included following our literature search. Data and results from these studies are outlined in Table 1. The largest study had a sample size of 42 patients and the smallest study, had a sample size of 8.

Some inferences can be made based on the data analyzed. Most subjects (71) were males. There were no reported dropouts from the treated group due to adverse events. All studies showed a significant reduction in BMI post-interventions (Figure 1) with a mean difference of -2.08 (-3.06, 1.10, I^2 = 78%). All six studies showed a significant reduction (P < 0.05) in steatosis (Table 1). A significant reduction or resolution of hepatic steatosis was seen in 93 of the 102 in the intervention group (91.2%). Three of the 6 included studies had both pre- and post-intervention liver biopsies, and these studies were included in the Forest plot (Figure 2) to allow for a standardized comparison of intervention outcomes; they showed a significant reduction in steatosis with a mean difference of -21.22 (-27.02, -15.43, I^2 = 56%).

Majority of donors who underwent weight loss interventions successfully underwent living liver donation (Figure 3) with the rate being at 88.5% (74.5-95.3%, I^2 = 42%). Prospective donors, who did not undergo donor hepatectomy, were either waiting to donate at study conclusion or had either steatohepatitis (2 in Choudhary group, 1 in Doyle group), inadequate Graft weight/recipient weight ratios (GRWR) (1 in Nakamuta group, 1 in Doyle group) or recipient causes for not donating (2 recipient deaths in the Nakamuta group).

Post-operative biliary complications (Figure 4) in the intervention group were not significantly different compared to control (non-intervention) donors with odds ratio of 0.96 [(0.14, 6.69), I^2 = 0]. The overall post-operative donor, graft, and recipient outcomes in the diet treated donors were also not significantly different when compared to non-diet treated donors. All studies were limited by their small sample size, and some by their retrospective study design.

DISCUSSION

With increasing rates of LT and a stagnant donor pool, the annual wait list removals

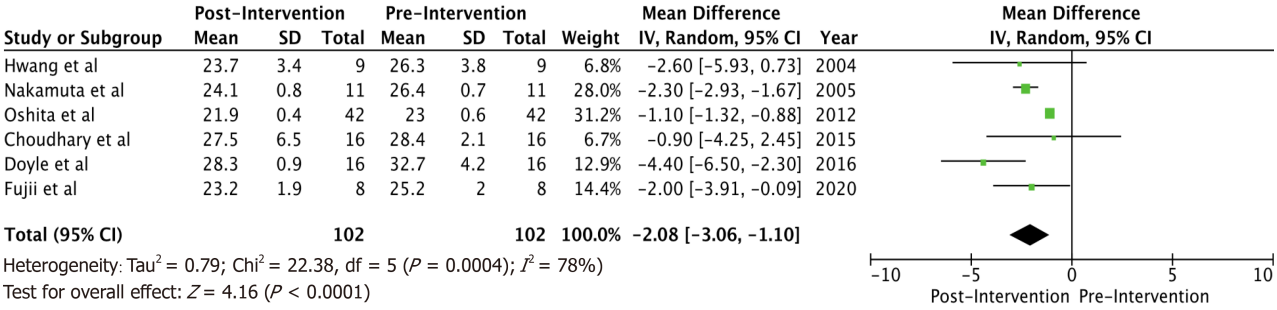


Figure 1 Forest plot showing mean difference in body mass index post-pre intervention in the intervention groups. Mean difference = -2.08 (-3.06, 1.10, I^2 = 78%), with a significantly lower post-intervention body mass index as compared to the pre-intervention body mass index. CI: Confidence interval; SD: Standard deviation.

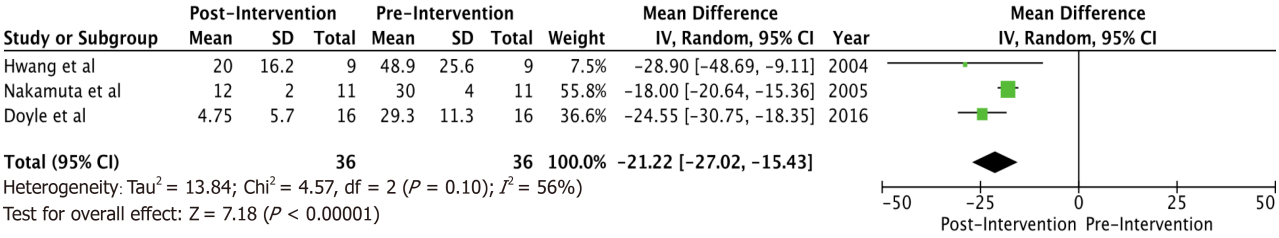


Figure 2 Forest plot showing mean difference in steatosis post-pre intervention. Mean difference = -21.22 (-27.02, -15.43, I^2 = 56%), with significantly lower post intervention steatosis as compared to pre intervention steatosis. CI: Confidence interval; SD: Standard deviation.

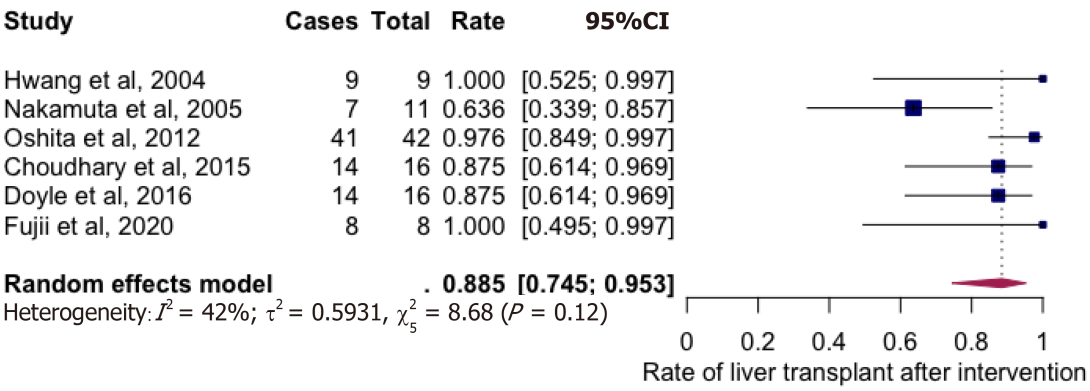


Figure 3 Living liver donation rates after weight loss interventions. Rate = 88.5% (74.5%-95.3%, I^2 = 42%). CI: Confidence interval.

have remained high. The use of extended criteria donors including those with steatotic livers to expand the donor pool is a viable option in expanding the donor pool. The data on donor, graft and recipient outcomes in grafts used for potential donors with steatotic livers who have undergone weight loss interventions, is however sparse. Compounding this issue is the increasing rates of obesity has made donor safety and successful recipient outcomes, an even greater challenge.

We, therefore, analyzed current literature on the role of short-term dietary interventions in preparing potential donors with hepatic steatosis for LDLT with the aim of safely and effectively expanding the donor pool and improving donor and recipient outcomes. Studies included, have been summarized in Table 1.

In study number 1 by Fujii *et al*[15], 8 potential donors were examined from October 2009 to August 2015. Exclusion criteria were age greater than 65 and steatohepatitis. Steatosis was diagnosed based on a liver to spleen (L/S) ratio of < 1.1 and/or hepatic attenuation of < 55 Hounsfield units (HU) on non-enhanced CT. Donors without fatty liver (n = 21) during the study period, were selected as a control group. Treatment efficacy was serially evaluated and when L/S was ≥ 1.1, and hepatic attenuation was ≥ 55 HU, a liver biopsy was performed. When macrovesicular steatosis of < 10% was confirmed, donors were taken up for partial hepatectomy. A significant reduction in

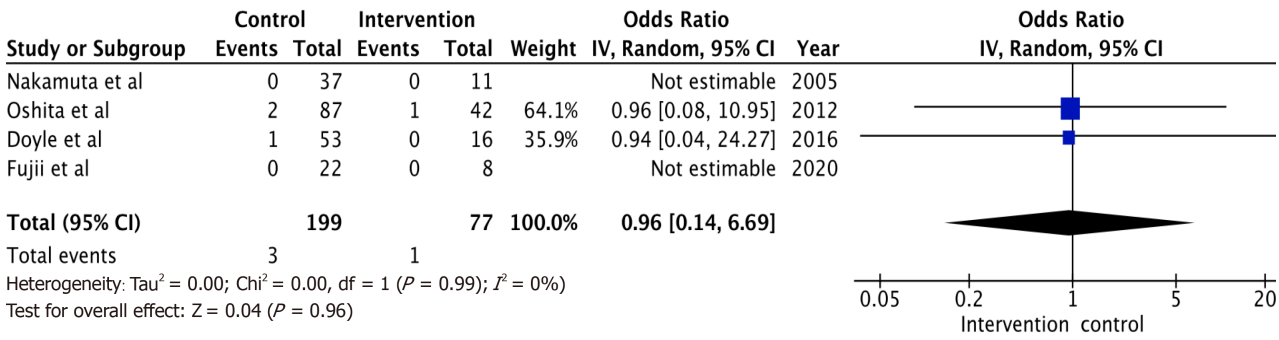


Figure 4 Odds ratio comparing rate of donor post-operative biliary complications in intervention group compared to control donors - odds ratio 0.96 (0.14, 6.69), I^2 = 0.

mean BMI (25 ± 2.0 to 23.2 ± 1.9, P = 0.0009) and L/S ratio [0.95 (0.62-1.06) to 1.2 (1.12-1.46), P = 0.003] were seen. All 8 in the study group showed < 10% steatosis on intra-operative biopsy and underwent partial donor hepatectomy. No major complications (Clavien grade IIIa or greater) were seen. No significant difference in graft function were observed between the 2 groups with 100% Graft and patient survival at 3 mo. They concluded that preoperative treatment for fatty liver was effective and treated potential donors can undergo LDLT without jeopardizing donor safety.

Study number 2 from University of Toronto by Doyle *et al*[16], retrospectively analyzed 16 potential donors from September 2011 to December 2014. Subjects were followed until September 2015. Potential donors with nonalcoholic steatohepatitis (NASH) were excluded and those with steatosis of > 10% who underwent treatment with Optifast, were included. Baseline pre-treatment liver biopsies were performed in the first 8 but after observing promising preliminary results, the authors proceeded directly to dietary intervention in the remaining 8, based on imaging. All underwent liver biopsies at treatment completion. A targeted BMI reduction of 10%, guided treatment duration. The control group (n = 53) included all non-Optifast donors had intraoperative liver biopsy showing < 10% macrovesicular steatosis, as part of the Adult-to-Adult Living Donor Liver Transplantation Cohort Study (A2ALL) consortium. The pre-intervention mean BMI of Optifast donors was 32.7 kg/m^2 [95% confidence interval (CI): 30.5-34.9 kg/m^2] and was higher than non-Optifast donors at 26.4 kg/m^2 (95%CI: 25.4-27.4 kg/m^2; P < 0.001).

Optifast was well tolerated and all 16 completed intervention. The mean BMI decreased to 28.3 kg/m^2 (95%CI: 26.3-30.2 kg/m^2; P < 0.001). All post-intervention biopsies demonstrated ≤ 10% macrovesicular steatosis with mean steatosis reduction from 29.3% to 4.75% (P < 0.001). Fourteen underwent partial hepatectomy with no reported donor mortality and no significant difference in surgical complications (P = 0.11), Clavien scores (P = 0.28), or mean length of stay (P = 0.82) between recipients of both groups. The authors concluded that Optifast can potentially eliminate or significantly reduce steatosis in donors being evaluated for LDLT, with donor and recipient outcomes equivalent to outcomes in non-steatotic donors.

Study number 3 by Choudhary *et al*[17], from July 2010 to January 2015, prospectively analyzed 16 potential liver donors. They had pre- and post-intervention liver biopsies and imaging and the study group was selected from a potential donor pool of 188 biopsy proven NAFLD. Exclusion criteria were metabolic syndrome, NASH, or steatosis of > 30%. Liver attenuation index (LAI, defined as liver attenuation minus splenic attenuation), was used for initial screen of steatosis. Prospective donors with LAI of 0 - 5 or liver attenuation < 53 HU (indicating steatosis), and presence of dyslipidemia or BMI > 28 kg/m^2, underwent biopsy. Donors with a remnant volume of < 30% also had biopsy and had repeat biopsy prior to hepatectomy.

The mean weight loss was 7 ± 4.3 kg with a significant post-intervention BMI reduction (P = 0.006) and improvement in LAI (P = 0.008). A median decrease in steatosis from 15% to 5% was seen in fifteen, including normalization in 7. Two donors had steatohepatitis, steatosis > 20% with borderline liver remnant and did not undergo liver donation. Fourteen underwent liver donation with all donors and their recipients having an uneventful post-operative course. The authors concluded that, in motivated younger liver donors with no comorbidities, steatosis is reversible in a short duration by aggressive lifestyle modifications.

Study number 4 by Oshita *et al*[18] compared outcomes of diet treated (n = 42) to non-diet treated donors (n = 87), from April 2003 to March 2010. Steatosis was assessed by pre-intervention L/S ratio and post-intervention biopsy. Pre-intervention exclusion criteria were diabetes mellitus and L/S ratio of ≥ 1.2. Post-intervention exclusion criteria were macrovesicular steatosis of > 20%.

BMI was reduced from 23.3 ± 0.6 to 21.9 ± 0.4 kg/m^2 (P < 0.0001). ALT, γ-GTP, and total cholesterol showed significant improvements (P = 0.0128, 0.0016, and 0.0004, respectively). Forty in the intervention group had stage 0/1 fibrosis with ≤ 20% steatosis and one had stage 2 fibrosis. One had inflammation and did not undergo liver donation. Forty-one treated donors underwent LDLT with no significant differences in perioperative lab data and complications (Clavien grading), including recipient biliary complications compared to controls. Overall, 1-, 3-, and 5-year recipient survival were not significantly different between the study and control groups (P = 0.455). The authors concluded that with appropriate selection criteria, use of diet-treated donors is feasible and safe with respect to donor and recipient outcomes.

Study number 5 by Nakamuta *et al*[19], tested short-term weight loss interventions on 11 potential donors with ≤ 30% combined microvesicular and macrovesicular steatosis. All had pre- and post-intervention liver biopsies. The control group included 37 donors without hepatic steatosis. The study was conducted from May 2003 to July 2004.

A significant reduction in steatosis (30% ± 4% to 12% ± 2%, P = 0.0028) and BMI (26.4 ± 0.7 kg/m^2 to 24.1 ± 0.8 kg/m^2, P = 0.0033) was seen. All had post-intervention normalization of liver enzymes, total cholesterol, and triglycerides. Seven underwent LDLT and one at study conclusion was waiting for donation. No adverse postoperative events were observed in study group donors or recipients with no difference in graft function. The authors concluded that short-term interventions are effective in reducing steatosis and can contribute to a safer LDLT.

In study number 6 by Hwang *et al*[20], from January 2001 to December 2002, 9 potential liver donors were examined. Exclusion criteria were a combined macro- and microvesicular steatosis of > 30% and or alcohol intake > 40 gm/wk. All underwent pre- and post-intervention liver biopsies and CT assessment of steatosis. In addition, all in the study group had intra-operative liver biopsies. All except one potential donor had pre-intervention elevation in LFTs. All nine in the intervention group showed a significant reduction in BMI (25.3 ± 3.8 to 23.7 ± 3.4, P = 0.0001) and in steatosis (48.9% ± 25.6% to 20.0% ± 16.2%, P = 0.006). All nine underwent donor hepatectomy with an uneventful post-operative course recovered and all recipients survived at 15 mo post-transplantation (study completion). They concluded that short-term weight loss in donors reduces steatosis and can contribute to expanding the donor pool.

Prior research has shown that hepatic steatosis adversely affects donor and recipient outcomes in LT and increases the likelihood of graft damage[21,22]. Marsman *et al*[23] reported that transplantation of livers with up to 30% steatosis resulted in a decreased 4-mo graft survival and 2-year patient survival rate. These findings, along with several other studies showing adverse outcomes with steatotic grafts[7-9], has led to the current practice of excluding potential overweight or obese donors with more than 10% macro vesicular steatosis[24]. In an analysis of the A2ALL database, only 15% of all living donors had a BMI of 30 or more[25]. As per UNOS database, in 2019, of the 874 donor livers discarded, 650 (74%) were in donor BMIs of 25 or more[1].

A few studies have used overweight or obese donors. Knaak *et al*[26], showed that donors with BMI of > 30 but < 35, had equivalent outcomes to non-obese donors. However, all potential donors with > 10% hepatic steatosis were excluded from their study. Also, certain donor characteristics separated them from other LDLT programs, including the use of Graft with higher GRWR in the obese donor group (mean of 1.42 ± 0.44%), a number much higher than the standard practice of using a GRWR cutoff of ≥ 0.8% and the greater use of male donors who tend to have larger liver volumes.

To avoid graft size mismatch, preoperative donor liver volumetry is done using the standardized GRWR. The donor graft weight is derived from CT volumetric assessment of the proposed graft to be harvested and the recipient's required standard liver volume (SLV) is calculated from the recipient's body weight[27]. GRWR is then expressed as the ratio of graft volume (expressed in kg) to the recipient's SLV calculated from the recipient's weight. Calculating GRWR is important in preventing overestimation of the donor's standard liver volume (that can result in excessive hepatic resection and consequent liver failure) and in preventing underestimation of the recipient's standard liver volume that could lead to small-for-size syndrome. The generally accepted GRWR threshold is 0.8%. Some authors have proposed the lowering of threshold to between 0.6 to 0.8% under specific circumstances including

donor age < 45 years, MELD score < 20, no graft steatosis and specific anatomic graft requirements. In such highly select cases, using a lower GRWR threshold in combination with grafts with no steatosis could lead to safe expansion of the donor pool with additional decrease in donor morbidity by preferentially selecting left lobe over right lobe grafts.

Calorie restriction, weight loss, and exercise are still recommended as the initial treatment for fatty liver. In a recent randomized control trial using paired biopsies of 261 patients with NASH who underwent dietary and lifestyle changes for a duration of 52 wk, 72 (25%) achieved resolution of steatohepatitis, 138 (47%) had reductions in NAFLD activity score (NAS) and 56 (19%) had regression of fibrosis[28]. The degree of weight loss correlated independently with all NASH histology. In those who achieved 10% or more weight loss, 90% had resolution of NASH and 45% had regression of fibrosis[28].

As our analysis has shown, there is promising data regarding short term interventions in decreasing or eliminating macro-vesicular steatosis, turning marginal steatotic donors to low-risk donors, and in positively impacting donor and recipient outcomes. All six studies included in this review (Table 1), however, are limited by their small sample size. All studies except one[15], used liver biopsy to quantify steatosis pre-donor hepatectomy and used non-invasive modalities for fat estimation, as an adjunct. Only one of the included studies is in Western population[16] making it difficult to extrapolate findings to our patient population. In addition, study inclusion and exclusion criteria were varied and so were the interventions. Despite these variabilities, most in the study groups tolerated the interventions well, and showed no increase in donor, graft or recipient morbidity or mortality as compared to non-diet treated donors.

Overall, the combination of short-term dietary intervention with low calorie diet (most studies had < 1200 kcal/d) for a duration ranging from 4 to 12 wk with exercise, and/or pharmacotherapy, was safe, well tolerated, and showed good treatment adherence. These interventions were effective in significantly reducing donor BMI with a pooled weighted difference of -1.6 (-4.4 to -1.1, CI of 0.95) and significantly reduced liver steatosis, leading to successful liver donation (88.5%) in the diet treated group. With respect to complications, diet-treated donors did extremely well, with only one donor in the Oshita group having Clavien grade III biliary stenosis. Outcomes of recipients who received grafts from diet-treated donor were not significantly different from recipients of grafts from non-diet treated donor. Grafts from diet treated donors functioned similarly to grafts from donors without obesity. The use of diet-treated donors is feasible with respect to safety of the donor and the outcome of the recipient in LDLT when strict selection criteria are used.

CONCLUSION

Short term dietary interventions, in conjunction with exercise and pharmacotherapy, is feasible and safe with good donor adherence. Our study has shown that such interventions significantly reduce and, in some help resolve hepatic steatosis in potential donors undergoing evaluation for LDLT. We conclude that, carefully selected steatotic diet treated living liver donors have equivalent donor, graft and recipient outcomes compared to those receiving grafts from non steatotic donors. It therefore has the potential to safely expand the donor pool and consequently, decrease the number of wait list removals.

ARTICLE HIGHLIGHTS

Research background

The rates of liver transplantation having increasing but the donor pool has largely remained stagnant leading to high removals from liver transplant waitlists. Living donor liver transplantation (LDLT) using fatty liver could potentially be used to expand the donor pool. However, due to negative effects of steatosis on Graft and recipient outcomes, current practice is to exclude overweight or obese donors with steatosis livers. Data on feasibility, efficacy, and safety of using weight loss interventions marginal donors to low-risk donors is lacking. The aim of the study was to evaluate the feasibility safety and efficacy of short-term weight loss interventions in converting marginal living liver donors to low-risk donors.

Research motivation

Data on safety, efficacy and donor, graft and recipient outcomes when using short term weight loss interventions to convert marginal steatotic liver grafts in LDLT, to low-risk grafts, is lacking. With continuing shortage of organs for transplantation, we looked into the safety and efficacy of using treated steatotic donors, for LDLT.

Research objectives

We did a meta-analysis on the feasibility, safety, and efficacy of weight loss interventions in converting marginal living liver donors to low-risk donors and analyzed the perioperative donor, graft and recipient outcomes.

Research methods

We performed a systematic review and meta-analysis on studies examining the role of short-term weight loss interventions in potential living liver donors with hepatic steatosis with the aim of increasing liver donation rates and improving donor, graft, and recipient outcomes.

Research results

A total of 6 studies with 102 potential donors were included. Most subjects were males (n = 71). All studies showed a significant reduction in body mass index post-intervention with a mean difference of -2.08 (-3.06, 1.10, I^2 = 78%). A significant reduction or resolution of hepatic steatosis was seen in 93 of the 102 (91.2%). Comparison of pre- and post-intervention liver biopsies showed a significant reduction in steatosis with a mean difference of -21.22 (-27.02, -15.43, I^2 = 56%). The liver donation rates post-intervention was 88.5 (74.5, 95.3, I^2 = 42%). All donors who did not undergo LDLT had either recipient reasons or had fibrosis/steatohepatitis on post intervention biopsies. Post-operative biliary complications in the intervention group were not significantly different compared to controls with an odds ratio of 0.96 [(0.14, 6.69), I^2 = 0]. The overall post-operative donor, graft, and recipient outcomes in treated donors were not significantly different compared to donors with no steatosis.

Research conclusions

Our study has shown that using liver grafts from potential living liver donors with hepatic steatosis undergoing short term weight loss interventions, have comparable donor, graft, and recipient outcomes, to donors with no hepatic steatosis.

Research perspectives

Use of appropriate short term weight loss interventions in living liver donors is a feasible, safe, and effective tool in turning marginal donors with liver steatosis to low-risk donors and therefore can help in expanding the donor pool.

REFERENCES

1 **U S. Department of Health and Human Services**. Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data; 2020. Database: Organ Procurement and Transplantation Network [Internet]. [cited 3 February 2020]. Available from: https://optn.transplant.hrsa.gov/data/view-data-reports/national-data/#

2 **Hwang S**, Lee SG, Lee YJ, Sung KB, Park KM, Kim KH, Ahn CS, Moon DB, Hwang GS, Kim KM, Ha TY, Kim DS, Jung JP, Song GW. Lessons learned from 1,000 living donor liver transplantations in a single center: how to make living donations safe. *Liver Transpl* 2006; **12**: 920-927 [PMID: 16721780 DOI: 10.1002/Lt.20734]

3 **International Registry in Organ Donation and Transplantation**. Donation Activity Charts; 2019. Database: IRODaT – DTI Foundation [Internet]. [cited 3 February 2020]. Available from: https://www.irodat.org/?p=database

4 **Moriarty S**. The Most Active Living Donor Liver Transplant Programs. UNOS. [cited 3 February 2020]. Available from: https://unos.org/news/improvement/most-active-living-donor-liver-transplant-programs/

5 **Trotter JF**, Wachs M, Everson GT, Kam I. Adult-to-adult transplantation of the right hepatic lobe from a living donor. *N Engl J Med* 2002; **346**: 1074-1082 [PMID: 11932476 DOI: 10.1056/NEJMra011629]

6 **Brown RS Jr**, Russo MW, Lai M, Shiffman ML, Richardson MC, Everhart JE, Hoofnagle JH. A survey of liver transplantation from living adult donors in the United States. *N Engl J Med* 2003; **348**: 818-825 [PMID: 12606737 DOI: 10.1056/NEJMsa021345]

7 **Imber CJ**, St Peter SD, Handa A, Friend PJ. Hepatic steatosis and its relationship to transplantation.

Liver Transpl 2002; **8**: 415-423 [PMID: 12004340 DOI: 10.1053/jlts.2002.32275]
8 **Chu MJ**, Dare AJ, Phillips AR, Bartlett AS. Donor Hepatic Steatosis and Outcome After Liver Transplantation: a Systematic Review. *J Gastrointest Surg* 2015; **19**: 1713-1724 [PMID: 25917535 DOI: 10.1007/s11605-015-2832-1]
9 **Wang Z**, Hisatake G, Yang L. Liver-specific deceased donor risk indices. *Hepatol Res* 2014; **44**: 159-164 [PMID: 24033790 DOI: 10.1111/hepr.12228]
10 **Gabrielli M**, Moisan F, Vidal M, Duarte I, Jiménez M, Izquierdo G, Domínguez P, Méndez J, Soza A, Benitez C, Pérez R, Arrese M, Guerra J, Jarufe N, Martínez J. Steatotic livers. Can we use them in OLTX? *Ann Hepatol* 2012; **11**: 891-898 [PMID: 23109453]
11 **Robertson C**, Archibald D, Avenell A, Douglas F, Hoddinott P, van Teijlingen E, Boyers D, Stewart F, Boachie C, Fioratou E, Wilkins D, Street T, Carroll P, Fowler C. Systematic reviews of and integrated report on the quantitative, qualitative and economic evidence base for the management of obesity in men. *Health Technol Assess* 2014; **18**: v-vi, xxiii [PMID: 24857516 DOI: 10.3310/hta18350]
12 **Ogden CL**, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011-2012. *JAMA* 2014; **311**: 806-814 [PMID: 24570244 DOI: 10.1001/jama.2014.732]
13 **Rinella ME**, Alonso E, Rao S, Whitington P, Fryer J, Abecassis M, Superina R, Flamm SL, Blei AT. Body mass index as a predictor of hepatic steatosis in living liver donors. *Liver Transpl* 2001; **7**: 409-414 [PMID: 11349260 DOI: 10.1053/jlts.2001.23787]
14 **Clavien PA**, Barkun J, de Oliveira ML, Vauthey JN, Dindo D, Schulick RD, de Santibañes E, Pekolj J, Slankamenac K, Bassi C, Graf R, Vonlanthen R, Padbury R, Cameron JL, Makuuchi M. The Clavien-Dindo classification of surgical complications: five-year experience. *Ann Surg* 2009; **250**: 187-196 [PMID: 19638912 DOI: 10.1097/SLA.0b013e3181b13ca2]
15 **Fujii Y**, Kawamura N, Zaitsu M, Watanabe M, Goto R, Kamiyama T, Taketomi A, Shimamura T. Outcome of Living-Donor Liver Transplantation Using Grafts from Donors Treated for Fatty Liver. *Ann Transplant* 2020; **25**: e920677 [PMID: 31919339 DOI: 10.12659/AOT.920677]
16 **Doyle A**, Adeyi O, Khalili K, Fischer S, Dib M, Goldaracena N, Dillon J, Grant D, Cattral M, McGilvray I, Greig P, Ghanekar A, Lilly L, Renner E, Levy G, Selzner N. Treatment with Optifast reduces hepatic steatosis and increases candidacy rates for living donor liver transplantation. *Liver Transpl* 2016; **22**: 1295-1300 [PMID: 27314458 DOI: 10.1002/lt.24495]
17 **Choudhary NS**, Saraf N, Saigal S, Gautam D, Lipi L, Rastogi A, Goja S, Menon PB, Bhangui P, Ramchandra SK, Soin AS. Rapid Reversal of Liver Steatosis With Life Style Modification in Highly Motivated Liver Donors. *J Clin Exp Hepatol* 2015; **5**: 123-126 [PMID: 26155039 DOI: 10.1016/j.jceh.2015.04.002]
18 **Oshita A**, Tashiro H, Amano H, Kobayashi T, Onoe T, Ide K, Takaki S, Takahashi S, Arihiro K, Chayama K, Ohdan H. Safety and feasibility of diet-treated donors with steatotic livers at the initial consultation for living-donor liver transplantation. *Transplantation* 2012; **93**: 1024-1030 [PMID: 22495493 DOI: 10.1097/TP.0b013e31824c9e25]
19 **Nakamuta M**, Morizono S, Soejima Y, Yoshizumi T, Aishima S, Takasugi S, Yoshimitsu K, Enjoji M, Kotoh K, Taketomi A, Uchiyama H, Shimada M, Nawata H, Maehara Y. Short-term intensive treatment for donors with hepatic steatosis in living-donor liver transplantation. *Transplantation* 2005; **80**: 608-612 [PMID: 16177634 DOI: 10.1097/01.tp.0000166009.77444.f3]
20 **Hwang S**, Lee SG, Jang SJ, Cho SH, Kim KH, Ahn CS, Moon DB, Ha TY. The effect of donor weight reduction on hepatic steatosis for living donor liver transplantation. *Liver Transpl* 2004; **10**: 721-725 [PMID: 15162464 DOI: 10.1002/Lt.20172]
21 **Seki S**, Kitada T, Yamada T, Sakaguchi H, Nakatani K, Wakasa K. In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. *J Hepatol* 2002; **37**: 56-62 [PMID: 12076862 DOI: 10.1016/s0168-8278(02)00073-9]
22 **Seifalian AM**, Chidambaram V, Rolles K, Davidson BR. In vivo demonstration of impaired microcirculation in steatotic human liver grafts. *Liver Transpl Surg* 1998; **4**: 71-77 [PMID: 9457970 DOI: 10.1002/Lt.500040110]
23 **Marsman WA**, Wiesner RH, Rodriguez L, Batts KP, Porayko MK, Hay JE, Gores GJ, Krom RA. Use of fatty donor liver is associated with diminished early patient and graft survival. *Transplantation* 1996; **62**: 1246-1251 [PMID: 8932265 DOI: 10.1097/00007890-199611150-00011]
24 **Levy GA**, Selzner N, Grant DR. Fostering liver living donor liver transplantation. *Curr Opin Organ Transplant* 2016; **21**: 224-230 [PMID: 26867047 DOI: 10.1097/MOT.0000000000000280]
25 **Trotter JF**, Wisniewski KA, Terrault NA, Everhart JE, Kinkhabwala M, Weinrieb RM, Fair JH, Fisher RA, Koffron AJ, Saab S, Merion RM; A2ALL Study Group. Outcomes of donor evaluation in adult-to-adult living donor liver transplantation. *Hepatology* 2007; **46**: 1476-1484 [PMID: 17668879 DOI: 10.1002/hep.21845]
26 **Knaak M**, Goldaracena N, Doyle A, Cattral MS, Greig PD, Lilly L, McGilvray ID, Levy GA, Ghanekar A, Renner EL, Grant DR, Selzner M, Selzner N. Donor BMI >30 Is Not a Contraindication for Live Liver Donation. *Am J Transplant* 2017; **17**: 754-760 [PMID: 27545327 DOI: 10.1111/ajt.14019]
27 **Kiuchi T**, Kasahara M, Uryuhara K, Inomata Y, Uemoto S, Asonuma K, Egawa H, Fujita S, Hayashi M, Tanaka K. Impact of graft size mismatching on graft prognosis in liver transplantation from living donors. *Transplantation* 1999; **67**: 321-327 [PMID: 10075602 DOI: 10.1097/00007890-199901270-00024]
28 **Vilar-Gomez E**, Martinez-Perez Y, Calzadilla-Bertot L, Torres-Gonzalez A, Gra-Oramas B,

Gonzalez-Fabian L, Friedman SL, Diago M, Romero-Gomez M. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. *Gastroenterology* 2015; **149**: 367-378. quiz e14-15 [PMID: 25865049 DOI: 10.1053/j.gastro.2015.04.005]

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